ROLES FOR PLD2 IN GROWTH FACTOR-MEDIATED SIGNAL TRANSDUCTION IN EL4 LYMPHOMA CELLS

by

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of MANPREET SINGH CHAHAL find it satisfactory and recommend that it be accepted.

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Chair

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ROLES FOR PLD2 IN GROWTH FACTOR-MEDIATED SIGNAL TRANSDUCTION IN EL4 LYMPHOMA CELLS

Abstract

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Phospholipase D2 (PLD2) generates phosphatidic acid through hydrolysis of phosphatidylcholine. Murine lymphoma EL4 cells lacking endogenous PLD2 expression present a unique model to elucidate the role of PLD2 in signal transduction. Stable overexpression of active PLD2 in EL4 cells does not effect basal proliferation, although expression of inactive PLD2 decreases basal proliferation. Active PLD2 leads to increased basal activation of Akt. Overexpression of active PLD2 leads to enhanced migration in response to serum, however a significant decrease in migration occurs in cells expressing inactive PLD2, as compared to parental cells. Active PLD2 enhances invasion in response to serum, while the opposite is observed with inactive PLD2.

Both parental EL4 cells and PLD2 transfectants express endogenous epidermal growth factor receptor (EGFR). Levels of EGFR protein are increased in cells expressing active PLD2, as compared to parental cells. EGF stimulates proliferation of EL4 cells expressing active PLD2, but not parental cells or cells

expressing inactive PLD2. PLD2 knockdown (via siRNA) reduces EGF-mediated proliferation in C5 cells to control levels. EGF-mediated proliferation and migration in cells expressing active PLD2 is mediated by the PI3K/Akt pathway. EGF-induced invasion is enhanced in cells expressing active PLD2, as compared to parental cells.

In order to elucidate genes modulated by PLD2, we used V7 and C5 cells in a microarray experiment. We identified 102 genes differentially expressed 2 to 16.5 fold in response to overexpressed PLD2. SOCS2, a protein with an inverse relationship with the EGFR, was selected as a gene of interest. Differential expression of SOCS2 was validated at the protein level, showing that overexpression of PLD2 leads to down-regulation of SOCS2.

Overall, the results of this study show that PLD2 enhances Akt activation, migration, and invasion in EL4 cells. PLD2 leads to increased EGFR expression, and acts in concert with EGFR to enhance mitogenesis and invasion. The results indicate that inactive PLD2 exerts inhibitory effects. The microarray study contributes to a broader understanding of the cellular role of PLD2.

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KEY TO ABBREVIATIONS

ARF:	small GTP-binding protein
Akt/PKB:	protein kinase B; kinase activated via PI3K
BPC:	BODIPY-phosphatidylcholine
C-Src:	intracellular tyrosine kinase
DMEM:	Dulbecco's modified Eagle's medium
Erk:	extracellular signal-related kinase
EGF:	epidermal growth factor
EGFR:	epidermal growth factor receptor
FAK:	focal adhesion kinase
FGF:	fibroblast growth factor
GPCR:	G protein-coupled receptor
GTP:	guanosine triphosphate
HA:	hemaglutinin
HKD:	conserved catalytic domains in phospholipase D
MAPK:	mitogen activated protein kinase
MEK:	MAPK/Erk kinase; dual function kinase that activates Erks
MMP:	matrix metalloprotinease
mTOR:	mammalian target of rapamycin
PA:	phosphatidic acid
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PBt:	phosphatidylbutanol

PC: phosphatidylcholine

PH: pleckstrin homology

PIP2: phosphatidylinositol bisphosphate

PI3K: phosphoinsositide-3 kinase

PKC: protein kinase C

PLA: phospholipase A

PLC: phospholipase C

PLD: phospholipase D

PMA: phorbol 12-myristate 13-acetate

PX: phosphoinositide-binding domain

Pyk2: protein tyrosine kinase 2 beta

Raf: protein serine/threonine kinase that activates MEK

Ras: small GTP-binding protein that activates Raf

RasGRP: Ras guanyl releasing protein

RGS: regulator of G-protein signaling

Rho: small GTP-binding protein involved in cytoskeletal reorganization

RT-PCR: reverse transcriptase-polymerase chain reaction

SDS: sodium dodecyl sulphate

SEM: standard error of measurement

siRNA: small interfering RNA

SOCS: suppressor of cytokine signaling

SOS: son of sevenless

TLC: thin layer chromatography

LIST OF FIGURES

DEDICATION

I would like to dedicate this work to my family and friends. The love, support, and encouragement that they have given me over the years has made all this possible. Their words of encouragement have helped me when I have needed it the most.

I would especially like to thank my parents, Malkiat and Mohinder Chahal, for their unconditional love and support to follow and achieve my goals and dreams.

CHAPTER I

GENERAL INTRODUCTION

1.1 PHOSPHOLIPASES

Phospholipases are enzymes that catalyze the hydrolysis of phospholipids (2). There are four major classes of Phospholipases (A, B, C, and D) which are grouped based on the specific phospholipid bond that they cleave (2). Phospholipase A1 and A2 cleave *sn1* and *sn2* acyl chains respectively (Figure 1-1). Phospholipase B cleaves both *sn1* and *sn2* acyl chains (Figure 1-1). Phospholipase C cleaves at the amino terminus side of the phosphate group, releasing diacylglyerol (Figure 1-1). Phospholipase D cleaves at the carboxyl terminus side of the phosphate group, leading to generation of phosphatidic acid (Figure 1-1).

Phospholipase A1 is expressed intracellularly and extracellularly and plays a role in lysophospholipid and fatty acid signaling (3). Phospholipase A2, which exists in multiple isoforms, is expressed intracellularly and secreted and plays a role in generation of fatty acid metabolites, remodeling of membrane phospholipids, and regulation of many inflammatory signaling processes (4). Phospholipase B is expressed intracellularly and extracellularly, is involved in membrane phospholipid synthesis, and maintains cell wall integrity in fungi (5). Phospholipase C comprises the largest class of phospholipases and plays a role in variety of signaling functions through production of second messengers such as diacylglycerol and inositol triphosphate (6).

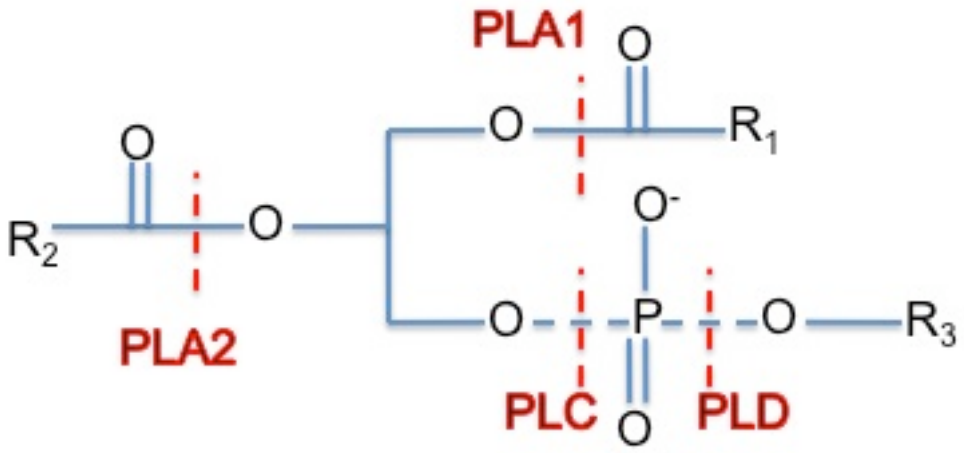

Figure 1-1: Classification of phospholipases. Phospholipases cleave a phospholipid molecule at specific sites. Red broken lines indicate the site of cleavage by the associated phospholipases. PLB cleaves at the sites targeted by both PLA1 and PLA2.

1.2 PHOSPHOLIPASE D

Phospholipase D (PLD) is an enzyme that catalyzes the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline (1). There are two isoforms of human PLD, PLD1 (120-kDa) and PLD2 (105-kDa); both share 51% amino acid sequence (7). PLD expression has been detected in many species including bacteria, yeast, plants, and humans (7). The mammalian PLD2 enzyme is expressed in most tissues including blood, bone, brain, heart, kidney, liver, and ovary (8). PLD2 plays a role in various cellular processes such as vesicle trafficking, exocytosis, cytoskeleton reorganization, cell survival, proliferation, and tumorigenesis (7,8). However, the exact mechanism by which PLD2 modulates its tumorigeneic functions is under investigation.

The PLD2 gene was cloned from a B-cell library in 1998 (9). Northern blotting demonstrated PLD2 expression in all tissue with the greatest expression found in the brain and lungs (8). The lowest expression was discovered in skeletal muscle and liver, however some cells were founds to have no detectable expression at all (7). FISH analysis was used to map PLD2 to the human chromosome 17 (7). Later studies investigated the role of PLD2 in vesicle trafficking and exocytosis in immune cells; and in leukocyte migration and function (7).

1.3 PHOSPHOLIPASE D2 STRUCTURE

The primary structure of PLD2 includes defined regulatory domains, the PX and PH domains flank a highly conserved PLD catalytic motif (7,10) (Figure 1-2). The PX domain is a lipid-binding domain that plays a role in regulation and translocation of PLD2 (10). The PH domain of PLD2 plays a role in localization and does not appear to regulate its activity. The catalytic domain of PLD2 consists of four conserved motifs (7). Motifs two and four contain the highly conserved HKD domains that form the active site leading to the production of PA (7). A mutation of the histidine residues in the HKD domain renders the enzyme non-functional (1,7).

1.4 PHOSPHOLIPASE D2 LOCALIZATION AND SIGNALING

PLD2 is localized at the plasma membrane and in endocytic vesicles (7,11). Some studies have also shown localization of PLD2 in cytosol, peri-nuclear regions, and the Golgi apparatus (2). Localization of PLD2 in the nucleus and the Golgi apparatus is controversial and still under investigation (12). Following agonist stimulation, PLD2 translocates from general plasma membrane to membrane ruffles or cytosol (10).

PLD2 activity is regulated by many factors including lipids, kinases, and small GTP binding proteins (7). In vitro, PLD2 has high basal activity compared

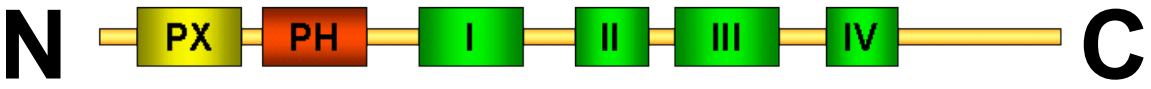

Figure 1-2: Structure of phospholipase D2. The PX and PH domain lie at the amino terminus and participate in protein-protein interactions. The conserved domains I to IV form the catalytic site resulting in generation of PA.

to PLD1 (7). Many of the factors regulating PLD2 activity work downstream of activation of G-protein coupled receptors, growth factors, and receptor tyrosine kinases (7). PIP2 regulates activity of PLD2 by binding at the PH domain and between conserved domains two and three in the PLD catalytic motif (7,13). Epidermal growth factor receptor (EGFR), upon agonist stimulation, interacts with PLD2 and causes its activation (14). PLD2 is also negatively regulated by many factors. Negative modulators identified thus far include beta-actin, actin binding protein fodrin, synucleins, munc-18-1, and alpha actinin (7,15). Most of these negative factors play a role in the actin regulation, indicating a tightly controlled role of PLD2 in cytoskeleton reorganization. PLD2 is also known to undergo post-translational modifications such as phosphorylation and lipidation; however, the functional significance of these modifications is not well understood (16).

PLD2 participates in protein-protein interactions via its PX, PH, and conserved domains and thereby regulates protein activity. PLD2 protein-protein interactions often take place independent of PA generation. The PH domain interacts and regulates the GTPase cycle of dynamin (17). The PX domain is involved in recruiting the Grb2/SOS complex during activation of the Ras/Raf/Erk signaling cascade (17). The PX and PH domains play a role together in interacting with PKC (17). Rho GTPase interacts with PLD at the carboxyl terminal, outside of the conserved catalytic domains that contribute to the generation of PA (17). Small GTPases of the ARF family interact with PLD2 across the conserved domains (7). Many phospholipids also interact directly with

PLD2 via its PX domain, such as PIP2 and PIP3 (7). Mutation studies are currently underway that will add to the already known PLD2 protein-protein interactions.

1.5 PHOSPHATIDIC ACID

PA is the phospholipid produced by the hydrolytic action of PLD2 (7,18). It is a putative lipid second messenger that can modulate signaling, either directly or through its metabolites such as diacylglycerol and lysophosphatidic acid (19). Some of the signaling effects modulated by PA are mediated by the membrane recruitment of cytosolic proteins (18). Several PA interacting proteins have been identified such as sphingosine 1-phosphate kinase, Raf-1, cAMP-specific phosphodiesterase and mammalian target of rapamycin (mTOR) (18,20). Identification of PA interacting proteins was accelerated with the discovery of putative PA binding domain in the kinase domain of Raf-1 protein (19,21). The interaction of PA with Raf-1 can aid recruitment of Raf-1 to the membrane, is proposed to help activate the Ras/Raf/Erk signaling cascade (21). However, the overall significance of PA as a messenger molecule is still under investigation.

1.6 EPIDERMAL GROWTH FACTOR RECEPTOR AND PHOSPHOLIPASE D2

EGFR is a trans-membrane protein tyrosine kinase that belongs to the ErbB family of receptor protein kinases (22). EGFR modulates many signaling

processes including apoptosis, cell survival, cytoskeleton rearrangement, and development (22,23). The extracellular domain of the EGFR binds epidermal growth factor (EGF), a polypeptide growth factor, as well as related peptides (22). Ligand binding induces dimerization of the receptor, followed by protein kinase activation that results in auto-phosphorylation (22). The activated receptor can activate many signaling cascades downstream (22). However, EGFR can also undergo trans-activation, in which a ligand binding to another receptor, such as a G-protein coupled receptor (GPCR), can lead to the activation of the EGFR through activation of intracellular protein tyrosine kinases, or via release of EGFR ligands (24). EGFR-mediated signaling is dysregulated in many epithelial cancers, promoting tumor growth and progression (25,26). Many novel anti-cancer therapeutics have been developed to inhibit EGFR-mediated signaling either by blocking EGFR activation, or by down-regulating the receptor (22,26). EGFR has also been implicated in the regulation of PLD2 activity (14). Studies have established that, in HEK 293 cells, PLD2 is constitutively associated with EGFR (26). Stimulation of the EGFR with EGF leads to stimulation of PLD2 activity. Stimulation with EGF induces tyrosine phosphorylation of PLD2, however the functional significance of this phosphorylation is still under investigation (14).

1.7 PHOSPHOLIPASE D2 AND CANCER

PLD2 can regulate various signaling processes such as proliferation, adhesion, survival, apoptosis and tumor transformation (7,27). A role for PLD2 in

proliferation has been suggested by studies that show elevation of PLD2 activity upon treatment of cells with growth factors such as EGF, FGF, and insulin (28). Elevation of PLD2 activity and expression has been reported in various cancers including renal cancer, colon cancer, and breast cancer (27,29,30). Further, a polymorphism of the PLD2 gene has been associated with increased prevalence of colorectal cancer in the Japanese population (29). Increased PLD2 expression contributes to the transformation of fibroblasts overexpressing tyrosine kinases, such as c-Src or epidermal growth factor receptor (14,31). Additionally, elevated PLD2 expression inhibits apoptotic signals via a p53 independent pathway through inhibition of p21 in rat fibroblasts and breast cancer cells while promoting survival signals through the mTOR pathway (32). PLD2 has also been shown to bypass cell cycle arrest programs that are often activated by apoptotic pathways, thereby leading to tumor progression (27,32).

Studies have also implicated PLD2 activity in the regulation of metastasis. PLD2 modulates various processes dysregulated during tumor invasion, such as actin cytoskeleton reorganization, adhesion, and migration (27). In v-Src transformed rat fibroblasts, overexpression of PLD2 significantly increases the size of cell protrusions (31). However, opposite effects (elimination of cell protrusions) were observed in the same study upon overexpression of catalytically inactive PLD2 (31). PLD2 activity has been implicated in tumor invasion. MDA-MB-231 breast cancer cells with highly elevated PLD2 activity have been shown to invade Matrigel in *in vitro* experiments, however, breast

cancer cells with very low PLD2 activity, such as MCF-7, invade poorly (27). Elevated PLD2 activity has also been implicated in increased protease secretion, a hallmark of invasive cancer cells. Overexpression of PLD2 in mouse fibroblasts causes an increase in MMP-2 secretion; a further increase in MMP-2 secretion was observed upon stimulation with an agonist that activates PLD2 (33).

The findings discusses above suggest that PLD2 plays a critical role in many cellular processes such as cell growth, cell survival, cell proliferation, apoptosis, cell migration and adhesion (7). Dysregulation of these processes can result in progression of normal cells to those with a malignant phenotype. Although existing data suggest that PLD2 plays a role in many stages of tumorigenesis, including transformation, progression, and invasion, although the exact molecular mechanism is not well understood (27). Elevated PLD2 activity has been found in various cancers including breast, colon, and kidney, suggesting that PLD2 could be a valuable therapeutic target in the quest to developing novel cancer therapeutics.

1.8 EL4 LYMPHOMA CELLS AND PRELIMINARY PLD2 STUDIES

EL4 cells, derived from a murine lymphoma, lack endogenous expression of PLD2 mRNA and protein (1). Previous work in our lab established EL4 cells stably transfected with catalytically active hPLD2, designated as C5 cells (1). Cells stably transfected with catalytically inactive hPLD2 were designated as D3

cells (1). In both cases, the PLD2 constructs contain a hemaglutinin (HA) tag to allow identification using an anti-HA antibody (1). These cell lines were used to investigate the role of PLD2 in adhesion, migration, and invasion. Expression of catalytically active PLD2 resulted in increased spreading and elongation of transfected cells, while inactive PLD2 produced the opposite effect (1). Inactive PLD2 also inhibits cell adhesion, migration, and serum-induced invasion. Cells expressing active PLD2 formed more tumors in syngeneic mice, as compared to parental cells or cells expressing inactive PLD2 (1).

This project further investigates the mechanisms of PLD2-induced cell adhesion, migration, and invasion, using the EL4 model system. The goal of this project was to elucidate signal transduction pathways downstream of PLD2 activation that leads to increased adhesion, migration, and invasion. This goal was accomplished using signal transduction studies, as well as microarray studies in order to indentify potential downstream targets that play a role in PLD2-induced increased adhesion, migration and invasion.

1.9 HYPOTHESIS AND PROJECT OVERVIEW

The major hypothesis addressed in this dissertation is that PLD2 plays a critical role in facilitating growth factor-mediated signal transduction, thus enhancing tumorigenesis. This project examines PLD2 and its role in a variety of signaling pathways.

PROJECT OVERVIEW

Aim I. To characterize the effects of PLD2 overexpression on EGFR expression and EGF-induced signaling events.

Previous data from our lab indicated that overexpression of PLD2 in EL4 cells leads to increased FAK phosphorylation, elongated phenotype, increased tumor formation and metastasis in a mouse model (1). To further investigate events that contribute to increased tumorigenesis by PLD2 we tested the basal proliferation, invasion, and migration in parental cells and cells expressing active and inactive PLD2. An unexpected finding was that murine lymphoma EL4 cells overexpressing PLD2 have increased basal levels of EGFR protein expression. We used PCR techniques to validate increased basal levels of EGFR expression at the mRNA level. In order to establish that PLD2-induced increased basal expression of EGFR is not an event specific only to EL4 cells, we transfected PLD2 into an ovarian cancer cell line (OVCAR3) and characterized EGFR

expression at protein level. Next, we characterized the effects of EGFR agonist stimulation on EL4 cells overexpressing PLD2 using cell proliferation, invasion, and signaling pathway activation studies.

Aim II. To determine effects of PLD2 overexpression on cellular gene transcription

Studies in our lab and others have shown that PLD2 overexpression leads to increases in tumor formation, invasion, and metastasis (1,27). We performed a microarray experiment using mouse arrays to look at gene expression of cancer cells stably transfected with PLD2, while using parental cells as control. The resulting data were analyzed to identify genes of potential interest. Differential protein expression was validated using Western blotting.

CHAPTER II

EFFECTS OF PHOSPHOLIPASE D2 ON BASAL EL4 PROLIFERATION, INVASION, AND MIGRATION

(These data were included in Knoepp et al., Mol. Pharmacol., June 3, 2008 (1))

2.1 SUMMARY

Phospholipase D2 (PLD2) generates phosphatidic acid through hydrolysis of phosphatidylcholine. Murine lymphoma EL4 cells lacking endogenous PLD2 expression present a unique model to elucidate the role of PLD2 in signal transduction. In the current study, we investigated the effects of PLD2 overexpression on proliferation, migration, and invasion. Overexpression of active PLD2 does not effect basal proliferation, however expression of inactive PLD2 leads to decreased basal proliferation in EL4 cells. PLD2 leads to increased basal activation of Akt when compared to parental cells and cells expressing inactive PLD2. Overexpression of active PLD2 leads to enhanced migration in C5 cells in response to serum, however a significant decrease is observed in D3 cells expressing inactive PLD2. PLD2 enhances invasion in C5 cells in response to serum, while the opposite is observed in D3 cells. Taken together, the data show that PLD2 enhances Akt activation, migration and invasion in lymphoma cells. The results also indicate that inactive PLD2 exerts inhibitory effects that are likely mediated by protein-protein interactions.

2.2 INTRODUCTION

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid (PA), a lipid mediator (1,7). There are two isoforms of human PLD, PLD1 and PLD2 (7). PLD2 expression has been detected in many organisms and in most mammalian tissues (8). PLD2 activity is regulated by lipids, kinases, and small GTP binding proteins (7,10). PLD2-generated PA modulates signaling either by binding to proteins directly, or through its metabolites (18). PA interacting proteins include sphingosine 1-phosphate kinase, Raf-1, cAMP-specific phosphodiesterase, mammalian target of rapamycin (mTOR), and p70S6K (18,20,34).

PLD2 regulates various cellular processes such as proliferation, adhesion, and survival (7). Treatment with growth factors such as FGF, EGF, and insulin leads to an increase in PLD2 activity (28). Increased PLD2 expression contributes to the transformation of fibroblasts overexpressing tyrosine kinases, such as c-Src or epidermal growth factor receptor (14,31). Elevated PLD2 activity has been found in various cancers including breast, colon, and kidney (27,29,30). MDA-MB-231 breast cancer cells with high levels of PLD2 activity invade Matrigel *in vitro*, however, breast cancer cells with very low PLD2 activity, such as MCF-7, are less invasive (27).

PLD2 appears to play a critical role in many cellular processes such as cell growth, cell survival, cell proliferation, apoptosis, cell migration, and adhesion (7). Dysregulation of many of these processes is critical for progression of normal cells to cells with a malignant phenotype, however, the exact molecular mechanisms are not well understood (27). Previous studies from our lab have shown that overexpression of catalytically active PLD2 in cells lacking PLD2 results in increased spreading and elongation of transfected cells, while inactive PLD2 produces the opposite effect (1). Cells expressing active PLD2 form more tumors in syngeneic mice, as compared to parental cells or cells expressing inactive PLD2 (1).

In the current study, we utilized EL4 cells expressing PLD2 to elucidate effects of PLD2 on proliferation, migration, and invasion. Our results show that EL4 cells expressing catalytically active PLD2 have increased Akt activation, migration, and invasion.

2.3 MATERIALS AND METHODS

Cell culture: Clonal EL4 cells were maintained in RPMI-1640 (GIBCO BRL, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) as previously described (1). All cells were grown in 5% CO2/95% air at 37°C on standard tissue culture dishes. Stably transfected EL4 cells were maintained in the presence of 0.25 mg/ml G418 (Calbiochem), except that they were

maintained in medium without G418 for 24 hours prior to experiments. Du145 and OVCAR-3 cells were grown in RPMI-1640 with 10% FBS, PC3 cells in F12K with 10% FBS, and PC12K cells (on Primaria plates) in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS.

Membrane PLD2 activity: PLD2 activity was determined as previously described (1). Briefly, cells were grown overnight in serum-free media and collected by scraping following treatment. Cells were lysed by sonication and membranes were separated by centrifugation at 100,000xg. Membranes (5μg protein) were incubated with BODIPY-phosphatidylcholine (B-PC), in the presence of 5% butanol, for 60 minutes at 30°. The samples were spotted on plastic-backed silica gel TLC plates and developed using methanol/chloroform/water/acetic acid (45:45:10:2 v/v). Products were imaged using a phosphorimager.

Immunoblotting: Following treatment, cells were rinsed with 1X PBS, and harvested by scraping. Cells were collected by centrifugation at 1,200Xg. Whole cell lysates were prepared in lysis buffer containing 20 mM Tris (pH 7.4), 1% Trition-X-100, 150 mM NaCl, 1 mM EGTA, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Coomassie blue reagent (Pierce Chemical, Rockford, IL) was used to determine protein concentrations. Equal amounts of protein (100 μg) were loaded in each lane of a 10% polyacrylamide gel, separated by SDS-PAGE, and transferred to PVDF membranes. Membranes

were incubated with primary antibodies and developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Anti-phosho-Akt and anti-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Blots were quantified using NIH Image software.

Cell proliferation: Equal numbers ($2X10^5$) of cells from each cell line were grown in 6-well plates in triplicates. Cells were grown for the indicated times. Cells were collected by scraping, and viable cells were counted by hemacytometer using Trypan blue to stain non-viable cells.

Cell migration: Cell migration was measured using a modified Boyden chamber method (BD Biosciences, San Jose, CA). To the upper wells of the chamber, cells overexpressing PLD2 and parental cells were added at a concentration of 100,000 cells in serum-free media. To the lower wells, media with 10% FBS was added. Following 24 hours, the membranes were fixed and stained with the Diff-Quik® dye kit. Cells that migrated into the lower wells in response to serum were counted using a microscope.

Cell invasion: *In vitro* cell invasion was measured using a modified Boyden chamber method (BD Biosciences, San Jose, CA). To the upper wells of the chamber, cells overexpressing PLD2 and parental cells were added at a concentration of 100,000 cells in serum-free media. To the lower wells, media with 10% FBS was added. Invasion was measured as the ability of cells to pass

through Matrigel (BD Biosciences), an artificial extracellular matrix that was used to coat the upper well. The membranes were fixed and stained with the Diff-Quik® dye kit. Cells that invaded into the lower wells were counted using a microscope.

Statistical analysis: All experiments were repeated; representative results from three independent experiments are shown. For quantified data, values were expressed as mean ± S. D. of values obtained. Statistical significance was assessed by Student's t-test, using InStat software (GraphPad).

2.4 RESULTS

Basal PLD2 activity in various cancer cell lines

In previous work, our lab generated EL4 cells stably transfected with catalytically active and inactive PLD2 (1). EL4 cells are good model for this study, as they lack detectable expression of PLD1 or PLD2 protein and mRNA. Full length PLD2 was generated by RT-PCR from RNA extracted from PC-3, a human prostate cancer cell line (1). EL4 V7 cells transfected with catalytically active PLD2 were designated as C5 cells, and cells transfected with catalytically inactive PLD2 were designated as D3 cells (1). PLD2 activity of these cells was determined using membrane PLD assay. Results were compared to PC12K (neuronal cell line), PC3 and Du145 (prostate cancer cell lines), and OVCAR3 (ovarian cancer cell line). All of these cell lines have been used in our lab

previously for studies of agonist-induced PLD2 activation. In this *in vitro* assay, production of PBt is correlated with PLD activity (35). Our results show that, of the EL4 cells, only C5 cells have PLD2 activity, as expected (Figure 2-1). PLD activity of C5 cells is similar to that of PC-3, the source of the transfected PLD2 (Figure 2-1). All other cancer cells display PLD2 activity to varying degrees (Figure 2-1). Overall, our results indicate that C5 EL4 cells have PLD2 activity and this activity level is comparable to that in PC-3, a cancer cell with detectable levels of endogenous PLD2 activity. PC-3 cells have been used previously in our lab for studies of the effects of physiologic agonists on PLD2 (36,37). Hence, C5 cells express physiologically relevant levels of PLD2 activity.

Basal proliferation of EL4 cells overexpressing PLD2

PLD2 regulates various processes such as proliferation, adhesion, and survival (7). Treatment with growth factors such as FGF, EGF, and insulin leads to an increase in PLD2 activity (28). We asked whether transfection of PLD2 in EL4 cells would alter their ability to proliferate. We used parental V7 cells, cells transfected with active PLD2 (C5), and cells transfected with inactive PLD2 (D3) in a proliferation assay for 12-48 hours (Figure 2-2). Our results indicated that, from 36 to 48 hours, V7 and C5 cells grow at similar rates (Figure 2-2). In contrast, D3 cells have a decreased proliferation during the same time (Figure 2-2). Our results suggest that increased expression of active PLD2 does not contribute to PLD2-enhanced proliferation. However, expression of inactive PLD2 leads to decreased proliferation in response to serum.

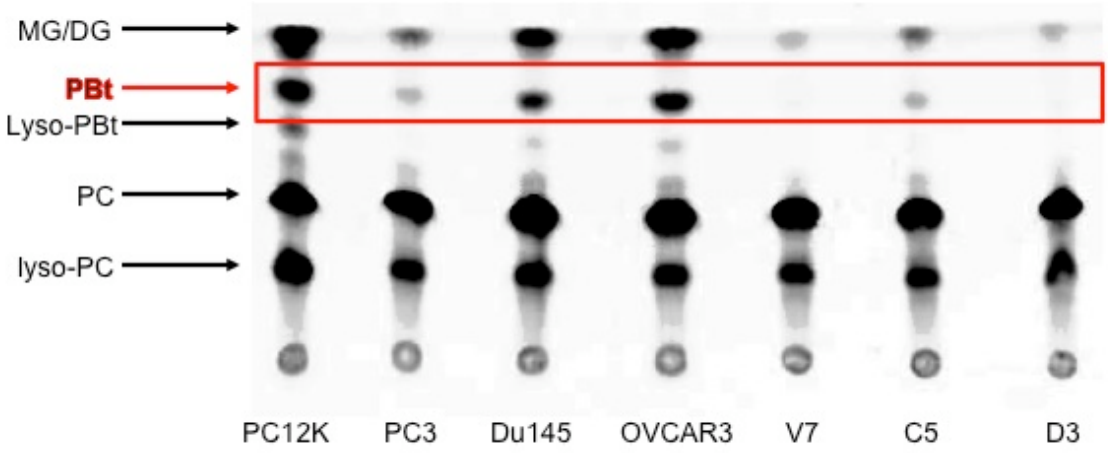

Figure 2-1: Membrane PLD activity in various cancer cell lines. Cells were plated overnight in medium without serum. Cells were collected, sonicated, and membranes were separated. Equal amounts (5 ug) of membranes were incubated with BODIPY-PC, and 0.5% butanol for 60 minutes at 30°. The samples were spotted on TLC plates and developed. Products were visualized using a phosphorimager.

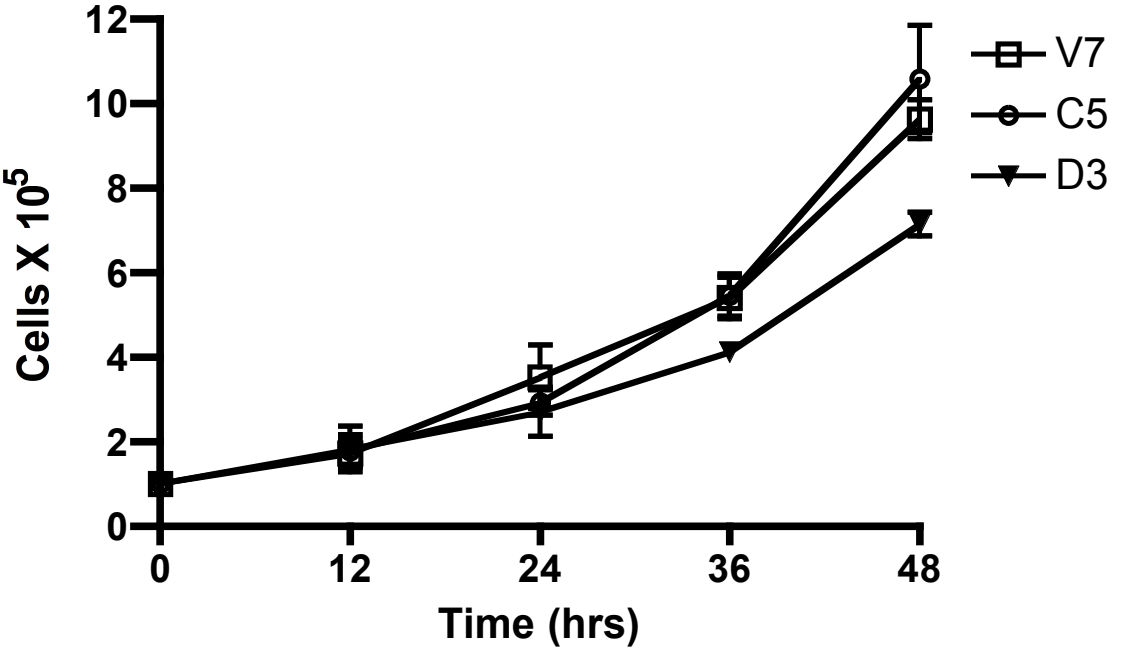

Figure 2-2: Effects of active and inactive PLD2 on EL4 cell proliferation. Equal numbers of cells were plated in 24 well plate in triplicate. Cells were then incubated in serum-containing media. At 12, 24, 36, and 48 hours, cells were collected by scraping and viable cells were counted.

Basal Akt activation in EL4 cells.

We next examined a signaling pathway that might contribute to PLD2-mediated cellular effects. The PI3K/Akt pathway modulates cell proliferation and survival (38). We asked whether PLD2 overexpression would modulate PI3K/Akt pathway in EL cells. We investigated basal Akt activation in V7, C5, and D3 cells. Our results indicate that there is basal Akt phosphorylation in all EL4 cells (Figure 2-3). However, C5 cells expressing active PLD2 exhibit increased Akt activation compared to parental cells and cells expressing inactive PLD2. In this experiment, D3 cells expressing inactive PLD2 show decreased Akt activation compared to V7 cells. However, repeated experiments with quantification showed that such a decrease is not statistically significant. Overall, overexpression of active PLD2 leads to increased Akt activation in EL4 cells.

Effects of serum treatment on EL4 cell migration

PLD2 is known to modulate adhesion, and Akt activation is known to play a role in cellular migration and invasion (1,38). Hence, we examined migration in EL4 cells in response to serum. Our results indicate that V7 and C5 cells migrate at the same rate under basal conditions (Figure 2-4). However, D3 cells demonstrate reduced migration under basal conditions. Further, upon treatment with 10% serum, there is significant increase in migration in V7 and C5 cells when compared to untreated cells. No significant change in migration is observed in D3 cells as compared to untreated cells (Figure 2-4). These results indicate that PLD2 activity plays a role in cellular migration.

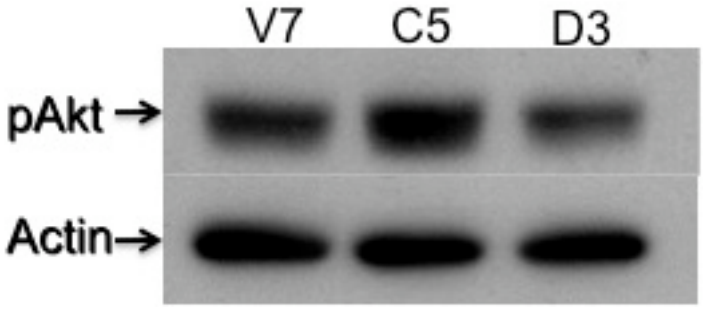

Figure 2-3: Basal Akt activation in EL4 cells. Equal numbers of cells were plated overnight in serum-starved media. Cells were collected by scraping and whole-cell lysates were prepared. Equalized aliquots of protein were loaded and separated on a SDS-PAGE gel. Blots were probed with phospho-Akt and actin antibodies. The anti-phospho Akt antibody detects activated form of Akt.

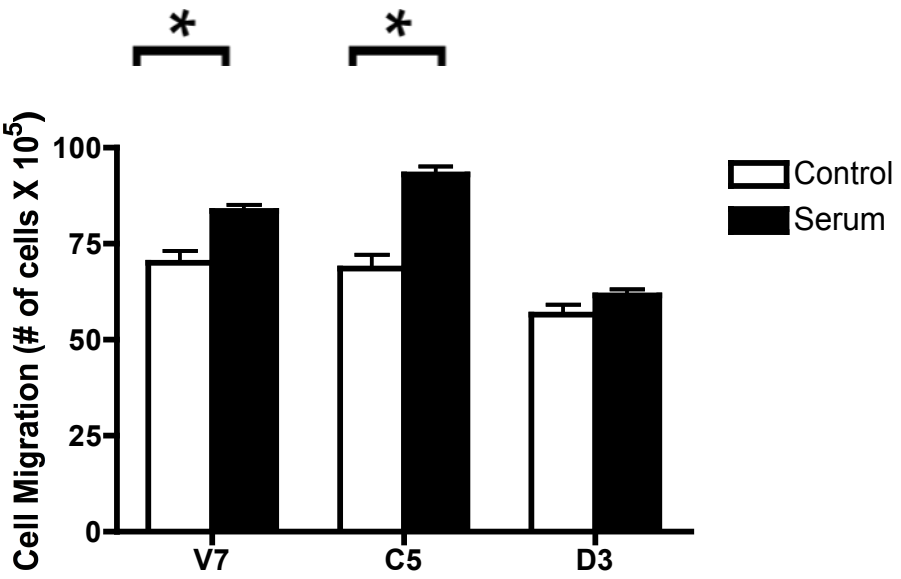

Figure 2-4: Serum-induced EL4 cell migration. Equal numbers of cells from the indicated cell lines were incubated in triplicate in serum-free medium in the upper chamber of the modified Boyden chamber. The bottom chambers contained medium with or without 10% serum. After 24 hours of migration, cells on the bottom the wells were stained and counted. The asterisk indicates values that were significantly different ($p<0.05$).

Effects of serum treatment on EL4 Cell Invasion

Invasion of PLD2-transfected cells was also examined. Modified Boyden chambers coated with Matrigel were used and cells allowed to invade for 24 hours. Our results indicate that, under basal conditions, there is no significant difference in invasion of V7, C5, and D3 cells (Figure 2-5). Upon treatment with 10% serum, there is a significant increase in invasion of both V7 and C5 cells. C5 cells show the highest level of invasion. However, D3 cells do not invade in response to serum. These results indicate that overexpression of catalytically active PLD2 enhances the invasion potential of EL4 cells. In contrast, inactive PLD2 inhibits serum-induced invasion.

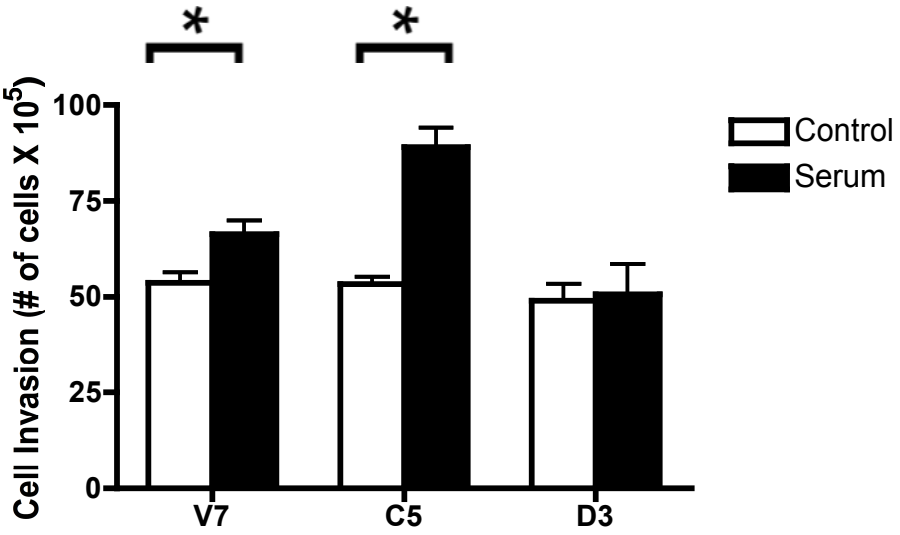

Figure 2-5: Serum-induced EL4 cell invasion. Equal numbers of cells from the indicated cell lines were incubated in triplicate in serum-free medium in the upper chamber of the modified Boyden chamber coated with Matrigel. Bottom chambers contained medium with or without 10% serum. After 24 hours of invasion, cells on the bottom of the inserts were stained and counted. The asterisk indicates values that were significantly different ($p<0.05$).

2.5 DISCUSSION

This study explored the effects overexpression of PLD2 on proliferation, migration, invasion, and signal transduction in EL4 cells. Previous studies have indicated that PLD2 plays a role in proliferation, adhesion, survival, and apoptosis (1). Our lab has generated and characterized multiple EL4 cell lines based on their response to PMA (39). EL4 cells are a good model to study effects of PLD2, as these cells are unusual in that they lack detectable levels of PLD mRNA and protein expression (1). Consequently, it is possible to study the effects of transfected PLD2 without any influence of PLD1.

We began by investigating the PLD2 activity in EL4 cells and comparing to that of other cancer cell lines. Our results indicate that, among the EL4 cell lines, only C5 cells exhibit PLD2 activity (Figure 2-1). PLD2 transfected in EL4 cells was cloned from PC-3 cells, and both cell lines have comparable levels of PLD2 activity (Figure 2-1). This result indicates that we were able to achieve physiologically relevant levels of PLD2 activity in C5 cells. The relatively modest level of PLD2 activity was unexpected, since the transfected PLD2 was driven by CMV promoter. The resulting activity suggests either that the promoter function was compromised or that post-transcriptional events moderate the levels of PLD2 maintained in the cells.

We investigated the effects of overexpressing PLD2 in EL4 cells on cellular proliferation under basal conditions. Our results indicate that there is no significance difference in proliferation of parental V7 cells and cells overexpressing catalytically active PLD2 in the presence of serum (Figure 2-2). In contrast, cells expressing catalytically inactive PLD2 exhibited significantly reduced proliferation when compared to V7 and C5 cells (Figure 2-2). These results indicate that PLD2 activity plays an important role in proliferation of EL4 cells. Our results are comparable to a previous study (40) in which PLD2 overexpression led to increased proliferation of another cell type.

We also investigated potential signaling pathways that may be influenced by overexpression of PLD2. Previous studies have shown that production of PA modulates activity of mTOR and p70S6K, proteins that participate downstream in the PI3K/Akt signaling cascade (34). Further, PLD2 has been known to modulate PIP3 and PIP2 levels (13). Since both of these lipids are known to modulate PI3K activity, we investigated basal Akt activation in EL4 cells. Our results indicate that overexpression of catalytically active PLD2 leads to an increased basal Akt activation compared to parental cells and cells expressing catalytically inactive PLD2 (Figure 2-3). In contrast, overexpression of active PLD2 does not enhance Erk activation (1).

PLD2 and PI3K/Akt pathways modulate many processes including migration and invasion (27,38). We investigated effects of PLD2 on serum-

induced migration in EL4 cells. Our results indicate that there is a significant increase in migration in V7 and C5 cells when compared to untreated cells (Figure 2-4). There is no change in migration of D3 cells in response to serum (Figure 2-4). Although studies implicating a direct role on PLD2 in migration are limited, studies have shown that overexpression of PLD2 in cells with endogenous PLD2 leads to increased cell protrusions, while inactive PLD2 has the opposite effects (31). Similarly, serum-induced invasion is significantly increased in V7 and C5 cells in response to serum, but no change is observed in D3 cells (Figure 2-5).

The results in this study present evidence that PLD2 plays a role in proliferation, migration, and invasion. Overexpression of catalytically active PLD2 leads to significant increase in migration and invasion. However, overexpression of catalytically inactive leads to decreased proliferation, migration, and invasion. The results of the catalytically inactive PLD2 were unexpected, since this construct is usually viewed as a “dominant negative” protein that can counteract the effects of endogenous PLD2. Since EL4 cells do not express detectable PLD2, the prominent effects of the inactive form suggests that it may interfere with signaling events independent of blocking any activity of endogenous PLD2. Thus, the result provides rationale for developing therapeutic agents modeled after inactive PLD2 that can be used to treat various cancers.

CHAPTER III

EFFECTS OF PHOSPHOLIPASE D2 ON EPIDERMAL GROWTH FACTOR RESPONSE IN EL4 LYMPHOMA CELLS

3.1 SUMMARY

Phospholipase D2 (PLD2) generates phosphatidic acid through hydrolysis of phosphatidylcholine. PLD2 is postulated to play a role in enhancing tumorigenesis. The epidermal growth factor receptor (EGFR) has been shown to interact with PLD2. Murine lymphoma EL4 cells lacking endogenous PLD2 expression present a unique model to elucidate the role of PLD2 in signal transduction. In the current study, we investigated effects of PLD2 on EGF-mediated responses. RT-PCR and immunoblotting showed that both parental EL4 cells and PLD2 transfectants express endogenous EGFR mRNA and protein. Levels of EGFR mRNA and protein are increased in cells expressing active PLD2, as compared to parental cells or cells expressing inactive PLD2. EGF stimulates proliferation of EL4 cells transfected with active PLD2, but not parental cells or cells transfected with inactive PLD2. PLD2 siRNA reduces EGF-mediated proliferation in C5 cells to control levels. EGF-mediated proliferation in cells expressing active PLD2 is modulated by the PI3K/Akt pathway. EGF-induced invasion through a synthetic extracellular matrix is enhanced in cells expressing active PLD2, as compared to parental cells or cells expressing inactive PLD2. Taken together, the data suggest that PLD2 acts in concert with EGFR to enhance mitogenesis and invasion in lymphoma cells.

3.2 INTRODUCTION

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid (PA), a lipid second messenger (1,7). There are two isoforms of human PLD, PLD1 and PLD2 (7). PLD2 expression has been detected in many organisms and in most mammalian tissues (8). PLD2 activity is regulated by lipids, kinases, and small GTP binding proteins (7,10). Following agonist stimulation, PLD2 translocates from general plasma membrane to membrane ruffles or cytosol (7,10). PLD2-generated PA modulates signaling either by binding to proteins directly, or through its metabolites (18). PA interacting proteins include sphingosine 1-phosphate kinase, Raf-1, cAMP-specific phosphodiesterase, mammalian target of rapamycin (mTOR), and p70S6K (18,20,34).

PLD2 regulates various processes such as proliferation, adhesion, survival, apoptosis and tumor transformation (7). Treatment with growth factors such as FGF, EGF, and insulin leads to an increase in PLD2 activity (28). Increased PLD2 expression contributes to the transformation of fibroblasts overexpressing tyrosine kinase, such as c-Src or epidermal growth factor receptor (14,31). Elevated PLD2 activity has been found in various cancers including breast, colon, and kidney (27,29,30). MDA-MB-231 breast cancer cells with high levels of PLD2 activity invade Matrigel in *in vitro* experiments, however, breast cancer cells with very low PLD2 activity, such as MCF-7, are less invasive

(27). Elevated PLD2 activity has been implicated in increased protease secretion, a hallmark of invasive cancer cells. Specifically, overexpression of PLD2 in mouse fibroblasts caused an increase in MMP-2 secretion; a further increase in MMP-2 secretion was observed upon stimulation with a PLD2 agonist (33).

Epidermal growth factor receptor (EGFR) is a transmembrane protein tyrosine kinase that belongs to the ErbB family of receptor protein kinases (22). EGFR-mediated signaling is dysregulated in many epithelial cancers, promoting tumor growth and progression (25,26). EGFR has been implicated in the regulation of PLD2 activity (14). For example, PLD2 is constitutively associated with EGFR in HEK 293 cells (14,26). Stimulation of EGFR with EGF leads to stimulation of PLD2 activity (26). Further, stimulation with EGF induces tyrosine phosphorylation of PLD2 (26); the functional significance of this phosphorylation is still under investigation.

Although existing data suggest that PLD2 plays a role in many stages of tumorigenesis, including transformation, progression, and invasion, the exact molecular mechanisms are not well understood (7,14). Previous studies from our lab have shown that overexpression of catalytically active PLD2 in EL4 lymphoma cells lacking PLD2 results in increased spreading and elongation of transfected cells, while inactive PLD2 produces the opposite effect (1). Cells expressing active PLD2 form more tumors in syngeneic mice, as compared to parental cells or cells expressing inactive PLD2 (1). In the current study, we

utilized EL4 cells expressing PLD2 to elucidate the mechanisms of PLD2 modulated mitogenesis and invasion. Our results show that EL4 cells expressing catalytically active PLD2 have increased expression of EGFR and exhibit an enhanced response to EGF.

3.3 MATERIALS AND METHODS

Materials: EGF was purchased from Sigma (St. Louis, MO). PD158780 and LY294002 were purchased from Calbiochem (San Diego, CA).

Cell culture: Clonal EL4 and OVCAR3 cells were maintained in RPMI-1640 (GIBCO BRL, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) as previously described (1). All cells were grown in 5% CO2/95% air at 37°C on standard tissue culture dishes. Stably transfected EL4 cells were maintained in the presence of 0.25 mg/ml G418 (Calbiochem), except when cells were used for experiments, when they were maintained in medium without G418 for 24 hours prior to experiments.

Immunoblotting: Following treatment, cells were rinsed with 1X PBS, and harvested by scraping. Cells were collected by centrifugation at 1,200Xg. Whole cell lysates were prepared by lysing cells in lysis buffer containing 20 mM Tris (pH 7.4), 1% Trition-X-100, 150 mM NaCl, 1 mM EGTA, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl

fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Coomassie blue reagent (Pierce Chemical, Rockford, IL) was used to determine protein concentrations. Equal amounts of protein (100 μg) were loaded in each lane of a 10% polyacrylamide gel, separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were incubated with primary antibodies and developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Anti-EGFR, anti-phosho-Akt, and anti-Actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Blots were quantified using NIH Image software.

Semi-quantitative PCR: Total RNA was extracted using the RNeasy Mini kit from Qiagen. Genomic DNA was removed from total RNA by treating with DNase 1 from Invitrogen. Concentration and purity of total RNA were determined by absorbance at 260 nm and by $OD_{260/280}$ ratio, respectively. Equal amounts (2 μg) of total RNA were used for cDNA synthesis primed by an EGFR anti-sense primer (41) using the Thermoscript kit according to the manufacturer's instructions (Invitrogen). PCR was performed using equal amounts of cDNA and EGFR primers in a MyiQ cycler (BioRad) using a PCR kit (Invitrogen) according to the manufacturer's instructions. The linear ranges of PCR amplification for EGFR and 18s RNA was determined as described previously (42).

PLD2 transfection: OVCAR3 cells were transiently transfected with a human HA-PLD2 construct (1) using Lipofectamine 2000 (Invitrogen) reagent in OPTI-MEM

media for 48 hours according to the manufacturer's instructions. Following transfection, cells were grown in serum-containing media for 48 hours.

Cell proliferation: Equal numbers ($2X10^5$) of cells from each cell line were grown in 6-well plates in serum-containing media for 24 hours. Prior to treatment of triplicate wells, cells were serum starved overnight. Following treatment for 24 or 48 hours, cells were collected by scraping, and viable cells were counted by hemacytometer using Trypan blue to stain non-viable cells.

Membrane PLD2 activity: PLD2 activity was determined as previously described (1). Briefly, cells were grown overnight in serum-free media and collected by scraping following treatment. Cells were lysed by sonication and membranes were separated by centrifugation at 100,000xg in an ultracentrifuge. Membranes (5µg protein) were incubated with BODIPY-phosphatidylcholine (B-PC), in the presence of 5% butanol, for 60 minutes at 30°. The samples were spotted on plastic-backed silica gel TLC plates and developed using methanol/chloroform/water/acetic acid (45:45:10:2 v/v). Products were imaged using a phosphorimager.

Cell invasion: *In vitro* cell invasion was measured using a modified Boyden chamber method (BD Biosciences, San Jose, CA). To the upper wells of the chamber, cells overexpressing PLD2 and parental cells were added at a concentration of 100,000 cells in serum-free media. To the lower wells, media

with 10% FBS was added. Invasion was measured as the ability of cells to pass through Matrigel (BD Biosciences), an artificial extracellular matrix that was used to coat the upper well. The membranes were fixed and stained with the Diff-Quik® dye kit. Cells that invaded into the lower wells were counted using a microscope.

PLD2 siRNA: Equal number of C5 cells were plated in a six well plate and grown overnight. Cells were grown for 48 hours in the presence of 30 nM PLD2 siRNA (Ambion) transfected using *Trans*IT reagent (Mirus). Equal numbers of cells were then plated for proliferation assay. Cells were serum starved 24 hours prior to EGF treatment. Serum-starved cells were treated in triplicate with and without 10 nM EGF for 24 hours. Following treatment, cells were collected by scraping and viable cells were counted by hemacytometer using Trypan blue to stain non-viable cells. Data are expressed as percent of control number of cells, mean ± SD.

Statistical analysis: All experiments were repeated; representative results from three independent experiments are shown. For quantified data, values are expressed as mean ± S. D. of values obtained. Statistical significance was assessed by Student's t-test, using InStat software (GraphPad).

3.4 RESULTS

Expression of EGFR in EL4 Lymphoma Cells

Although PLD2 has been shown to interact with the EGFR (14,26), the expression of EGFR in lymphomas or lymphoma cells has not been reported previously. Therefore, we investigated whether EGFR is expressed in EL4 cells. Equal amounts of protein lysates from V7, C5 (V7 + catalytically active PLD2), and D3 (V7 + catalytically inactive PLD2) cells were subjected to immunoblotting with an anti-EGFR antibody. The results show that all three cell lines express EGFR protein (Figure 3-1). Basal levels of EGFR protein are increased in cells expressing active PLD2 (C5) as compared to parental V7 cells and cells expressing inactive PLD2 (D3) (Figure 3-1). In contrast, EGFR protein levels are decreased in D3 cells expressing inactive PLD2 (Figure 3-1).

We next examined EGFR mRNA expression in EL4 cells. In order to determine the linear range of amplification, a PCR reaction was performed using C5 cDNA and EGFR primers. The PCR reaction was stopped at cycles 25, 28, 31, 34, 37, and 40. PCR products were separated on an agarose gel and visualized with ethidium bromide (Figure 3-2). Band intensity was determined using densitometry. The results showed that the cycle 34 resulted in PCR products that were within the linear range of amplification for EGFR primers.

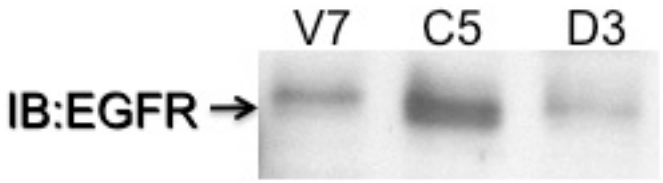

Figure 3-1: EGFR protein expression in EL4 Cells. Equal number of cells were plated and grown overnight without G418. Cells were collected by scraping and lysed. Equal amounts of protein lysate from all three cell lines were separated using SDS-PAGE and probed with anti-EGFR antibody.

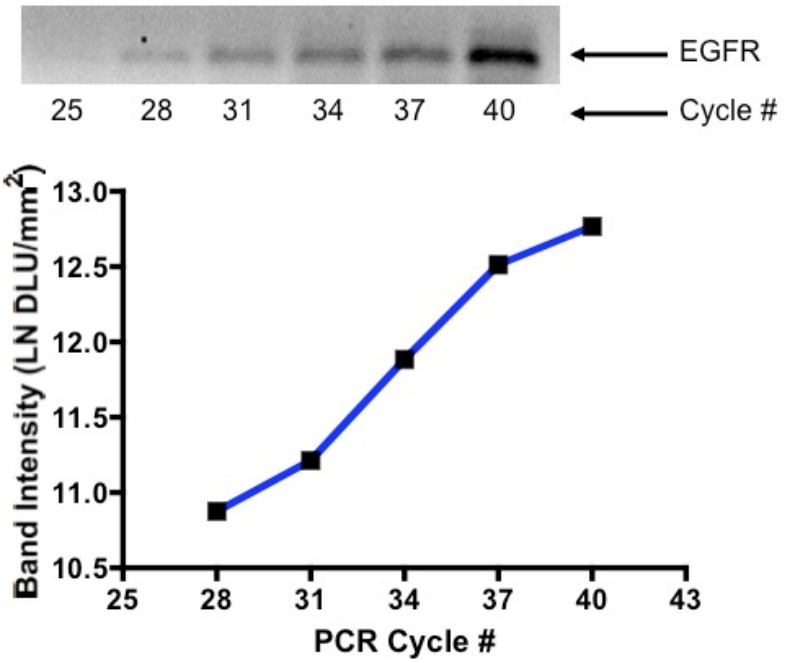

Figure 3-2: Linear range of amplification for EGFR primers in C5 cells. Total RNA was extracted from C5 cells. Equal amounts of total RNA were used in a PCR reaction containing EGFR primers. PCR products were separated on an agarose gel and visualized with ethidium bromide. Quantified data are presented in the graph.

Forward and reverse primers specific for murine EGFR were used in a polymerase chain reaction (PCR) to amplify cDNA from V7, C5, and D3 EL4 cells at cycle number 34 in the linear range for amplification of EGFR. The results show that all cell lines express EGFR mRNA (Figure 3-3). Basal levels of EGFR mRNA are increased in cells expressing active PLD2 (C5) as compared to parental V7 cells (Figure 3-3). In contrast, EGFR mRNA levels are decreased in D3 cells expressing inactive PLD2 (Figure 3-3).

In order to ensure that the PCR product amplified using EGFR primers is not another member of the EGFR family, the product was excised from the gel, cloned, and sequenced at the Genomics Core facility at Washington State University. The sequencing data was used in a nucleotide BLAST search (data not shown). The excised PCR product was found to be 99% identical to both murine EGFR transcript variants 1 and 2 mRNA (43). The results show that indeed the amplified PCR product is EGFR and not another member of the EGFR family. Hence, overexpression of PLD2 leads to increased levels of basal EGFR.

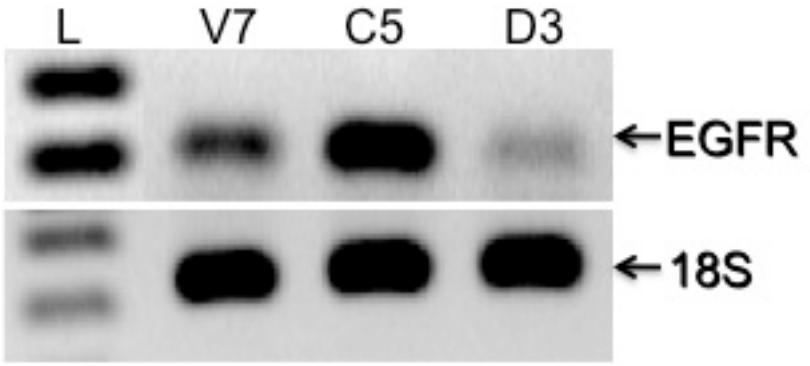

Figure 3-3: EGFR mRNA expression in EL4 cells. Total RNA was extracted from all three cell lines. Equal amounts of total RNA were used in a PCR reaction for 34 cycles containing EGFR and 18S primers. PCR products were separated on an agarose gel and visualized with ethidium bromide.

Overexpression of PLD2 in OVCAR3 Ovarian Cancer Cells

Our results indicate that overexpression of PLD2 in EL4 cells, which lack endogenous PLD2, leads to an increase in basal levels of EGFR protein and mRNA. To test whether this response occurs in other cell types, we transiently overexpressed PLD2 in OVCAR3 cells. This human ovarian cancer cell line expresses endogenous PLD2 (Figure 2-1) and previous work in the lab had shown that PLD2 could be over-expressed in these cells by transient transfection (44). Following transfection, whole-cell lysates were blotted for EGFR protein. The results show that OVCAR3 cells overexpressing HA-tagged PLD2 have higher basal levels of EGFR protein as compared to control cells (Figure 3-4). Overall, results from Figure 3-1 and 3-4 indicate that overexpression of PLD2 results in increased expression of EGFR in a cell-independent manner.

Effects of EGF treatment on EL4 Cell Proliferation

Since overexpression of PLD2 results in increased expression of basal EGFR, the next question asked was whether this increase was physiologically significant. To address the function of EGFR in EL4 cells, we treated V7, C5, and D3 cells with EGF, and assessed EL4 cell proliferation. Cells were serum starved for 24 hours, and then treated with and without 10 nM EGF for 24 hours. Our results indicate that EGF induces a significant increase in proliferation of cells overexpressing catalytically active PLD2 (C5) (Figure 3-5). However, there was no significant increase in cell proliferation in response to EGF in parental cells (V7), or in cells expressing catalytically inactive PLD2 (Figure 3-5). Overall, these

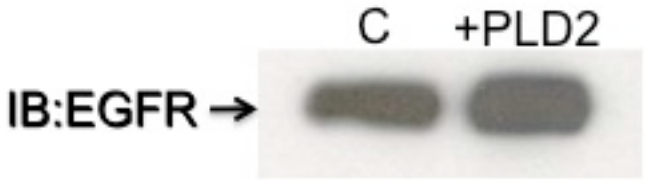

Figure 3-4: Effects of PLD2 overexpression on EGFR levels in OVCAR3 cells. Equal number of cells were grown overnight and then transfected with HA-PLD2 construct using Lipofectamine reagent. Following transfection, cells were grown in serum-containing media for 48 hours and collected. Equal amounts of whole-cell lysates were separated on SDS-PAGE, transferred to PVDF membrane, and blotted with anti-EGFR antibody.

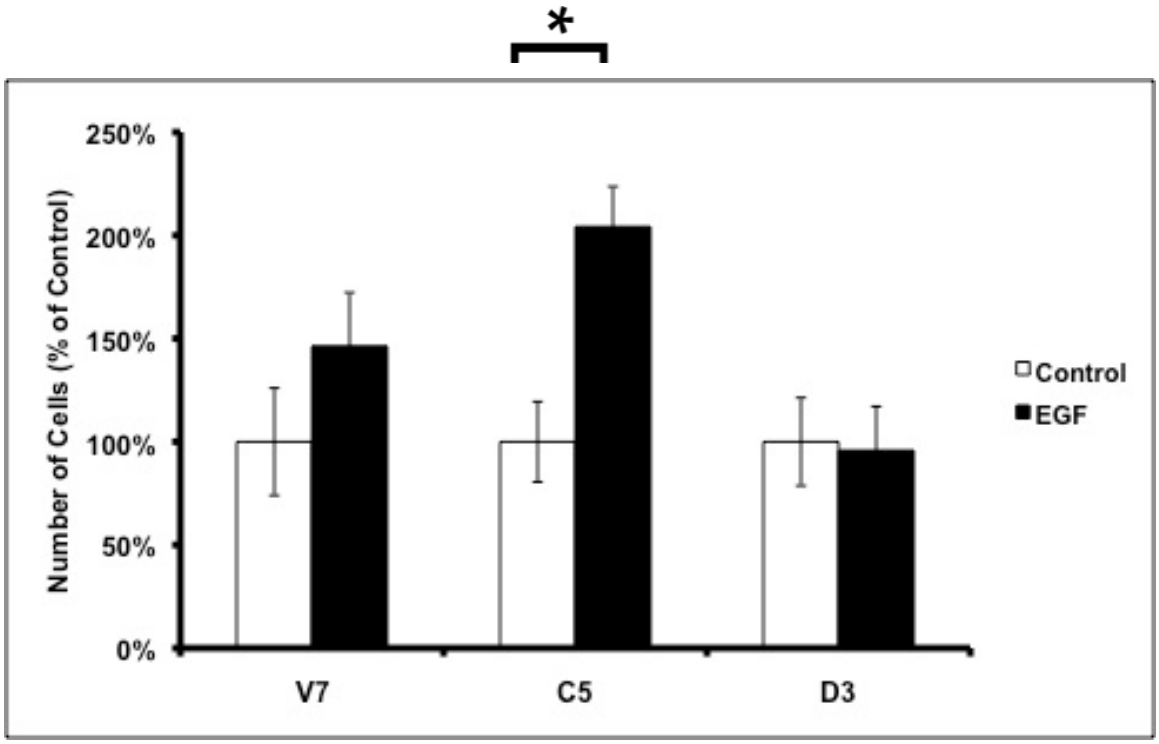

Figure 3-5: EGF-induced EL4 Cell Proliferation. Equal number of cells were plated in triplicate wells overnight in serum-free media. Cells were treated with or without 10 ηM EGF for 24 hours. Cells were collected and counted. Each data point represents the mean ± S.D. of values from triplicate wells. The asterisk indicates values that were significantly different ($p<0.05$).

results indicate that overexpression of PLD2 leads to an increase in cell proliferation upon EGF stimulation. These results also indicate that the EGFR is functional in EL4 cells.

Effects of PLD2 siRNA on EGF-induced Proliferation in C5 cells.

Our results indicated that overexpression of PLD2 leads to increased expression of EGFR and enhanced proliferation in response to EGF. We next investigated whether a reduction in PLD2 expression in C5 cells would result in reduced proliferation in response to EGF. We incubated equal numbers of serum-starved C5 cells with or without PLD2 siRNA for 48 hours. PLD2 activity was tested *in vitro* and the results suggest that cells incubated with PLD2 siRNA have reduced PLD2 activity as expected (Figure 3-6). However, this reduction in PLD2 activity was observed once, subsequent experiments failed to repeat this result.

Equal numbers of siRNA treated cells (same experiment as shown in Figure 3-6) were used for a proliferation assay in response to EGF. Cells were plated overnight in the absence of serum and then treated with or without EGF for 24 hours. Our results indicate that control and PLD2 siRNA treated cells proliferate at a similar rate in the absence of EGF treatment (Figure 3-7). Treatment with EGF resulted in significantly increased proliferation of control cells. However, cells treated with PLD2 siRNA did not proliferate in response to EGF. These preliminary results suggest that reduced levels of PLD2 results in

Figure 3-6: PLD2 activity in C5 cells with or without PLD2 siRNA. Equal number of C5 cells were plated and grown overnight without serum. Cells were incubated with or without 30 nM of PLD2 siRNA for 48 hours. Cells were collected and membrane PLD2 activity was determined.

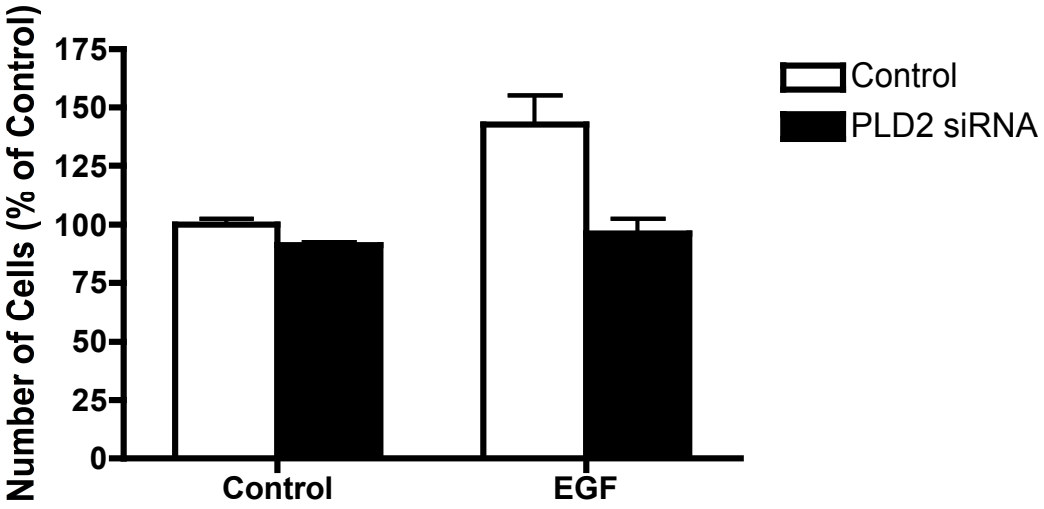

Figure 3-7: EGF-induced proliferation of C5 cells treated with PLD2 siRNA. Equal number of cells were plated and serum-starved overnight. Cells were incubated with or without 30 nM of PLD2 siRNA for 48 hours. Equal numbers of cells were again plated overnight in the absence of serum and treated with or without 10 nM EGF for 24 hours. Cells were collected and viable cells were counted.

decreased response to EGF-mediated proliferation in C5 cells. However, since the effects of the siRNA treatment were not consistent, we turned to other approaches to further examine the role of PLD2.

Effects of inhibitors on EGF-induced C5 Cell Proliferation

EGFR activation leads to the recruitment and activation of downstream targets that facilitate cell growth (22). In particular, EGF activates the PI3K/Akt and Ras/Raf/Erk pathways, leading to cell proliferation (22). In order to determine if these pathways are involved in the EGF-induced increase in proliferation in C5 cells, we treated C5 cells with or without various signal transduction inhibitors in the absence or presence of 10 nM EGF for 24 hours (Figure 3-8). The results indicate that treatment with an EGFR kinase inhibitor, PD15878, reduces EGF-induced C5 proliferation to control levels (Figure 3-8). Treatment with a PI3K inhibitor, LY290042, reduces both basal and EGF-induced C5 proliferation to below control levels (Figure 3-8). Treatment with a MEK inhibitor, U0126, also reduces cell proliferation below control levels; treatment with EGF is able to rescue this decrease in proliferation (Figure 3-8). Overall, results from Figure 3-8 indicate that both the PI3K/Akt and Erk pathways are necessary for EGF-induced proliferation in C5 cells. In subsequent studies we pursued only the PI3K/Akt pathway in PLD2-mediated effects due to its known role in both proliferation and migration.

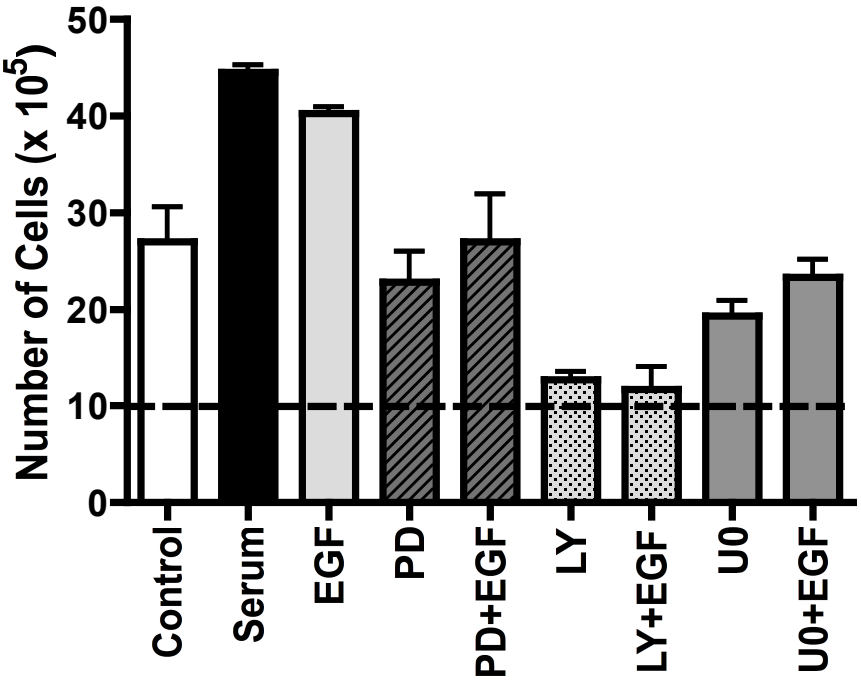

Figure 3-8: Effects of various inhibitors on EGF-induced C5 cell proliferation. Equal numbers of cells were plated overnight and serum-starved. Cells were treated 10% serum (positive control), 10 nM EGF, 1 pM PD15878, 10 nM EGF plus 1 pM 15878, 25 uM LY290042, 10 nM EGF plus 25 uM LY290042, 10 uM U0126, and 10 nM EGF plus 10 uM U0126. Cells were collected and viable cells were counted.

Time Course of EGF-induced Akt activation in EL4 cells

In order to further investigate the role of the PI3K/Akt pathway in EGF response, we examined the time course for Akt activation upon EGF treatment in all three EL4 cell lines (Figure 3-9). Cells were treated with 10 nM EGF from 0 to 60 minutes. Whole cell lysates were immunoblotted for phospho-Akt. The results show that C5 cells with catalytically active PLD2 have significantly higher basal phospho-Akt as compared to parental V7 cells and cells expressing catalytically inactive PLD2 (D3), confirming our previous findings (1). This experiment shows that EGF stimulation leads to a time-dependent increase in phospho-Akt levels only in V7 and C5 cells, and not in D3 cells. The magnitude of EGF response is enhanced in C5 cells as compared to V7 cells. Overall, cells expressing catalytically active PLD2 have increased levels of basal phospho-Akt. Expression of catalytically inactive PLD2 suppresses EGF-mediated Akt activation.

Effects of EGF-induced Cell Invasion in EL4 Cells

Previous studies from our lab have shown that overexpression of catalytically active PLD2 results in increased spreading and elongation of transfected cells (1). These are morphological changes that are characteristic of invasive cells. We therefore investigated the effects of EGF on invasion in EL4 cells expressing active or inactive PLD2. We used a modified Boyden chamber assay to assess EL4 cell invasion. Our results indicate that cells overexpressing catalytic active PLD2 display significantly increased cell invasion in response to

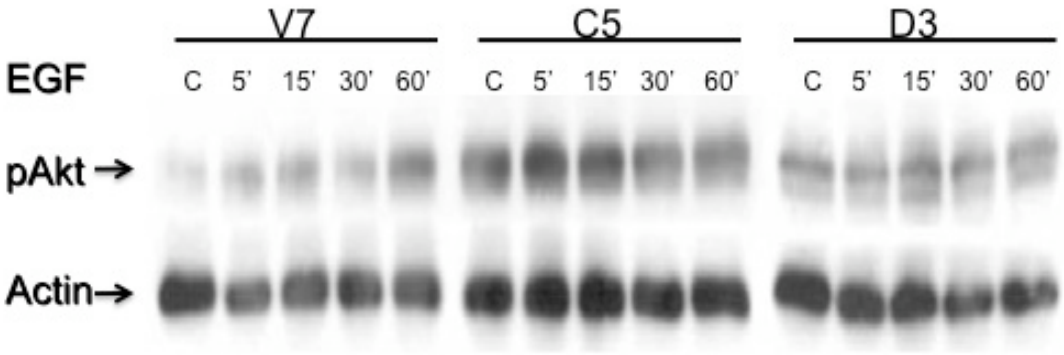

Figure 3-9: Time course of EGF-induced Akt activation in EL4 Cells. Equal numbers of cells were plated overnight and serum starved. Cells were treated with 10 nM EGF for the indicated times and collected. Whole-cell lysates were prepared and separated by SDS-PAGE. Blots were probed with anti-EGFR and anti-actin antibodies .

serum and EGF compared to control (Figures 3-10, 3-11). Parental V7 cells also display an increase in invasion in response to serum and EGF (Figure 3-10, 3-11). In contrast, no invasion is observed in D3 cells expressing catalytically inactive PLD2 in response to serum and EGF when compared to control (Figures 3-10, 3-11).

3.5 DISCUSSION

This study presents a new relationship between PLD2 and EGFR. Previous studies have shown that EGFR can regulate PLD2 activity (14,26). Studies have also shown that PLD2 and EGFR co-localize, and that EGFR activation leads to an increase in PLD2 activity (14,26). We show for the first time that overexpression of PLD2 leads to an increase in EGFR expression. This is also the first study that we are aware of that demonstrates EGFR expression in a lymphoma cell line. Further, we also provide data suggesting that expression of active PLD2 leads to enhanced EGF response in EL4 cells.

Our lab has generated and characterized multiple EL4 cell lines based on their response to PMA (39). V7 cells are PMA-resistant, and do not activate Erks efficiently in response to PMA, unlike PMA sensitive cells (39). The difference in PMA sensitivity is largely due to a lack of RasGRP expression in PMA-resistant cells (45). EL4 cells are difficult to transfect. In order to validate our initial finding that PLD2 increased basal EGFR expression, we transiently overexpressed

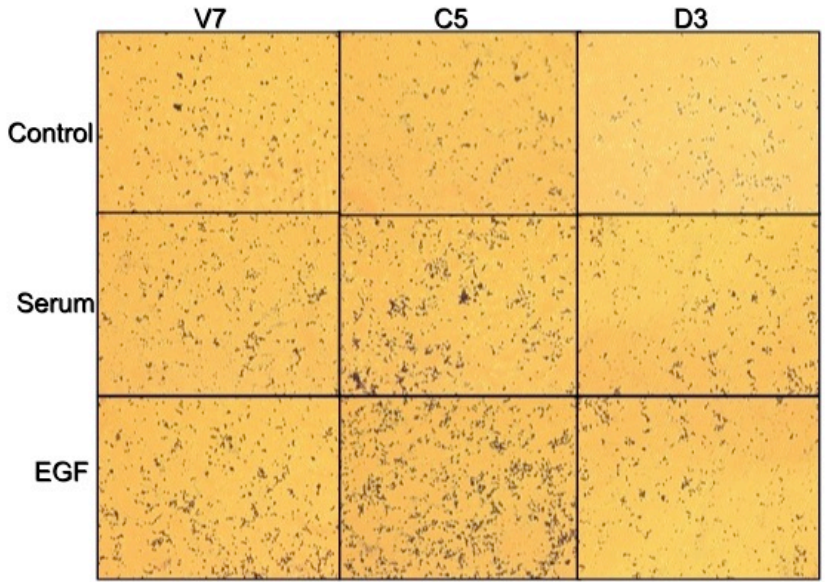

Figure 3-10: EGF-induced invasion of EL4 cells. Equal numbers of cells were added to the upper chamber of trans-well inserts coated with Matrigel. To the bottom chamber of the inserts, either 10% serum was added as positive control or 10 nM EGF was added to serum-free media. Following 24 hours of treatment, invaded cells were fixed, stained, and photographed.

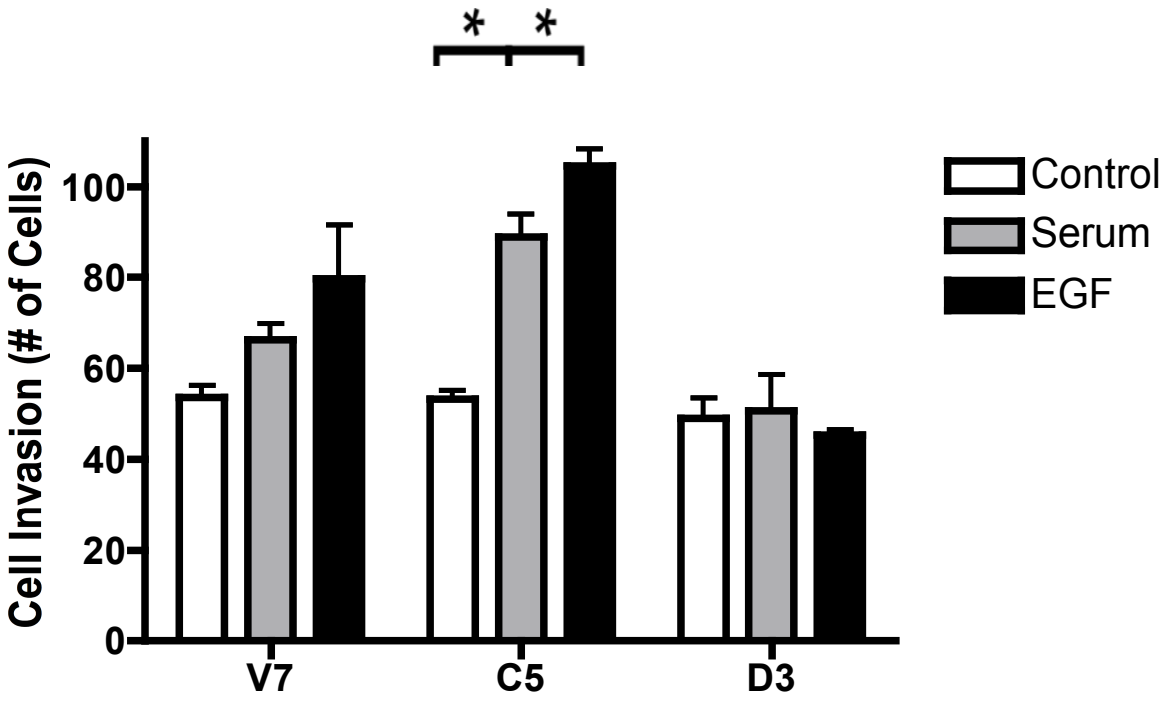

Figure 3-11: EGF-induced invasion of EL4 cells. Equal numbers of cells were added to the upper chamber of trans-well inserts coated with Matrigel. To the bottom chamber of the inserts, either 10% serum was added as positive control, or 10 nM EGF was added to serum-free media. Following 24 hours of treatment, invaded cells were fixed, stained, and counted. Each data point represents the mean ± S.D. of values from triplicate wells. The asterisk indicates values that were significantly different ($p<0.05$).

PLD2 in OVCAR3 cells (Figure 3-4). OVCAR3 cells are easier to transfect, and have been used in our laboratory previously (44). The results indicate that a PLD2-induced increase in EGFR expression is not exclusive to EL4 cells. Although we did not investigate the mechanism by which PLD2 induces an increase in EGFR expression, our microarray data (Chapter 4) indicate that PLD2 leads to downregulation of Suppressor of Cytokine Signaling (SOCS). SOCS4 has been known to increase EGFR degradation (46). Alternatively, or perhaps in a complimentary manner, the corresponding increase in EGFR mRNA in PLD2-transfected cells suggests that PLD2 alters signaling events in a manner that upregulates EGFR mRNA expression.

We verified the functionality of the EGFR in EL4 cells via cell proliferation assays. Our results indicate that EL4 C5 cells proliferate in response to EGF (Figure 3-5). These results demonstrate that indeed the EGFR in EL4 cells is functional and responds to EGF treatment. Further, our results indicated that EL4 cells expressing catalytically active PLD2 have a significant increase in EGF-induced proliferation compared to parental cells and cells expressing catalytically inactive PLD2 (Figure 3-5). This result maybe due in part to the fact that C5 cells have approximately two-fold higher expression of EGFR compared to V7 and D3 cells, providing increased EGFR to be activated by EGF. However, it is also likely that the presence of PLD2 enhances EGF response. This latter conclusion is supported by the negative effects of catalytically inactive PLD2 on EGF response (Figures 3-9, 3-10, and 3-11).

The PLD2-induced increase in EGFR expression and EGF response were further examined by transfecting C5 cells with PLD2 siRNA and then investigating EGF-induced proliferation. Our results suggest that transfection with PLD2 siRNA for 48 hours can result in decreased PLD2 activity in C5 cells (Figure 3-6). Treatment of PLD2 siRNA treated cells with EGF results in decreased proliferation when compared to control cells (Figure 3-7). These preliminary results suggest that PLD2 siRNA reverses the enhanced EGF response resulting from overexpression of PLD2. Further experiments are needed to validate this conclusion.

EGF-induced proliferation involves activation of downstream pathways such as the PI3K/Akt and Ras/Raf/Erk pathways (22). We investigated the role of these pathways in EGF-induced proliferation of EL4 cells. Our results indicate that the PI3K/Akt pathway is critical for EGF-induced EL4 cell proliferation (Figure 3-8). Our signal transduction results (Figure 3-9) further support the fact that the PI3K/Akt pathway plays an important role in C5 cells in response to EGF stimulation. Based on Figures 3-8 and 3-9, we conclude that EGF-induced proliferation in C5 cells is mediated through the PI3K/Akt pathway. Other groups have also demonstrated that PLD2 can modulate activation of the PI3K/Akt pathway (47); hence our data are consistent with these previous results. However, the prominent role of PLD2 in EGF-mediated Akt activation is novel to the current study.

Our study establishes that EGF induces invasion in EL4 cells, and that cells expressing catalytically active PLD2 demonstrate the greatest increase in cell invasion upon EGF stimulation (Figure 3-10 and 3-11). These results support results from our laboratory and others demonstrating that PLD2 modulates various cellular events in a manner that can promote tumor progression (1,7). In conclusion, our study presents a new role of PLD2 with respect to EGFR, a target of various anti-cancer drugs. Specifically, PLD2 enhances EGFR expression and EGF responses in a manner that leads to increased mitogenesis and invasion.

CHAPTER IV

EFFECTS OF PHOSPHOLIPASE D2 ON GENE EXPRESSION IN EL4 LYMPHOMA CELLS

4.1 SUMMARY

Phospholipase D2 (PLD2) generates phosphatidic acid through hydrolysis of phosphatidylcholine. PLD2 is postulated to play a role in enhancing tumorigenesis. Murine lymphoma EL4 cells lacking endogenous PLD2 expression present a unique model to elucidate the role of PLD2 in signal transduction. In the current study, we used parental (V7) and cells stably transfected with catalytically active PLD2 (C5) in a microarray experiment to elucidate genes modulated by PLD2. The analysis identified 102 genes differentially expressed due to overexpression of PLD2. The differential expression ranged from 2 to 16.5 fold. Based on existing literature, SOCS2 was selected as a gene of particular interest that is decreased in response to PLD2. Differential expression of SOCS2 was validated at the protein level, showing that overexpression of PLD2 leads to down-regulation of SOCS2. Other interesting genes were also identified for future study. Overall, this study contributes to a broader understanding of the cellular effects modulated by PLD2.

4.2 INTRODUCTION

Phospholipase D2 (PLD2) catalyzes the hydrolysis of phosphatidylcholine (PC) to produce phosphatidic acid (PA), a lipid second messenger (1,7). PLD2 expression has been detected in many organisms and in most mammalian tissues (8). PLD2 activity is regulated by lipids, kinases, and small GTP binding proteins (7,10). Following agonist stimulation, PLD2 translocates from general plasma membrane to membrane ruffles or cytosol (7,10). PLD2-generated PA modulates signaling either by binding to proteins directly, or through its metabolites (18). PA interacting proteins identified thus far include sphingosine 1-phosphate kinase, Raf-1, cAMP-specific phosphodiesterase, mammalian target of rapamycin (mTOR), and p70S6K (18,20,34).

PLD2 regulates various processes such as proliferation, adhesion, survival, apoptosis and tumor transformation (7). Treatment with growth factors such as FGF, EGF, and insulin leads to an increase in PLD2 activity (28). Elevated PLD2 activity has been found in various cancers including breast, colon, and kidney (27,29,30). PLD2 appears to play a critical role in many cellular processes such as cell growth, cell survival, cell proliferation, apoptosis, cell migration, and adhesion (7). Dysregulation of many of these processes is critical for progression of normal cells to cells with a malignant phenotype, however, the exact molecular mechanisms are not well understood (14).

Previous studies from our lab have demonstrated that overexpression of catalytically active PLD2 in cells lacking PLD2 results in increased spreading and elongation of transfected cells, while inactive PLD2 produces the opposite effect (1). Cells expressing active PLD2 form more tumors in syngeneic mice, as compared to parental cells or cells expressing inactive PLD2 (1). However, the mechanism of PLD2-induced mitogenesis, migration, and invasion are not well understood.

In the current study, EL4 cells overexpressing catalytically active expressing PLD2 were used in a microarray experiment to further elucidate mechanisms of PLD2-modulated mitogenesis, migration, and invasion. Our results show overexpression of PLD2 leads to differential expression of 102 genes. The potential roles of these genes in PLD2-mediated signaling were considered.

4.3 MATERIALS AND METHODS

Cell culture: Clonal EL4 cells were maintained in RPMI-1640 (GIBCO BRL, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) as previously described (1). All cells were grown in 5% CO2/95% air at 37°C on standard tissue culture dishes, except for WT2, which were grown on suspension culture plastic (Corning). Stably transfected EL4 cells were

maintained in the presence of 0.25 mg/ml G418 (Calbiochem), except that all cells were maintained in medium without G418 for 24 hours prior to experiments.

RNA preparation: Total RNA was extracted from serum-starved cells using the RNeasy Mini kit from Qiagen. Genomic DNA was removed from total RNA by treating with DNase 1 from Invitrogen. Concentration and purity of total RNA were determined by absorbance at 260 nm and by $A_{260/280}$ ratio, respectively. RNA integrity was determined separating 2 ug of total RNA on a denaturing ethidium bromide containing agarose gel and visualized using a Gel Doc imaging system (BioRad).

Microarray: Differential gene expression was determined using an Affymetrix (Santa Clara, CA) mouse 430 2.0 array containing 39,000 mouse transcripts, at the WSU genomics core facility by Derek Pouchnik. Two duplicate arrays were used for each cell line. High quality total RNA (5 ug) from each cell line was used to synthesize cDNA that was biotinylated, hybridized to the microarray, and scanned. Results were normalized, log transformed, and analyzed using Genesifter data analysis system. Pairwise analysis was conducted with minimum 2-fold threshold. Statistical significance was determined using t-test with Benjamini and Hochberg correction.

Immunoblotting: Following treatment, cells were rinsed with 1X PBS, and then harvested by scraping. Cells were collected by centrifugation at 1,200Xg. Whole

cell lysates were prepared by lysing cells in lysis buffer containing 20 mM Tris (pH 7.4), 1% Trition-X-100, 150 mM NaCl, 1 mM EGTA, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Coomassie blue reagent (Pierce Chemical, Rockford, IL) was used to determine protein concentrations. Equal amounts of protein (100 μg) were loaded in each lane of a 10% polyacrylamide gel, separated by SDS-PAGE, and transferred to PVDF membranes. Membranes were incubated with primary antibodies and developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Anti-SOCS2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

4.4 RESULTS

Integrity of RNA used for microarray

Total RNA was extracted from each cell line and treated with DNase to remove genomic DNA. Equal amounts of RNA from all cell lines were separated on a denaturing agarose gel to establish RNA integrity. Denaturing agarose gel separates 28s and 18s rRNA; the RNA is considered not degraded if the band intensity of 28s is two-fold that of 18s rRNA. Our result indicates that RNA from all three cell lines was of good quality, as the 28s rRNA band was two-fold that of 18s rRNA for all three cell lines (Figure 4-1).

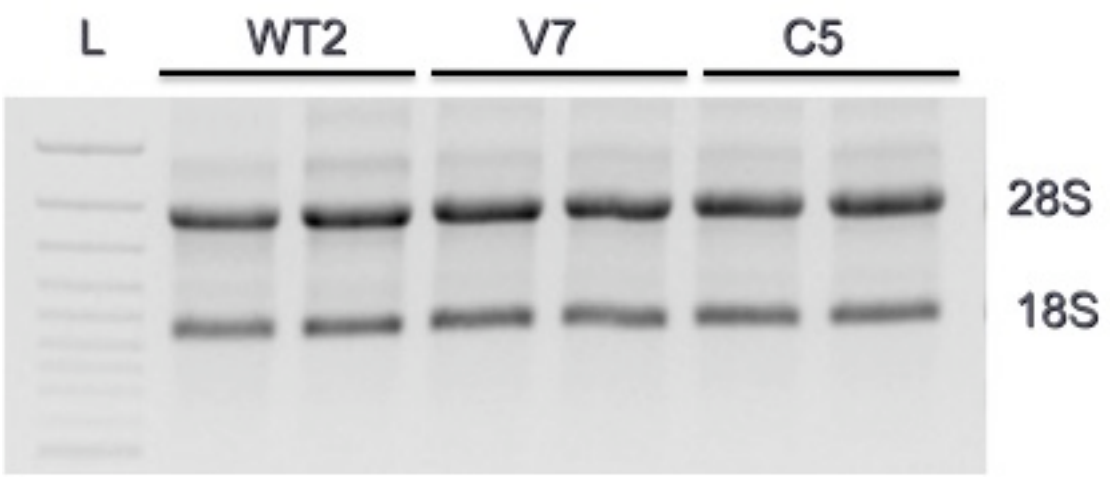

Figure 4-1: Integrity of RNA prepared from EL4 cells. Equal numbers of cells were plated and grown overnight. Cells were collected and total RNA was extracted. Equal amounts of total RNA from all three cell lines (duplicate samples) was separated using a denaturing agarose gel and visualized with ethidium bromide.

Differential expression of genes in EL4 cells: WT2 vs. V7

We performed microarray analysis on WT2, V7, and C5 EL4 cells. WT2 and V7 cells are used in the lab to study mechanisms of PMA response (39). WT2 cells are PMA sensitive, and V7 cells are PMA resistant (39). V7 and C5 cells are of interest to study the effects of PLD2 overexpression (1). No study has been published to date exploring the effects of PLD2 on differential expression of genes in a mammalian genome.

When gene expression was compared between WT2 and V7 cells, over 2400 genes were differentially expressed between the two cell lines. Table 1 lists several genes differentially expressed minimum 2-fold change between WT2 and V7 cells. FAK, Pyk2, PKCeta, and RasGRP1 genes were selected because these genes or their products had already demonstrated to be differentially expressed between WT2 and V7 cells (45,48,49). The results indicate that, for Fak, Pyk2, and PKCeta, the differential expression observed in microarray analysis corresponds to differences in mRNA and protein expression observed in previous studies from our lab. Differential expression of RasGRP1 was observed previously at the protein level but not at the mRNA level (45). Thus, the results shown in Table 1 validate the utility of the microarray method.

Table 1: Genes differentially expressed between WT2 and V7 cells. Genesifter microarray analysis tools were used to analyze the differential expression of genes between WT2 and V7 cells. “Ratio” stands for fold change in gene expression. “Exp in V7” refers to the trend of expression of respective genes in V7 cells as compared to WT2 cells.

	Ratio	**Exp in V7**	**p-value**	**Gene ID**	**Gene Name**
1	11.58	Up	2E-03	AI528614	Protein tyrosine kinase 2 (FAK)
2	4.73	Down	1E-02	BB354696	RAS guanyl releasing protein 1
3	2.97	Down	1E-02	AV026976	Protein tyrosine kinase 2 beta (Pyk2)
4	2.68	Down	2E-03	BM243756	Protein kinase C, eta

Differential expression of genes in EL4 cells: V7 vs. C5

Genesifter microarray analysis was utilized to determine the PLD2-induced differential expression of genes in C5 cells as compared to V7 cells. Table 2 lists the genes differentially expressed between V7 and C5 cells. The results indicate that there are 102 genes differentially expressed between V7 and C5 cells. The list includes mostly unique genes, expressed sequenced tags (EST), and DNA segments with unknown function. The magnitude of the differential expression ranges from 2 to 16.5 fold. We decided to select a gene from the list in order to validate the result at the protein level. We selected Suppressor of Cytokine Signaling 2 (SOCS2) due its role in negatively regulating signal transduction (50), its role in EGFR modulation (46,51), and because its expression is silenced in ovarian and breast cancer cells due to promoter hypermethylation (51,52). The microarray analysis results indicate that SOCS2 gene expression is down-regulated in C5 cells by 2.81 fold at the mRNA level (Table 1). Immunoblotting was performed to investigate the expression of SOCS2 protein in V7 and C5 cells (Figure 4-2). Our results indicate that SOCS2 expression is decreased in C5 cells as compared to V7 cells. Thus, the immunoblot results validate the microarray results.

4.5 DISCUSSION

This study explores the effects of PLD2 on differential expression of genes using a microarray analysis. To our knowledge, this is the first study of its kind

Table 2: Genes differentially expressed between V7 and C5 cells. Genesifter microarray analysis tools were used to analyze the differential expression of genes between V7 and C5 cells. “Ratio” stands for fold change in gene expression. “Exp in C5” refers to the trend of expression of respective genes in C5 cells as compared to V7 cells.

	Ratio	Exp in C5	p-value	Gene ID	Gene Name
1	16.47	Up	0.001	BB139986	Regulator of G-protein signaling 18
2	16.25	Up	0.002	BB139986	Regulator of G-protein signaling 18
3	10.03	Down	0.010	AV028445	BTB (POZ) domain containing 3
4	7.34	Down	0.007	BB709312	Beta galactoside alpha 2,6 sialyltransferase 2
5	6.44	Down	0.008	BG074320	Rho GTPase activating protein 29
6	6.18	Down	0.005	BB369191	DNA segment, human D4S114
7	5.89	Up	0.011	AF022072	Growth factor receptor bound protein 10
8	5.63	Up	0.005	AW553532	Leprecan-like 1
9	4.54	Down	0.001	AV271771	Pleckstrin homology domain containing, family G (with RhoGef domain) member 4
10	4.3	Down	0.013	AW490498	RIKEN cDNA 1810053B01 gene

11	4.27	Down	0.008	BB794831	Phospholipase C, beta 1
12	3.81	Down	0.017	BM935811	Integrin alpha 6
13	3.54	Down	0.017	AK012075	WD repeat and FYVE domain containing 3
14	3.53	Down	0.021	NM_024406	Fatty acid binding protein 4, adipocyte
15	3.36	Down	0.032	NM_007697	Cell adhesion molecule with homology to L1CAM
16	3.28	Down	0.046	NM_007650	CD5 antigen
17	3.26	Down	0.000	BF228318	Fibulin 2
18	3.23	Down	0.000	AI326975	Gene model 131, (NCBI)
19	3.23	Down	0.006	AF266478	Interphotoreceptor matrix proteoglycan 1
20	3.21	Up	0.015	BG067039	Angiomotin
21	3.2	Down	0.014	AI528824	Placental protein 11 related
22	3.12	Down	0.005	AF316014	Deoxynucleotidyltransferase, terminal
23	3.12	Down	0.002	NM_007470	Apolipoprotein D
24	3.11	Down	0.017	BC002148	Fatty acid binding protein 4, adipocyte
25	3.01	Down	0.021	BB160593	Deoxynucleotidyltransferase, terminal
26	3.01	Down	0.034	BB333100	RNA binding motif protein 11
27	2.99	Down	0.004	NM_022886	Sciellin
28	2.99	Down	0.040	AK013588	Mus musculus adult male hippocampus cDNA, RIKEN full-length enriched library, clone:2900024I17:ELAV (embryonic lethal, abnormal

					vision, Drosophila)-like 4 (Hu antigen D), full insert sequence.
29	2.99	Down	0.001	BM935811	Integrin alpha 6
30	2.97	Down	0.012	BB314774	Leucine-rich repeat LGI family, member 1
31	2.96	Down	0.001	BB404920	expressed sequence C78643
32	2.92	Up	0.004	AK018112	RIKEN cDNA 9930013L23 gene
33	2.91	Down	0.031	D45203	DNA segment, human D4S114
34	2.9	Down	0.008	NM_008376	GTPase, IMAP family member 1
35	2.82	Down	0.003	BC011229	Flavin containing monooxygenase 1
36	2.81	Down	0.019	NM_007706	Suppressor of cytokine signaling 2
37	2.8	Down	0.015	AI528824	Placental protein 11 related
38	2.77	Down	0.011	AW548096	RIKEN cDNA 5730593F17 gene
39	2.76	Down	0.013	BB160593	Deoxynucleotidyltransferase, terminal
40	2.71	Down	0.010	NM_010488	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 4 (Hu antigen D)
41	2.65	Up	0.006	BI082172	expressed sequence AI197429
42	2.56	Down	0.004	NM_019511	Receptor (calcitonin) activity modifying protein 3
43	2.56	Up	0.004	NM_008526	Killer cell lectin-like receptor

					subfamily B member 1D
44	2.56	Up	0.011	NM_007406	Adenylate cyclase 7
45	2.54	Down	0.015	BG069663	Yippee-like 2 (Drosophila)
46	2.51	Down	0.029	AV332226	Transcribed locus
47	2.49	Up	0.003	AF342896	Killer cell lectin-like receptor subfamily B member 1D
48	2.42	Up	0.002	BQ032752	Stanniocalcin 1
49	2.4	Down	0.003	M58045	2,3-cyclic nucleotide 3 phosphodiesterase
50	2.38	Up	0.020	BB746807	Adenylate cyclase 7
51	2.38	Down	0.034	BB134767	expressed sequence AW228608
52	2.38	Down	0.020	AK013588	Mus musculus 13 days embryo head cDNA, RIKEN full-length enriched library, clone:3110037G03:ELAV (embryonic lethal, abnormal vision, Drosophila)-like 4 (Hu antigen D), full insert sequence.
53	2.37	Down	0.021	BE692283	RIKEN cDNA C030013G03 gene
54	2.36	Down	0.047	NM_008321	Inhibitor of DNA binding 3
55	2.36	Up	0.005	AF342896	Killer cell lectin-like receptor subfamily B member 1D
56	2.35	Down	0.023	NM_133902	Serine dehydratase-like
57	2.34	Down	0.016	NM_011252	RNA binding motif protein, X chromosome
58	2.34	Down	0.004	BB354684	Tribbles homolog 2 (Drosophila)

59	2.33	Down	0.000	NM_011157	Serglycin
60	2.32	Down	0.030	NM_010511	Interferon gamma receptor 1
61	2.32	Up	0.008	AV294178	Killer cell lectin-like receptor subfamily B member 1D
62	2.28	Down	0.023	BM234719	EH-domain containing 3
63	2.28	Down	0.032	NM_022315	SPARC related modular calcium binding 2
64	2.27	Up	0.047	BB829614	Transcribed locus
65	2.21	Down	0.043	NM_011581	Thrombospondin 2
66	2.2	Up	0.023	AW108488	Exocyst complex component 3-like
67	2.2	Down	0.009	AV005759	ESTs, Weakly similar to COXD MOUSE CYTOCHROME C OXIDASE POLYPEPTIDE VIA-HEART PRECURSOR (M.musculus)
68	2.19	Down	0.010	NM_023892	Intercellular adhesion molecule 4, Landsteiner-Wiener blood group
69	2.19	Up	0.025	AF342896	Killer cell lectin-like receptor subfamily B member 1D
70	2.18	Up	0.040	AK004119	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
71	2.18	Down	0.002	BG966217	Immunoglobulin kappa chain variable 1 (V1)
72	2.18	Down	0.006	BI107286	Immunoglobulin kappa chain variable 1 (V1)
73	2.17	Down	0.003	AK003736	Mus musculus 18 days

					embryo whole body cDNA, RIKEN full-length enriched library, clone:1110017B05:hypothetical protein, full insert sequence.
74	2.17	Down	0.042	BE335796	Ubiquitin-like domain containing CTD phosphatase 1
75	2.17	Down	0.013	NM_009344	Pleckstrin homology-like domain, family A, member 1
76	2.14	Down	0.016	BC025116	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4
77	2.13	Down	0.035	NM_007394	Activin A receptor, type 1
78	2.12	Down	0.007	NM_009943	Cytochrome c oxidase, subunit VI a, polypeptide 2
79	2.12	Down	0.023	BI555209	Fer-1-like 3, myoferlin (C. elegans)
80	2.11	Down	0.025	BB327301	Transmembrane protein 16K
81	2.1	Down	0.001	BG067119	RIKEN cDNA 4833420G17 gene
82	2.1	Down	0.018	BG070237	DNA segment, Chr 19, ERATO Doi 652, expressed
83	2.1	Down	0.015	AK005093	ethanol induced 1
84	2.1	Down	0.000	NM_018767	CD160 antigen
85	2.09	Down	0.030	AA986082	Tubulin, beta 2b
86	2.08	Down	0.015	AU045688	CD160 antigen
87	2.07	Down	0.002	BB251922	cyclic nucleotide

					phosphodiesterase 1
88	2.07	Down	0.000	BF319593	RIKEN cDNA 1700001J04 gene
89	2.06	Down	0.027	BB209605	RIKEN cDNA A430093F15 gene
90	2.06	Down	0.016	BE851919	Transcribed locus
91	2.06	Down	0.006	NM_010386	Histocompatibility 2, class II, locus DMa
92	2.06	Down	0.001	AK008566	Glucosamine-phosphate N-acetyltransferase 1
93	2.06	Down	0.047	AV110584	MARCKS-like 1
94	2.05	Down	0.040	BM936366	Ubiquitin specific peptidase 3
95	2.05	Down	0.006	AK007161	RIKEN cDNA 4933425D22 gene
96	2.05	Down	0.000	BG063148	Expressed sequence AA407270
97	2.04	Up	0.011	BM243756	Protein kinase C, eta
98	2.04	Down	0.006	X14388	T-cell receptor beta, joining region
99	2.03	Down	0.003	BB349707	RAB3D, member RAS oncogene family
100	2.02	Down	0.024	AV066321	Serine peptidase inhibitor, Kazal type 4
101	2	Up	0.011	AF301018	Chemokine (C-X-C motif) receptor 6
102	2	Down	0.015	NM_015786	Histone cluster 1, H1c

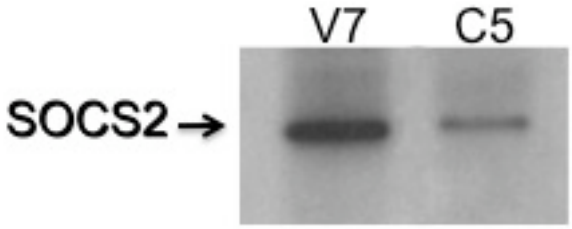

Figure 4-2: Expression of SOCS2 in EL4 cells. Equal number of cells were plated and grown overnight in serum-free medium. Cells were collected and whole-cell lysates were prepared. Equalized aliquots of protein were and separated on a SDS-PAGE gel. Blots were probed with SOCS2 antibody.

that utilizes a microarray to study the effects of PLD2 in any cell type. We used the parental (V7) cells and cells overexpressing catalytically active PLD2 (C5) in the microarray experiment. The subsequent microarray data analysis indicates that there are 102 genes differentially expressed as a result of overexpression of PLD2.

Our lab has an extensive background in investigating PMA response in EL4 cells (39). We have previously developed PMA sensitive (WT2) and PMA resistant (V7) cells to study PMA response (39). From our microarray analysis we selected 4 genes that we have previously reported to be differentially expressed between WT2 and V7 cells (Table 1). Our microarray results for three of the four genes matched to the expression results reported previously. This validates our microarray results. The fourth gene, RasGRP1, is greatly reduced in V7 cells at the protein level, although a previous semi-quantitative RT-PCR study did not reveal a difference in mRNA expression (45). The microarray results suggest that further analysis of mRNA of RasGRP1 using real time PCR is needed.

Literature search of the genes differentially expressed between V7 and C5 cells indicate that majority of them are not known to interact with, or be modulated by, PLD2. Some of the genes are of particular interest with respect to PLD2-mediated signaling. For example, Regulator of G-protein signaling 18 (RGS18) is up-regulated in C5 cells by 16.5 fold. Expression of RGS18 is restricted to bone marrow derived cells (53). RGS18 is known to inhibit signal

transduction cascades initiated by certain Gi modulated GPCRs (54). It also modulates response of dendritic cells to chemokines (54). In general, RGS proteins are known to be up-regulated in response to excessive signaling through particular GPCR pathways (54). Since PLD2 can be activated via GPCRs, the up-regulation of RGS18 may represent a compensatory response by cells overexpressing PLD2. Similarly, we noted that PLCβ1 is down-regulated in C5 cells. Agonists that activate PLD2 typically also activate PLC isoforms (55). Again, the down-regulation of PLCβ1 may compensate for the overexpression of PLD2. We attempted to perform immunoblots for both RGS18 and PLCβ1 to validate the down-regulation observed in the microarray, but did not achieve satisfactory results. However, this is very likely due to problems with immunoblotting method. Continued analysis of the microarray results will likely reveal additional genes of interest, providing a basis for future studies.

SOCS2 is another gene of interest. SOCS2 participates in regulation of immune cell response to cytokines (50). It is down-regulated in C5 cells by 2.8 fold (Table 1). Additionally, our immunoblotting results validate the microarray results in that they demonstrate a decrease in C5 cells as compared to V7 cells (Figure 4-2). SOCS2 is involved in bone formation, somatic growth, and modulates response to infection (50). Expression of SOCS2 is silenced in ovarian and breast cancer cells (51,52). SOCS2 protein contains a centralized SH2 domain and a conserved SOCS box domain at the C terminal that participates in ubiquitination (50). The SOCS2 SH2 domain participates in

protein-protein interaction with phospho-tyrosine residues on intracellular domains of cytokine receptors (50). SOCS2 expression is inversely related with EGFR expression (51). Another member of SOCS family SOCS4, has been shown to promote EGFR degradation (46). Hence, downregulation of SOCS2 may contribute to the observed increase in EGFR expression observed in Chapter 3. Further experiments will be needed to explore this possibility.

Our study establishes, for the first time, a list of genes that are potentially modulated by PLD2. Additionally, this study provides information on pathways previously not known to be modulated by PLD2. Microarray technology provides a good tool to study genome wide differential expression under various experimental conditions. However, microarray results for specific genes can often not be validated at the protein level, diminishing their utility. We were able to validate SOCS2 differential expression at the protein level. In the future, SOCS2 may help to elucidate the mechanism of the PLD2-induced increase in EGFR expression. In conclusion, our study presents a novel protein modulated by PLD2 that may contribute to PLD2-mediated effects, and also reveals other signaling proteins that may provide insight into the overall role of PLD2 in cancer cells.

CHAPTER V

GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 GENERAL DISCUSSION

Phospholipase D2 is a lipid-metabolizing enzyme that plays an emerging role in cellular process including vesicle trafficking, exocytosis, cytoskeleton reorganization, cell survival, proliferation, and tumorigenesis (7,14). However, the exact mechanism by which PLD2 modulates its tumorigeneic functions is still under investigation. Previous study in our lab explored the role of over-expressing PLD2 in EL4 cells lacking endogenous PLD expression (1). The results from this study indicated that PLD2 modulates cell elongation, invasion, and metastasis (1). The goal of this project was to further elucidate the mechanisms of PLD2-induced cellular response in a tumor cell model.

Previous studies have suggested the PLD2 modulates cellular proliferation (40). We explored the effects of over-expressing PLD2 on proliferation of EL4 cells. Overexpression of active PLD2 did not enhance proliferation, however, inactive PLD2 had the opposite effect. The latter result was unexpected and indicates that PLD2 plays a role in cellular proliferation. However, the mechanism by which inactive PLD2 reduces cellular proliferation in the context of cancer cells remains to be determined. We hypothesize that PLD2 participates in productive protein-protein interactions, and that inactive PLD2 engages in similar but non-productive interactions, thereby exerting negative effects.

Overexpression of PLD2 leads to increased Akt activation under basal conditions (1). Cross-talk between PLD2 and the PI3/Akt pathways has been demonstrated previously. Studies have indicated the involvement of PLD2 activity in regulation of mTOR and p70S6K, proteins that lie downstream of Akt activation (34). PLD2 also modulates levels of PIP3 and PIP2, lipids that modulate PI3K activity (13). Thus our results support existing data and confirm the role of PLD2 in PI3K/Akt pathway activation. Previous studies have suggested that PLD2 plays a role in invasion and migration (1). Our results support existing data; PLD2 leads to increased migration and invasion, while inactive PLD2 has the opposite effect. Once again results from the inactive PLD2 were unexpected, in the context of a lack of endogenous PLD2 and suggest that PLD2 activity is critical for mediating migration and invasion. Cells probably compensate for lack of PLD2 expression using compensatory pathways. However in the presence of inactive PLD2, signaling cascades converging on PLD2 maybe unable to yield signaling effects downstream. In other words, inactive PLD2 has a greater negative effect than a lack of PLD2 protein itself, presumably via protein-protein interactions.

We elucidated a new relationship between PLD2 and EGFR. Previously it was known from limited reports that EGFR regulates PLD2 activity and that PLD2 and EGFR co-localize (14, 21). Our results demonstrate for the first time that PLD2 leads to an increase in EGFR expression at the mRNA and protein levels. Even though we did not elucidate the mechanism of PLD2-induced increase in

EGFR expression, a literature search suggested that SOCS protein might play a role (46,51). Our data show that SOCS2 is down-regulated with PLD2 overexpression, and data from others indicate that SOCS2 expression is inversely related to EGFR expression (51). PLD2 also leads to enhanced EGF response in terms of proliferation and invasion. In contrast, inactive PLD2 has negative effects on EGF response. Our results also suggest that activation of Akt plays a critical role in PLD2-mediated proliferation.

Overall, this project elucidated mechanisms of PLD2-mediated proliferation, migration, and invasion. We elucidated a novel relationship between PLD2 and EGFR, in which PLD2 leads to overexpression of EGFR and enhances EGF response. We also indentified genes, including SOCS, previously not known to be modulated by PLD2.

5.2 FUTURE DIRECTIONS

The results from this project demonstrate that PLD2 leads to increase in EGFR expression and EGF response. However, we did not elucidate the mechanism by which PLD2 modulates EGFR expression. Our microarray results identified SOCS2, a protein that is down-regulated by PLD2 and whose expression is inversely correlated with EGFR (51). Existing studies present strong evidence that SOCS2 might be involved in PLD2-mediated increase in EGFR expression (46). In order to test this hypothesis, SOCS2 could be

overexpressed in C5 cells and then expression of EGFR tested at the mRNA and protein levels. We would expect that overexpression of SOCS2 will lead to reduced levels of EGFR in C5 cells. Since PLD2 mediates an increase in EGFR expression at the mRNA level as well, we could investigate the effects of PLD2 overexpression on expression and localization of transcription factors that play a role in EGFR transcription. The effect of PLD2 on the turnover rate of EGFR also needs to be examined.

Our results also indicate that SOCS2 expression is decreased in cells expressing catalytically active PLD2. In order to elucidate whether the down-regulation of SOCS2 is modulated by increased PLD2 expression or activity, expression of SOCS2 could be investigated in cells expressing inactive PLD2 while comparing to parental and cells expressing active PLD2. If downregulation of SOCS2 is similar in C5 and D3 cells, it would indicate that levels of SOCS2 are effected by PLD2 expression and not its activity. Levels of SOCS2 expression could also be modulated in EL4 cells by knockdown approaches and the effects on EGFR levels analyzed.

PLD2-mediated signaling events are initiated by PA and through direct protein-protein interactions (7). PA, either by binding to proteins directly, or through its metabolites, modulates many signaling events; many PA interacting proteins have been discovered (18,20,34). Our microarray study is a good start to explore genes affected by PLD2. The microarray study did not include D3 cells

expressing inactive PLD2; such data would be very informative. Future studies exploring proteins that directly interact with PLD2 and modulate signaling events is warranted. To accomplish this, we could use C5 and D3 cells, immunoprecipitating PLD2 with anti-HA antibody and then separating associated proteins using a 2D gel. Mass spectrometry could then be used to sequence PLD2 interacting proteins. By using C5 and D3 we can also learn about proteins whose interaction with PLD2 is dependent on PLD2 activity.

Our study indicates that inhibition of PLD2 activity leads to various anti-cancer effects. Results from our study and those of others suggests that anti-PLD2 therapeutics would be promising anti-cancer drugs. Based on our studies inactive PLD2 leads to decreased proliferation, migration, and invasion as compared to C5 cells and parental V7 cells lacking PLD2 expression. Hence, our study suggests that drugs targeting PLD2 activity would be more beneficial than drugs targeting PLD2 expression. To date, primary alcohols have been used to "inhibit" PLD by subverting the enzyme reaction, but they do not actually stop enzyme activity. Few published studies suggest that curcumin and AEBSF inhibit PLD, however, these inhibitors are not specific for PLD2 (56,57).

Results presented in this study provide a rationale to pursue anti-PLD2 activity compounds as potential anti-cancer drugs. Our study also suggests that PLD2 expression and activity could be potentially used as markers to indentify tumors that are particularly susceptible to anti-PLD2 therapies. Additionally, anti-

PLD2 therapeutics could be used in combination with anti-EGFR drugs for enhanced anti-cancer effects.

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Oral Presentations:

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