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The Role of Phospholipase D (PLD) and Grb2 in Chemotaxis

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THE ROLE OF PHOSPHOLIPASE D (PLD) AND GRB2 IN CHEMOTAXIS

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

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B.S., Indiana University of Pennyslvania, 2006

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WRIGHT STATE UNIVERSITY

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<u>December 19, 2008</u>

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER
MY SUPERVISON   BY   <u>Katie   J.   Knapek</u> ENTITLED
<u>The Role of Phospholipase D (PLD) and Grb2 in Chemotaxis</u>
BE   ACCEPTED   IN   PARTIAL   FULFILLMENT   OF   THE
REQUIREMENTS   FOR   THE   DEGREE   OF Master   of Science.

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ABSTRACT

Knapek, Katie J. M.S., Department of Biological Sciences, Microbiology and Immunology Program, Wright State University, 2008. The Role of Phospholipase D (PLD) and Grb2 in Chemotaxis.

Phospholipase D (PLD) is an enzyme that hydrolyzes phosphatidylcholine yielding choline and phosphatidic acid. PLD is activated by mitogens (lead to cell division) and motogens (leading to cell migration). PLD is known to contribute to cellular proliferation and deregulated expression of PLD has been implicated in several human cancers. PLD has been found to play a role in leukocyte chemotaxis and adhesion as studied through the formation of chemokine gradients. We have established a model of cell migration comprising three cell lines: macrophages RAW 264.7 and LR-5 (for innate defense), and fibroblast COS-7 cells (for wound healing). COS-7 cells respond to EGF, while the other cell lines respond to MIP-1α and MCP-1.

Transfection of cells with either PLD1-WT or PLD2-WT constructs leads to increased cell chemotaxis. PLD2-WT is better ($\geq$1.5-fold) at increasing chemotaxis than PLD1 WT. Phospholipase inactive mutants in the HKD domain have a negative effect on chemotaxis. Two PLD2 mutants in the PX domain, Y169F and Y179F, known to inhibit the ability of PLD2 to bind through an SH2 domain, failed to potentiate chemotaxis. Conversely, mutation created near, but not in, the SH2 recognition domain in PLD2 (PLD2 Y165F) did not impair the positive effect on chemotaxis observed for the WT. We have demonstrated also here that a protein-protein interaction between PLD2 and Grb2 is needed to enhance chemotaxis. Thus, PLD, signaling through Grb2, is a key regulator of the functionality of the three cells studied. This may pay an important role in facilitating wound healing and innate defense capabilities of our body.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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I. LITERATURE REVIEW

History and Catalyic Activity of Phospholipase D

Phospholipase D (PLD), an enzyme that breaks down phosphatidylcholine (PC) at the ester linkage between phosphate and the choline group, (Figure 1) was discovered in plants by Hanahan and Chaikoff (1-3). PLD was later found in animal cell lines in response to extracellular stimuli (3-5). This enzyme has thus far been identified in bacteria, protozoa, fungi, plants and animals (5).

PLD is an enzyme capable of carrying two types of reactions: hydrolysis (described above) and transesterification. Transesterification reactions use primary-alcohols (i.e. butanol or ethanol) as phosphatdiyl-group acceptors. Phospholipase D can be indirectly measured through the transphosphatidylation activity, whereby the resultant phosphatidylalcohols are produced. In transesterification reactions the primary alcohol replaces the water molecule and becomes the nucleophilic acceptor. These alcohols are not normally found in biological membranes, and they are only produced by PLDs (3, 4).

PLD is also involved in the regulation of essential cellular functions largely due to the production of second messengers such as phosphatidic acid (PA) and ultimately diacylglycerol (DAG) (3-10). Once produced, PA is involved in many cellular functions, ranging from cytoskeletal rearrangement, phagocytosis, vesicle trafficking, exocytosis, and neuronal and cardiac stimulation (3, 6, 9, 11, 12).

PLD (1 & 2) Structure

There are two isoforms of the mammalian PLD gene, PLD1 and PLD2. PLD1 cDNA was cloned from HeLa cells and encodes a 1074-amino acid 120 kDa protein, whereas

PLD

R1-O
R2-O

$$\text{O} \atop \| \atop \text{O-P-O-CH}_2\text{-CH}_2\text{-N-(CH}_3)_3 \atop | \atop \text{O}^-$$

PC

Hydrolysis
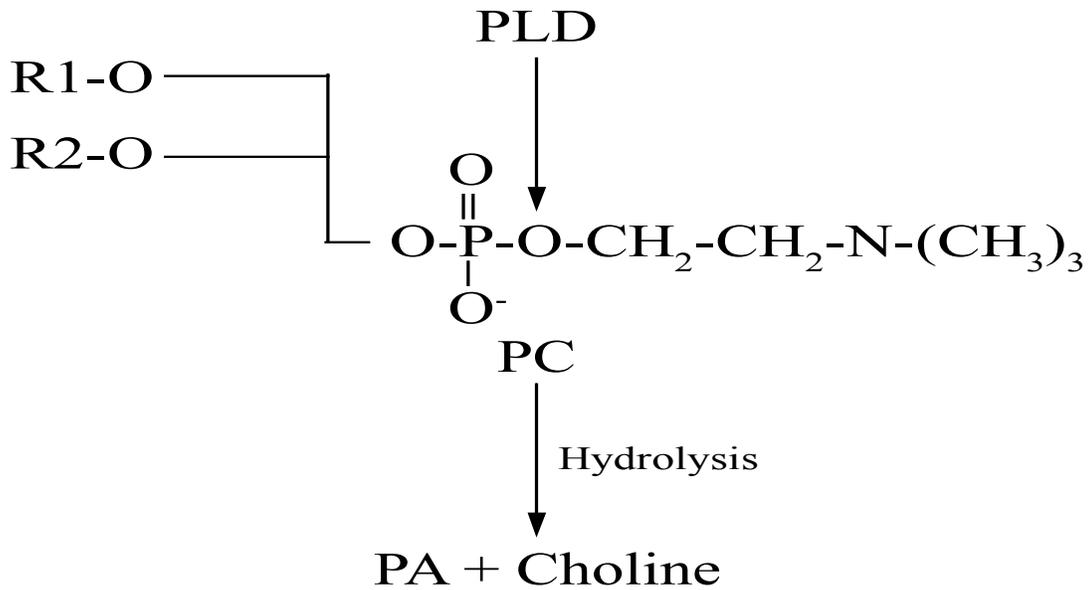
PA + Choline

Figure 1:

Illustration of the catalysis of the phosphodiester bond of phosphatidylcholine (PC) by

Phospholipase D (PLD). Hydrolysis of PC is broken down into phosphatidic acid (PA)

and choline (C) (Adapted from 3).

PLD2 is a 933-amino acid 106 kDa protein (3, 6). PLD1 is located on the long arm (q) of chromosome 3(3q26) (13). PLD2 is located on the short arm (p) of chromosome 17 (17p13) (14). PLD1 and 2 share ~50% amino acid homology (6, 11). There are two splice variants of PLD2 which are, PLD2a and PLD2b. The variants of PLD2 are indistinguishable when comparing their function. Splice variants of PLD1 include: PLD1a1, PLD1a2, PLD1b1 and PLD1b2 (6, 9). PLD1 and 2 contain two invariable sequences referred to as HKD motifs (HxKx4Dx($_6$)GSxN), which are responsible for their enzymatic activity (10, 15) (Figure 2). Both PLD enzyme isoforms have several conserved regions such as a PIP$_2$ binding site, a pleckstrin homology (PH) domain, and a *phox* homology (PX) domain located at their N-termini (3, 10, 15). The PH domain of PLD2 enables it to bind SH2/SH3-containing tyrosine kinases (15). The PH domain of PLD1 mediates protein to phospholipid interactions as well as protein to protein interactions (11). The PX domain, composed of 100-140 amino acids, has been found in other proteins related to membrane trafficking and cell signaling (16). Similarities and differences are shown in Figure 3.

Intracellular Localization of PLD

PLD1 localizes in the nuclear envelope, endoplasmic reticulum (ER), Golgi apparatus, transport/secretory vesicles and the plasma membrane (3, 16). In HeLa cells, a human cervical cancer line, Hiroyama and Exton found that PLD1 localizes primarily in the perinuclear site, as well as in the *trans*-Golgi apparatus, multivesicular endosomes and late endosomes (17). However, PLD1 was rarely found in early endosomes (17). The authors used a GFP-tagged PLD1 co-localized with Anti-CD63 staining in the
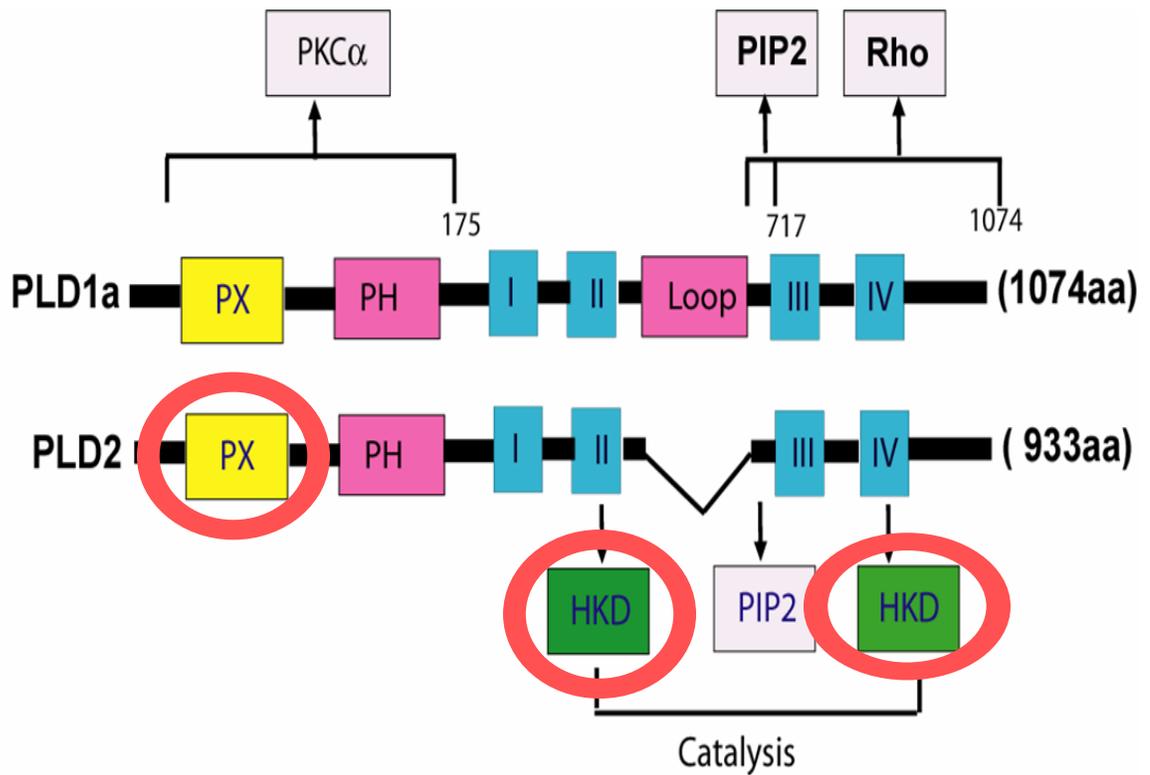
Figure 2:

Schematic representation of PLD1a and PLD2 genes showing the two HKD domains, the PX domain as well as the PH domain (7). Areas circled in red are areas of interest pertaining to this thesis. A notable difference between these genes is the "loop" in PLD1 that is not in PLD2.

MAMMALIAN PLD1 and PLD2
- Share ~50% homology
- The two invariable sequences $HxK(x)_4D(x)_6GSxN$
        ("HKD" domains) are needed for activity

    PLD1
- Chromosome 3q.26 (Humans); 137 kb gene; 31 exons
- Splice variants: a, b, a_2, b_2; PLD1a (longest) = 1,074 AA
- 120 kDa protein
- Subcellular location: Perinuclear, Golgi, ER, endosomes
- Requires PIP_2, ARF/Rho and PKC for activity

    PLD2
- Chromosome 17p.13 (Humans); 16 kb gene; 25 exons
- Splice vaiants; PLD2a, b, c; PLD2a (longest) = 933 AA
- 105 kDa protein
- Subcellular location: Cell membrane, cytosol
- Constitutively active (in vivo and in vitro)
- Requires PIP_2 but not ARF/Rho; PKC activates minimally

Figure 3:

Basic similarities and differences of the chromosomal location, their splice variants, size,

location, and activators of PLD1 and PLD2.

perinuclear area (17). Cluster of Differentiation-63 is a gene encoded by a protein specific for the cell-surface and mediates signal transduction which has roles in motility, growth, cell development and activation. The anti-CD63 antibody was used to determine the location of multivesicular endosomes as well as late endosomes.

There seem to be conflicting results of PLD1 localization in the Golgi apparatus. One explanation for this difference was that different cell lines were used. Using HeLa cells, Hiroyama and Exton used WGA-conjugated Texas-Red and anti-mannosidase II as markers for the *trans*-, *cis*-, and medial-Golgi apparatus. WGA-Texas-Red had 20% localization with GFP-PLD1, but there was no co-localization with the anti-mannosidase II. This indicates that PLD1 localizes in the *trans*-Golgi apparatus, but not in the *cis*- or medial-Golgi (17). ConA-conjugated Texas-Red was used to stain the endoplasmic reticulum (ER) to illustrate that PLD1 does not localize in the ER (17). Colley *et al.* found PLD1 to be localized in the Golgi apparatus of REF-52 cells (rat embryonic fibroblast); however, they used antibodies specific for the *trans*-Golgi apparatus only. Colley and others found that PLD1 may localize in the endoplasmic reticulum of REF-52 cells (18).

Using the same markers to determine PLD1 localization, Hiroyama and Exton also determined localization of PLD2. Whereas PLD1 was found to be in intracellular membranes, PLD2 was found primarily in the plasma membrane (3, 17, 18). PLD2 is predominant around the cell periphery in comparison to PLD1 (17, 18). A notable difference that PLD2 has over other plasma membrane proteins in REF-52 cells is that overexpressed PLD2 results in filopodia function. In one study, activated human Ras was injected into REF-52 cells and did not induce the production of ventral cellular

projections like those seen in cells with overexpressing PLD2 (18). Ras is a signal transduction protein that regulates actin skeletal integrity, proliferation, differentiation, adhesion, apoptosis and migration. Ras also is able to activate Raf/MEK/ERK pathway, PI3K, or Ral-GEF leading to cellular migration through the production of lamellopidia and filopodia.

Regulator Molecules of PLD1 & PLD2

PLD1 and PLD2 isoforms are highly regulated. The low basal activity of PLD1 versus the high basal activity of PLD2 share similarties in how they are regulated, but there are also differences to be noted (3, 7, 15). One of the crucial activators of PLD1 is phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$) (3, 19-21). The interaction of PLD and lipids is highly dependent on PIP$_2$ at least *in vitro* (10). PIP$_2$ is a polybasic molecule. The conserved region located within the C-terminus of the PLD subdomain is the specific region apparently responsible for the regulation of a lipid (i.e. polyphosphoinositide) that PLD can bind (10, 19, 20).

Phosphatidic acid (PA) is a signaling lipid resulting from PLD activity and is a precursor to diacylglycerol (DAG), an endogenous activator of protein kinase C (PKC). Protein kinase C is another regulator of PLD1, and its isoforms (α and β) can directly activate this enzyme (11, 22). The different isoforms of PKC associate with different areas of the PLD1 protein. PKC isoform ζ requires amino acids 1-323 to activate PLD1 (3). Certain growth factors (EGF, PDGF) have been found to activate PLD through PKC (10, 11). PLD1 is also activated by GTPases of Rho, Ral, and ADP ribosylation factor families (ARF) (10, 20).

Phospholipase D2 is activated by PIP_2 and PKC to a minimal extent (9). It has been suggested that PLD2 is required for the transportation of membrane receptors from endosomes back to the plasma membrane through the mediation of Arf6 (23). This was first proposed because PLD2 was found to localize mainly in the plasma membrane.

Functions of PLD

Functions of PLD have been studied in a variety of cell types and in a number of cellular functions. For instance, PLD has been found in macrophages, which has been correlated to phagocytosis of PLD containing macrophages (19). To support this theory conversely, dominant negative forms of PLD1 and PLD2 were found to inhibit phagocytosis of macrophages (19).

As part of the innate immune system, neutrophils, as well as fibroblasts, can produce superoxide that directly kills bacteria (7, 24). Activation of *phox* leads to generation of superoxide radicals which can then produce H_2O_2. This H_2O_2 can then be converted into HOCl or other toxic products through other systems (i.e. halide-myeloperoxidase system) (25). PLD is a regulator of NADPH oxidase, which is sequentially activated by PA and DAG (24). After adhesion of neutrophils to the endothelium, the oxidative burst and degranulation are activated by the action of various cytokines (26). While PLD promotes the ability of cells to initiate defense mechanisms, the inhibition of PLD results in a diminished ability of cells to adhere (6).

Taking adhesion one step further, the mechanism of chemotaxis can be examined. Chemotaxis or direction migration is the ability of a cell to move toward a chemoattractant or chemical stimuli. Chemoattractants aid to direct cell migration toward

a specific location to function in defense. An *in vitro* study involving neutrophils was conducted to allow for transendothelial migration towards an extracellular matrix (26). Cells that migrated through the membrane and subsequently adhered to this surface were then found to synthesize PA and DAG (26).

Some cytokines shown to promote the ability of cells to chemotax include: IL-8, fMLP (formyl-met-leu-phe), and ENA-78 (epithelial neutrophil-activating peptide-78) which are specific for neutrophils. Extracellular growth factor (EGF) is a cytokine that is able to stimulate fibroblasts.

There are also chemokines specific for monocytes and macrophages, which include: macrophage inflammatory protein 1-α (MIP-1α), monocytic chemotactic protein-1 (MCP-1) and macrophage-colony stimulating factor (M-CSF). These cytokines specific for the cell types listed will be discussed in further detail.

History and Role of Grb2

Growth factor receptor bound protein 2 (Grb2) is a 25 kDa protein composed of two SH3 domains and one SH2 domain (27, 28). The crystal structure of the SH2 domain of the Grb2 c-src is a central antiparallel β-sheet that has two α-helices on either side (29). Peptide binding is mediated by the β-sheet, intervening loops and one of the α-helices (29). As seen with the stimulation of Grb2 by EGF, Grb2 binds to the EGF receptor either directly or indirectly through phosphotyrosine-containing proteins through its SH2 domain (15, 27). Other proteins with phosphotyrosine (pTyr) residues include PLD, Shc, FAK (focal adhesion kinase), Syp and IRS-1. What has been noticeable with these pTyr residues is they are bound to Grb2 in β-turn conformations (27).

The two SH3 domains of Grb2 are known to associate with proline-rich areas of other proteins (28). Son of sevenless (Sos), a protein rich in proline, associates with the SH3 domain of Grb2. Sos promotes GTP loading of Ras and leads to the activation of Ras effectors (15). This Grb2/Sos complex has multiple functions, associated with embryogenesis, cancer, regulation of the cytoskeleton, cell differentiation and DNA synthesis (28, 30). Grb2 is a cytoplasmic protein and is able to transmit signals into the cytoplasm (31). When Grb2 is stimulated with EGF, for example, cytoplasmic Grb2 located in the cytoplasm relocates to the plasma membrane (28).

Overexpression of PLD2 and Grb2 has been found in different types of cancer, possibly leading to its ability to metastasize. For example, increased amounts of Grb2 have been correlated with formation of tumors in the liver of mice as well as in human breast cancer cells (27). By inhibiting the Grb2 binding domains with a potential binding blocker it may be possible to reduce the production of metastatic cells.

Interaction of PLD2 and Grb2

PLD was found to be elevated in cells which have been transformed by several oncogenes which include v-Src, v-Ras, v-Raf, and v-Fps, leading to the belief that there is a chronic turnover of PLD-dependent phosphatidylcholine (32). More recently, Grb2 has been identified as a regulator of PLD2. Novel research has revealed that the tyrosines of PLD2 Y^{169} and Y^{179} are needed in order for Grb2 to interact with PLD2 (Figure 4) (15). These two residues were found to play differing roles in the function of PLD2. Point mutation of tyrosine-169 diminishes the enzymatic activity of PLD2, whereas mutation of the tyrosine-179 diminishes the ability of PLD2 to regulate tyrosine phosphorylation (15,

28). Both tyrosines are located in the PX domain of PLD2, but only the Y^{179} residue is conserved in mammalian PLD2 (15). *In vivo* studies firmly established the involvement of the latter tyrosines in Sos recruitment, possibly through Grb2. Supporting this idea is the knowledge that the SH3 domains of Grb2 interact directly with Sos (15). A double mutant, PLD2 Y169/179F was not able to bind to the SH2 domain of Grb2 or activate the Ras pathway (28).

Transfection of a stably-induced short hairpin Grb2 (shGrb2) plasmid silenced endogenous Grb2 in COS-7 cells, suppressing PLD2 activity (28). Di Fulvio and others studied the role of Grb2 in PLD activity further by determining whether activity could be salvaged by transfecting silenced Grb2 cells with a plasmid refractory to shGrb2 plasmid referred to as xGrb2^{SiL} (28). The cells stably transfected with the shGrb2 plasmid were then studied using a mutant that was deficient in the SH2 domain, xGrb2^{SiL}R86K (28). The results showed that the refractory plasmid transfected into COS-7 shGrb2 cells was able to rescue PLD2 activity however, transfection of a Grb2 plasmid lacking the SH2 domain failed to rescue PLD2 activity. The actual mechanism of Grb2 regulating PLD2 is poorly understood, however the current proposal identifies the tyrosine residues at 169 and 179 as the binding sites for the Grb2 SH2 domain (15, 28).

Once the tyrosine residues mentioned interact with the SH2 domain of Grb2, the SH3 domain of this protein recruits tyrosine kinases and phosphatases, which have been found to be important to the functionality of PLD2 (33). Following this idea, a mutant plasmid, GSTGrb2 P49/206L, was constructed which had the two SH3 domains of Grb2 deleted, resulting in decreased PLD2 activity when transiently transfected into mammalian cells (15).

Figure 4:

Sequence of PLD2 showing PX, PH, and HKD domains. Red "Y" indicates functional tyrosines.

Later, it was found that decreased activity of PLD2 did not affect the ability of Grb2 to bind PLD (15, 33). PLD2 lipase inactive mutants (K444R, K758R, K444/758R) were constructed, transfected into cells and protein was immunoprecipitated from cell lysates using antibodies to Grb2. Western blot analysis was then performed using antibodies against the tagged lipase inactive mutants. Western blotting revealed bands corresponding to Grb2 binding to the PLD2 mutants thus showing that PLD2-Grb2 binding and activity are not directly associated (15).

EGF, MIP, MCP, M-CSF, IL-8, fMLP, and ENA-78 Chemoattractants

Epidermal growth factor (EGF) triggers two main biological functions in fibroblasts, namely proliferation and differentiation (34, 35). EGF also serves other functionsm such as cell rounding, ruffling, actin cytoskeletal reorganization, filopodia extension and the most important for research conducted herein, cell motility (35). EGF is a polypeptide that has two sets of anti-parallel β-sheet conformations with negligible α-helical arrangement (34). Tyrosines that are autophosphorylated at the EGF receptor C-terminus allow for the binding of Src 2 (SH2) and phosphotyrosine binding (PTB) domains (35). It has previously been reported that receptor kinase activity along with at least one of the C-terminal tyrosine autophosphorylation sites are both required for cell movement (36). In addition to the latter requirements, both phospholipase C-γ and protein kinase C (PKC) have been linked to EGF and its ability to enhance cell motility (37, 38).

Monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and macrophage-colony stimulating factor (M-CSF) are chemoattractants

found to increase cell motility among monocytes/macrophages. MCP-1 and MIP-1α, which have a C-C structure, activate monocytes and induce directional migration (39). Aside from the ability of MCP-1 to stimulate chemotaxis, it is also able to stimulate monocytes to become cytostatic for some tumor cells lines, increase levels of intracellular calcium and induce monocytes to release superoxide anions and lysosomal enzymes (39).

MIP-1α proteins act through G-protein-coupled cell surface receptors that are expressed by monocytes/macrophages (40). MIP-1α binds to chemokine receptors leading to a variety of cell functions including: chemotaxis, degranulation, phagocytosis and mediator synthesis. Signaling events that are initiated by the previously stated G-protein complex lead to its dissociation into two subunits: Gα and Gβγ. These two subunits then activate the PI3K pathway and PLC, relatively (40). Upon activation of PLC, an influx of Ca^{2+} activates a protein kinase C isoform (41).

M-CSF plays essential roles in the ability of monocytes to survive, proliferate, differentiate and mature (41). M-CSF acts by binding to cell surface receptors (CSF-1R) (42). These cell surface receptors are encoded by the c-fms proto-oncogene. Upon the binding of M-CSF, the CSF-R1 dimerizes, and the tyrosine kinase domain is activated, resulting in transphosphorylation of the receptor. Grb2 and PI-3 kinase, two proteins containing SH2 domains, bind to two of the four phosphotyrosyl residues created (43).

IL-8 and ENA-78 are strong chemoattractants for neutrophils, leukocytes that play critical roles in angiogenesis, tumor development and wound repair (44, 45). IL-8 and ENA-78 are grouped into a CXC subfamily, where the first cysteine is preceded by the Glu-Leu-Arg (ELR) sequence of amino acids, thus these chemokines are part of a group referred to as ELR$^+$CXC (46). These CXC chemokines signal through seven

transmembrane G-protein coupled receptors. Specifically, IL-8 is able to bind to CXCR1 and CXCR2 sites, while and ENA-78 binds with a stronger affinity to the CXCR2 receptor site (47). The CXCR-1 receptor site for IL-8 has been shown to activate PLD,1 and CXCR-2 has been shown to activate PLD2. Like the chemokines previously discussed, these also signal through cystolic Ca^{2+} changes as well as through activation of phospholipase D, subsequent chemotaxis, and exocytosis (48).

N-formyl-methionine-leucine-phenylalanine (fMLP) is a bacterial derived tripeptide that stimulates neutrophils to migrate towards bacteria (49). FMLP is known to be produced by enteric flora and contributes to inflammatory bowel disease. Receptors for this peptide have been found in both monocytes and neutrophils (48). All of the chemoattractants described were used to stimulate migration of COS-7, RAW 264.7, LR-5 and dHL-60 cells, as well blood isolated neutrophils in the current study.

The Physiological Importance of Cellular Migration

Normalcy of migration is found in the ability of leukocytes to move toward foreign invaders of the body in phagocytic and immunogenic responses. Fibroblasts and endothelial cells migrate to aid wound repair and become collagen around the wounded area (50, 51). Migration is also important in embryogenesis and angiogenesis (52). Cellular migration may be involved in not only the normal physiological state of some cells, but also in the pathological state. With regard to abnormal migration, the most common example is viewed in metastasis, whereby tumor cells migrate from the initial tumor site into the circulatory system where they can then eventually locate to another site in the body (50). Before migration can occur, cells must orient themselves to

establish a distinct cell front and rear. The orientation of the cell depends on the inflammation site and location of the chemical stimuli or chemoattractants being released (50, 53).

PA, the product of PLD, has been shown to mediate chemotaxis, as increasing concentrations of PA lead to a more enhanced rate of cell migration (53). Rac, a small G-protein downstream target of PLD during actin cytoskeleton rearrangement, is also involved in cellular migration (54). Actin cytoskeletal rearrangement is subsequently involved in migration, phagocytosis, and axonal growth mitogenesis, morphological change and superoxide production (54). While it is still unknown whether PLD is a primary or secondary regulator of migration, it has been shown by Lehman, *et al.* that PLD is a mediator for chemotaxis in COS-7 cells, but it does not affect chemokinesis. Through the inhibition of PLD, membrane ruffling and formation of lamellipodia structures are blocked.

In the murine lymphoma cell line EL4, Knoepp, *et al.* found that activated PLD2 promotes phosphorylation of FAK and Akt, leading to cell-substrate adhesion (54, 55). However, while inactivated PLD2 inhibits adhesion, migration, proliferation and tumor invasion, it does not alter the basal level of FAK and Akt phosphorylation (55). Thus, it is not through these molecules that the inhibitory effects of PLD act (55). Although PA does play a role in cell migration, the specific mechanisms involved are not completely understood and more precise structure-function studies are needed.

II. HYPOTHESIS AND SPECIFIC AIMS

Preliminary observations in our lab have implicated PLD (as part of a complex intracellular signaling network) in leukocyte migration. IL-8 and related chemokines stimulate PLD activity and promote chemotaxis. There is a body of evidence of a role for PLD in chemotaxis in the lab after studies with neutrophils. However, the experimental use of these cells is somewhat limited in that these cells are not amenable to molecular biology manipulation (e.g. DNA/RNA transfection) (53). This has been addressed in this thesis by the use of leukocyte cell lines that are both transfectable and mobile.

Hypothesis

We propose that specific amino acids in the PLD molecule are critical for the triggering of cell chemotaxis that is mediated by an interaction between PLD and specific signaling proteins.

Specific Aims

In order to test the hypothesis, we designed the following four specific aims and conducted experiments directed at testing the main hypothesis.

AIM 1: Identify a suitable cell line that is amenable to transfection and can exhibit chemotaxis. Neutrophil, monocyte/macrophage and fibroblast cell lines were studied utilizing a multitude of methods to determine the proper cell line to use for optimal transfection efficiency. Also, different methodologies to study chemotaxis were invoked to establish a suitable technique.

AIM 2: Characterize a PLD/chemotaxis relationship by transfection of PLD plasmids. Plasmid DNA constructs containing the exon for wild-type PLD (1 and 2) were transfected into cells either by electroporation or using lipofectamine/plus reagents

and the chemotactic response was quantitated. Next, plasmids containing lipase dead mutants (PLD1 K860R, PLD2 K444R, PLD2 K758R, PLD2 K444/758R), previously constructed in our lab (15), were transfected to determine whether the enzymatic activity of PLD is required for chemotaxis. Specific chemokines were used to study chemotaxis for specific cell lines depending on the cell type (EGF for COS-7; MIP-1α for LR5). In addition, immunofluorescence microscopy was used to determine possible intracellular location of PLD.

AIM 3: Identify PLD mutants that enhance or suppress chemotaxis. PLD mutants (PLD2 Y169F, PLD2 Y179F, PLD2 Y169/179F, PLD2 Y165F) that had been previously constructed in our lab (15, 28, 58) were studied to examine the effect of a particular mutation on the ability of cells to chemotax.

AIM 4: Characterize a protein that can associate with PLD and induce chemotaxis. Cells were tranfected with Grb2 mutants (Grb2, Grb2[Res]WT, shGrb2 and xGrb2 R86K), previously constructed in our lab (15, 28, 58) to determine if overexpression of Grb2 increased chemotaxis, if silencing endogenous Grb2 decreased chemotaxis or if Grb2 binding to PLD could increase chemotaxis.

III. MATERIALS AND METHODS

Materials

Reduced sodium bicarbonate DMEM, RAW 264.7 and COS-7 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). HPBMC human peripheral blood monocytes and LGM-3 growth medium were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Raw 264.7/LR-5 cells were obtained from Dr. Dianne Cox of Albert Einstein College of Medicine Yeshiva University. Peripheral blood neutrophils and monocytes were isolated from donated human whole blood. Histopaque-1077 was obtained from Sigma Aldrich (St. Louis, MO). RPMI 1640 1x was obtained from Mediatech (Manassas, VA). Lipofectamine transfection reagent, plus reagent and Opti-MEM were purchased from Invitrogen Co. (Carlsbad, CA) Superfect transfection reagent was obtained from Qiagen (Valencia, CA). MIP-1α, MCP-1, M-CSF, EGF, IL-3, and ENA-78 were from PeproTech Inc. (Rocky Hill, NJ). Anti-Protein G agarose, Anti-PLD and Anti-Myc tag monoclonal antibodies were obtained from Millipore (Temecula, CA). c-Myc antibody conjugated (AC) agarose mouse monoclonal IgG beads, HA-tag AC agarose mouse monoclonal IgG2a beads and β-actin antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HA-tag mouse mAb was from Cell Signaling (Danves, MA). Triton X- 100, phalloidin-FITC conjugate (conjugate from *Amanita phalloide*) and sodium citrate solution were purchased from Sigma (St. Louis, MO). Enhanced chemiluminescence (ECL) western blotting detection reagents and Percoll/RediGrad were purchased from GE Healthcare (Piscataway, NJ). Nucleofector electroporation device, transfection solution V, and GFP plasmid (pmax-GFP, 3486 bp) were from Amaxa Inc., (Gaithersburg, MD).

Methods

RAW 264.7 RAW 264.7/LR5 Cell Culture, Viability, and Transfection

RAW 264.7 cells are adherent cells. Upon receipt, cells were thawed quickly, washed 1x with pre-warmed complete growth medium and centrifuged (800 rpm for 7 minutes). Supernatant was removed, cells were resuspended in fresh complete growth medium (CGM) consisting of reduced sodium bicarbonate DMEM, 20% FCS (fetal calf serum) and 1% gentamycin and transferred to tissue culture coated flasks and maintained at 37°C in 5% CO_2. After 2-3 passages, 10% NCS (newborn calf serum) was substituted for 20% FCS in the CGM. Cells were fed with fresh CGM every three to four days and, once confluent, were split 1:4 (cell:media) ratio. Trypan blue exclusion test was used to determine cell viablility. Viability of cells prior to transfection was > 90%. RAW 264.7 cells were serum starved one day prior to transfection. Cells ($\sim 3 \times 10^6$ per transfection) were lifted from flask surfaces using cell dissociation buffer, then diluted with an equal volume of media, pelleted at (700 rpm for 5 minutes) and resuspended in 100µl of Amaxa nucleofection solution V containing the plasmid DNA (1-5µg) of interest. Cells, plasmid DNA and solution V were placed in an electroporation cuvette and then subjected to an appropriate electric current for an appropriate length of time by the electroporation instrument. Transfected RAW 264.7 cells were plated in 6-well non-tissue coated culture plates containing 2ml/well CGM (no antibiotics). Experiments were conducted 28.5 hours post-transfection. LR5 cells were transfected using superfect reagent (Qiagen). One day prior to transfection LR5 cells were split into 6-well non-tissue coated culture plates and maintained in CGM (antibiotic and 10% NCS). At the time of transfection cells were between 80-85% confluent. A mixture of SuperFect

reagent, plasmid DNA (1-5μg) and CGM (no serum) was incubated for 10 minutes at room temperature and then added drop-wise onto the adherent monolayer of cells to a final volume of 2ml per well. Cells were incubated with transfection reagent and plasmid for ~3 hours after which cells were washed 3 times with CGM, and incubated an additional 28.5 hours in the presence of CGM prior to experimental use.

COS-7 Cell Culture, Viability, and Transfection

COS-7 cells, like LR-5 and RAW 264.7 cells, grow as an adherent monolayer. Cultures were established from a frozen stock by washing thawed cells 1x with pre-warmed DMEM and placed in coated tissue culture flasks in CGM. Cells were maintained in 75cm^2 tissue culture coated flasks and, once confluent cells were trypsinized, counted and subcultured in 6-well culture plates 1 day prior to transfection. Viability was determined by trypan blue exclusion. On the day of transfection, cells were washed 2x with warm PBS to remove serum and returned to 37°C and 5% CO_2 with 1ml per well of pre-warmed Opti-MEM (serum free media). Lipid-DNA complexes were prepared by mixing lipofectamine reagent, plasmid DNA, plus reagent and Opti-MEM and incubating at room temperature for 10 minutes according to manufacturer's instructions (Invitrogen). Transfection mixtures were added drop-wise to the plated cultures. Cells were incubated in the transfection media at 37°C and 5% CO_2 for 3 hours. Transfection media was replaced with 2ml CGM (no antibiotics) after washing 1x with CGM (no antibiotics). Cells were allowed to grow for 48-55 hours prior to harvesting for experimentation.

Neutrophil and Monocyte Peripheral Blood Isolation

Similar to English and Anderson, blood was drawn in a syringe containing 10% sodium citrate and then slowly added to 6% dextran saline (56). Blood was allowed to settle (30 minutes), top phase removed, sedimented (~ 700 ref for 3 minutes) and pellet resuspended in saline. A ficoll (Histopaque) gradient was used to separate leukocytes and red blood cells (RBCs). Following centrifugation (10000 ref for 15 minutes), the supernatant containing the pellet consisting of RBCs and polymorphonuclear cells (PMNs) was resuspended in 1ml saline, then diluted in ice-cold sterile water to lyse remaining RBCs and returned to isotonic conditions with an equal volume of 1.8% sodium chloride. Following centrifugation (7 ref for 3 minutes), the resulting pellet consisting primarily of neutrophils was resuspended in HBSS/Hepes buffer. The cell concentration was adjusted for experimentation and diluted with the proper volume of RPMI media.

Chemotaxis

Adherent cells were detached using 25% Trypsin/EDTA or a non-enzymatic cell dissociation buffer for COS-7 or RAW cells, respectively. A hemocytometer was used to count cells, and trypan blue exclusion was used to determine cell viability. 24-well plates and Transwell inserts were pre-wetted with DMEM containing 0.5% BSA. Cell concentration was adjusted to 5×10^4 cells per 400µl of DMEM containing 0.5% BSA per insert. 600µl of DMEM containing 5% BSA was added to the bottom of the well prior to placing the cells and insert in the well. Either 10 nM macrophage inflammatory protein (MIP-1α) for RAW 264.7 and RAW 264.7/LR5 cells or 100 ng/µl EGF for COS-

7 cells were used as chemoattractants. The murine macrophage cells lines were allowed to chemotax for 3.5 hours and COS-7 cells chemotaxed for 1 hour at 37°C, 5% CO_2. Afterward, inserts were removed and cells that had migrated to the bottom were fixed with 4% paraformaldehyde per each well. Cells that migrated to the bottom were then allowed to settle and adhere before being counted. Cells were then counted using an inverted microscope at 20x. The average numbers of 6 counts per well were taken and data is quantified.

Cell Lysate Preparation

Lysates of cells were taken at 28 and 48 hours after transfection for RAW/LR-5 cells and COS-7 cells respectively. Lysates were prepared using a lysis buffer containing 0.4% Titron X-100 and protease inhibitors, aprotinin and leupeptin (referred to as SLB). Cells were washed 2x with ice cold PBS, then scraped from wells and pelleted. Cell pellets were resuspended in 150 µl of ice cold SLB and sonicated twice for 10 seconds. An aliquot was removed from each sonicate to determine protein concentration using BioRad protein assay and total protein was detected at O.D. 595 nm using an Amersham BioTrack II plate reader.

Western Blotting

Transfected cell sonicates were subjected to SDS-PAGE using Pierce 4-20% Precise Protein gels and Trichrom Ranger Prestained Molecular Weight Markers. Gels were run at 120 Volts for approximately 45 minutes. Proteins from the gel were transferred to PVDF membranes at 60mA for 2 hours. The membranes were incubated in

5% BSA/TBS-T at 4°C (blocking buffer) overnight. Western blotting was carried out using the following dilutions of primary antibodies in blocking buffer: 1:1000 anti-Myc (rabbit or mouse IgG), 1:1000 anti-HA (rabbit or mouse IgG), 1:1500 anti-Grb2 (mouse IgG), 1:3000 anti-β-Actin (mouse IgG). Washed blots were then incubated in 1:3000 dilutions of the appropriate secondary antibody-HRP conjugate. After washes with TBS-T Amersham's ECL reagent was used to activate the horseradish peroxidase, and x-ray film was exposed to the immunoblots. Film exposures of the blots were taken and developed 24-48 hours after the incubation of the membrane in primary antibody. Blots were then mounted in a cassette and autoradiography film was used to visualize the blots.

Immunofluorescence Microscopy

RAW 264.7/LR5 cells transfected with myc- or HA-tagged plasmids were fixed onto 22mm^2 glass coverslips by adding 1ml of 4% paraformaldehyde to each coverslip for at least 10 minutes. Paraformaldehyde was aspirated off and cells were permeabilized with 1 ml 0.5% Triton X-100 in PBS for 10 minutes. Cells fixed on coverslips were then blocked in IF blocking buffer (10% newborn calf serum and 0.1% Triton X-100 in PBS) for 4 hours. Coverslips were then incubated overnight at 4°C in the dark in a 1:1000 dilution of anti-HA-FITC or anti–myc FITC IgA conjugate in PBS. Next, coverslips were washed 3 times for 5 minutes in 1 ml of PBS. Actin staining was carried out using a 1:200 dilution of Phalloidin TRITC in PBS for 2 hours at room temperature. Following washing, a final stain using DAPI, diluted in 1:2000 PBS, was used to stain cell nuclei by incubating coverslips for 5 minutes and then once again washing with PBS. Excess liquid was aspirated off and cells were washed a final time. Air-dried coverslips were

then adhered to glass microscope slides (cell side down) using Vectashield mounting medium for fluorescence (Vector Laboratories, Inc.) and were visualized on a Nikon Eclipse 50i inverted microscope with MetaVue software.

Construction of Cerulean-C1-Grb2 WT and Cerulean-N1-Grb2 WT

1. Backbone plasmids Cerulean-C1 and Cerulean-N1

Construction of chimeric plasmids began with *Homo sapiens* growth factor receptor-bound protein 2 (Grb2) cDNA. P-Cerulean-N1 and -C1 plasmids were provided as a gift by Dr. Joel Swanson, University of Michigan. Purified DNA on filter paper was eluted in a sterile eppendorf tube with 30 μl ultrapure water. A 1 μl aliquot of the resulting plasmid DNA solution was used to transform DH5α competent cells. Bacteria were screened for kanamycin (K^+) resistance on K+ LB plates. Isolated colonies were picked and used to inoculate 5 ml luria broth (LB-K^+). The *Escherichia coli* were grown overnight at 37^oC with 350 rpm aeration until the cultures were turbid. Plasmids Cerulean-C1 and Cerulean-N1 were purified using Qiagen QIAprep Spin Miniprep kit.

2. PCR amplification of Grb2 WT and mutant Grb2 R86K cDNA

Grb2 and its mutant Grb2 R86K (the 86 amino acid R was mutated to K) were cloned into pcDNA 3.1 to yield pcDNA-Grb2 WT and pcDNA-Grb2R86K. The Grb2 WT and Grb2 R86 fragments were amplified using Pfu polymerase (Primers used are in Table 1). Primers were purified using PCR for thirty-six cycles: 95^oC, 2 minutes, 95^oC, 45 seconds, 55^oC, 1 minute, 72^oC, 6 minutes and 72^oC, 10 minutes. Final products were stored at 4^oC indefinitely. The PCR product was gel purified with Qiaquick Gel Extraction Kit, then ligated to pCR-Blunt II-TOPO with Zero Blunt TOPO PCR cloning

Forward primer	GAGTCGACATGGAAGCCATC	Tm=55.6
Reverse primer 1	ATCCGCGGTTAGACGTTC	Tm=54.8 for mCFP-C1
Reverse primer 2	CCGCGGGACGTTCCGGTTCAC	Tm=65.4 for mCFP-N1

Table 1:

Primers used in ligation for Grb2 WT and Grb2 R86K.

kit according to manufacture's instructions. The ligation reactions were kept on ice and transformed into DH5α competent cells. Colonies positive for the plasmids were screened on LB/K+ agar plates. Isolated colonies were used to inoculate luria broth (LB, K+) and cultures were grown overnight at 37°C with 350 rpm aeration. Mini preparation of purified plasmid DNA was performed for Grb2 WT and Grb2 R86K ligated into the TOPO plasmid. Plasmid verification was performed using PCR and restriction enzyme digestion (RED).

3. Digestion and ligation

The TOPO-Grb2WT and TOPO-Grb2R86K plasmids were digested with restriction enzyme Sal I and Sac II, sequentially. During this time, the backbone plasmids containing the fluorescent protein coding region, Cerulean C1 and Cerulean N1, were also digested using these same two restriction enzymes. The digested fragments (backbone fragments, Grb2 WT and Grb2 R86K) were purified with the gel purification kit listed above, and the backbone fragments were treated with T4 DNA polymerase to avoid plasmid self-ligation. The Cerulean and Grb2 fragments were ligated overnight at 4°C with T4 DNA ligase. The ligation product obtained was used to transform DH5α cells which were then spread onto a LB/K+ agar plate. Colonies that grew on K+ plate were picked randomly and used to inoculate to 5 ml LB/K+ media for plasmid extraction.

4. PCR and Restriction Enyzme Digestion (RED) for positive colony identification

The Grb2 forward and backward primers were used for PCR identification for the recombinant plasmids. The PCR positive plasmids were extracted using Qiagen QIAspin Miniprep Kit and subjected to RED. Size and restriction map of each of the plasmids was verified by RED (~ 5300 bp). Plasmids identified as recombinant ones were sequence verified.

5. Maxiprep for the verified plasmids

     E. coli colonies containing verified plasmids were grown in 2L LB media and maximum preparations of the purified DNA plasmids were made using Mo Bio UltraClean Plasmid Prep Kit. Concentrations and purity of plasmid DNA was determined by spectrophotometry at OD260/280. Purified plasmid DNA was used for transient transfections.

IV. RESULTS

AIM 1: Identify a suitable cell line that is amenable to transfection and can exhibit chemotaxis.

We chose for this thesis three cell types: two macrophage-derived lines (RAW 264.7 and LR-5) and a fibroblast-derived cell line (COS-7). We wanted first to understand if the cells were amenable to DNA/RNA transfection and if they were responsive to chemoattractants. Available strategies for transfecting cells include lipofectamine and plus reagent, superfect and electroporation solution V (Amaxa, Gaithersburg, MD) for COS-7, LR-5 and RAW 264.7 cells respectively. These products are suitable (at different levels) to use on the adherent cells studied. Prior to transfection, cells were found to be >90% viability by trypan blue exclusion. Following transfection, viability remained stable except when using electroporation with RAW 264.7 cells, which had an average viability of 20% post-transfection. Several different methodologies for measuring chemotaxis were studied including boyden chamber densitometry and Transwell quantitation (Figure 5). Only human peripheral white blood cells studied (neutrophils and monocytes/macrophages) were found to yield positive results in the Boyden chamber, which were used initially as a positive control for chemotaxis (50) (Figure 5A). Figure 2 is a representation of the Boyden chamber after cells adhered to the underside of the membrane and following were Wright-stained to show areas where neutrophils adhered to the membrane.

Cancer cell lines used were capable of giving positive results only in Transwell experiments. Transwells for experimentation were loaded with 5×10^5 cells per well in a volume of 400µl. A representative procedure of transwell chemotaxis is shown in Figure
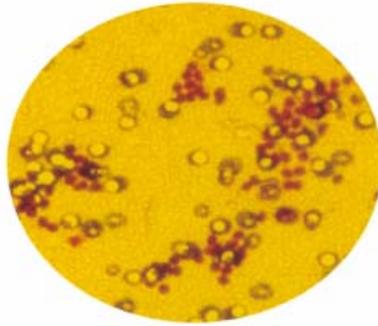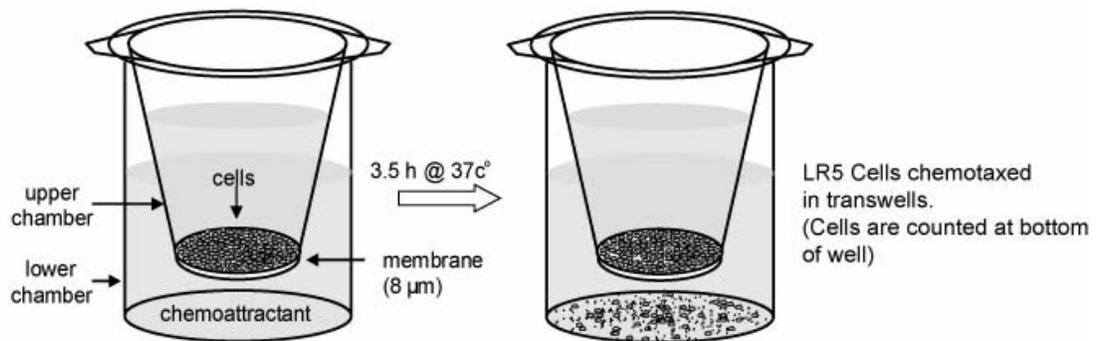
Figure 5:

Methodologies used in performing chemotaxis. (A) An example of a 12 μm pore size filter membrane with cells stained with Wright Stain. (B) Transwell basic procedure that has 2 compartments separated by a semi-permeable membrane.

3B using LR-5 as the experimental cell line. It was established that transwells were the best means to study a multitude of cell lines and their reactions to chemoattractants.

A. Establish the chemotactic response of neutrophils to standard chemoattractants.

The upper compartments of the Boyden chamber were loaded with neutrophils, while the lower compartments received varying amounts of IL-8 (1.5, 2.5, 12.5 and 25 nM), fMLP (15, 150, and 300 nM), and ENA-78 (10, 30, and 50 nM) (Figure 6) (44, 49). Through this experiment IL-8 and FMLP showed neutrophils extracted from human blood were able to stimulate chemotaxis in a dose-dependent manner best. Neutrophils that were tested showed significant migration in the Boyden chamber in all concentrations of FMLP as well as IL-8 at 12.5 and 25 nM (Figure 7). All concentrations of fMLP were significant and they showed hyperbolic responses.

B. Study the chemotactic response of three motile cell lines through dose and time dependency.

1. RAW 264.7 Cells, a macrophage cell line that responds to MIP and MCP.

RAW 264.7 cells are a murine macrophage cell line obtained from ATCC. Cells used in these experiments were between passages ten and fifteen. RAW 264.7 cells can be stimulated by many chemoattractants including: MIP-1α, MCP-1α, and M-CSF. The RAW 264.7 cells were found to be stimulated best by MIP-1α at a 10 nM concentration for three hours rather than a 10nM concentration of MCP-1 (Figure 8A). MCP-1α was found to stimulate chemotaxis but not to the magnitude of MIP-1α. For the studies conducted herein, MIP-1α was the chemoattracant of choice used to stimulate RAW
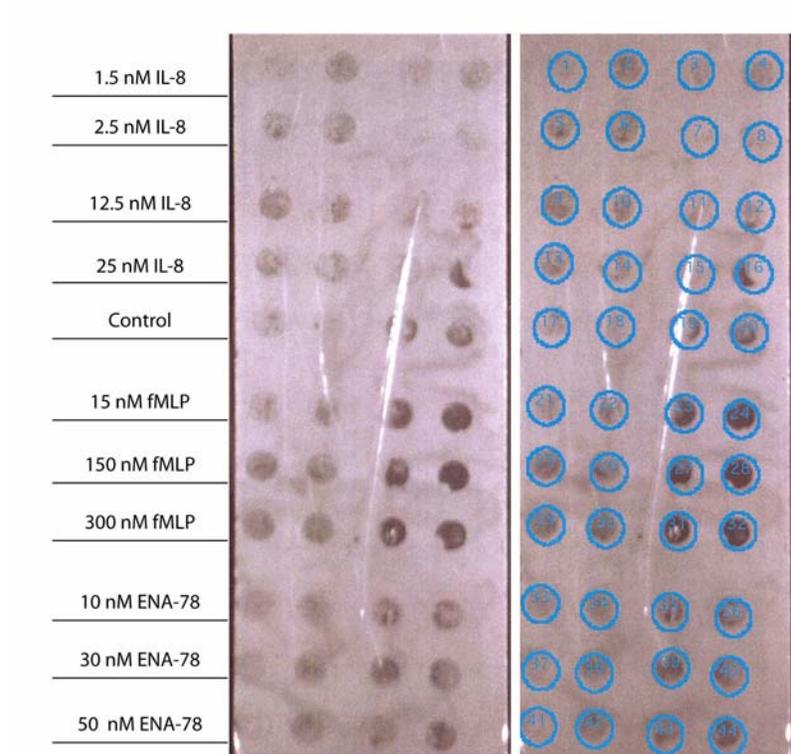
Figure 6:

Analysis of a Boyden chamber membrane after chemotaxis. Peripheral blood neutrophils were resuspended in RPMI-based chemotaxis bufer at a concentration of 5×10^5 cells/mL density and placed in the upper chamber of a 5 μm pore membrane in the original Boyden chamber. Bottom wells contained either buffer only or varying amounts of IL-8, fMLP, or ENA-78.
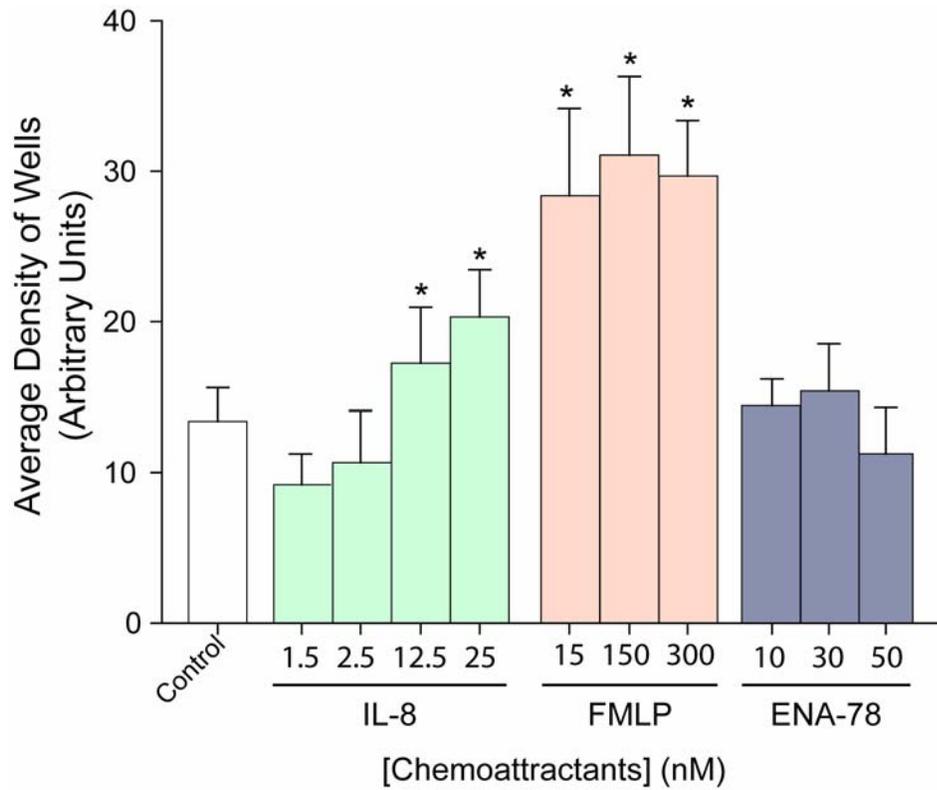
Figure 7:

Chemotactic response of human blood neutrophils toward the indicated concentration and type of chemoattractants. (* indicates p-value < .05 with respect to "control".)
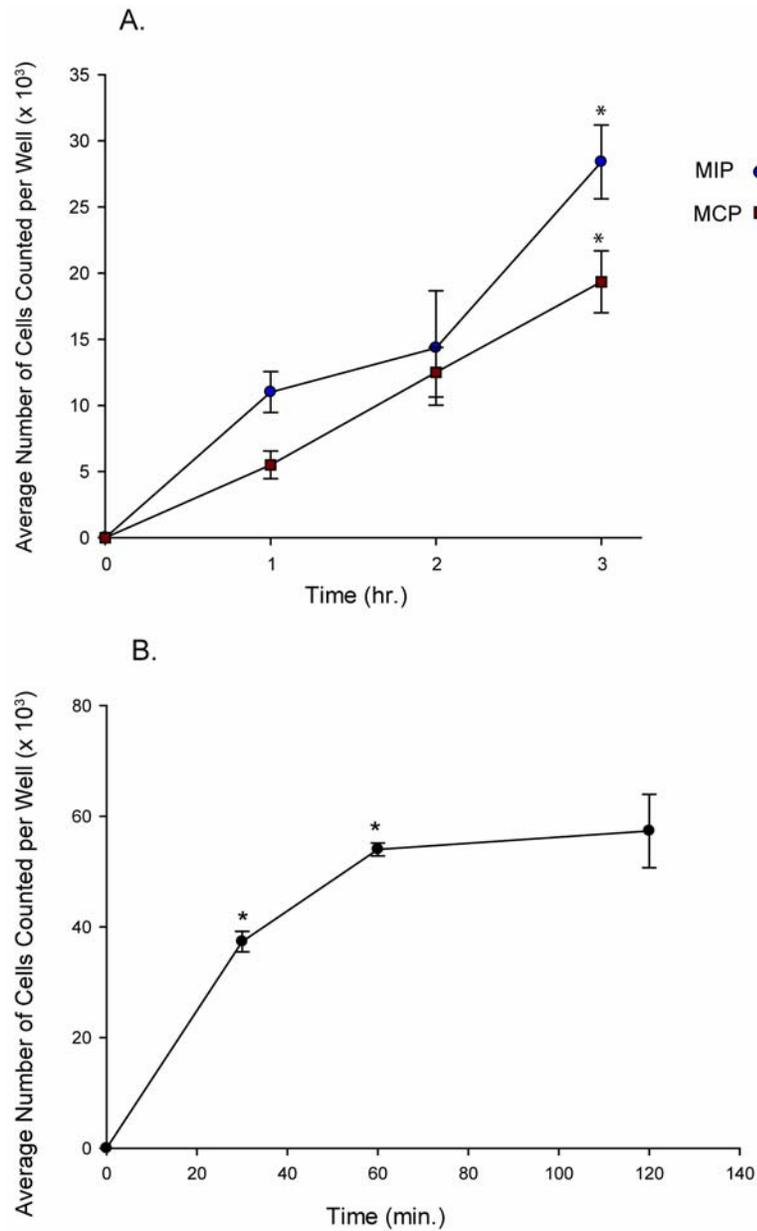
Figure 8:

Time-dependency of chemotaxis (A) RAW 264.7 cells were studied in a time-dependent manner using 10 nM of MIP (blue circles) and 10 nM MCP (red squares). (B) COS-7 cells were studied at an EGF concentration of 100 ng/μl at 30 minute increments over a period of 120 minutes.

264.7 and LR-5 cells due to its significance (Figure 9B and C). M-CSF could also be used to stimulate chemotaxis (data not shown).

2. LR-5 cells, also a macrophage cell line which is stably expressing the Lac repressor and are proven to be more mobile than the parental RAW 264.7 cells.

A dose response experiment was also performed using LR-5 cells, which were provided by Dr. Diane Cox, Albert Einstein School of Medicine Yeshiva University, NY. Dr. Cox's laboratory found LR-5 cells to be more mobile than RAW 264.7 using live-cell microscopy. LR-5 cells are murine macrophages that stably express the Lac repressor derived from RAW 264.7. Cells were used for dose and time experiments between passages ten and fifteen. Like the RAW 264.7 cells, LR-5 cells, are found to be stimulated by the same chemoattractants (Figure 9C).

3. COS-7 cells, a fibroblast cell line that is stimulated by EGF.

COS-7 cells are fibroblast cells, which grow in the presence of EGF (33). COS-7 cells were used in cell migration to develop a model for wound healing. Fibroblasts synthesize collagen and maintain the structural integrity of connective tissue and we have found that EGF could also act as a chemoattractant. Dose and time dependent experiments were used with COS-7 cells between passages ten and twenty. Dose and time response experiments revealed experimental optimization at 100 ng/µl EGF and for one hour.

We have established a model of cellular migration that is comprised of three cell lines that exceed our expectations when compared to neutrophils. RAW 264.7 is a murine macrophage cell line that responds very well to MIP-1α and to a lesser extent, MCP-1 in a dose and time dependent manner. As reported by the laboratory that first
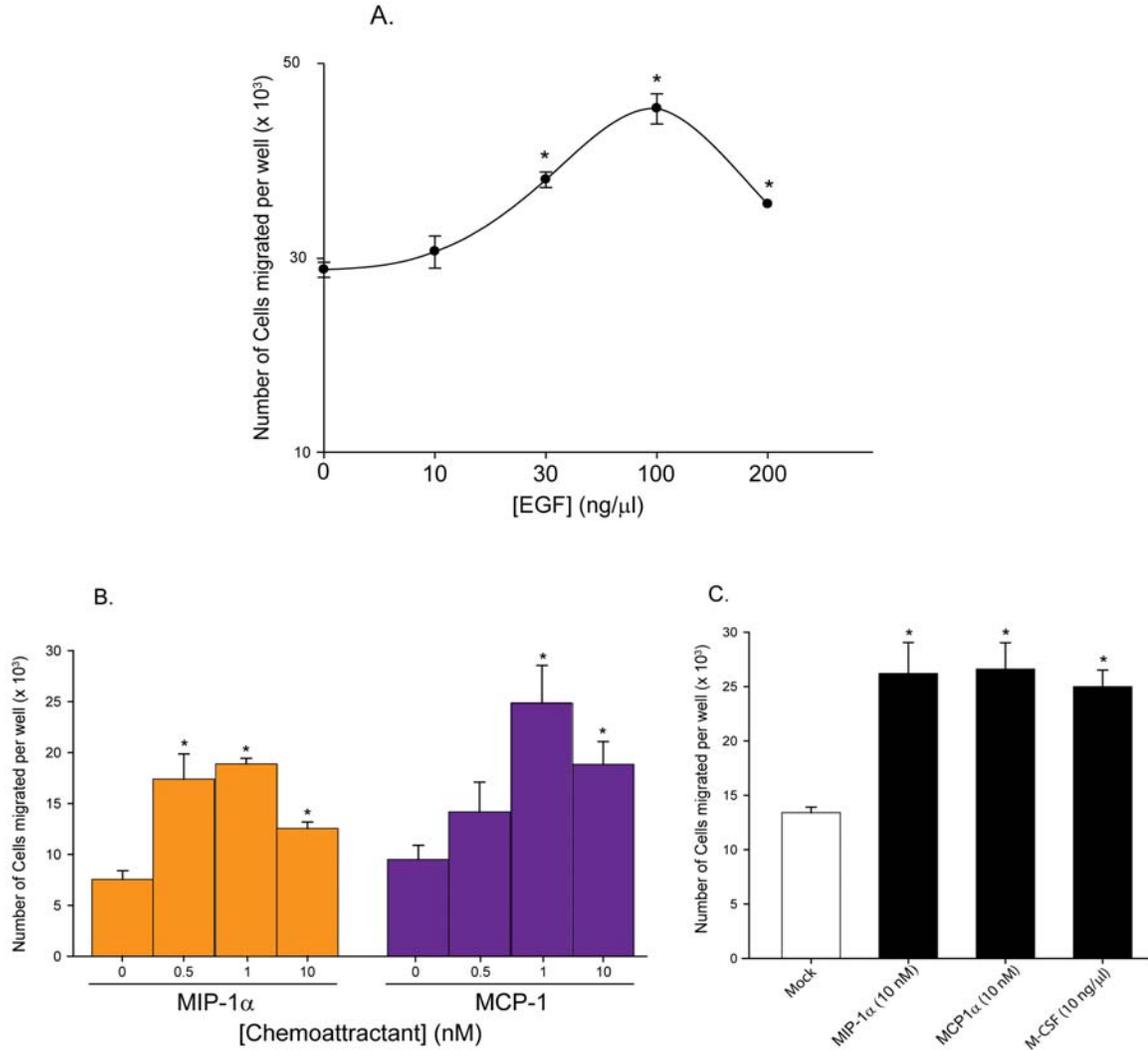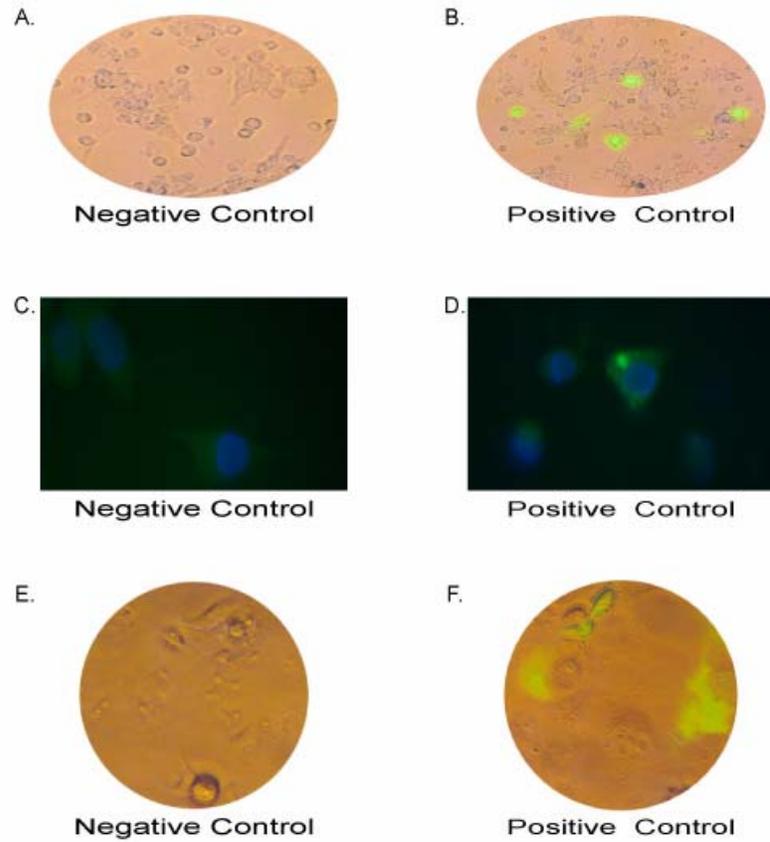
Figure 9:

Dose response of chemotaxis. (A) COS-7 (B) RAW 264.7 and (C) LR-5 cells stimulated with their respective chemoattractants. COS-7 cells were chemotaxed for 1 hour while RAW 264.7 and LR-5 cells were chemotaxed over a period of 3 hours. (* are $p<0.05$ with respect to no chemoattractant (B) or Mock (C).)

identified them (Dr. Diane Cox, Albert Einstein School of Medicine, New York) LR-5s is a murine macrophage cell line stably expressing the lac repressor that is: (1) more mobile and (2) more easily transfectable than the parental counterpart RAW 264.7 cells. Like the previous macrophage cell line mentioned, LR-5 cells also respond to MIP-1α and MCP-1 in a similar manner. COS-7 cells on the other hand are fibroblastic in nature and migrate towards EGF in a dose dependent manner as illustrated (Figure 9C).

AIM 2: Characterize a PLD/chemotaxis relationship by transfection of PLD plasmids.

Cells utilized in the following section were transfected with green fluorescent protein (GFP) to determine transfection efficiency using the strategies indicated in Aim 1 (Figures 10 and 11). RAW 264.7 cells were electroporated using amaxa nucleofector solution V at $3x10^6$ cells per condition, which resulted in 25% viability and 18% average transfection efficiency for plasmids (Figure 11). Transfection efficiency and viability was greater for LR-5 and COS-7 cells than that of RAW 264.7. Viabilities of the latter cells lines (LR-5 and COS-7) were both >90% following transfection with GFP. Transfection efficiencies of these cell lines were 40% and 83%, respectively.

COS-7 cells were transfected with phospholipase D (PLD) 1 and 2 wild-type plasmids in phCMV and pcDNA 3.1 vectors to determine if an overexpression of PLD was able to increase chemotaxis. To create a control for this experiment, empty vector plasmids were transfected into COS-7 cells and compared with the mock control and the PLD plasmids. The empty vector transfections were not significantly different from the mock (Figure 12). These results conclude that transfection alone does not alter the cell in any way structurally or metabolically that adversely or directly impacts chemotaxis. We

Figure 10:

Control experiments with Green Fluorescent Protein (GFP)-expressing cells. (A) and (B) RAW 264.7 cells, (C) and (D) LR-5 cells, (E) and (F) COS-7 cells (A,C,E) mock transfected and (B, D, F) GFP transfected using Amaxa Solution V, Superfect and Lipofectamine and Plus reagent respectively.
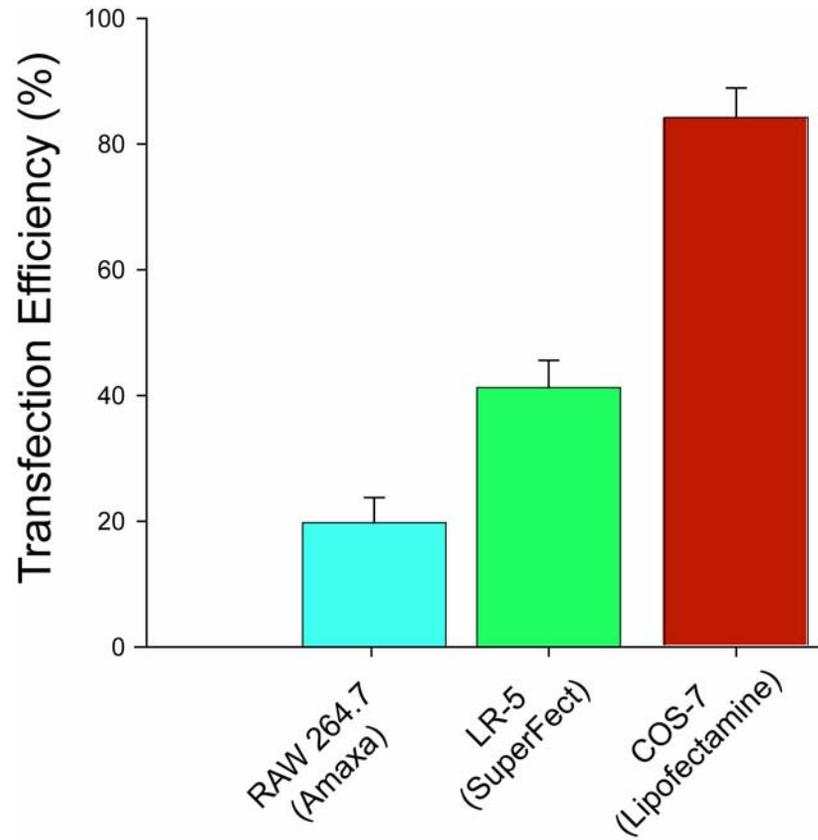
Figure 11:

Transfection efficiency of pmax GFP using manufacturer's recommended methodologies and parameters for the respective cell lines.
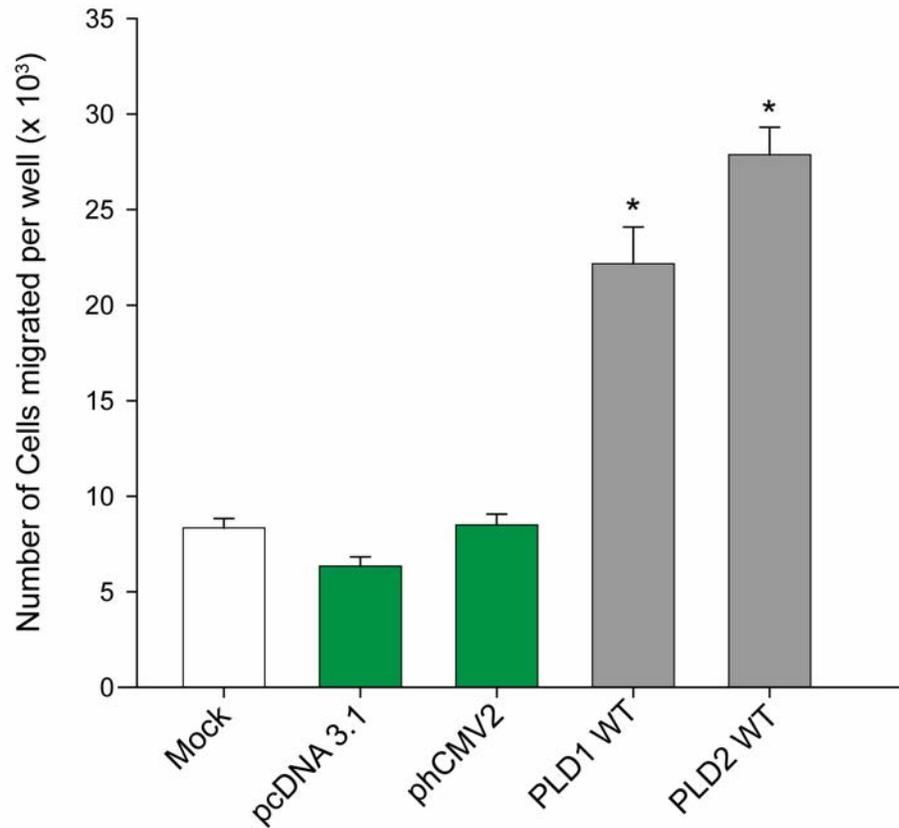
Figure 12:

Control experiment ruling out a side effect of the empty vectors. COS-7 cells were transfected with the PLD1 and PLD2 mutants and their respective empty vectors (phCMV2 and pcDNA 3.1) to show that chemotaxis is not a mere product of transfection. (* are p-values of <0.05 with respect to Mock.)

can then conclude that overexpression of phospholipase D leads to a 2-fold increase in chemotaxis and since no attempts were made to separate expressing cells from non-expressing cells, it may be reasonable to conclude that the observed effect in enhanced chemotaxis could very well be underestimated.

A. Transfect cells with PLD1 and PLD2 (WT) constructs. Analyze cells studied through immunofluorescence, assess the transfection efficiency of cells and run controls with empty vectors to show no specificity toward transfection.

COS-7 cells transfected with the PLD1 and PLD2 WT constructs enhance chemotaxis when stimulated with EGF (at p-values <0.05) and these results foreshadow the cellular changes induced by PLD during EGF-mediated chemotaxis. PLD is an enzyme that has been connected to leukocyte function and migration. The cells studied in this thesis initially have small amounts of endogenous PLD. Through transfection of the PLD2 WT plasmid, stimulation of MIP-1α or MCP-1 for ten minutes leads to structural changes, which were visualized using immunofluorescence (Figure 13). After transfection of PLD2 the cell remains in an elongated morphology, but when stimulated with a chemoattractant, the cell cytoskeleton changes from an elongated shape to compact and migratory (57).

COS-7, RAW 264.7 and LR-5 cells were transfected with a multitude of plasmids that were previously constructed by researchers within our laboratory. The PLD1 K830R, PLD2 K444R, PLD2 K758R, and PLD2 K444/758R constructs are mutants that are lipase dead. Figure 14 is a schematic representation of PLD1 and PLD2 lipase dead constructs. The lipase dead mutant on the PLD1 isoform is located within the second HKD domain of the gene. The PLD2 isoforms lipase dead mutants (444 and 758) are
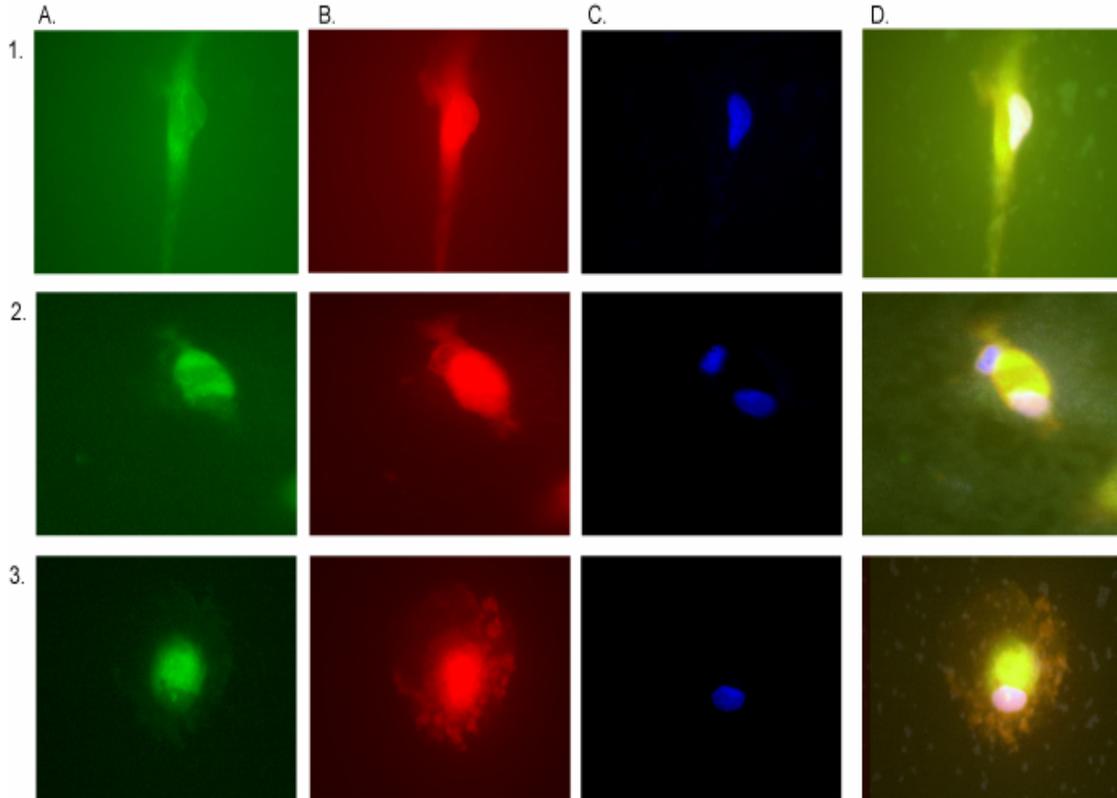
Figure 13:

Visualization of PLD in human macrophages. Human macrophages (from Cambrex Walkersville, MD) were transfected with the PLD2 WT construct tagged with myc. Column A is stained with a FITC antibody against myc to show fluorescence. Column B is Phalloidin TRITC antibody that reveals β-actin. Column C are the nuclei of cells stained with DAPI. Column C is the overlay of A,B,C where the areas of light green are areas of PLD expression. Row 1 of these cells is a PLD2 WT transfected cell with no stimulation. Row 2 is a PLD2 WT transfected cell with MIP-1α stimulation for ten minutes. Row 3 is a PLD2 WT transfected cell with MCP-1α stimulation for ten minutes.
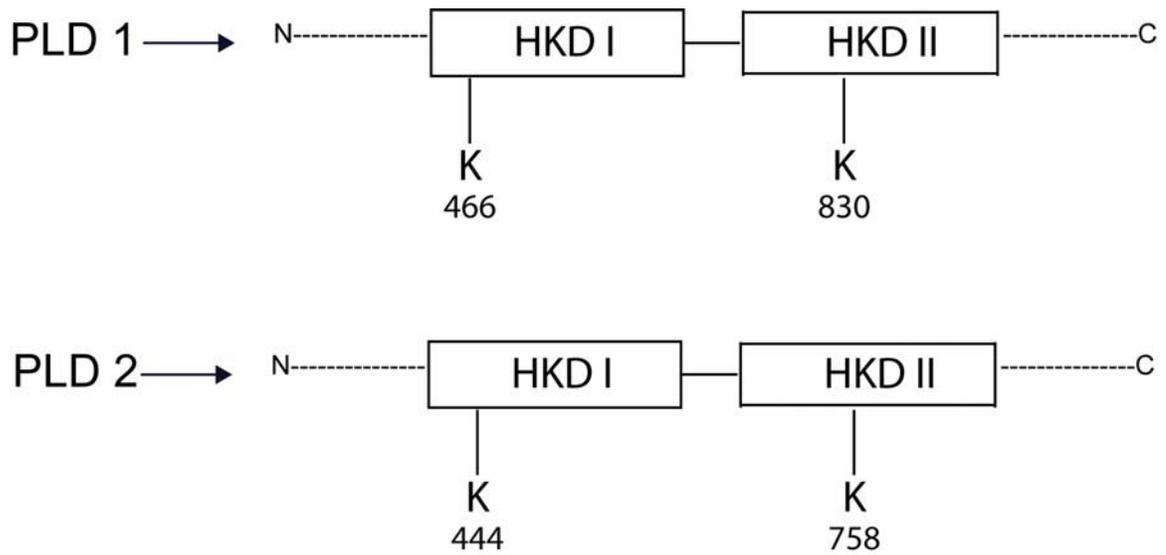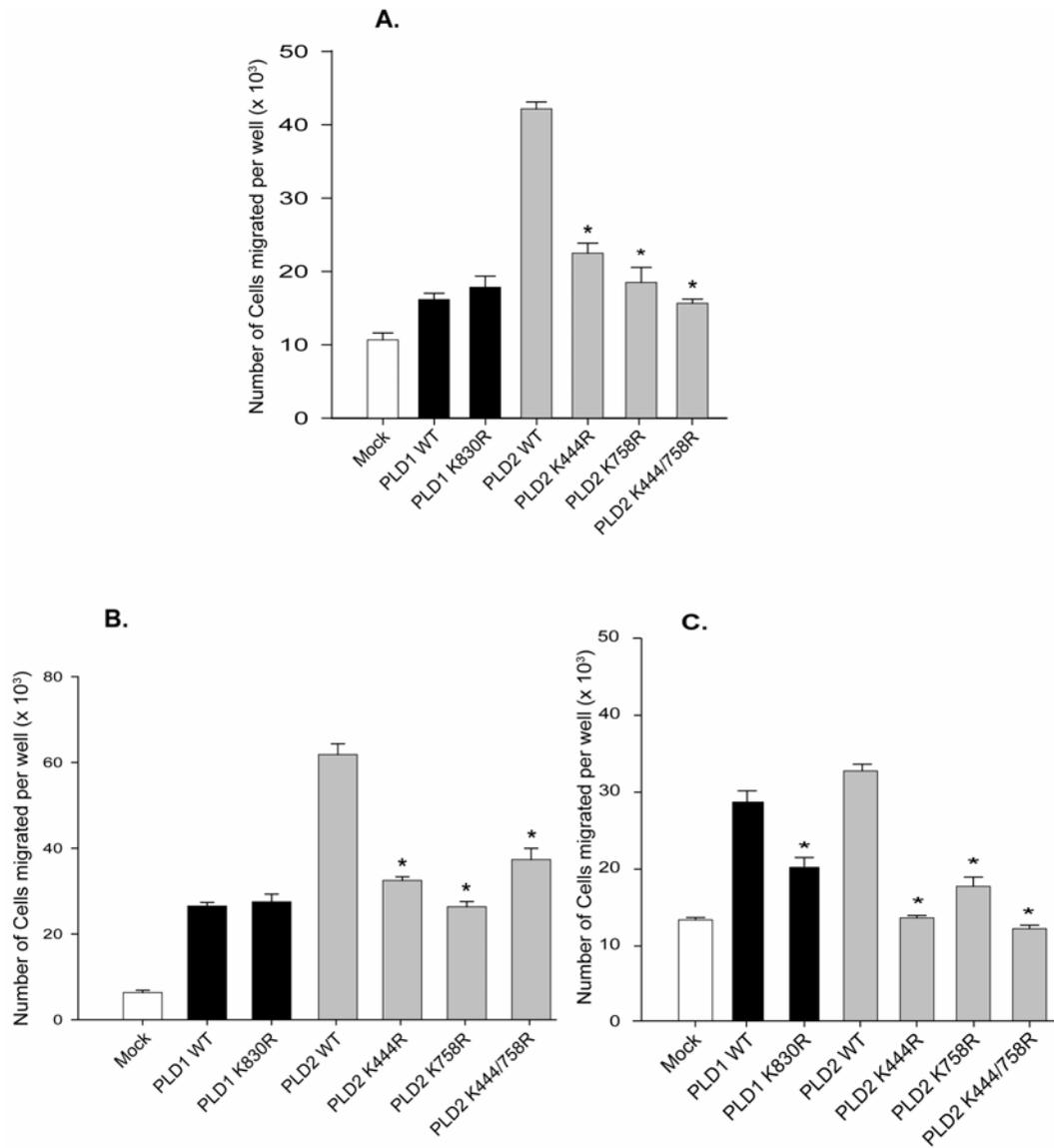
Figure 14:

Phospholipase D isoforms showing locations of the lipase dead mutants on PLD1 and

PLD2 isoforms. HKD domains are numbered according to their order on the gene.

located in the first and second HKD domains respectively. The HKD $(HxKx4Dx_{(6)}GSxN)$ domains of PLD are invariable regions that are responsible for their enzymatic activity of PLD (10, 15). The difference between these two regions is a loop section between the first and second HKD motif of PLD1 (Figure 2).

B. Study chemotaxis

Chemotaxis assays were conducted in COS-7, RAW 264.7, and LR-5 cells. These cells both play roles in the innate immune response. Both cells lines migrate to injured areas within the body. Fibroblasts aid in wound healing by synthesizing the production of collagen. These cells are important in maintaining the structural integrity of connective tissues. Fibroblasts transfected with PLD2 WT construct significantly enhanced chemotaxis when stimulated with EGF, a four-fold increase when compared to mock (Figure 15A). All other plasmids were significantly less mobile than the PLD2 WT construct possibly detailing that the HKD domain of PLD2 may be an important region of the protein in chemotaxis. Because there was not a significant difference between mock, PLD1 WT and PLD1 K830R, the HKD domain of PLD1 WT may not be as involved in chemotaxis as PLD2 may be.

Macrophages are antigen-presenting cells that have the ability to phagocytose debris and dead cells. Macrophages are monocytes that have migrated to tissues in the case of injury or foreign invasion (bacteria). In both macrophage cell lines studied, (RAW 264.7 and LR-5) PLD2 WT was able to increase the rate of chemotaxis as in COS-7 cells (compare Figures 15B and 15C to Figure 15A). All PLD2 lipase inactive mutants were significantly reduced compared to the PLD2 WT plasmid in both macrophage cell lines. Unexpectedly in LR-5 cells, PLD1 WT was significantly different

44

Figure 15:

Study of chemotaxis after transfection with PLD constructs. (A) COS-7, (B) RAW 264.7, and (C) LR-5 cells with PLD1 and PLD2 WT constructs and lipase inactive mutants of PLD1 and PLD2 ("KR"). Values are the means $\pm$ the SEM of three experiments and asterisks indicate significant difference from values obtained with corresponding WT PLD (*, $p < 0.05$).

from the mock and PLD1 K830R suggesting that in this cell line the first HKD domain of PLD1 may be involved in chemotaxis contrary to COS-7 and RAW 264.7 results.

C. Run controls by immunoblotting to determine equal expression of plasmids.

β-actin immunoreactivity was used as a gel loading control to show equal protein expression of transfected COS-7 cell lysates. PLD1 WT and PLD1 KR constructs were transfected equally to yield equal expression of PLD1 (Figure 16A). PLD2 WT and KR constructs also had relatively equal expression of PLD2 (Figure 16B). In LR-5 cells lipase inactive mutants PLD2 K444R and PLD2 K444/758R were not significantly different from the mock. In conclusion transfection of PLD2 WT constructs into RAW 264.7, LR-5, and COS-7 cells leads to an increase in chemotaxis. Conversely, after transfection of PLD1 WT chemotaxis is enhanced only in LR-5 cells. The phospholipase inactive mutants residing in the HKD domain(s) of the isoforms have no enhancement effect on chemotaxis (compare Figure 20 and Figure 15).

AIM 3: Identify new PLD mutants that enhance or suppress chemotaxis.

A. Transfect cells with YF point mutants in the Phox domain of PLD and study chemotaxis.

Cell lines studied were transfected with mutants with a point mutation at a tyrosine. The sites of the point mutations are represented in Figure 17. After phosphorylation, tyrosines at the 169 and 179 sites are able to bind to the SH2 motif of other proteins. Transfection of all YF mutants was found to be significantly different than PLD2 WT in all cell lines (Figure 18). The ability of tyrosines in the PX domain of PLD2 to be phosphorylated by various kinases has a large effect on the ability of cells to chemotax. In LR-5 cells, the Y165F mutant significantly increases chemotaxis when

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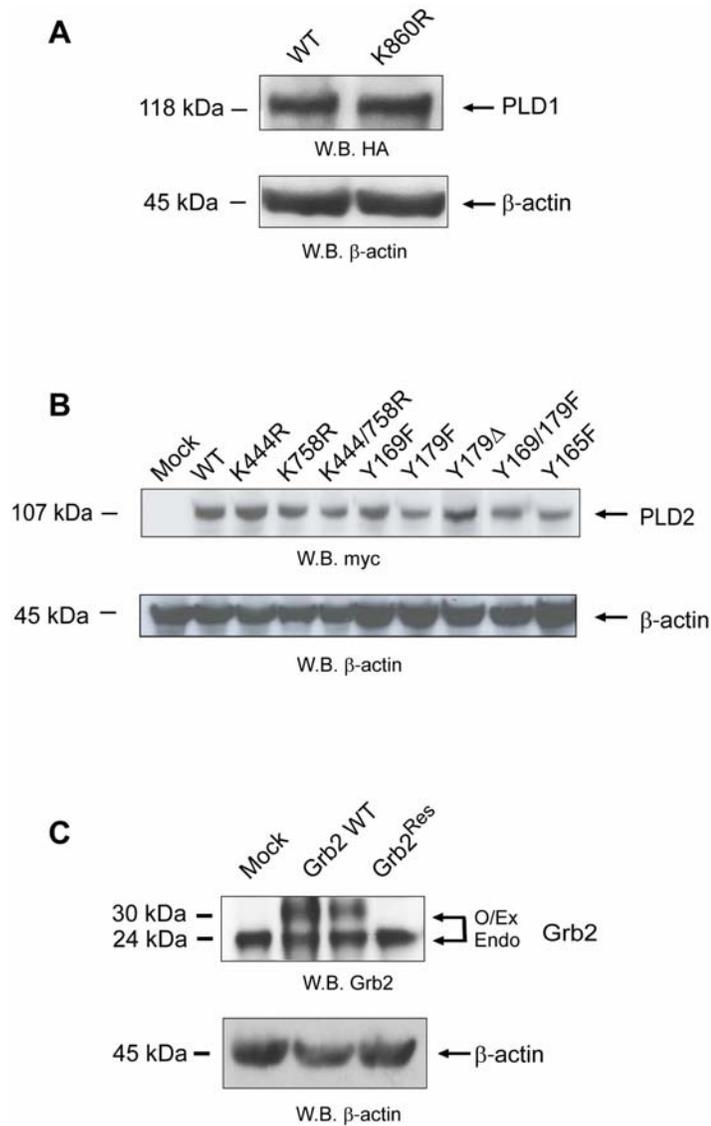
Figure 16:

Western blots of transfected plasmids in COS-7 cells to show similar levels of expression. (A) PLD1 WT and PLD1 KR expression. (B) PLD2 WT, PLD2 KR, and PLD2 YF expression. (C) Grb2 WT and Grb2^{Res} WT expression after transfection. PLD1 WT and KR are tagged with HA, while PLD2 WT and its mutants are tagged with myc. Grb2 constructs are not tagged and thus an antibody specific for Grb2 was used. Western-blots probed were transfected with β-actin to show equal loading. O/Ex = overexpressed. Endo = endogenous.
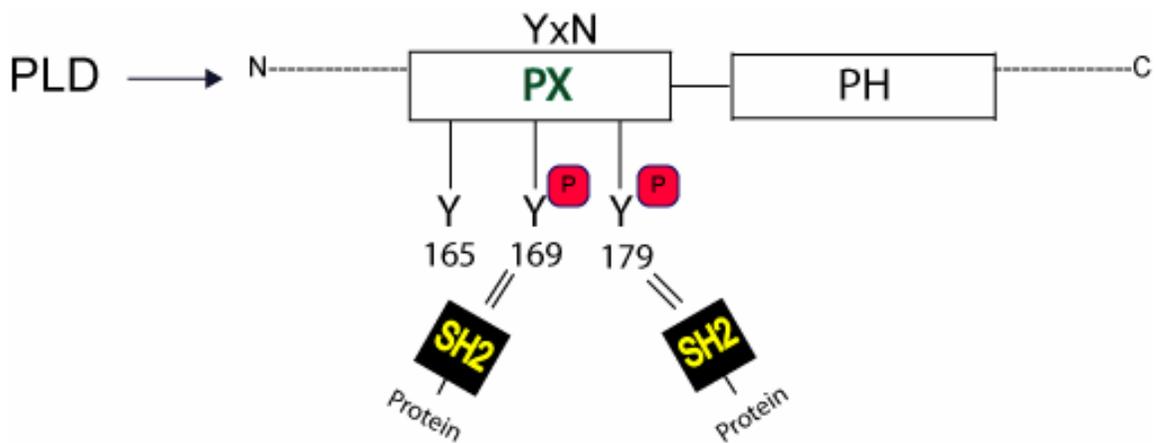
Figure 17:

Schematic representation of YF mutantions of the PLD2 gene. After phosphorylation, Y169 and Y179 sites are able to bind an SH2 region of other proteins.
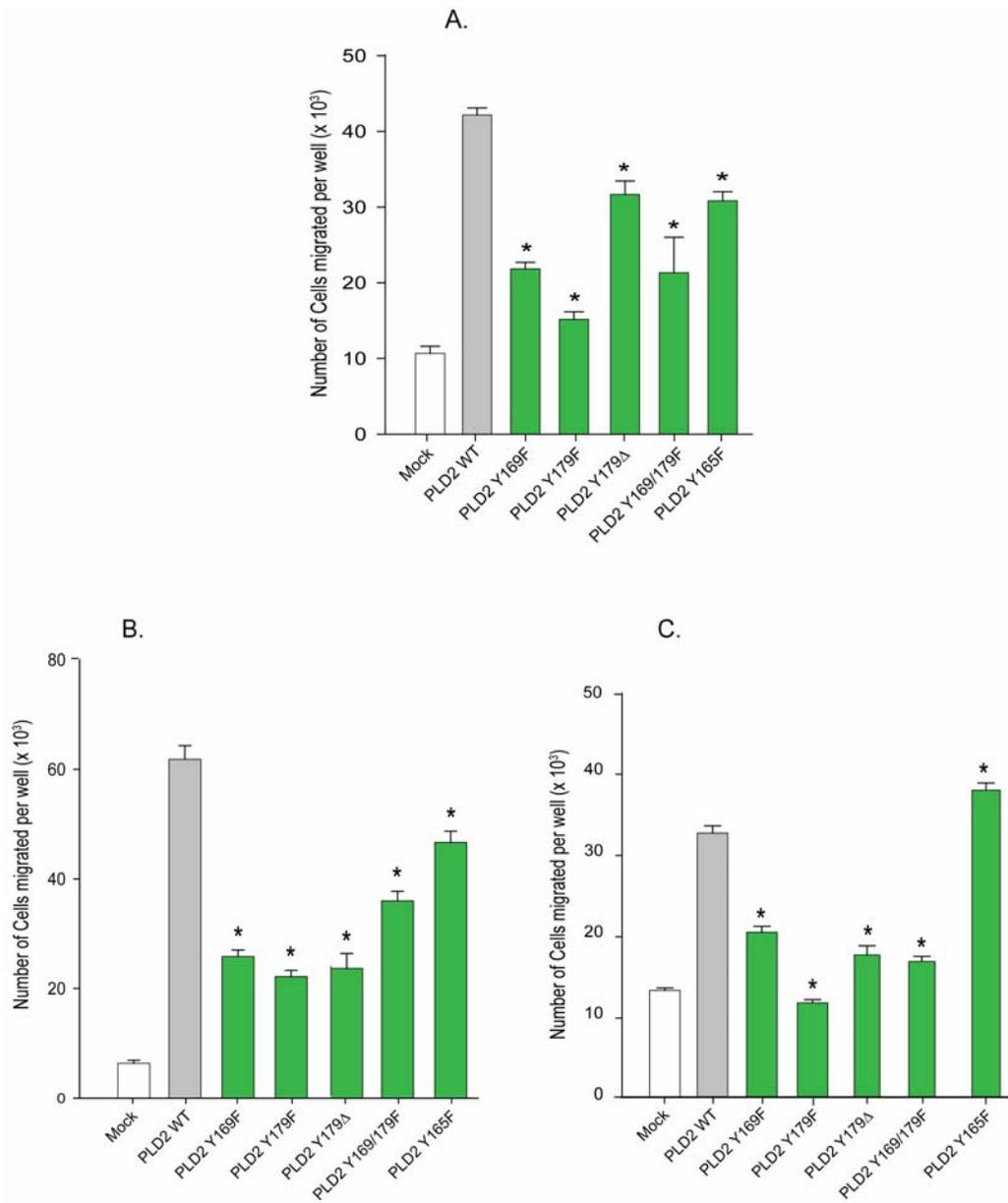
Figure 18:

Chemotaxis after cell transfection with PLD2 WT and YF mutants. (A) COS-7 cells (B) RAW 264.7 and (C) LR-5 cells. Values are the means $\pm$ the SEM of three experiments and asterisks indicate significant difference from values obtained with corresponding WT PLD (*, $p < 0.05$).

compared to PLD2 WT (Figure 18C). All mutants transfected into the RAW 264.7 and COS-7 cells had a chemotaxis result significantly less than PLD2 WT construct, but significantly greater than the mock.

B. Run controls of the YF mutants by immunoblotting and immunofluorescence.

Immunoblots of the YF mutants were performed to ascertain that chemotaxis was due to overexpression of the PLD2 constructs and not because of an unequal expression of protein (Figure 16B). Plasmids were compared with a mock transfection of COS-7 cells, which have little to no endogenous PLD. COS-7 cells were also transfected with PLD2 WT and PLD2 Y165F that express a yellow fluorescent protein (YFP) upstream of the PLD protein of interest (Figure 19). PLD2 is identified by the yellow portions around the nucleus.

C. Investigate whether enzymatic activity of PLD is necessary for chemotaxis

Enzymatic activity of PLD was determined using the procedure in the Materials and Method section of this thesis. PLD1 K830R has little to no enzymatic activity when compared to PLD1 WT (Figure 20), but its ability to chemotax is not significantly different than either of the PLD1 constructs in COS-7 and RAW 264.7 cells (Figure 15A and B). The reduced cell migration of the lipase inactive mutants of PLD2 may correlate to the significant decrease in enzymatic activity between these mutants and the PLD2 WT. Mutation on 165 does not affect the activity of the enzyme (and mutation does not effect chemotaxis). Mutations of the 169 and 179 amino acids are part of the SH2 binding motifs in the PX domain and it can be theorized that chemotaxis is mediated by the ability of PLD to bind through those sites. However, only mutation on 169 affects the enzymatic activity. Therefore, the effect of 169 on chemotaxis can be mediated through
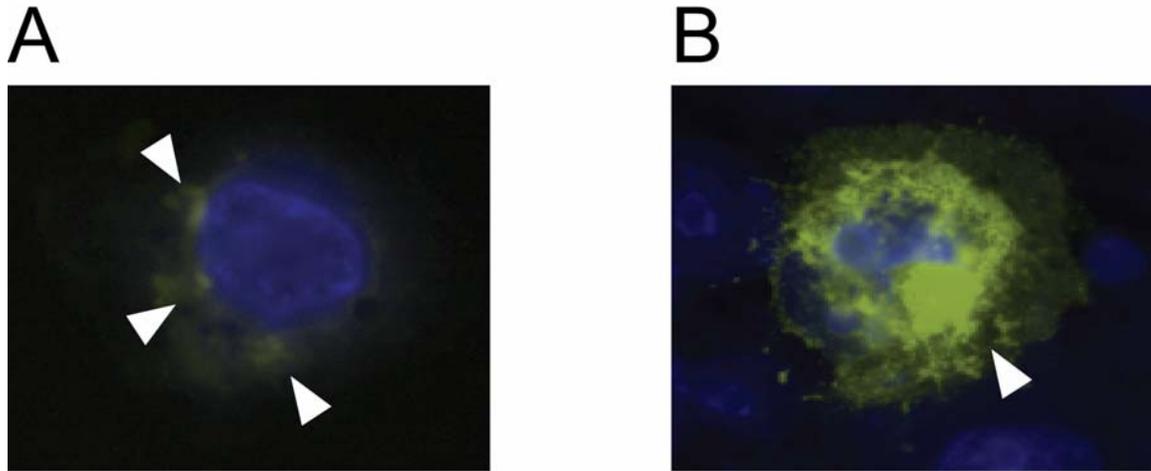
Figure 19:

Fluorescence of cells transfected with PLD chimeras. COS-7 cells transfected with (A) YFP-PLD2 WT and (B) YFP-PLD2 Y165F chimeras. Yellow fluorescence indicates PLD2 localization (white arrow heads). Blue is DAPI staining of the nuclei.
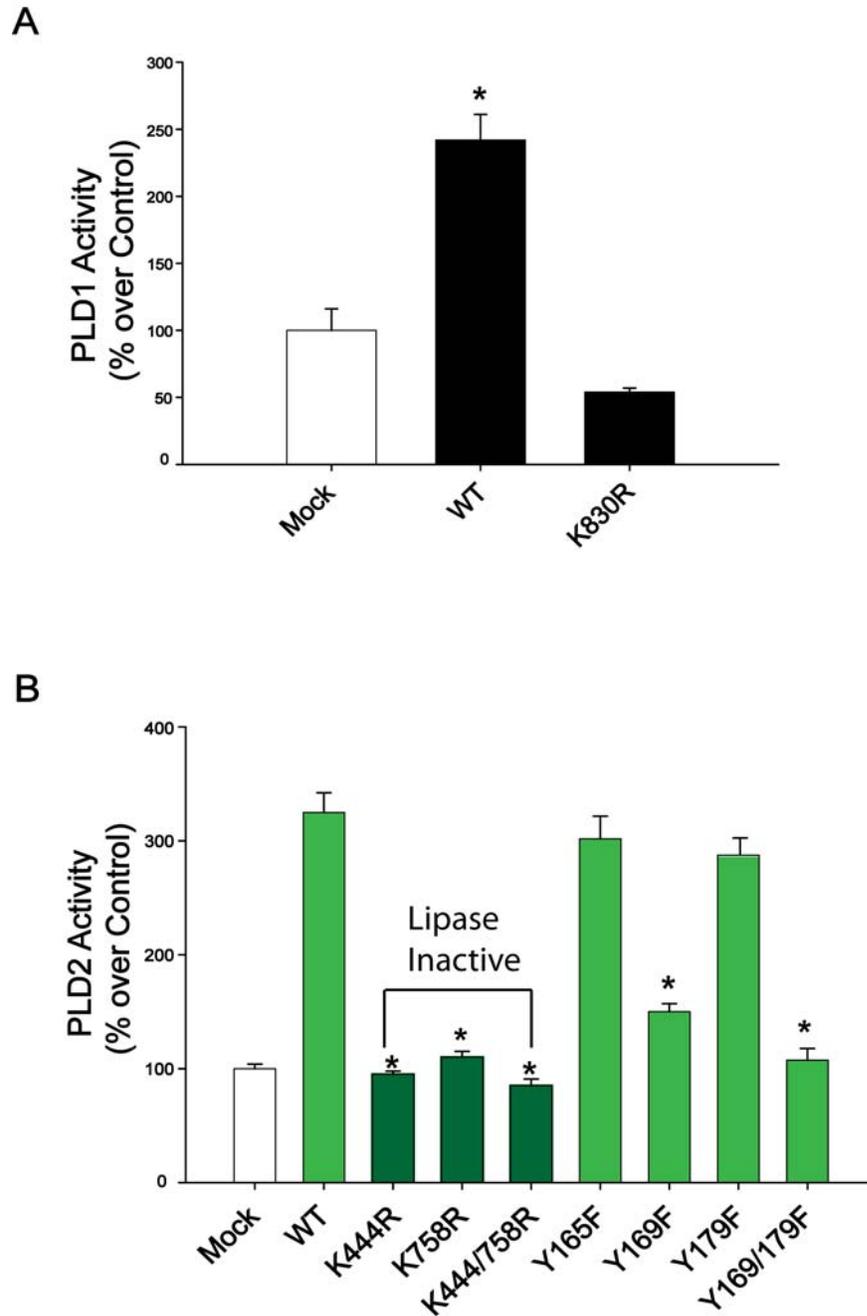
Figure 20:

PLD enzymatic activity of cells after transfection with PLD constructs. (A) PLD1 WT and KR and (B) PLD2 WT, KR, and YF constructs in COS-7 cells. In (A) * is p-value <0.05 with respect to Mock values. In (B) * are p-values <0.05 with respect to WT values.
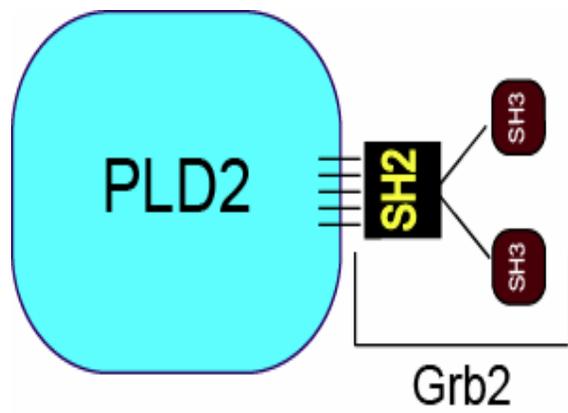
Figure 21:

Schematic representation of PLD binding to the SH2 region of the Grb2 protein.

PA while 179 mediates only protein-protein interactions.

AIM 4: Characterize a new protein that can associate with PLD and induce chemotaxis.

It was proposed that the 169 and 179 tyrosines of PLD2 served as the binding domain of an SH2 motif (Figure 20), such as that of the growth receptor binding domain 2 protein (Grb2) (Figure 21) (15, 28). The SH3 domain of Grb2 is able to recruit tyrosine kinases and phosphatases which have been found to be important to the functionality of PLD2 (33). The Grb2 protein has been found in many types of cancer possibly leading to an increased ability of a cell to metastasize.

A. Transfect cells with Grb2 constructs and study chemotaxis.

Western Blotting of Grb2 constructs were transfected equally to yield equal expression of exogenous Grb2, which is the upper band of Figure 16C while the lower band is endogenous Grb2. The exogenous Grb2 has a molecule weight due to the Xpress-tag on the Grb2 plasmids. β-actin immunoreactivity was used a loading control. In all 3 cell lines (Figure 22) we observed a consistently significant increase of chemotaxis after Grb2 transfection. Conversely, when LR-5 cells were transfected with a deficient SH2 domain, as with Grb2 R86K (Figure 22C), chemotaxis was completely negated similar to mock (Figure 22). The mock and Grb2 R86K results were not significantly different this indicates that at least a component of chemotaxis is dependent on Grb2.

B. Determine a possible association of PLD and Grb2 through immunoprecipitation and immunofluorescence.
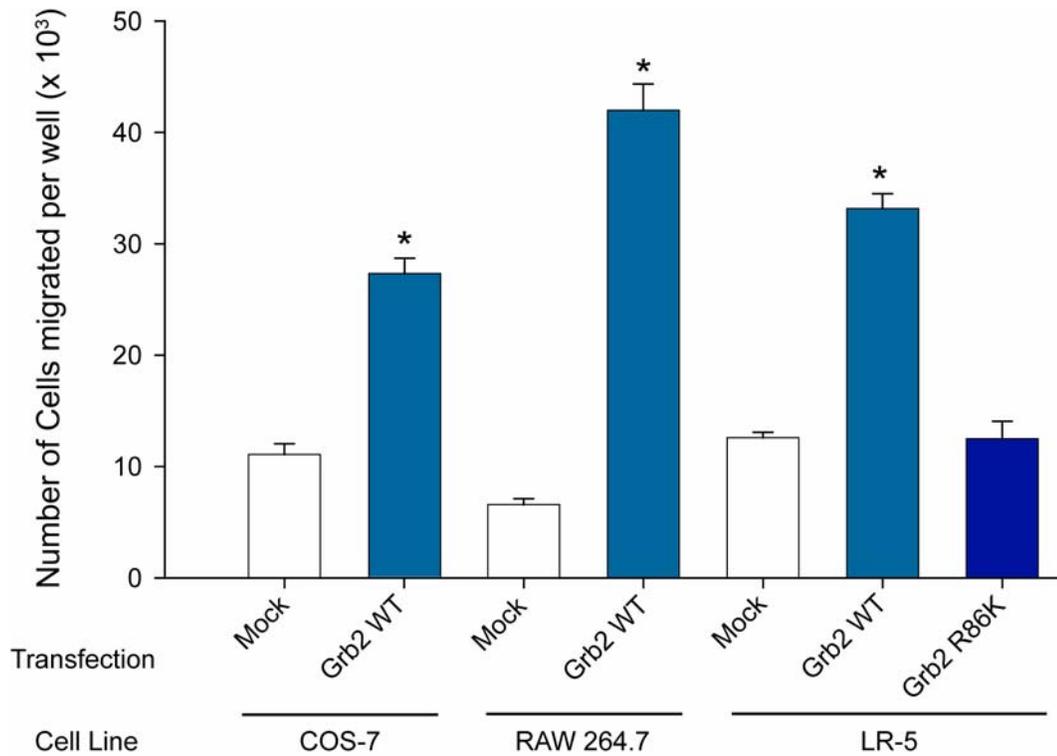
Figure 22:

Chemotaxis of cells after transfection with Grb2 constructs. COS-7, RAW 264.7, and LR-5 cells were used.   Values are the means $\pm$ the SEM of three experiments and asterisks indicate significant difference from values obtained with corresponding WT PLD (*, p-value are < 0.05 with respect to their Mock controls).
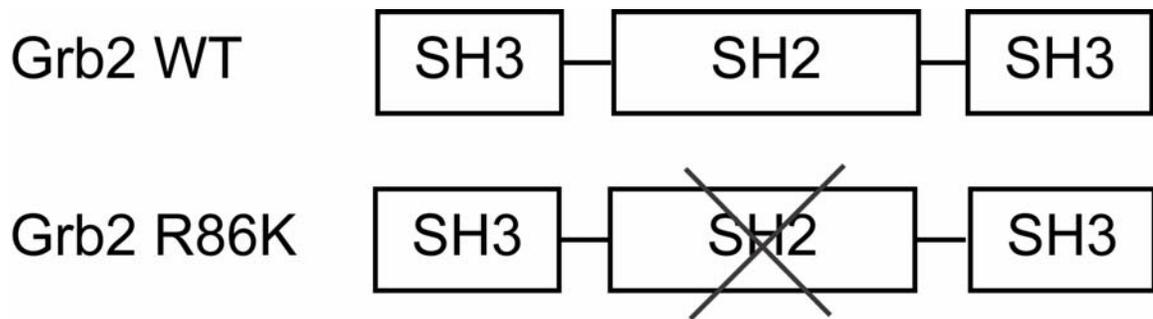
Figure 23:

Schematic representation of Grb2 WT and Grb2 R86K constructs (15).
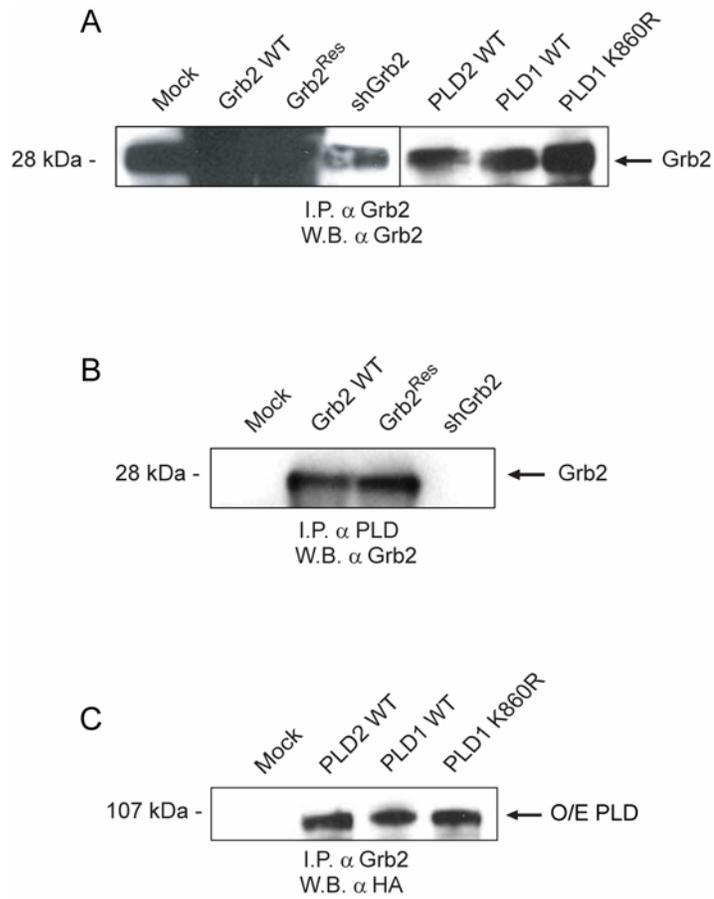
Figure 24:

COS-7 cells immunoprecipitated (IP) with (A) anti-Grb2 and western blotted for anti-Grb2; (B) IP with anti-PLD and western blotted for anti-Grb2; and (C) IP with anti-Grb2 and western blotted for anti-HA. O/E = overexpressed.

Cell lysates were prepared after transfection of PLD2 WT, PLD1 WT, PLD1 K830R constructs, as well as the Grb2-WT, Grb2^{Res} (a variation of Grb2, also WT) and shGrb2 (silencing) constructs. Immunoprecipitation (IP) was performed to detect a relationship between PLD(1 or 2) and Grb2. IP with a Grb2 antibody pulled down endogenous and overexpressed Grb2 in cells untransfected or transfected with recombinant Grb2 and then immunoprobed with Grb2 antibody (Figure 24A). Endogenous Grb2 was 100 % in the mock, whereas shGrb2 was approximately 70 % silenced. Endogenous Grb2 was also detected in lysates of cells transfected with PLD1 or PLD2 WT constructs and subsequently pulled down using the Grb2 antibody and then immunoprobed for Grb2 on a western blot (Figure 24A).

Cells transfected with Grb2 recombinants were immunoprecipitated with anti-PLD, which binds endogenous PLD, and were then immunoprobed with anti-Grb2 (Figure 24B). This indicates that endogenous PLD is able to bind, with greater affinity, to overexpressed Grb2 rather than endogenous Grb2, since there is evidence of Grb2 immunoreactivity in the Grb2 WT and Grb2^{Res} WT lanes only. IP of PLD2 did not pull down endogenous Grb2, as shown in the mock or shGrb2 transfected samples. Next, PLD2 WT, PLD1 WT and PLD1 K830R constructs were transfected into cells, which were then immunoprecipitated with a Grb2 antibody and immunoblotted with anti-HA (specific for overexpressed PLD2 or PLD1 proteins). Immunoreactivities here show binding between endogenous Grb2 and overexpressed PLD (Figure 24C). COS-7 cells were transfected with CFP-Grb2 WT and CFP-Grb2 R86K plasmids to show cellular localization of the Grb2 protein (Figure 25), which appears to localize in the cytoplasm (27).
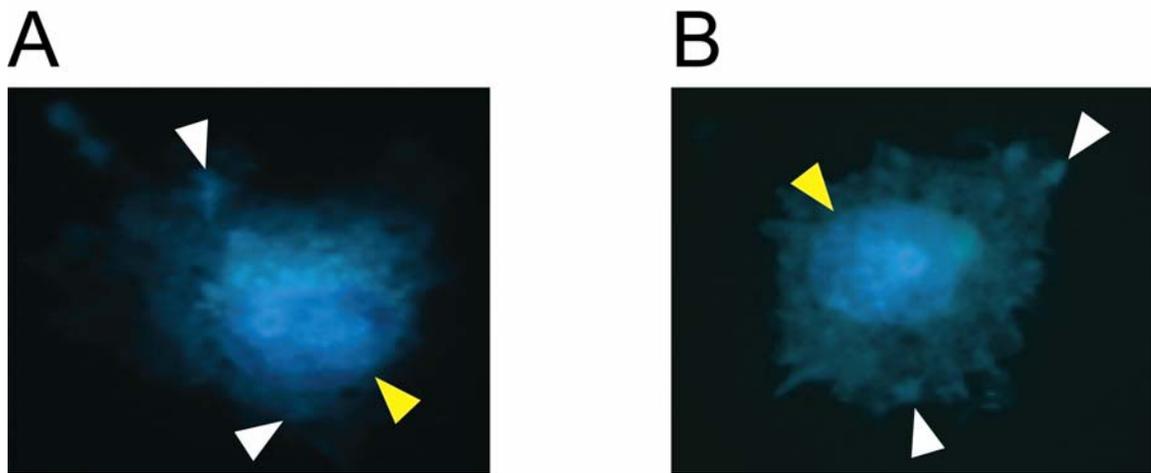
Figure 25:

Immunofluorescence of transfected of COS-7 cells with (A) CFP-Grb2 WT and (B) CFP-Grb2 R86K. Lighter blue area, Grb2, localizes in the cytoplasm of the cell (white arrowheads). Darker blue is DAPI staining of the nuclei (yellow arrowhead).
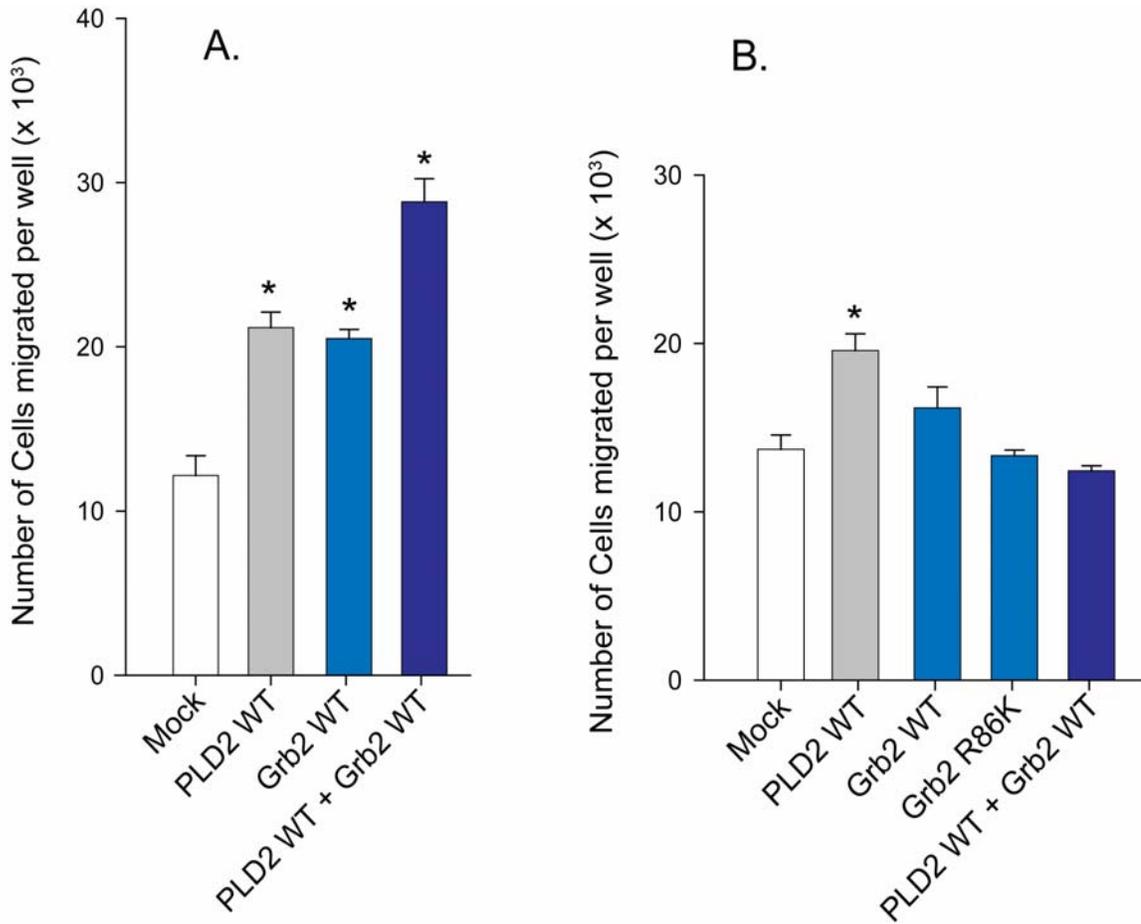
Figure 26:

Co-transfection (A) and Silencing (B) of cells, and chemotaxis in LR-5 cells. (* = Significance compared to Mock p<0.05)

C. Silence endogenous Grb2 using shGrb2 plasmid.

Grb2 is an SH2-bearing protein and we have observed that transfection of cells with Grb2 constructs leads to a significant increase in chemotaxis (Figures 22). In preliminary experiments, we wanted to ascertain if co-transfection of Grb2 and PLD2 would result in a non-additive or an additive or in a synergistic effect on chemotaxis. Figure 26A indicated that PLD2 and Grb2 result in a modest additive effect. Also preliminary experiments were performed to determine if, by removing, all of the endogenous Grb2 using shGrb2 whether chemotaxis would still occur. LR-5 cells were transfected with the shGrb2 plasmid two days prior to transfection with PLD2 WT, Grb2 WT, Grb2 R86K plasmids (Figure 26B). In Grb2 silenced cells, all samples were significantly less migratory than the PLD2 WT plasmid. The additive effect of PLD2 and Grb2 co-transfection observed in Figure 26A is negated in silenced cells (Figure 26B).

Through the experiments conducted here, we assume an association between PLD2 and Grb2. Grb2, a protein containing SH2 domains, when transfected into cells has significantly greater chemotaxis than the mock, which is only significantly less than that of PLD2 WT, unless they are co-transfected. By silencing Grb2, the above increase seen above with co-transfection is abrogated. During cell migration Grb2/PLD interaction is mediated by chemoattractants.

D. Proposed Model for participation of PLD in chemotaxis

Figure 27 presents our proposed model based on results in this thesis, along with those of other authors that could explain the participation of PLD and Grb2 in cell chemotaxis.
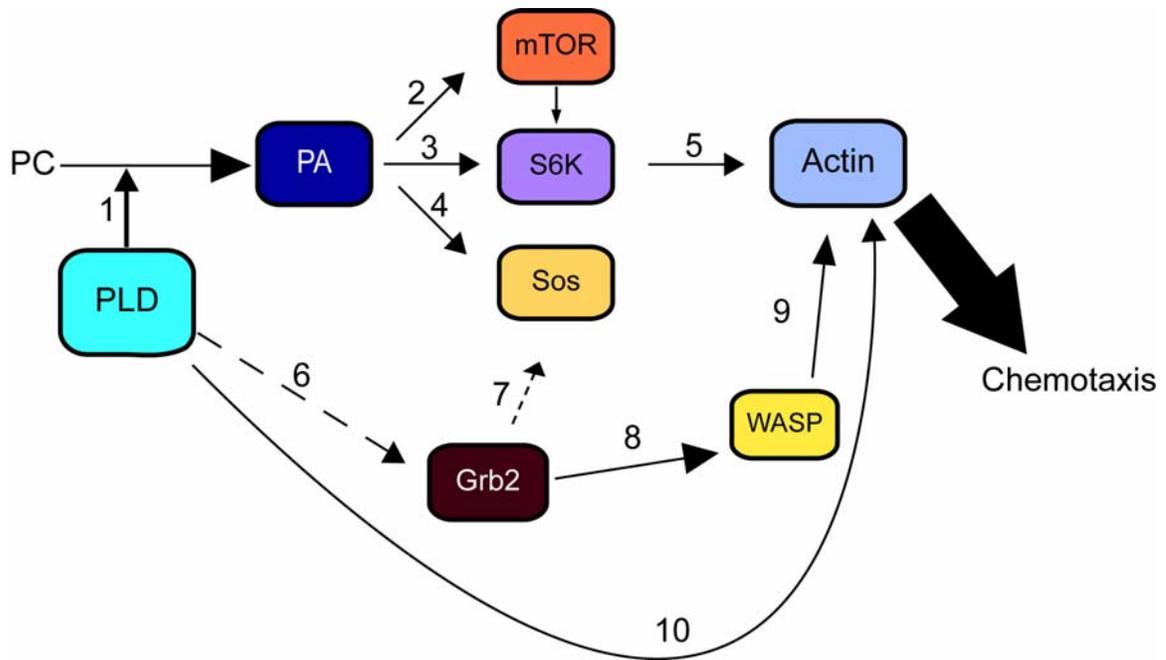
Figure 27:

Proposed model for the participation of PLD2 and Grb2 in chemotaxis. Three major pathways are considered here. First [1-5], PLD uses its activity to produce PA that binds to target proteins mTOR [2], S6K [3] or Sos [4] (60-62). S6K has been proposed to stimulate actin polymerization [5] (63). Results in this thesis seem to indicate that Y^{169} is involved in enzyme activity (PA production) leading to chemotaxis. Second [6-9], PLD, independently of its activity, can bind to either Sos or Grb2 [6, 7] (8, 15). Results in this thesis indicate that Y179 is involved in a PLD2-Grb2 protein-protein interaction irrespective of enzymatic activity. Grb2 can then possibly activate WASP [8] that also initiates actin polymerization [9]. Third, PLD can directly interact with actin [10] (64, 65).

V. CONCLUSIONS

1. We have established a model of cell migration comprising three cell lines, which compare favorable to neutrophils in regards to their chemotactic response: (a) RAW 264.7 cells, a macrophage cell line that responds to chemoattractants (MCP-1α and MIP-1α) in a dose and time dependent manner; (b) LR-5 cells, also a macrophage cell line that is more mobile than the parental RAW 264.7 cells; and (c) COS-7 cells, a fibroblast cell line that migrates toward EGF.

2. Transfection of cells (RAW 264.7), (LR-5), or (COS-7) with either PLD1 or PLD2 expression constructs leads to an increase in cell chemotaxis. PLD2 is better (≥ 1.5-fold) at inducing chemotaxis than PLD1.

3. Phospholipase inactive mutants in the HKD domain, particularly PLD2 KR constructs, are 2-fold less chemotactic than PLD2 WT, but not significantly different to mock transfected cells.

4. PLD2 has two functional tyrosines, (Y^{169} and Y^{179}) that, when mutated, lead to diminished chemotaxis when compared to PLD2 WT. Since these are mutations of the SH2 binding motifs in the PLD2 PX domain, we conclude that chemotaxis is mediated by the PLD's ability to bind other proteins through these sites. Further, the activity of the enzyme (production of PA) seems to involve only Y^{169}.

5. We have discovered that Grb2's SH2 domain interacts with PLD2. Transfection of cells with Grb2 leads to an augmentation of chemotaxis. The overall conclusion is that triggering of cell migration by chemoattractants is mediated by PLD, which requires an association with Grb2.

VI. DISCUSSION

The results presented in this thesis fall within the overall study of signal transduction molecules: PLD1, PLD2, and Grb2 and their interaction within a cell. Three different cell lines (COS-7, RAW 264.7, and LR-5) were examined to determine if an association of PLD and Grb2 exists during chemotaxis and if it is constant between different cell lines. We wanted to use cells that were easily amenable to transfection and could chemotax. The cell lines used are known to play key roles in wound healing during innate immunity. We used macrophages and fibroblasts to accomplish Aim 1 of this thesis. Fibroblasts are cells that synthesize collagen in the event of injury to the body and also maintain connective tissue in animals. As for macrophages, they are able to phagocytose debris and bacteria. Macrophages are cells that present antigens to other cells in order to form antibodies against them.

Within Aim 1, we used RAW 264.7 cells, which are able to be transfected through electroporation, but result in a significant amount of cell death. This issue has been resolved at the laboratory of Dr. D. Cox (Albert Einstein College of Medicine) by creating LR-5 cells, from RAW 264.7 cells, that stably express the lac repressor allowing them to be transfected using superfect. This new cell line has greatly reduced cell death using electroporation or lipid-based transfection strategies. All cell lines studied were able to chemotax within a Transwell, especially when tranfected with the PLD2 WT plasmid.

Chemoattractants are able to increase the ability of PLD2 to increase the chemotactic response within the cell lines studied. COS-7 cells are able to be stimulated by EGF, whereas RAW 264.7 and LR-5 cells are stimulated by MIP-1α and MCP-1 in

chemotactic assays. COS-7 cells achieve maximum chemotaxis when stimulated by EGF for one hour. Macrophages require three hours for cells to thoroughly migrate through the membrane of a Transwell to reach the chemoattractant in the bottom of the well.

Transfection of the PLD2 WT plasmid causes a 4-fold increase over the mock transfected in chemotaxis in the cell lines studied. The PLD2 KR constructs have significant decreases in chemotaxis compared to the PLD2 WT plasmid indicating that PLD activity affects chemotaxis. The method to how these cell lines are transfected are all different and may leave each cell line in a different state before the chemotaxis procedure is carried out. We observed that immunofluorescence of RAW 264.7 cells stimulated with MIP-1α or MCP-1 left macrophages in a compact migratory shape. Experimentation of COS-7 and LR-5 cells using immunofluorescence and chemoattractant stimulation would need to be conducted in order to test this idea.

Several PLD2 point mutants have been developed in our laboratory (15, 28) and their ability to enhance or suppress chemotaxis was studied. We have found that Y^{169} and Y^{179} residues are essential for the association of PLD2 with Grb2 and that this interaction is independent of PLD2 activity (15, 28). Mutation of $Y^{16}9$ (to Y169F) diminishes enzymatic activity and mutation of Y^{179} (to Y179F) diminishes the ability to regulate tyrosine phosphorylation (28). It has also been noted that Y^{169} and Y^{179} residues may recruit the Grb2/Sos complex which leads to the activation of Ras effectors and consequently phosphorylation of MEK through Raf (15, 58). The double mutant, Y169/179F, was found to be catalytically inactive and was not able to interact with the Grb2/Sos complex or activate Ras (28). Mutation of Y^{169} may disable its ability to activate the Ras/MAPK pathway since its tyrosine can no longer be phosphorylated.

More so, it has been reported that Y^{179} regulates total tyrosine phosphorylation of PLD2 and that it uses the Ras/MAPK pathway to negatively monitor cellular proliferation (15). However, Di Fulvio, *et al.* have demonstrated that the Y179F plasmid construct leads to an increase in DNA synthesis (58). Overexpression of Y179F increased Ras activation, but did not increase cellular proliferation; the mechanism for this is unclear (15). We have found that both Y169F and Y179F mutants are unable to confer an increase in chemotaxis. Since Ras is also able to activate migration, the PLD2 Y^{179}/Grb2/Sos complex may keep chemotaxis on hold in a similar fashion to how it keeps cellular proliferation in check, if this is indeed the mechanism that is followed (15). Neither of these mechanisms are fully understood.

By comparing the transient transfection of Grb2 constructs, specifically Grb2 R86K, we can assume that Grb2 plays a role in chemotaxis. Overexpressed Grb2 R86K, which cannot bind to PLD through its SH2 domain, is not significantly different from the mock, but it is significantly different compared to the PLD2 WT or Grb2 WT plasmids. It has already been shown that PLD2 binds to Grb2 through its SH2 domain (15, 28, 58). Cells coexpressing PLD2 WT and Grb2^{Res} in stably silenced COS-7 cells were found to redistribute in the perinuclear Golgi region indicating that PLD2 requires Grb2 to localize to the Golgi after EGF stimulation (28). There may be a signal in the Golgi that leads to cellular migration, which is why we see this relocalization after stimulation by EGF.

The effect of a chemoattractant to alter not only localization, but also cell shape is seen earlier with immunofluorescence of macrophages after MIP-1α or MCP-1 of PLD2 WT transient transfections. Figure 11 shows that macrophages transfected with PLD2 WT maintain their elongated shape, but after chemoattractant stimulation the

cytoskeletons change to a compact migratory shape. Results of the same nature are seen in macrophage morphology after transfection of Rac $1/2^{-/-}$ (57). Membrane ruffling in lamellipodia is induced through actin polymerization by Rac (57). The Grb2/Sos complex leads to activation of Ras, but it may also lead to activation of another GTPase, Rac, through which macrophage chemotaxis functions (59).

The results presented in this thesis show that PLD is able to affect other signaling pathways that regulate chemotaxis independently of PLD activity. The PLD2 Y165F mutant is not part of the SH2 binding motif of Grb2 and thus does not affect binding. We saw that transfection of PLD2 Y165F leads to a migration that is relatively equal between the three cell lines. Transfection of the PLD2 WT construct does not cause equal migration between the cell lines. PLD2 WT enhances chemotaxis the most in RAW 264.7 cells. In RAW 264.7 cells transfection of PLD2 WT causes more migration when compared to COS-7 and LR-5 cells. We determined that transfection of macrophages with PLD2 WT plasmid and stimulation with a chemoattractant changes their morphology, but we would need to do immunofluorescence with the other two cell lines to determine if a morphological change also occurs.

In relation to COS-7 cells, we know that fibroblasts, by nature, are a slower migratory cell which may explain of PLD2 WT has less of an effect on their migration (35). Two tyrosines on PLD bind to Grb2 through its SH2 motif. It is through this association that PLD has an enhanced chemotactic response. When we alter the enzymes ability to bind to Grb2 the chemotactic response created with the PLD2 WT construct is diminished. As seen throughout this thesis, a specific chemokine that is used against a

specific cell line is not enough to cause a cell to migrate; it requires a signal from an overexpressed protein to increase the rate of chemotaxis.

VII. SIGNIFICANCE OF STUDY

The first cell line utilized in this study was COS-7. COS-7 cells are fibroblast cells, which respond to the growth factor EGF (33). COS-7 cells were used in the chemotaxis experimental setting of this thesis in order to develop a model for wound healing. Fibroblasts synthesize collagen and maintain the structural integrity of connective tissue and we have found that EGF could also act as a chemoattractant. The second (RAW 264.7) and third cell (LR-5) lines utilized in this study are both macrophage-derived. Macrophages are antigen-presenting cells that have the ability to phagocytose debris and dead cells. Macrophages are monocytes that have migrated to tissues in the case of injury or foreign invasion and play central roles in the innate immune response. We have demonstrated that the three cell lines (COS-7, RAW 264.7 and LR-5) are capable of chemotaxing when placed in the presence of an appropriate stimuli.

Phospholipase D is an enzyme that has been found to play many roles within mammalian cells and is able to directly or indirectly alter several cell signals. In this study, we have found that overexpression of PLD, particularly that of the isoform PLD2, enhanced a cell's ability to chemotax. We have demonstrated also here that a protein-protein interaction between PLD2 and Grb2 enhance chemotaxis. Thus, PLD, signaling through Grb2, is a key regulator of the functionality of the three cells studied. This may play an important role in facilitating wound healing and innate defense capabilities of our body.

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