REGULATION OF FIBRONECTIN ASSEMBLY BY PLC-γ1

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BCR	B-cell receptor
CHX	cycloheximide
Ca_i^{2+}	intracellular calcium
DAG	diacylglycerol
DOC	deoxycholic acid
ECM	extracellular matrix
EDA	extra domain A
EDB	extra domain B
EGFR	epidermal growth factor receptor
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
GEF	guanidine nucleotide exchange factor
IP_3	inositol 1,4,5 trisphosphate
IGF-1	insulin-like growth factor 1
MMP	matrix metalloproteinase
MAPK	mitogen-activated protein kinase
NFAT	nuclear factor activator of transcription
PBS	phosphate buffered saline
PDGFR	platelet-derived growth factor receptor
PH	pleckstrin homology
PIP_2	phosphotidylinositol 4,5-bisphosphate

PI-PLC	phosphoinositide specific phospholipase C
PLC-γ1	phospholipase C gamma 1
PMA	phorbol 12-myristate 13-acetate
RTK	receptor tyrosine kinase
SH2	Src homology 2
SH3	Src homology 3
siRNA	small interfering RNA
TCR	T-cell receptor
U73122	1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione
VEGFR	vascular endothelial growth factor receptor
V-region	variable region

CHAPTER I

INTRODUCTION

The focus of this dissertation is to determine the mechanistic role of PLC-γ1 in cellular adhesion and migration. The introduction contains an extensive review of PLC-γ1 literature, as well as an overview of the biology of cell adhesion and integrin signaling.

PLC-γ1 Family

Phospholipase C-γ1 (PLC-γ1) is a member of the phosphoinositide specific phospholipase C family that catalyzes the hydrolysis of plasma membrane phosphotidylinositol 4,5 – bisphosphate (PIP_2) to form the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG)(Figure 1). The former stimulates the release of Ca_i^{2+} from the endoplasmic reticulum and the latter signals downstream to activate protein kinase C. This enzyme family is composed of subfamilies denoted PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ and PLC-η and several of these contain multiple isoforms.

All members of the PLC family contain X and Y catalytic subdomains, but differ in the content of the other domains contained in the enzyme (Figure 2). The PLC-β subfamily consists of isozymes β1-β4 and contains a long 400 amino acid carboxy-terminal tail that is required for activation by the Gα subunit of heterotrimeric G proteins(Lee *et al.*, 1993; Wu *et al.*, 1993; Choi *et al.*, 2007).

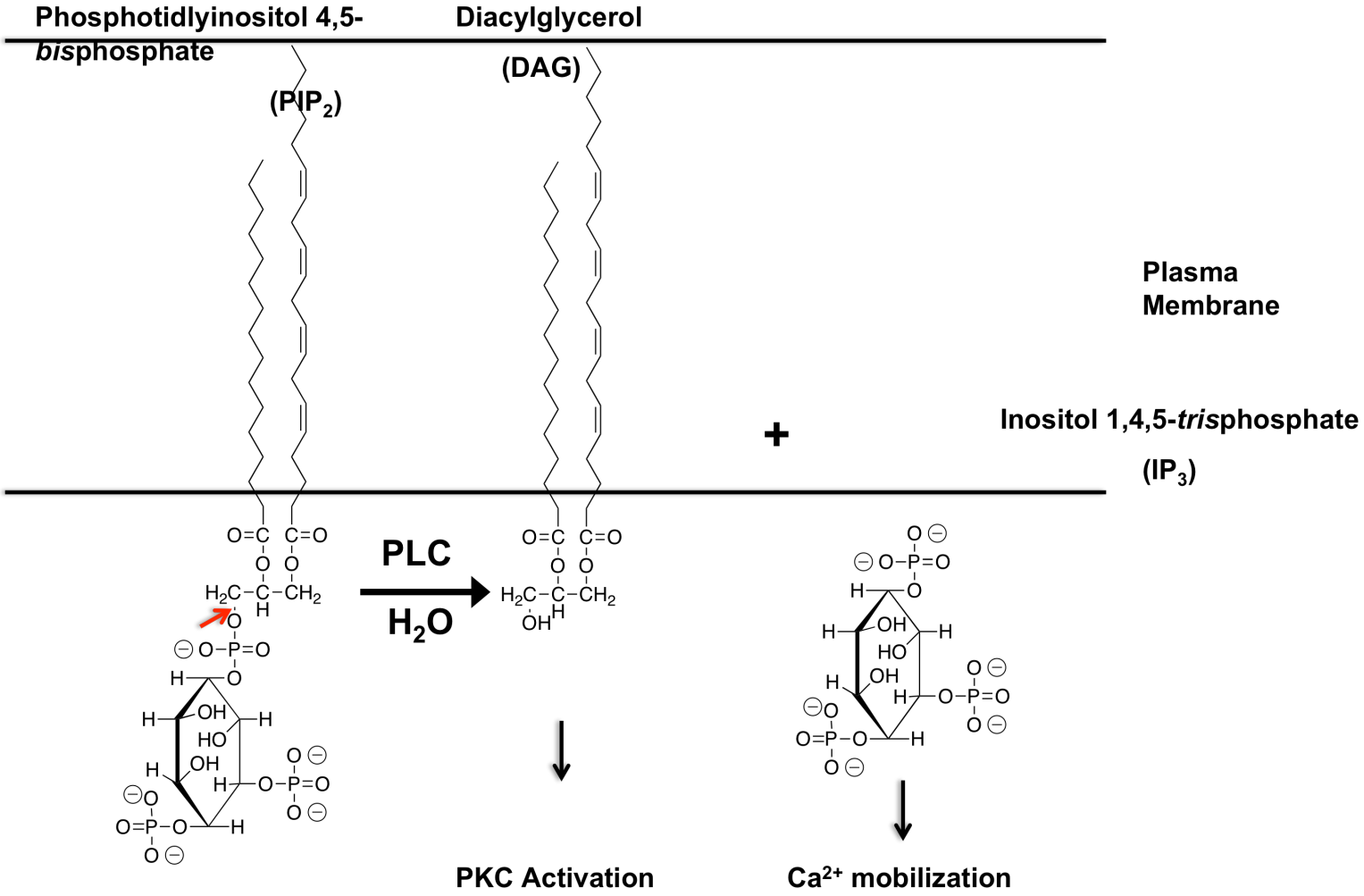


Figure 1. Hydrolysis of phosphotidylinositol 4,5 bisphosphate ($PIP_{2)}$ by phospholipase C (PLC) generates the second messengers diacylglycerol (DAG) and inositol 1,4,5 trisphosphate. DAG activates protein kinase C (PKC) and IP_3 stimulates the release of calcium from the endoplasmic reticulum. The red arrow on PIP_2 represents the site of hydrolysis.

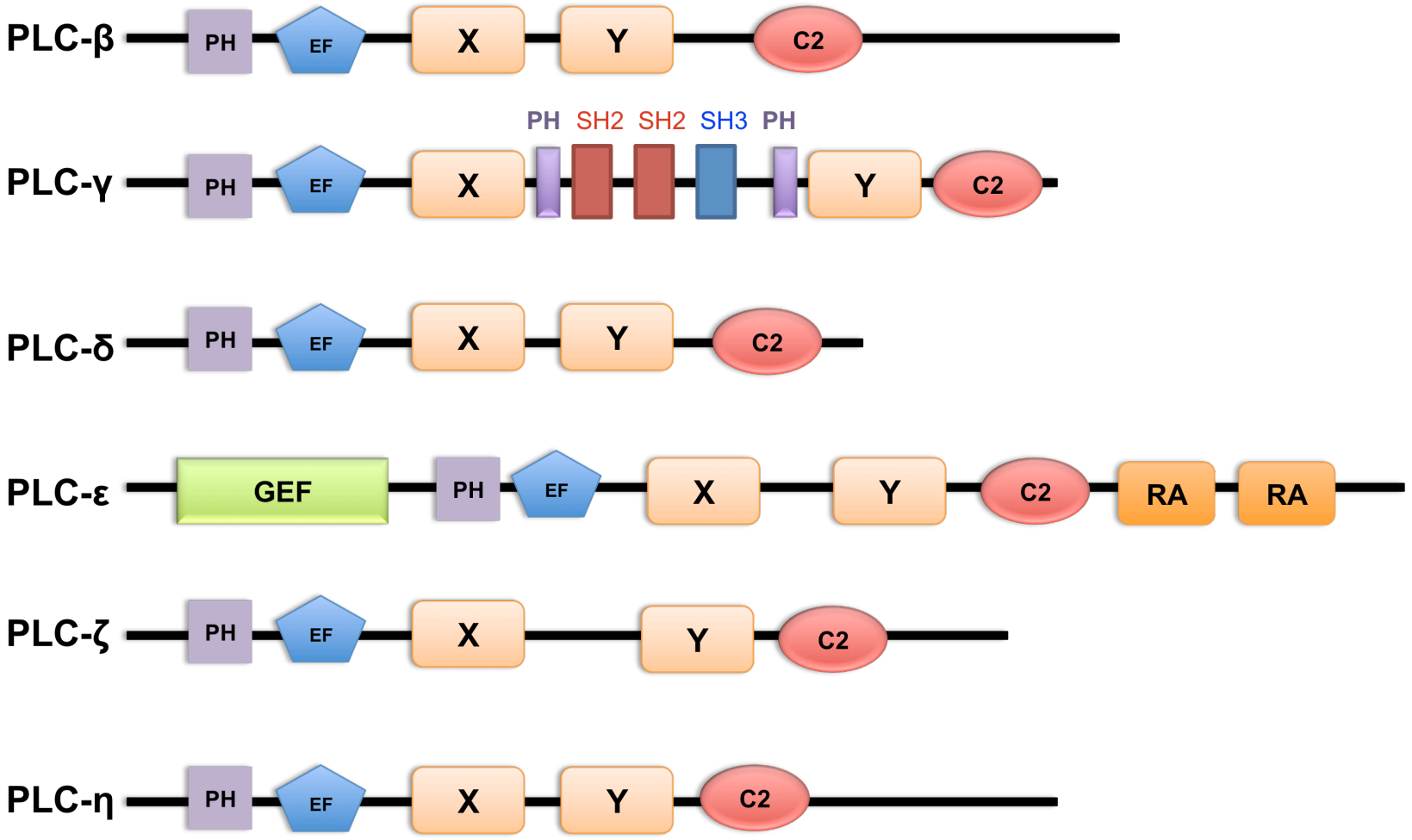


Figure 2. Domain structure of phospholipase C subfamilies. Abbreviations: PH, pleckstrin homology domain:EF, EF hand domain: X and Y catalytic subdomains: SH2, Src homology domain 2: SH3, Src homolgy domain 3: GEF, guanidine nucleotide exchange factor domain: RA, Ras association domain. Adapted from Choi et al. 2007 .

The PLC-δ subfamily is comprised of δ1-δ4 and is activated by changes in the Ca_i^{2+} concentration (Allen *et al.*, 1997). PLC-ε is a very unique family member in that it contains a guanine nucleotide exchange domain and two Ras associated domains, which allow for regulation by H-Ras, Rho, and $G\alpha_{12/13}$. In addition, the GEF (guanidine nucleotide exchange factor) domain of PLC-ε mediates GEF activity towards Rap1 independent of phosphoinositide hydrolysis (reviewed in (Wing *et al.*, 2003)). PLC-ζ is required for Ca^{2+} oscillations in sperm (Saunders *et al.*, 2002) and PLC-η is a neuron specific subfamily that includes isoforms η1 and η2 (Hwang *et al.*, 2005; Nakahara *et al.*, 2005).

The PLC-γ subfamily consists of the γ1 and γ2 isozymes, is activated by tyrosine kinases, and differs from other PLC family members in that it contains two Src homology 2 (SH2) domains and one Src homology 3 (SH3) domain flanked by a split pleckstrin homology (PH) domain (Rhee, 2001). PLC-γ1 and PLC-γ2 have about 50% sequence identity and contain the same structural organization. PLC-γ1 is ubiquitously expressed, while PLC-γ2 is limited to B-cells, platelets and a few other hematopoietic cells.

Both PLC-γ isozymes can be phosphorylated and activated by tyrosine kinases, such as Src and PDGFR (platelet derived growth factor receptor), and are also activated downstream of integrin activation (Sultzman *et al.*, 1991; Kanner *et al.*, 1993; Liao *et al.*, 1993; Blake *et al.*, 1994; Tvorogov *et al.*, 2005). This does not mean that the two isoforms are redundant. In B-cells and natural killer cells, which express both isozymes, loss of PLC-γ2 prevents maturation (Roifman and Wang, 1992; Hashimoto *et al.*, 2000; Wang *et al.*, 2000; Regunathan *et al.*, 2006). Lack of PLC-γ2 in platelets results in defective collagen induced platelet aggregation (Wang *et al.*, 2000). While it could be

argued that the defects seen in the *plcg2 -/-* cells are due to a decrease in total PLC-γ, overexpression of PLC-γ1 provides only partial rescue (Regunathan *et al.*, 2006). This evidence indicates that PLC-γ1 and PLC-γ2 are not redundant.

Structure of PLC-γ1

Much of the available knowledge regarding PLC-γ1 structure has been derived from the structure of PLC-δ (Essen *et al.*, 1996). All PLC isozymes contain two conserved domains, known as the X and Y catalytic subdomains, which are required for hydrolysis of PIP_2. The X and Y subdomains together form a triosephosphate isomerase-like barrel domain that contains the active site. Two histidine residues required for hydrolysis, His311 and His356, project side chains into the active site (Essen *et al.*, 1996; Ellis *et al.*, 1998). Other residues found within the hydrophobic ridges of the active site confer the requirement for Ca^{2+} and phosphoinositide specificity. PI-PLC isozymes require Ca^{2+} as a cofactor at the active site. The Ca^{2+} ion functions to lower the pKa of the 2-hydroxyl group of inositol to facilitate hydrolysis of PIP_2. The two catalytic subdomains, the C2 domain, and the EF hand domains all pack together to form the multidomain core, with the C2 domain acting as a bridge between the X:Y catalytic domain and the EF hand. The EF hand domain is composed of four EF hand motifs. It is not known whether these motifs actually bind Ca^{2+}, but the second EF hand is required for association with the C2 domain (Williams and Katan, 1996).

PLC-γ is unique in the PLC family in that it contains two SH2 domains and one SH3 domain flanked by a split PH domain (Figure 2). The SH2 domains are required for enzyme regulation in several ways. First, the SH2 domains associate with phosphorylated

tyrosines on receptor and non-receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) and Src (Rotin *et al.*, 1992; Ji *et al.*, 1999). Each of the N-and C-SH2 domains can individually recognize and bind phosphorylated receptors, but both SH2 domains are required for maximal activation and hydrolysis (DeBell *et al.*, 1999; Ji *et al.*, 1999). The second way in which the SH2 domain regulates activity is through intramolecular association with the split PH domain(DeBell *et al.*, 2007). In T-cells, association of the C-terminal SH2 domain with the N-terminal half of the split PH domain is required for negative regulation of the enzyme. Mutations that block this interaction constitutively activate the enzyme (DeBell *et al.*, 2007).

The SH3 domain mediates association with proline-rich sequences in proteins such as Cbl (Tvorogov and Carpenter, 2002; Graham *et al.*, 2003), dynamin 1 (Seedorf *et al.*, 1994), PIKE (Ye *et al.*, 2002) and FAKB (Kanner, 1996).The SH3 domain of PLC-γ1 has been reported to have GEF activity towards PIKE, a GTPase (Ye *et al.*, 2002). Other than this, the physiological significance of the SH3 domain is not well understood in terms of PLC-γ1 function.

PLC-γ1 contains two PH domains that confer specific binding to phosphoinositides as well as proteins. The N-terminal PH domain is responsible for membrane targeting and specifically binds the membrane phospholipid PIP_3. PI-3 kinase phosphorylates PIP_2 to generate PIP_3, which is reported to stimulate the translocation of PLC-γ1 to the membrane in some cells (Bae *et al.*, 1998; Falasca *et al.*, 1998). In addition, the N-terminal PH domain has been reported to bind proteins, such as β-tubulin (Chang *et al.*, 2005) and the neurofilament light chain; however, the significance of these interactions has yet to be elucidated (Kim *et al.*, 2006).

The split PH domain of PLC-γ1 flanks the SH2 and SH3 domains. While the split PH domain folds into a canonical PH domain, it does not bind phospholipids (Wen *et al.*, 2006). Instead, it has been proposed that this domain plays a role in PLC-γ1 enzyme activation. The idea that domains within PLC-γ1 negatively regulate enzyme activity first emerged after limited proteolysis of the enzyme resulted in stable association of the X and Y catalytic subdomains with an increased level of enzymatic activity (Fernald *et al.*, 1994). More recently, it has been shown that association of the C-terminal SH2 domain with the N-terminal portion of the split PH domain confers a regulatory mechanism. Mutations in the N- terminal split PH domain that abolish this interaction remove the phosphorylation requirement for activation (DeBell *et al.*, 2007). In addition to regulating PLC-γ1 enzyme activation, the split PH domain binds to other proteins, such as TRPC3, a receptor involved in agonist-induced Ca^{2+} entry, to mediate its cell surface expression (van Rossum *et al.*, 2005). The split PH domain has also been reported to bind elongation factor-1 alpha, although the function of this has not been determined (Chang *et al.*, 2002).

Activation of PLC-γ1

There are two main pathways for activation of PLC-γ1 in non-hematopoetic cells (Figure 3). In the first pathway, PLC-γ1 is activated through the association and tyrosine phosphorylation by an activated receptor tyrosine kinase. In the second pathway, integrins are activated through adhesion to the extracellular matrix. The activated integrin stimulates the activation of the non-recptor tyrosine kinase Src, which phosphorylates PLC-γ1 and activates the enzyme.

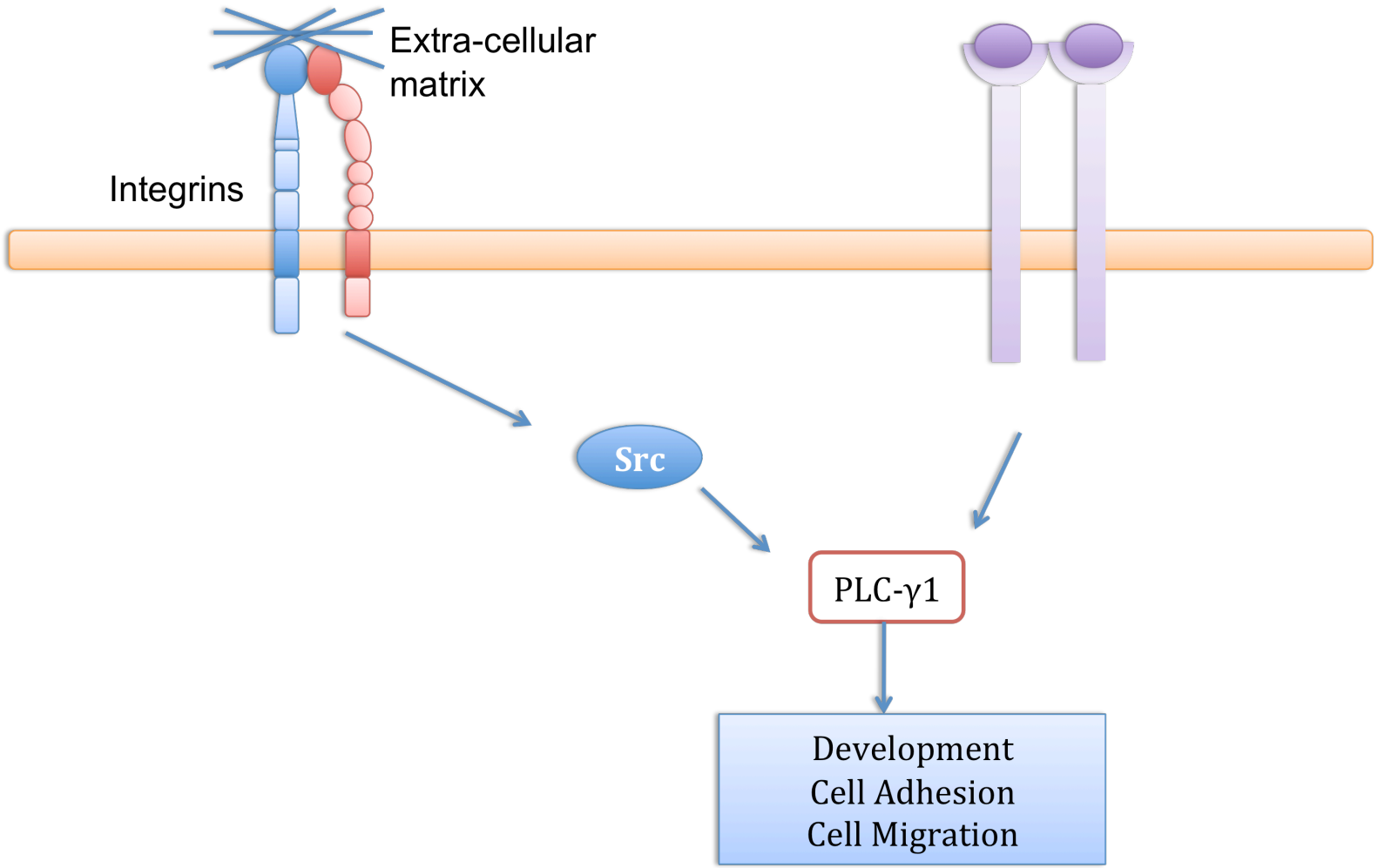


Figure 3. Activation of PLC-γ1 through integrin and receptor tyrosine kinase signaling

Stimulation with a growth factor results in the activation of the cognate receptor tyrosine kinase and subsequent phosphorylation and activation of PLC-γ1. EGFR (Wahl *et al.*, 1989a), PDGFR (Wahl *et al.*, 1989b), the Trk protooncogene (Vetter *et al.*, 1991), and Flk-1 (Takahashi and Shibuya, 1997) are examples of an extensive number of receptor tyrosine kinases that activate PLC-γ1. In contrast, the insulin receptor is unique in that it does not associate with nor stimulate phosphorylation of PLC-γ1 (Meisenhelder *et al.*, 1989; Nishibe *et al.*, 1990).

An activated tyrosine kinase can phosphorylate PLC-γ1 in several ways. First, The SH2 domains of PLC-γ1 bind directly to a phosphotyrosine residue on the activated tyrosine kinase. The SH2 domains on PLC-γ1 are specific for certain phosphotyrosine residues on certain receptors. For example, Y1021 and Y1009 of the PDGFR and Y1169 of the vascular endothelial growth factor receptor-1 (VEGFR1) are the major binding sites for PLC-γ1 (Valius *et al.*, 1993; Sawano *et al.*, 1997). The EGFR is an exception to this. No individual tyrosine residue on the EGFR is specific for PLC-γ1 or other SH2-containing molecules, although certain tyrosine residues are required for downstream cell responses, such as membrane ruffle formation (Sorkin *et al.*, 1992; Soler *et al.*, 1994; Nogami *et al.*, 2003).

PLC-γ1 can be phosphorylated on tyrosine residues 472, 771, 775, 783, and 1254 (Figure 4). Phosphorylation of tyrosine 472 has mainly been reported in vitro (Kim *et al.*, 1990) and phosphorylation of tyrosine 775 has been limited to immune receptor stimulation (Serrano *et al.*, 2005). Tyrosines 771, 783, and 1254 are all phosphorylated in response to EGF or PDGF stimulation (Kim *et al.*, 1990; Wahl *et al.*, 1990) but only

Y783 is absolutely required for activation of the enzyme (Kim *et al.*, 1991; Sekiya *et al.*, 2004).

The PLC-γ1 Knockout Mouse

Plcg1-/- mice were generated in 1997 (Ji *et al.*, 1997). The mutant mouse was embryonic lethal at around embryonic day 8.5 due to deficiencies in vasculogenesis and erythrogenesis, (Ji *et al.*, 1997; Liao *et al.*, 2002). Both of these deficiencies are likely to be due to the inhibition of signaling downstream of the Flk-1 VEGF receptor as mutations of the PLC-γ1/ MAPK binding site of Flk-1 also produced similar defects in vasculogenesis and erythrogenesis (Sakurai *et al.*, 2005). Because the *PLCg1* Null mice were embryonic lethal at such an early time point, and because PLC-γ1 is ubiquitously expressed, mouse embryonic fibroblasts were isolated from the Null mice for further study and immortalized using the 3T3 method (Ji *et al.*, 1998; Xu, 2005).

PLC-γ1 in Adhesion and Migration

The role for PLC-γ1 in adhesion and migration (Table 1) is probably the best described function for this protein. In the absence of PLC-γ1, cells are deficient in both the adhesion to fibronectin and the migration towards fibronectin through an unknown mechanism(Tvorogov *et al.*, 2005). The deficiency in adhesion is both time and concentration-dependent. At low concentrations of fibronectin, two-fold fewer *Plcg1* Null cells adhere to fibronectin than an add-back cell line.

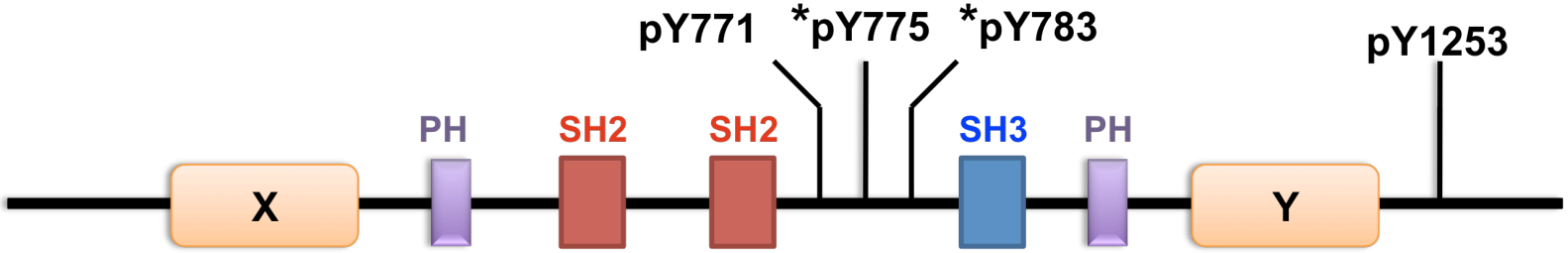


Figure 4. Tyrosine residues that are phosphorylated on PLC-g1. Y771, Y783, and Y1253 are phosphorylated after growth factor or T-cell stimulation. T-cell stimulation also results in phosphorylation on Y775. Asterisks * denote tyrosine phosphorylation that are required for enzymatic activity.

As the concentration of fibronectin substrate increases or the time of the adhesion assay increases, this difference in PLC-γ1-mediated adhesion is lost. This is not due to a difference in integrin expression, nor is there any difference in integrin clustering. In human prostate carcinoma cells, PLC-γ1 has been shown to regulate the expression of Rap 1 GEF, which activates the Rap 1 GTPase to promote cell adhesion(Peak *et al.*, 2008).

The deficiency in migration has been observed in multiple cell types through the activation of both integrins and receptor tyrosine kinases (Kundra *et al.*, 1994; Wells and Grandis, 2003; Tvorogov *et al.*, 2005). Several studies have attempted to define a mechanism for this, but none have provided a clear picture.

In Tvorogov *et al,.* 2005, *Plcg1* Null and Null + cells were stained with phalloidin to visualize actin stress fibers after spreading for two hours on fibronectin coated coverslips (Tvorogov *et al.*, 2005). The Null cells formed dense actin stress fibers while the Null + cells formed lamellipodia. These results were somewhat reminiscent of the work done by the Hall group in which Rho activation leads to stress fiber formation and Rac activation leads to lamellipodia (Hall, 1998). The role of PLC-γ1 in the regulation of GTPases has been investigated with varying results. In one study, a truncated 120 kDa, nuclear form of PLC-γ1 was reported to act as a guanine nucleotide exchange factor (GEF) for the small GTPase PIKE (PI3Kinase enhancer) and this functions to enhance mitogenesis (Ye *et al.*, 2002). In another study, the siRNA-mediated knockdown of PLC-γ1 decreased the levels of RAP GEF 1 and thus modulated activation of Rap 1 and adhesion of a prostate cancer cell line.

Table 1. Reports of PLC-γ1 in Adhesion and Migration

Effect of PLC-γ1	Model/ Cell Type	Treatment	References
Localization to focal adhesions	Endothelial Cells, Fibroblasts		(McBride, 1991 Plopper, 1995)
Required for Adhesion to ECM	Human Prostate Carcinoma	PLC-γ1 siRNA	(Peak ,2008)
Required for Adhesion to ECM	*Plcg1* Null/ Null + MEFs,		(Tvorogov 2005)
FAK Association During Adhesion	Hepatic Stellate Cells		(Carloni 1997)
Binds Integrin β1	CHO cells		(Vossmeyer ,2002)
Required for Migration	CHO cells	Mutated PLC-γ1 binding site of *PDGFR*	(Kundra ,1994)
	Plcg1 Null/ Null + MEFs		(Tvorogov, 2005; Jones 2005)
Required for Tumor Metastasis	Prostate Tumor Cells	Z peptide and U73122 treatment	(Kassis , 1999; Mamoune , 2004; Shepherd, 2006)
	Glioma Cells	Z fragment and U73122 treatment	(Khoshyomn , 1999)
	Breast Cancer Cells	Z fragment and U73122 treatment	(Shepard , 2006; Kassis., 1999)
	Head and Neck Cancer Cells	U73122	(Nozawa , 2008; Thomas, 2003)
	Head and Neck Cancer Cells	Z fragment	(Shepard , 2007)

Last is the report that PLC-γ1 negatively regulates Rac and CDC42 signaling in stimulated mast cells in a Ca^{2+} and PKC-dependent manner, thus facilitating changes in the actin cytoskeleton (El-Sibai and Backer, 2007). All of these reports depict a role for PLC-γ1 in the positive or negative regulation of small GTPases either through acting as a guanine nucleotide exchange factor (GEF) or by modulating the expression of a regulator protein. PLC-γ1 has been shown to regulate the expression of the immediate early genes *FIC*, *Cox2*, *KC*, *JE*, and *cFos,* which function as regulators of a variety of cell signaling pathways including gene transcription and mitogenesis (Liao *et al.*, 2001; Liao *et al.*, 2006).

PLC-γ1 in Cancer

Because there is a requirement for PLC-γ1 in adhesion and migration in many cell types, there has been much investigation as to what role PLC-γ1 might play in the progression of cancer. Over-expression of PLC-γ1 has been observed in breast cancer (Arteaga *et al.*, 1991) and colorectal cancer (Noh *et al.*, 1994; Lee *et al.*, 1995). Functionally, PLC-γ1 is required for tumor metastasis of multiple tumor cell types including breast (Kassis *et al.*, 1999; Shepard *et al.*, 2006), glioma (Khoshyomn *et al.*, 1999), head and neck (Thomas *et al.*, 2003; Shepard *et al.*, 2007; Nozawa *et al.*, 2008), ovarian (Sewell *et al.*, 2005), and prostate (Turner *et al.*, 1997; Kassis *et al.*, 1999; Mamoune *et al.*, 2004; Shepard *et al.*, 2006; Peak *et al.*, 2008) (Table 1). The studies that have identified a role for PLC-γ1 in these processes have all utilized various inhibitors of PLC-γ1 that are discussed in more detail in the chapter IV section on experimental models. Only one study identifies a mechanism for PLC-γ1 regulation in cancer and that is a role for regulation of the expression of Rap1 GEF, which activates Rap1 to stimulate

adhesion (Peak *et al.*, 2008). Although the mechanism for PLC-γ1 regulation of metastasis is not known, we can assume that it would be fairly similar to that of the integrin-mediated cell migration.

Integrins

Integrins are transmembrane receptors that bind extracellular matrix molecules and provide a link between the ECM and the cell cytoskeleton. Integrins function as heterodimers composed of one α and one β subunit. At present, there are 18 known α and 8 known β subunits that form 24 heterodimers through noncovalent interactions (Takada *et al.*, 2007). A hallmark of integrin biology is the ability of each integrin to recognize multiple specific ligands. Integrin heterodimers are not functionally redundant (Plow *et al.*, 2000). For example, both integrins α5β1 and α3β1 bind fibronectin. But of those two, only integrin α5β1 will assemble fibronectin into fibrils (Mao and Schwarzbauer, 2005).

Integrins transmit signals through the plasma membrane bidirectionally. Inside-out signaling results in a conformational change in the integrin that enables ligand binding and subsequent outside-in signaling (Luo *et al.*, 2007). One major example of inside-out signaling is through the protein, talin. The "head" of talin is thought to bind the cytoplasmic domain of the β1 integrin and disrupt the salt bridge between the α and β integrin subunits (Calderwood *et al.*, 1999; Vinogradova *et al.*, 2002; Tadokoro *et al.*, 2003; Calderwood, 2004). This results in a conformational change in the integrin heterodimer from a state of low affinity matrix binding to one of high affinity. Once in a state of high affinity, the integrin binds ligand and transmits signals across the plasma membrane. Integrin signaling mediates many cell actions such as adhesion, migration,

and fibronectin assembly. PLC-γ1 is activated downstream of integrin signaling and regulates cell adhesion and migration (Tvorogov *et al.*, 2005).

Fibronectin

In fibroblasts, integrin mediated adhesion to fibronectin results in the tyrosine phosphorylation of PLC-γ1 as well as an array of other signaling events. Fibronectin is a fibrous extracellular matrix protein that is secreted from a variety of cells and assembled into matrix by integrins α5β1 and αvβ3 (Leiss *et al.*, 2008). Regulation of fibronectin secretion is best described at the mRNA level. The fibronectin promoter contains a cAMP responsive element (CRE) in addition to SP1, AP2 and EGR1 sites (Kornblihtt *et al.*, 1996; Liu *et al.*, 2000). The CRE element is responsible for both basal and serum-stimulated transcription, while the EGR1 transcription factor is known to stimulate increases in steady state fibronectin mRNA through binding to EGR1 sites (Kornblihtt *et al.*, 1996; Liu *et al.*, 2000; Baron *et al.*, 2005). EGR1 also stimulates transcription of TGF-β, which stimulates fibronectin transcription through the Jnk signaling cascade (Hocevar *et al.*, 1999). PLC-γ1 has been shown to regulate the expression of immediate early genes *FIC*, *Cox2*, *KC*, *JE*, and *cFos*. Since the described PLC-γ1 regulated immediate early genes are not known to regulate these reported sites on the fibronectin promoter, it is not known whether PLC-γ1 might regulate the transcription of fibronectin.

Once the fibronectin protein is translated, it is folded, dimerized and glycosylated in the endoplasmic reticulum (ER)(Choi and Hynes, 1979; Hynes and Yamada, 1982). Fibronectin is dimerized by the formation of two disulfide binds at the C-terminus and

dimerization is required for assembly into fibrils (Figure 5)(Choi and Hynes, 1979; Schwarzbauer, 1991).

The fibronectin molecule is composed of type 1, type II and type III domains as well as a variable domain (Figure 5). The type III extra domain A (EDA), extra domain B (EDB), and variable (V) domains are alternatively spliced (Schwarzbauer *et al.*, 1983). Cellular fibronectin contains the EDA and EDB domains while plasma fibronectin does not. The variable domain is required for dimerization and is expressed in at least one of the two fibronectin molecules in the dimer. In the absence of a variable domain, fibronectin is translated but not secreted (Schwarzbauer *et al.*, 1989).

Individual or adjacent domains within the fibronectin molecule mediate the interaction of fibronectin with other proteins or even with other fibronectin dimers (Leiss *et al.*, 2008). The type II domains mediate collagen binding while the first five type I domains, the 1st and 2nd type III domains, and the 12th-14th type III domains mediate intramolecular interactions between fibronectin dimers. The binding of integrins $\alpha5\beta1$ and $\alpha v\beta3$ to the fibronectin dimer is mediated by the 5th type I as well as the 9th and 10th type III domains, which contain an isoDGR, synergy sequence and RGD motif respectively. The RGD motif is the canonical motif for fibronectin binding and is required along with the PHSRN synergy sequence for fibronectin adhesion and assembly by integrin $\alpha5\beta1$(Leiss *et al.*, 2008). Integrin $\alpha v\beta3$ has been shown to bind the RGD sequence, but this sequence is not required for fibronectin assembly by this integrin. Instead, the isoDGR motif allows for fibronectin assembly by integrin $\alpha v\beta3$ and weakly binds integrin $\alpha5\beta1$(Takahashi *et al.*, 2007; Leiss *et al.*, 2008).

Fibronectin is secreted in a compact conformation that is mediated by electrostatic interactions(Johnson *et al.*, 1999). Secretion in this conformation likely prevents intermolecular interactions and cell aggregation prior to fibronectin assembly. Following secretion, the compact fibronectin dimer binds to the integrin and stimulates the formation of adhesion sites that link the integrin to the cytoskeleton. Linkage to the cytoskeleton is thought to produce tension on the fibronectin-bound integrin dimer, which causes it to unfold and expose sites within the molecule that allow for intermolecular association. Additional fibronectin dimers are unfolded by integrins and form intermolecular interactions with other fibronectin molecules, thus generating fibrils (Figure 6).

Study Aims

The aim of my study was to investigate whether PLC-γ1 regulates production of fibronectin matrix and what affect this has on cell behavior. Previous reports show that in the absence of PLC-γ1 signaling, there is an increase in actin stress fiber formation as well as a decrease in cell migration and tumor metastasis (Turner *et al.*, 1997; Wells and Grandis, 2003; Jones *et al.*, 2005; Tvorogov *et al.*, 2005). Actin stress fiber formation is required for the assembly of fibronectin matrix and increased fibronectin matrix is known to slow migration, inhibit metastasis, and facilitate cell aggregation during development(Lash *et al.*, 1984; Akamatsu *et al.*, 1996; Palecek *et al.*, 1997). Because of this, I hypothesize that PLC-γ1 negatively regulates the production of fibronectin matrix and that PLC-y1 regulation of matrix modulates cell behavior.

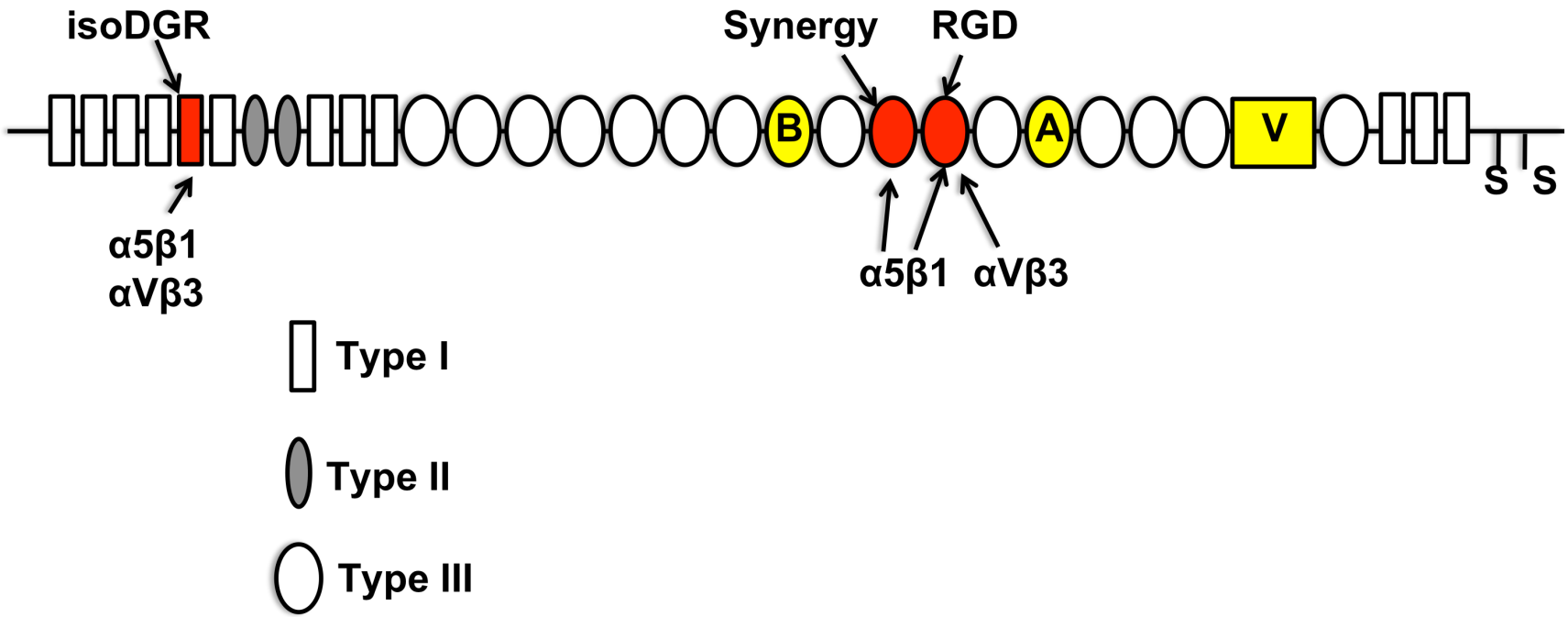


Figure 5. Domain structure of fibronectin. Fibronectin is composed of 12 type I, 2 type II, and 15 type III domains. Extra type III domains A and B and the V (yellow) domain are alternatively spliced. Individual domains within the fibronectin molecule mediate binding to integrins α5β1 and αvβ3 (Shown in red). C-terminal disulfide bonds are denoted by (S). Figure adapted from Leiss et al 2008.

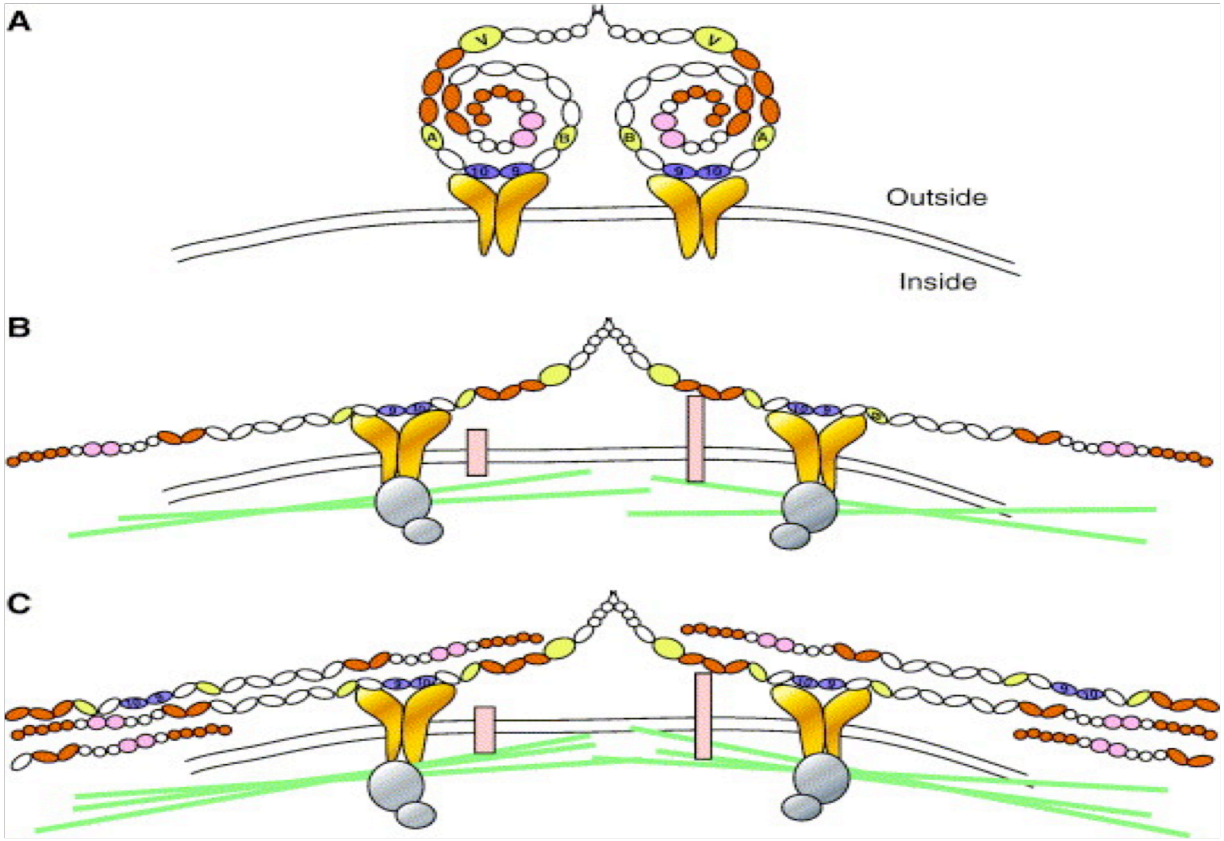


Figure 6. Integrin-mediated fibronectin assembly. A) The compact fibronectin dimer binds the integrin. B) Fibronectin binding results in the formation of adhesion complexes, reorganization of the cytoskeleton and the unfolding of the fibronectin dimer to expose fibronectin intermolecular association sites. C) Association of additional fibronectin molecules and formation of fibronectin fibrils. Taken from from Mao and Schwarzbauer, Matrix Biology 2005

CHAPTER II

MATERIALS AND METHODS

Materials: Antibodies for mouse anti-actin, mouse anti-vimentin, and mouse anti-fibronectin were purchased from Sigma Aldrich, while those to integrins α5 (5H10-27), β1 (9EG7), β1 (HA2/5), β3 (2C9.G2) and αV (RMV-7) were purchased from BD Biosciences. The rabbit anti-fibronectin used for immunoprecipitation was purchased from Santa Cruz Biotechnology. Cy3 conjugated goat anti-mouse was purchased from Jackson Immunoresearch and Alexa 647-conjugated goat anti-mouse was a product of Molecular Probes. Mouse and rabbit secondary antibodies used in western blotting were purchased from Licor Biosciences. CycloRGD (RGDfV), and cycloRAD (RADfV) were obtained from Biomol International. Peptides were dissolved at a concentration of 5ug/ml in 25% DMSO/PBS and used at a concentration of 250 μg/ml. The 70 kDa fibronectin fragment (McKeown-Longo and Mosher, 1985) was purchased from Sigma and dissolved in cell culture medium. The extracellular matrix and adhesion molecules oligo GEArray™ kit was purchased from SuperArray Bioscience Corporation.

Cell Culture and Staining: *Plcg1* Null and Null + immortalized mouse embryonic fibroblasts have been described previously (Ji *et al.*, 1999). Cells were cultured in DMEM supplemented with 10% FBS. In experiments using fibronectin-free FBS, fibronectin was removed by passing FBS through a gelatin-Sepharose column (GE Health Sciences). For cell staining, cells were fixed in 4% PFA and stained for fibronectin. Stained cells or hanging drop aggregates were visualized using a Zeiss LSM 510 confocal microscope.

Hanging Drop Assay: Null and Null + cells were detached using Accutase (Innovative Cell Technologies) and re-suspended at 500,000 cells/ ml in cell culture medium. The 70 kDa fragment, cyclic RGD or RAD peptides, or integrin β1 or β3 blocking antibodies were added at indicated concentrations. Drops (30 µl) were placed on the lid of a 24 well plate and the lid was inverted over the cell culture wells, which contained PBS to avoid evaporation of the hanging drop. Cells were cultured in the hanging drop overnight. Subsequently, hanging drops were photographed, pipetted 20 times to disrupt cell aggregates and then photographed again using a Leica inverted microscope with 10X objective.

Deoxycholic Acid (DOC) Solubility Assays: The DOCsolubility assays have been previously described (Wierzbicka-Patynowski and Schwarzbauer, 2002). In brief, 12 $X10^5$ cells were plated in 100 mm dishes in DMEM supplemented with 10% fibronectin-free FBS. After 4-24 hrs, the cells were lysed in 2% DOC lysis buffer (2% DOC, 20mM tris-CL pH 8.8, 2mM PMSF, 2mM EDTA, 2mM iodoacetic acid, and 2 mM N-ethylmaleimide) and lysates were passed through a 25 gauge needle and centrifuged (16,000 x g,20 min) at 4°C. The supernatant was removed and saved as the DOC soluble fraction, while the pellet was washed in DOC lysis buffer and then re-suspended in 2X LDS reducing sample buffer (Invitrogen). Protein levels were determined for DOC soluble fractions by BCA assay (Pierce). Equal amounts of DOC-soluble and -insoluble protein were resolved on a 4-12% SDS-PAGE gel.

Metabolic Labeling: Null and Null + cells were placed in labeling medium (methionine and cysteine-free DMEM, 10% FN free FBS, 20 μM unlabeled methionine, and 50 uCi/ml ^{35}S *in vitro* cell labeling mix (GE Healthsciences)). Cells were then harvested as described above and fibronectin was precipitated using mouse anti-human fibronectin (BD Biosciences). To measure levels of secreted fibronectin, conditioned medium was collected and phenylmethylsulfonyl fluoride was added to a final concentration of 2mM. Gelatin-Sepharose beads were added to adsorb fibronectin and the samples were incubated overnight. Beads were washed and 2X reducing sample buffer was added. DOC-soluble, DOC-insoluble, and conditioned medium samples were resolved on a 4-20% SDS-PAGE. The gel was dried and exposed to a Phosphorimager screen. Bands were quantified using Image J software.

For pulse experiments, cells were pulsed for 10 min with ^{35}S-methionine prior to harvesting the cells in TGH lysis buffer (1% Triton X 100, 10% glycerol, 50mM Hepes pH 7.2, 100mM NaCl). Lysates were sonicated and fibronectin was precipitated using rabbit anti-fibronectin. For pulse-chase experiments, cells were labeled for one hr with ^{35}S-methionine prior to a 2-hr chase. Conditioned medium was collected and radioactivity analyzed by scintillation counting. Radioactivity was normalized to protein levels.

Exogenous Assembly Assays: Bovine plasma fibronectin (Sigma) was biotinylated using the manufacturer's protocol (Pierce). Null and Null + cells were re-suspended at a concentration of 1.2 x 10 ^{5} cells/ml in DMEM supplemented with 10% FN-free FBS and biotinylated fibronectin (20μg/ml) and then plated in 60 mm dishes. After 4 hrs, the cells were lysed and DOC-soluble and -insoluble fractions were isolated. Fractions were resolved on 4-12% SDS-PAGE gels and transferred to a PVDF membrane. Membranes

were blotted with NeutrAvidin® HRP (Pierce) or Streptavidin IR680 (Licor inc) and visualized by chemiluminescence or an Oddysey fluorescence imager (Licor inc).

RNA Analysis: Cells were plated at equal density and cultured overnight. The next day, RNA was isolated using the Qiagen RNeasy mini kit. Total RNA was subjected to northern blot and QRT-PCR. For northern blots, the 683 bp human fibronectin probe was cut from plasmid pSP73 RFN 2375-6090 (provided by Dr. Jean Schwarzbauer, Princeton University) using EcoRV and BamHI. Blots were stripped and re-probed for cyclophilin as a loading control. Fibronectin QRT-PCR primers were purchased from Applied Biosystems. cDNA was generated using the iscript cDNA Synthesis Kit (BioRad). QRT-PCR was performed using IQ Supermix (BioRad) and the Biorad ICycler. Values were normalized to actin using the ΔΔcT method.

Oligo GEArray® for extracellular matrix and adhesion molecules: Cells were plated at equal density and cultured overnight in 10% FBS/ DMEM. The next day, total RNA was isolated using the Qiagen RNeasy mini kit. cDNA and biotin-16-UTP labeled cRNA were synthesized from 1 μg total RNA using the TrueLabeling-AMP™ 2.0 kit from SuperArray following the manufacturers instructions. cRNA was then purified using the ArrayGrade™ cRNA Cleanup Kit (SuperArray). Biotin-16-UTP labeled cRNA was hybridized to the extracellular matrix and adhesion molecule array following the manufacturers instructions. Signal was detected using the GEArray™ Chemiluminescent Detection Kit (SuperArray). Membranes were imaged using the BioRad Chemidoc XRS Camera with Quantity One software. Images were quantitated as follows. Each spot on the membrane was measured 3 times using Image J software. Background was subtracted from each membrane using empty "No Gene" spots. Housekeeping genes on each

membrane were used to normalize signals so that the membranes hybridized with Null and Null + cRNA could be compared. Hybridization was performed twice for each cell line using duplicate membranes.

CHAPTER III

REGULATION OF FIBRONECTIN SECRETION, ASSEMBLY AND CELL AGGREGATION BY PLC-γ1

Results

PLC-γ1 Deficiency Increases Cell Aggregation

Plcg1 Null and Null + cells were cultured in hanging drops overnight to examine cell aggregation. Briefly, 500,000 cells were re-suspended in 1 mL of cell culture media and 30 uL drops were placed on the lid of a 24 well plate. The lid was inverted onto a plate filled with PBS to prevent evaporation of the hanging drop and then hanging drops were cultured overnight. Subsequently, drops were photographed, pippetted twenty times to disrupt aggregates, and then photographed again. The data in Figure 7A demonstrate that the Null cells formed a single compact aggregate whereas aggregates formed by Null + cells were smaller and more numerous. Attempts to disperse Null cell aggregates by pipetting were unsuccessful, while similar attempts with Null + cells yielded a significant number of single cells or doublets (Figure 7B). These results show that cells deficient in PLC-γ1 display significantly increased aggregation under these culture conditions.

Cell aggregation is mediated by either cadherin -dependent cell:cell interactions and/or fibronectin-dependent cell:matrix interactions (Lash *et al.*, 1984; Robinson *et al.*, 2004). Fibronectin is the major matrix protein secreted by fibroblasts and following secretion, fibronectin is assembled into fibrils by integrins α5β1 andαvβ3 (Mao and Schwarzbauer, 2005). To determine whether the hanging drop cell aggregates contain assembled fibronectin, the aggregates were fixed and stained for fibronectin. Fibronectin was detected in the aggregates of both Null and Null + cells (Figure 7A Right Panels). As

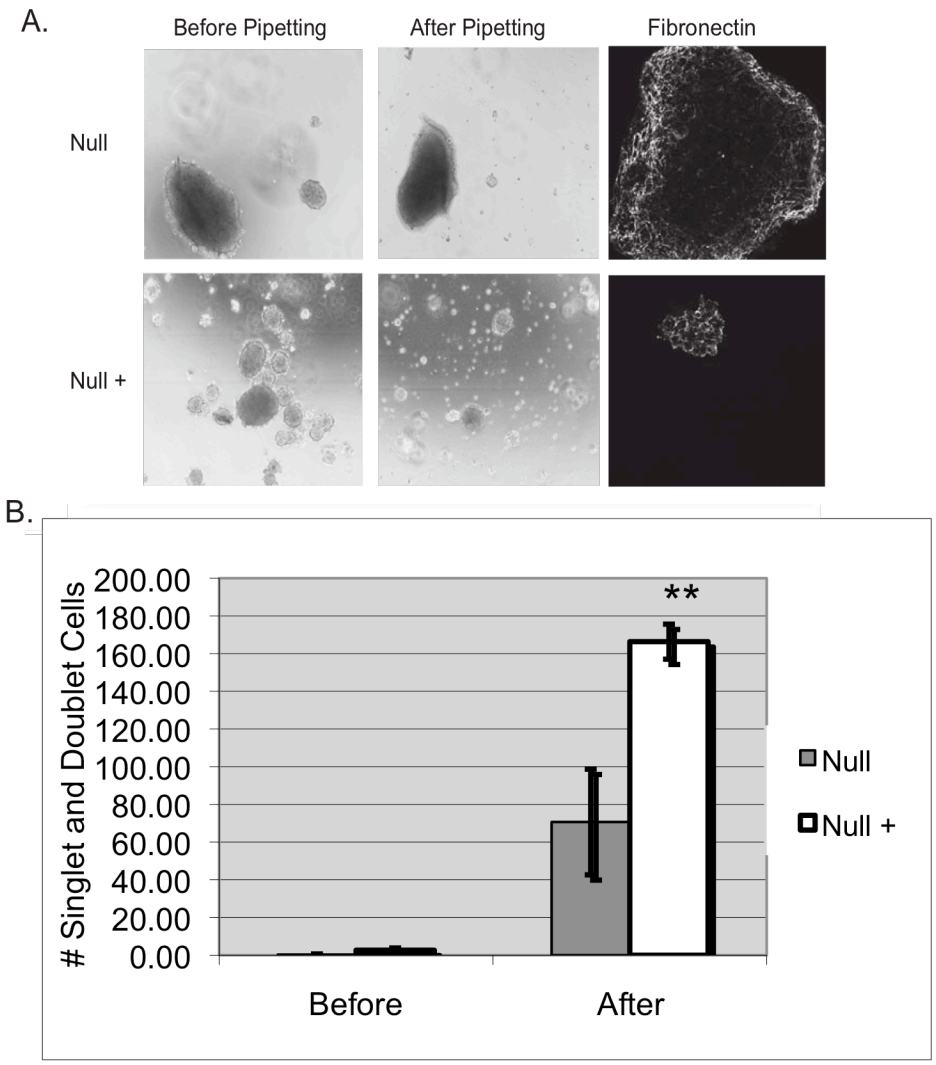


Figure 7. Assessment of cell aggregation by Null and Null + cells: A) Cells were detached and 5 X 10^5 cells were re-suspended in cell culture medium. 30 μl drops were added to the lid of a 24 well dish and hanging drops were cultured overnight. The next morning, drops were photographed (left panels), pipetted 20 times to disperse, and photographed again (middle panels). Pictures are representative images of multiple hanging drops. Cellular aggregates were stained for fibronectin and then visualized using a Zeiss confocal microscope (right panels). B) Quantification of the number of singlet's and doublets in photographs before and after pipetting. Values are the average of 5 different hanging drops. Error bars indicate standard deviation. Asterisk (**) denotes a significant difference (P = 0.005) in the values observed in the Null and Null + cells using the unpaired students *t* test.

cell aggregation could be due to cell-cell interactions, levels of N-cadherin were examined by western blotting using both Null and Null + cells. This demonstrated that Null and Null + cells express equivalent levels of N-cadherin, suggesting that differential expression of N-cadherin does not account for the differences observed in the two cell lines (Figure 8).

To determine whether fibronectin assembly contributes to the cell aggregation shown in the hanging drops of Figure 7, cells were treated with cyclic RGD peptide or the inactive cyclic RAD peptide to block fibronectin interaction with the integrins. The RGD motif is a conserved peptide sequence among many extracellular matrix molecules and is critical for ligand recognition by many integrins. It is commonly used to inhibit fibronectin assembly (Pierschbacher and Ruoslahti, 1984; Lash *et al.*, 1987; Nagai *et al.*, 1991; Sechler *et al.*, 1997; Robinson *et al.*, 2004; Feral *et al.*, 2007). As a control for nonspecific effects of the peptide, cells were treated with the functionally inactive cyclic RAD peptide that contains a glycine to alanine change. Additional controls included a vehicle control to control for any effects the vehicle (25% DMSO/ 75% PBS) may have on the cells. The vehicle, RGD, and RAD treated cells were all compared with untreated cells (Control).

As observed in Figure 7A, untreated Null cells formed larger aggregates than Null + cells (Figure 9). Vehicle treated or cyclic RAD treated cells mimicked the untreated cell lines, indicating that neither the vehicle nor inactive peptide has a significant effect on cell aggregation. Surprisingly, rather than preventing cell aggregation the cyclic RGD peptide actually increased cell aggregation in Null + cells to a level equivalent of the Null cells (Figure 9). In order to investigate the effectiveness of cyclic RGD peptide

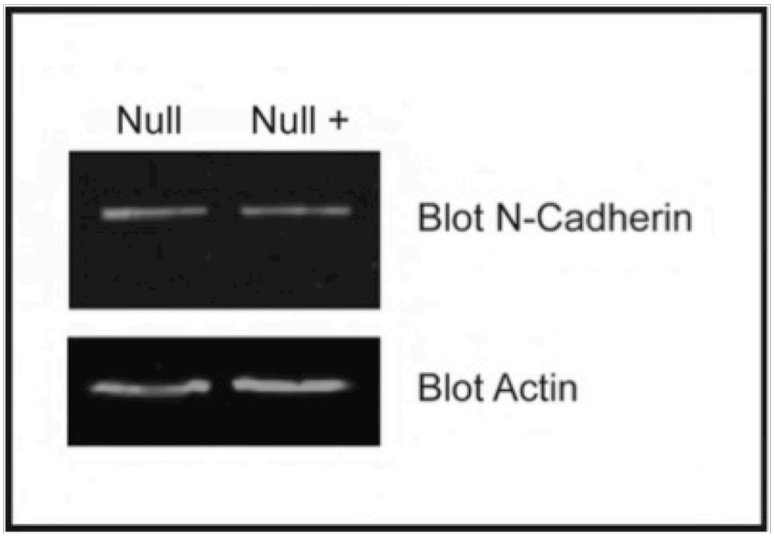


Figure 8. Expression of N-cadherin in Null and Null + Cells. Total cell lysates from Null and Null + cells were blotted for N-cadherin. Actin was used as a loading control.

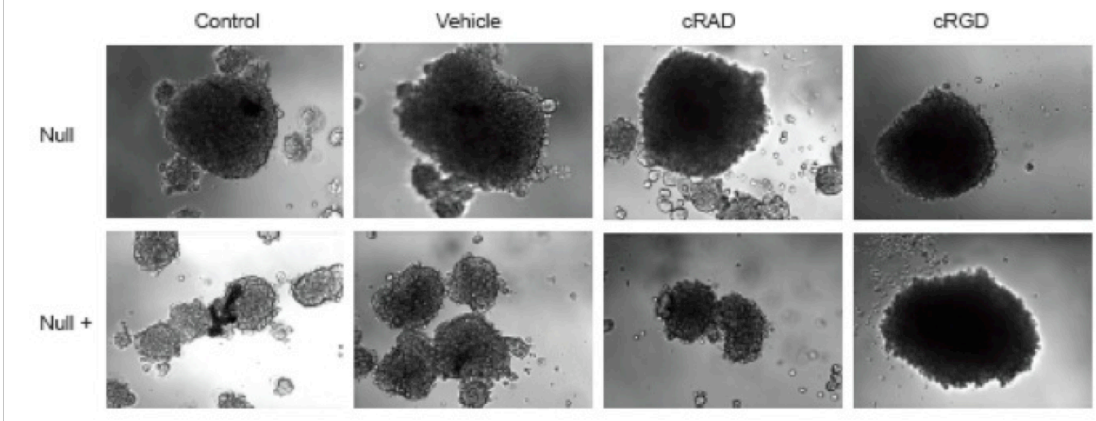


Figure 9. Hanging Drop Assay with cRGD and cRAD treatment. Null and Null + cells were treated with cyclic RGD, cyclic RAD, vehicle (25% DMSO/PBS), or left untreated, and then cultured in hanging drops overnight.

treatment, a fibronectin adhesion assay was performed on Null cells treated with cyclic RGD, cyclic RAD, or left untreated. Two different concentrations of peptides were used, [0.1 or 0.2 mg/ml], in order to determine whether inhibition of adhesion to fibronectin is concentration dependent. Pretreatment of Null cells with cyclic RGD resulted in approximately 25% and 42% inhibition of adhesion at treatment concentrations of 0.1 and 0.2 mg/ml respectively (Figure 10). This indicates that in a 30-minute experiment, the cyclic RGD peptides effectively block adhesion of Null cells to fibronectin. As the hanging drop assay occurs over 16 hours, it was necessary to investigate whether the cyclic RGD peptides were inhibiting the association of fibronectin with the cells over that longer time. Previous studies indicate that the effectiveness of the RGD peptides is limited to 6 or 7 hours under cell culture conditions (Lash *et al.*, 1987). To determine whether, in the context of the cell aggregation assay, the cyclic RGD peptides blocked the association of fibronectin with the cell surface, cyclic RGD treated aggregates were fixed and then stained for fibronectin. Cyclic RGD treated aggregates stained positive for fibronectin in a manner similar to control and cyclic RAD treated aggregates, indicating that the peptide was ineffective at blocking fibronectin interaction with the cell (Figure 11).

Since previous reports have indicated that RGD peptides are not stable during incubations longer than 6-8 hours and that cyclic RGD may be more specific for integrins αVβ3 than α5β1(Lash *et al.*, 1987; Pfaff *et al.*, 1994), a 70 kDa N-terminal fibronectin fragment, generated from proteolytic digestion of fibronectin, was used bock fibronectin assembly in hanging drops (McKeown-Longo and Mosher, 1985; Lash *et al.*, 1987; McDonald *et al.*, 1987; Pfaff *et al.*, 1994). This fragment includes the collagen and fibrin

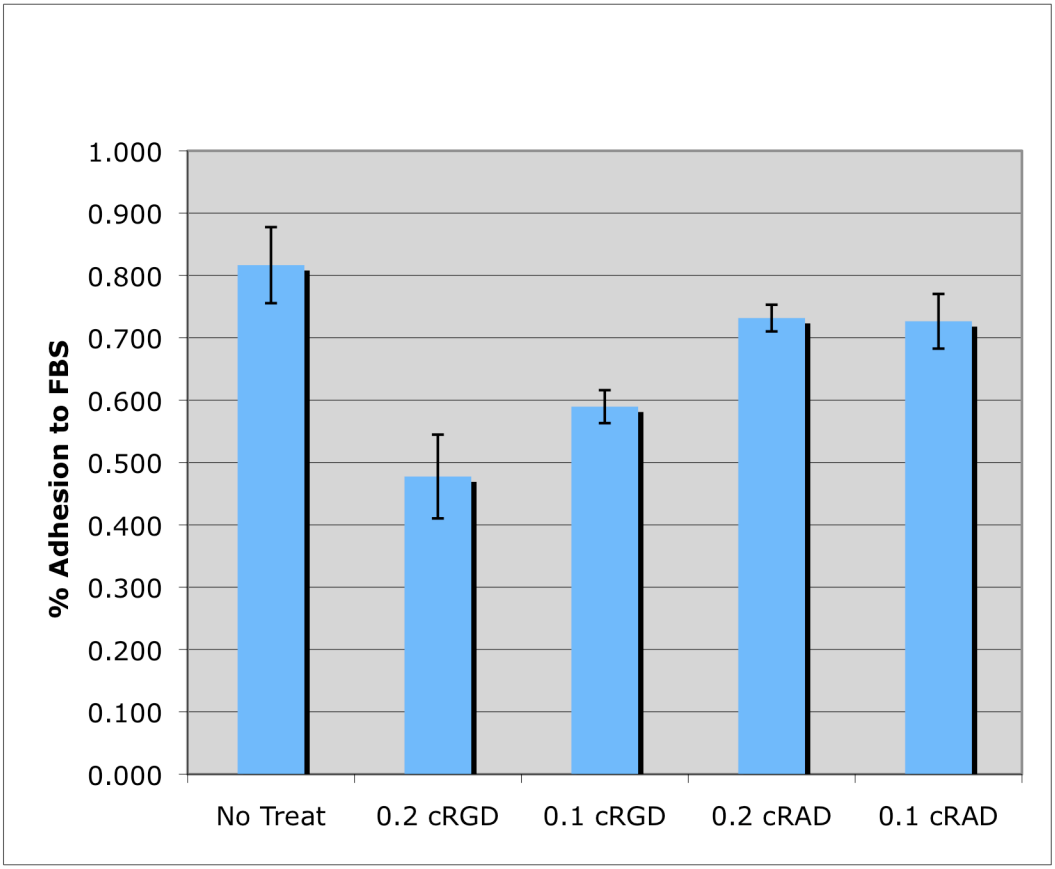


Figure 10. Effect of cyclic RGD and cyclic RAD peptides on adhesion of Null cells to 10ug/ml fibronectin for 30 minutes. Asterisk (*) indicates significant differences in values of untreated and cRGD-treated Null cells using an unpaired Student's t test. (*) indicates that the P-value is less than 0.05. (**) indicates that the P-value is less than 0.001.

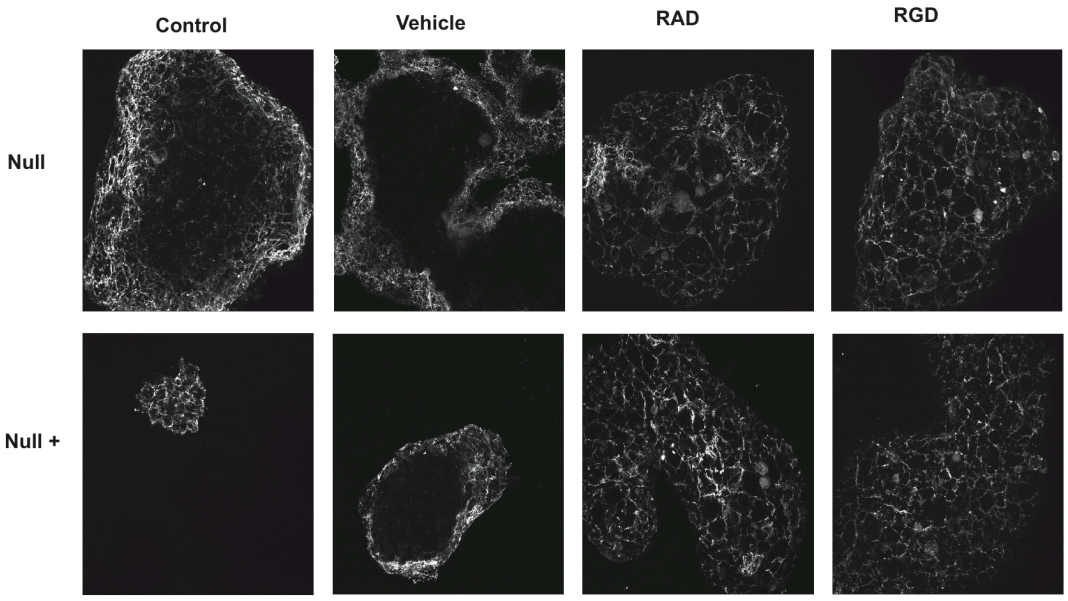


Figure 11. Fibronectin Staining of Null and Null + Aggregates. Null and Null + cells were treated with cyclic RGD, cyclic RAD, vehicle (25% DMSO/PBS), or left untreated, and then cultured in hanging drops overnight. Aggregates were fixed and stained for fibronectin.

domains of fibronectin and has been shown to block initiation of fibronectin assembly and also to bind integrin α5β1 (McKeown-Longo and Mosher, 1985; Takahashi *et al.*, 2007). Treatment of Null and Null + hanging drops with the 70 kDa fragment completely abolished cell aggregation at concentrations at or above 500 μg/ml. At a concentration of 100 μg/ml, this fragment decreased cell aggregation in the Null + cells, but had no effect on the Null cell aggregation when compared to untreated Null and Null + cells (Figure 12).

Influence of PLC-γ1 on Fibronectin Assembly

To determine whether fibronectin assembly is increased in Null cells, deoxycholic acid (DOC) assembly assays were performed. These assays are based on the insolubility of assembled fibronectin fibrils in DOC. Null and Null + cells were labeled with ^{35}S methionine for 0-24 hours to compare the assembly of endogenous fibronectin. Cells were lysed in 2% DOC lysis buffer and centrifuged (13,000 RPM for 30 minutes) in a microcentrifuge. The DOC-insoluble pellet was re-suspended and fibronectin was precipitated from both the DOC-soluble and -insoluble fractions using an antibody against fibronectin. The DOC-soluble fraction contains unsecreted fibronectin as well as fibronectin that has been secreted and is associated with the cell, but has not been assembled into fibrils yet. The DOC-insoluble fraction contains fibronectin that has been secreted and assembled into fibrils.

The results, shown in Figure 13 (top left panel), demonstrate that the Null cells assemble two-fold more fibronectin than Null + cells. An increased level of assembled fibronectin was also observed in the Null cells when the assembly of exogenous

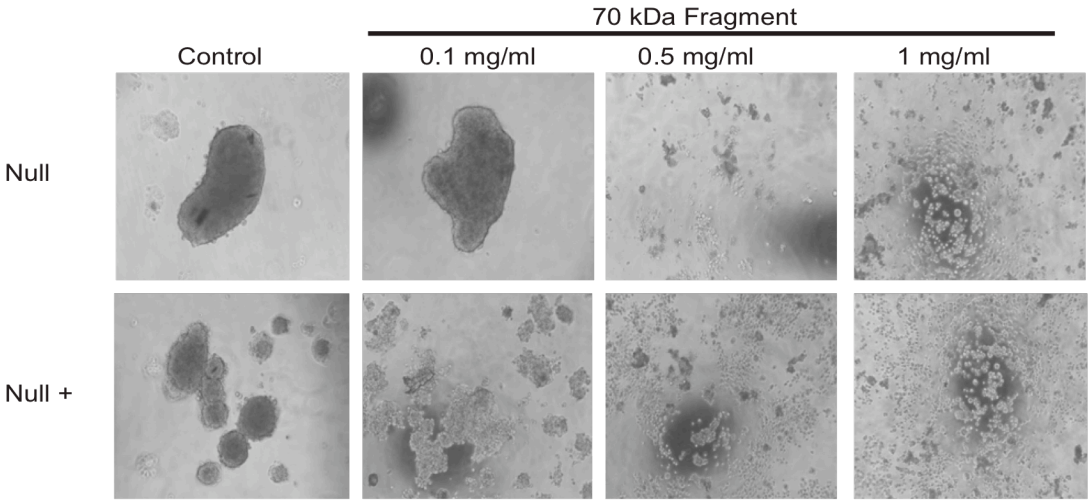


Figure 12. Treatment of Hanging Drops with 70 kDa fragment: Null and Null + hanging drops were treated as in Figures 1 and 2 except for the addition of 1000 µg/ml, 500 µg/ml, or 100 ug/ml 70 kDa fibronectin fragment.

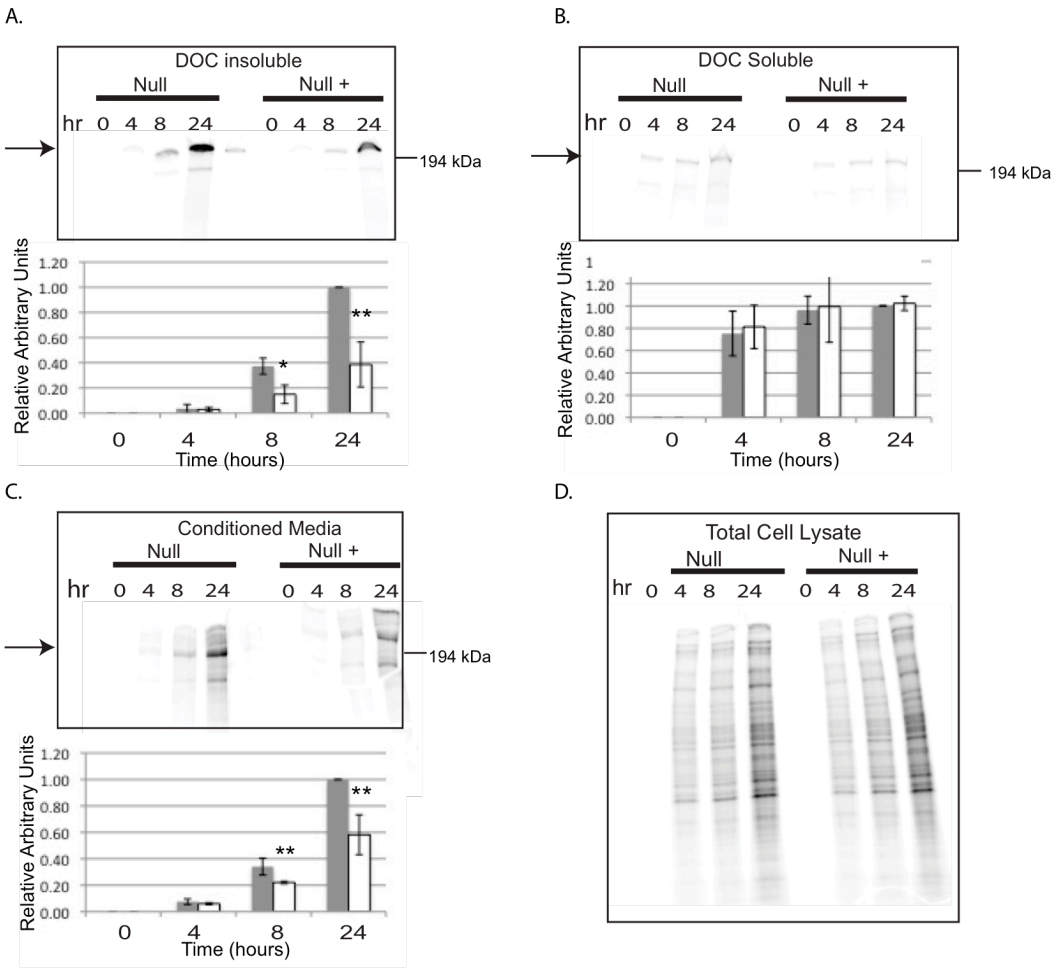


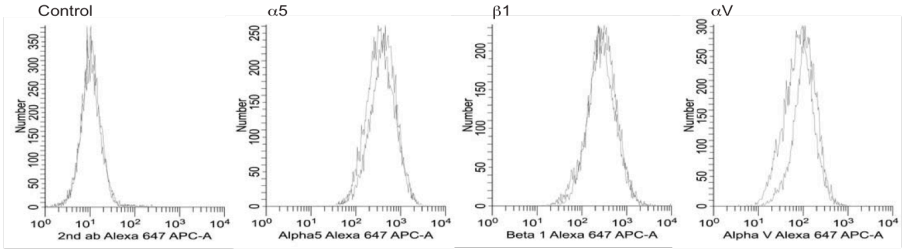
Figure 13. Assessment of fibronectin assembly in Null and Null + cells: Null and Null + cells were metabolically labeled with ^{35}S methionine for the times indicated. Parts A, B, and C: Top panels - phosphorimager image. Arrows indicate 220 kDa fibronectin; Bottom panels- densitometric analysis. Values are relative to Null cells at 24 hours. Gray bars represent Null cells and white bars represent Null + cells. A) DOC-insoluble fraction; B) DOC-soluble fraction; C) Conditioned media; D) Total cell lysate. Asterisk (*) indicates significant differences in values of Null and Null + MEF using a paired Student's t test. (*) indicates that the P-value is less than 0.05. (**) indicates that the P-value is less than 0.001.

fibronectin was measured (Figure 15A, control lanes). In this assay, cells are incubated in fibronectin free media in the presence of biotinylated fibronectin. Due to this, only the biotinylated fibronectin as well as the fibronectin endogenously secreted by cells is available for assembly into fibrils. Similar levels of fibronectin were detected in the DOC-soluble fraction (Figure 13; top right panel).

To determine whether Null and Null + cells differentially secrete fibronectin, fibronectin was precipitated from the conditioned medium using gelatin sepharose beads. Gelatin is denatured collagen and binds the N-terminal gelatin-binding domain of fibronectin. An increase in the level of labeled fibronectin in the Null cell conditioned medium was detected, indicating that PLC-γ1 may regulate levels of secreted fibronectin (Figure 13; bottom left panel). As a control, equal amounts of total cell lysates were run on a gel to ensure equal loading (Figure 13; bottom right panel). These results show that the increased cell aggregation observed with Null cells is correlated with the presence of fibronectin fibrils in the aggregates and increased fibronectin assembly.

Increased fibronectin assembly can reflect increased integrin or fibronectin expression. Previous work with these cells revealed similar integrin expression in the two cell lines (Tvorogov *et al.*, 2005). To confirm this, we compared the expression of integrins α5, αV, and β1 in Null and Null + cells by FACS analysis. Null and Null + cells were stained with antibodies against integrins α5, αV, or β1. As a control for background staining, cells were stained with only secondary antibody (Figure 14; control). Null cells express approximately 1.25-fold more integrin α5, 1.2-fold more β1, and 1.4-fold more αV as compared to Null + cells (Figure 11).

A.



B.

Integrin	Null	Null +	P value
α5	54.37±10.4	40.76±4.09	0.0042
β1	28.41±3.94	23.18±5.66	0.1781
αV	10.37±1.03	7.33±0.37	0.0088

Figure 14. Integrin surface expression in Null and Null + cells: A) Facs analysis of Null and Null+ cells stained for mouse integrins α5, β1, and αV. B) Statistical data from Facs analysis.

To determine which integrin pair is responsible for fibronectin assembly in these cells, exogenous assembly assays were performed with cells plated on either 10μg/mL fibronectin or 5 μg/ml vitronectin. If the cells utilize integrin α5β1 to assemble fibronectin, then plating cells on fibronectin would reduce the assembly of exogenous biotinylated fibronectin. However, if cells use integrin αVβ3, also known as the vitronectin receptor, for assembly, then plating cells on vitronectin should also reduce

fibronectin assembly. When the Null and Null + cells were plated on fibronectin, there was a significant decrease in fibronectin assembly in both cell lines, while plating on vitronectin had little to no effect on assembly (Figure 15). This indicates that, under these conditions, integrin α5β1 is responsible for fibronectin assembly in Null and Null + cells.

To test whether the α5β1 integrin is required for the cell aggregation observed in Figure 7, blocking antibodies to either integrin β1 or β3 were incorporated into the hanging drop assay (Figure 15C). Antibodies were added to the cell suspension prior to placing the hanging drops on the plastic lids and were incubated with the cells for the 16-hour duration of the assay. The β3-blocking antibody was selected as a control since the data in Figure 15A suggest that under serum culture integrin αVβ3 does not mediate fibronectin assembly in *Plcg1* Null and Null + cells. Integrin β1-blocking antibodies effectively reduced cell aggregation, while integrin β3 antibodies had no effect (Figure 15C). Treatment with both antibodies yielded similar results to integrin β1 antibody treatment, indicating that the β1, but not β3 integrin is required for the formation of tight aggregates in Null and Null + cells.

PLC-γ1 Negatively Regulates the Levels of Secreted Fibronectin

As *Plcg1* Null cells display an increased capacity to assemble fibronectin into fibrils and increased fibronectin was detected in the conditioned medium of the Null cells (Figure 13), it is possible that this reflects increased levels of fibronectin mRNA and/ or protein. To determine whether steady-state fibronectin mRNA levels differ in the two cell lines, RNA was extracted from Null and Null + cells and mRNA levels were compared using qRT-PCR and northern hybridazation. For fibronectin qRT-PCR, RNA was

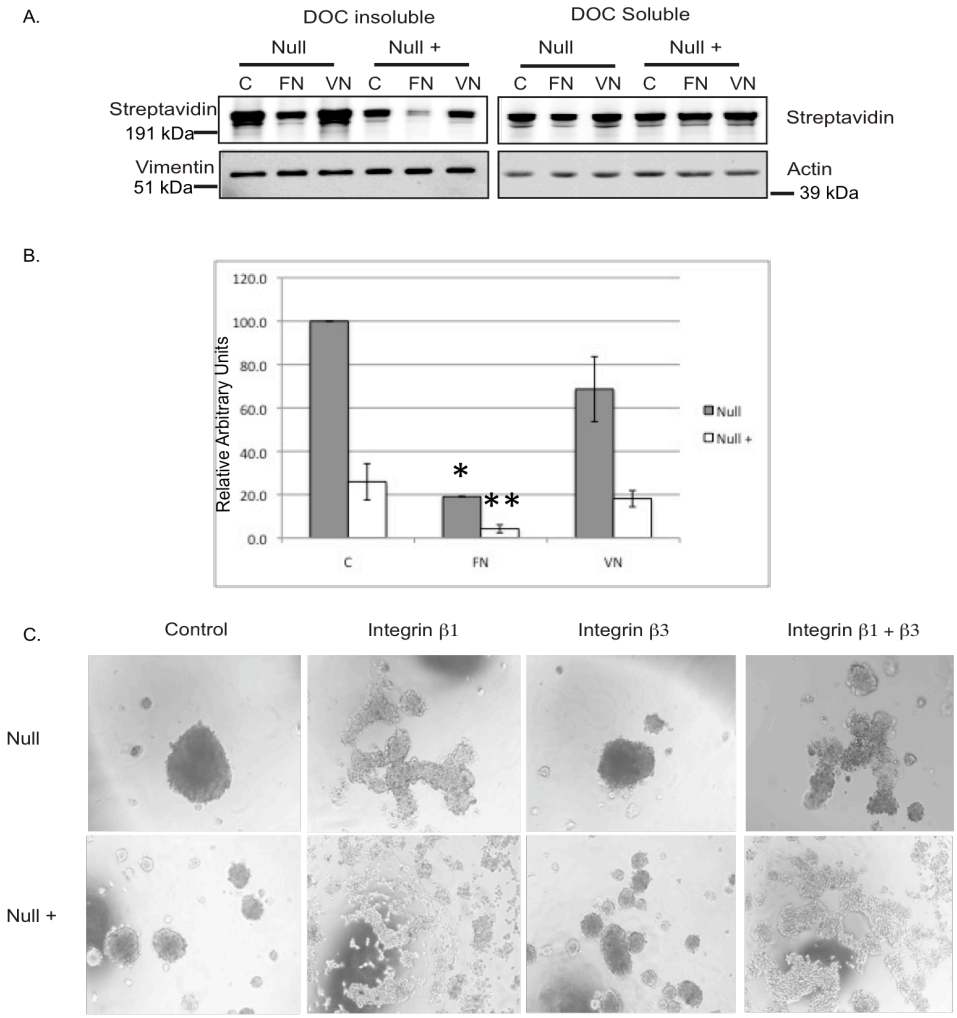


Figure 15. Fibronectin assembly is mediated by Integrin α5β1 in Null and Null + cells: A) Exogenous assembly assay; Cells were plated on 10ug/ml fibronectin or 5 ug/ml vitronectin in fibronectin free medium prior to the addition of biotinylated fibronectin. Cells were incubated overnight followed by collection of lysates and separation into DOC-soluble and insoluble fractions. Right panels: DOC-insoluble fractions. Biotinylated fibronectin is detected by blotting with IR Dye 680 Streptavidin. Vimentin serves as a loading control. Left Panels: DOC-soluble fraction: Actin serves as a loading control. B) Densitometric analysis of DOC insoluble fraction in panel A. Gray bars represent Null cells. White bars represent Null + cells. C) Hanging drop assay: Cells were treated as in figures 1 and 2 except for treatment with 100 µg/ml inhibitory antibodies against integrins β1 (HA 2/5), β3 (2C9.G2) or a combination of both. Single asterisk (*) denotes a significant difference (P< 0.0001) in values observed in the Null cells plated on plastic (control) verses fibronectin (FN). Double asterisks (**) denotes a significant difference(P=.0200) in the values of Null + cells plated on plastic verses fibronectin.

converted to cDNA using the i-script cDNA synthesis kit (BioRad) and then quantitative PCR was performed using a mouse fibronectin probe and normalized to actin levels using the ΔΔcT method. For northern blotting, a human fibronectin probe was used to detect fibronectin mRNA as human fibronectin is 94% homologous to mouse fibronectin. The data show that steady state fibronectin mRNA levels are equivalent in both cell lines using both qRT-PCR (Figure 16A) and northern blotting (Figure 17).

DOC assembly assays indicate that there may be an increase in fibronectin protein expression in the conditioned medium of Null cells (Figure 13). Therefore, fibronectin levels in the conditioned medium of Null and Null + cells were compared. Null and Null + cells were cultured in fibronectin-free medium in which fibronectin was removed from the FBS using a gelatin-Sepharose column. This enabled a comparison of only the endogenous fibronectin levels present in the conditioned medium. Consistent with previous data (Figure 13), these results show that the Null cells secrete 2-fold more fibronectin into the medium than Null + cells (Figure 16B). In addition, a fibronectin western blot of total cell lysates demonstrated an increase in fibronectin (Figure 16B). Total cell lysates contain both DOC-soluble and -insoluble fractions of fibronectin.

To determine whether the difference in fibronectin secretion could account for the difference in assembly, Null and Null + cells were pretreated with cycloheximide for 30 minutes and then subjected to an exogenous assembly assay (Figure 18). If the difference in endogenous secretion accounts for the increased fibronectin assembly observed in Null cells, the cycloheximide treatment should equalize assembly levels in Null and Null + cells. Cycloheximide treatment has been shown to have no effect on fibronectin assembly in untransformed fibroblasts (McKeown-Longo and Etzler, 1987; Pankov *et al.*, 2000).

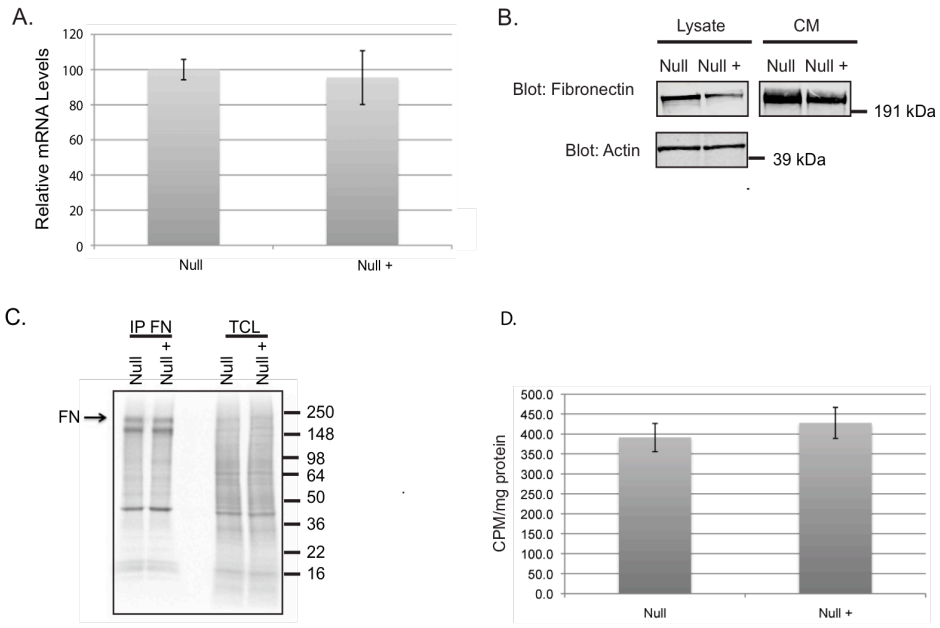


Figure 16. Comparison of fibronectin mRNA and protein levels: A) QRT-PCR of RNA extracted from Null and Null + cells. B) Null and Null + cells were plated and cultured overnight. Cell lystates were harvested in RIPA buffer and conditioned medium (CM) was collected. Equal amounts of protein were resolved by SDS-PAGE followed by detection with rabbit FN and mouse actin antibodies. C) Cells were pulsed for 10 minutes with ^{35}S methionine followed by lysis and Immunoprecipitation for fibronectin. Abbreviations FN Fibronectin; TCL total cell lysate D) Null and Null + were pulsed for ^{35}S methionine followed by a two hour chase. The levels of radioactivity were determined using scintillation counter and normalized to cell lysate protein levels.

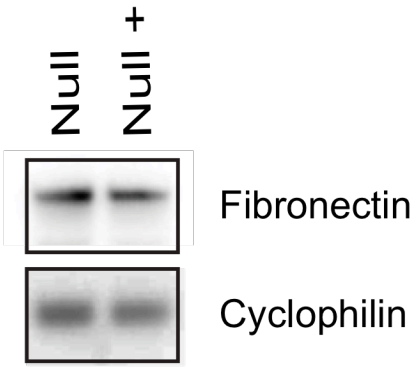


Figure 17. Northern hybridization using a fibronectin probe. RNA isolated from Null and Null + cells subjected to northern hybridization using a probe for fibronectin. The membrane was stripped and re-blotted for cyclophilin as a loading control

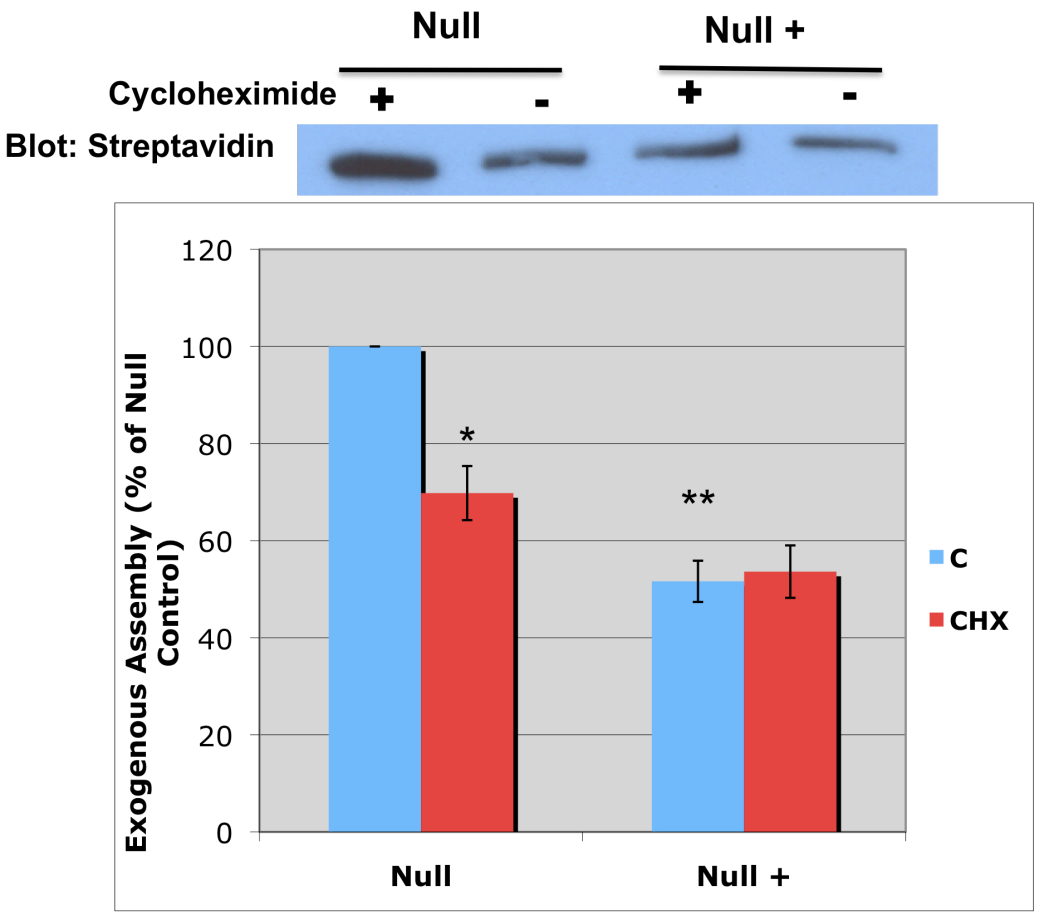


Figure 18 . Effect of Cycloheximide treatment on fibronectin assembly**.** Null and Null + cells were plated in fibronectin free medium with or without 10 µg/ml cycloheximide to block protein synthesis. 30 minutes later, 20 µg/ml biotinylated fibronectin was added to each plate and cells were incubated for 4 hours prior to lysis in 2% DOC as described in figure 2. Graph shows an average of 4 independent experiments. Error bars indicate standard deviation. Single asterisks (*) denotes significant difference between Null cells left untreated (C) or treated with cycloheximide (CHX). Double asterisks (**) denote significant difference between untreated Null and Null + cells. In both cases, statistical analysis was performed using a paired Student's *t* test and the P-value was less than 0.0001.

Cycloheximide treatment reduced assembly of exogenous fibronectin in the Null cells without affecting assembly in the Null + cells (Figure 18). This data supports the hypothesis that the increased fibronectin secretion in the Null cells may account for the increase in fibronectin assembly. However, there is the possibility that cycloheximide could be inhibiting the production of a protein that is negatively regulated by PLC-γ1 and increases fibronectin assembly.

If fibronectin mRNA levels are equivalent in both cell lines then it is possible that increased fibronectin translation in Null cells could produce the observed increase in fibronectin protein (Figures 13, 16B). To determine whether fibronectin translation is increased in Null cells, Null and Null + cells were pulse-labeled with ^{35}S-methionine for 10 minutes prior to lysis. Fibronectin was then immunoprecipitated using an antibody towards fibronectin. Previous studies have shown that it takes about 30 minutes for fibronectin to be translated, secreted and assembled into fibrils (McKeown-Longo and Mosher, 1985; McKeown-Longo and Etzler, 1987). The results, shown in Figure 16C, show that fibronectin protein production is equivalent in Null and Null + cells. Therefore, differences in fibronectin in the conditioned medium must be due to differences in secretion.

Because fibronectin secretion is increased in Null cells, it is important to determine whether this is a global effect on secretion, or whether the regulation is limited to fibronectin and possibly a few other unknown proteins. To determine whether PLC-γ1 exerts a global effect on protein secretion, a pulse chase experiment was performed. Cells were labeled with ^{35}S-methionine for 90 minutes, washed three times with phosphate buffered saline and then chased for 90 minutes in unlabeled medium. Conditioned

medium was collected and the radioactivity quantitated by scintillation counting. Null and Null + cells secreted equivalent levels of radiolabeled protein, indicating that PLC-γ1 selectively regulates the secretion of fibronectin (Figure 16D). As a control for equal loading, conditioned medium was run on a gel. The gel was dried and the radioactivity was imaged using a phosphorimager (data not shown).

cRNA Array for Extracellular Matrix Molecules Which May Be Regulated By PLC-γ1

In order to further investigate the role of PLC-γ1 in cell adhesion and migration, RNAs were isolated from *Plcg1* Null and Null + cells and subjected to a cRNA array for extracellular matrix molecules. Candidate genes are listed in Table 2. In order to confirm differential expression of candidate mRNAs, quantitative RT-PCR was performed on RNAs isolated from Null and Null+ cells using probes for matrix metalloproteinase 2 (MMP2), MMP3, procollagen Iα1, procollagen IIIα1, procollagen VIα1, procollagen VIα2, Adamts1, and thrombospondin 1 (Figure 16). Of these targets, increased mRNA levels of MMP2 and MMP3 were detected. Since increased MMP levels are generally associated with increased motility, rather than the decreased motility observed in *Plcg1* Null cells, the effect of PLC-γ1 on MMP mRNA levels was not pursued further(Tvorogov *et al.*, 2005). Contrary to array results, mRNA levels of procollagens were equivalent in Null and Null + cell lines procollagens were equivalent in Null and Null + cell lines.

Table 2. Summary of Array Results. Numbers indicate the ratio of Null/ Null + mRNA levels as determined in two replicate cRNA arrays. Numbers were obtained by dividing the integrated density obtained from the Null signal by the integrated density obtained from the Null + signal. (nd) stands for non detected.

Gene	Array 1	Array 2
MMP2	4.6	1.4
MMP3	5.5	nd
ProCollagen Iα1	3.5	1.3
Procollagen IIIα1	3.7	2.5
Procollagen IVα1	2.0	1.1
Procollagen VIα2	8.1	1.9
Thrombospondin 1	7.5	5.0
Fibronectin	1.1	1.5
Adamts1	2.7	2.2

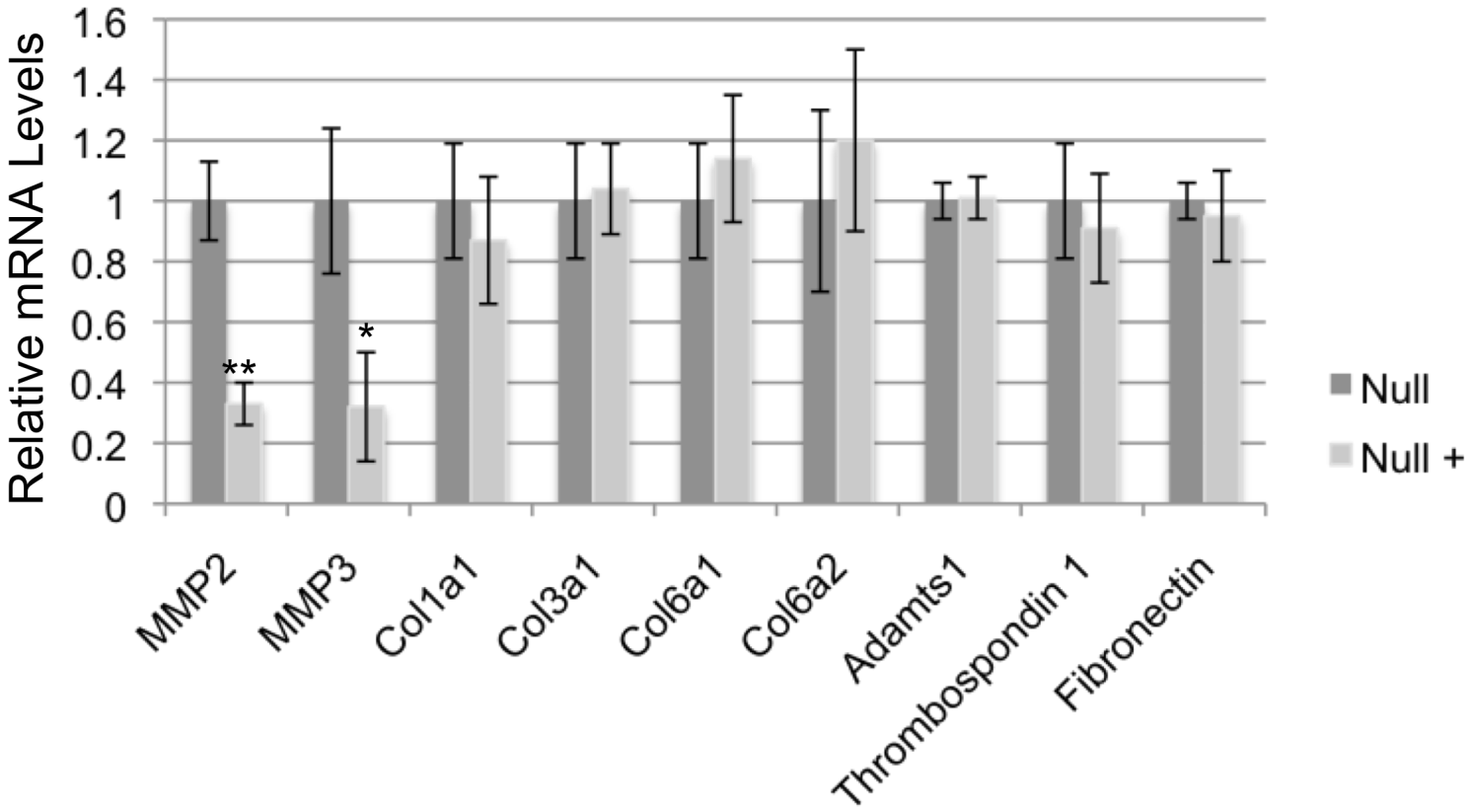


Figure 19. mRNA levels of candidate genes in *Plcg1* Null and Null+ cells. RNA was isolated from Null and Null + cells and subjected to quantitative RT-PCR. Relative mRNA determined using the ΔΔcT method. Single Asterisk (*) denotes statistical significance with a P-value of 0.0172. Double asterisk (**) denotes statistical significance with a P-value of 0.0014. Statistical analysis was performed using an unpaired Student's *t* test.

Discussion

While numerous reports have demonstrated a requirement for PLC-γ1 in cell adhesion and/or migration in various cell types (Kundra *et al.*, 1994; Wells and Grandis, 2003; Tvorogov *et al.*, 2005; Shepard *et al.*, 2006; Wang *et al.*, 2007), a mechanistic understanding of this requirement has not been elucidated. The data in this dissertation show that PLC-γ1 has a regulatory role in controlling the amount of fibronectin produced and assembled into fibronectin fibrils. As fibronectin is a major component of the fibroblast extracellular matrix, which mediates cell adhesion and migration, the level of fibronectin production needs to be tightly controlled to avoid an abnormal composition of extracellular matrix. There are several examples of aberrant matrix compositions that affect cell function (George *et al.*, 1993b; Sottile *et al.*, 1998; Sottile and Hocking, 2002; Sottile and Chandler, 2005). For example, when cell migration is measured on increasing concentrations of assembled fibronectin, migration levels exhibit a biphasic effect. Migration rates increase as fibril concentrations increase until an optimal concentration for maximum migration and above this increased concentrations of assembled fibronectin reduce migration (Morla *et al.*, 1994; Hocking and Chang, 2003; Li *et al.*, 2005; Smith *et al.*, 2006).

The data show that increased secretion correlates with increased fibronectin assembly (Figure 13). This is not a novel concept as increased fibronectin secretion stimulates the increased assembly of exogenous fibronectin (Bae *et al.*, 2004). This is likely due to the close proximity of the endogenous fibronectin to the integrin. The presence of a small amount of fibronectin assembly functions to seed assembly of the exogenous fibronectin. While I have shown that increased fibronectin assembly correlates

with increased secretion and that treatment with cycloheximide to block protein synthesis reduced exogenous assembly in Null cells to a level similar to that observed in the Null + cells (Figure 18), I have not proven that the increased assembly is caused by increased secretion. In order to examine this, fibronectin expression would have to be selectively inhibited in both cell lines and assembly of exogenous fibronectin compared.

The data in this dissertation show that while PLC-γ1 does not influence mRNA levels nor production of fibronectin protein within the cell, it does regulate the levels of fibronectin protein that is secreted. This increase in fibronectin secretion is not part of a global effect on secretion as both Null and Null + cell lines secrete equivalent levels of protein. The data also show that the increase in secreted fibronectin in cells genetically deficient in PLC-γ1 is accompanied by an increase in fibronectin assembly into fibrils. The increase in fibronectin assembly is observed in assays that rely on both the assembly of endogenous fibronectin as well as exogenous fibronectin. While the latter might indicate a separate role of PLC-γ1 in assembly, there is published data indicating that an increase in the levels of endogenous fibronectin does in fact result in an increase in the assembly of exogenous fibronectin into fibrils (Bae *et al.*, 2004; Huang *et al.*, 2008). The data would indicate that PLC-γ1 functions to set the limit for the maximal level of fibronectin secretion, and in the absence of this protein is oversecreted.

The fact that the absence of PLC-γ1 increases the level of secreted fibronectin protein, but not mRNA or protein production is novel. The second messengers formed by PIP_2 hydrolysis are well described for their capacity to effect signaling that impinges on gene expression. However, how these second messengers may affect post-translational processes is less well known.

CHAPTER IV

CONCLUSIONS

Here I have shown that in the absence of PLC-γ1, cells form larger aggregates that are mediated by integrin β1-fibronectin interactions. The increased aggregation is caused by increased fibronectin assembly, which is caused at least in part by increased fibronectin secretion. The increase in secretion is not accompanied by an increase in the levels of fibronectin mRNA or translated protein, nor is there an increase in the total secretion of proteins in the *Plcg1* Null cells.

Regulation of Secretion by PLC-γ1

The main question is "By what mechanism could fibronectin secretion be selectively regulated by PLC-γ1?" I believe that the most likely mechanism for specific regulation is by a molecular chaperone, which may regulate folding, dimerization, or even inclusion into secretory vesicles (Figure 20). There are several reasons for this. First, the existence of molecular chaperones that specifically assist in the folding of a particular protein have already been described(Anelli and Sitia, 2008). Second, a previous study on the fibronectin molecule has indicated a potential role for a molecular chaperone. Third, activation of PLC-γ1 has been shown to regulate gene expression through Ca^{2+} and PKC and fluctuations of the Ca^{2+} level have been shown to regulate the expression of molecular chaperones.

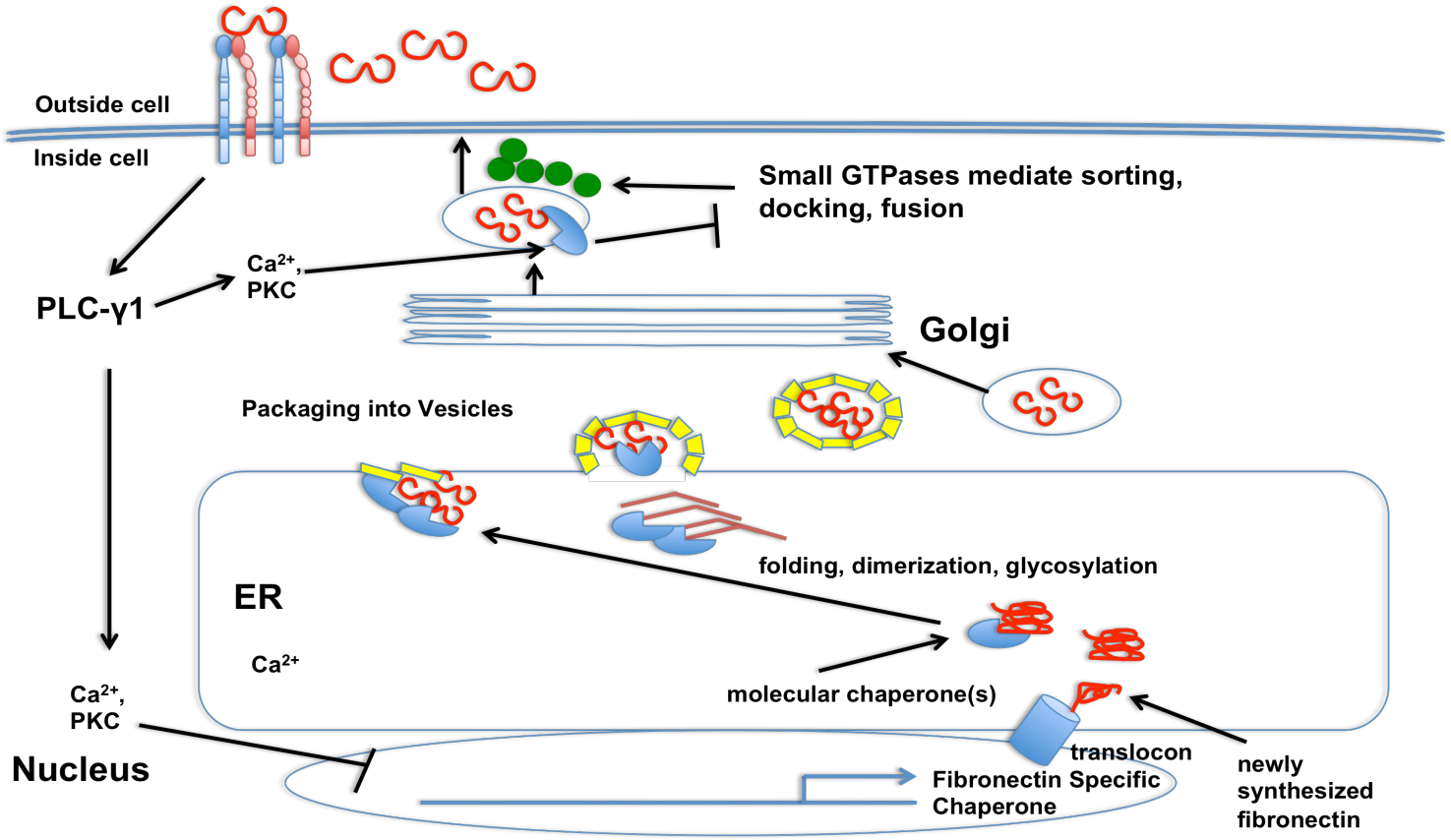


Figure 20. Possible Role of PLC-γ1 in Fibronectin Biosynthesis: The newly synthesized fibronectin is bound by a fibronectin specific molecular chaperone in the endoplasmic reticulum that mediates dimerization, glycosylation, and folding into a compact conformation. Because fibronectin is a soluble protein and would not interact with the Sec24 protein of the COPII machinery, the chaperone may mediate the inclusion of fibronectin into the budding vesicle. Alternatively, the chaperone may modulate activity of the small GTPases that mediate vesicle docking and fusion PLC-γ1 negatively regulates the expression or activation of this molecular chaperone through calcium signaling. In the absence of PLC-γ1, less chaperone is made or activated, therefore reducing the amount of secreted fibronectin.

There are protein specific molecular chaperones that mediate protein folding and secretion. Procollagen folding and secretion is mediated by a molecular chaperone termed Hsp47, which specifically binds procollagen through recognition of the triple helix (Nakai *et al.*, 1992). In the absence of this protein, procollagen is not secreted and assembled into matrix, but is aggregated within the ER (Ishida *et al.*, 2006). Overexpression of Hsp47 correlates with increased collagen secretion and matrix assembly (Taguchi and Razzaque, 2007). In the same way, a molecular chaperone could specifically mediate the secretion of fibronectin. A fibronectin-specific molecular chaperone might mediate proper folding, glycosylation and recruitment of a protein such as protein disulfide isomerase to mediate dimerization. The chaperone may also bind fibronectin and mediate insertion into budding vesicles through interactions with sorting proteins.

Fibronectin dimers are secreted in a compact conformation that most likely prevents the premature aggregation of fibronectin molecules both within the secretory pathway and in the conditioned medium prior to fibronectin assembly(Johnson *et al.*, 1999). A fibronectin-specific chaperone may be required to facilitate secretion of fibronectin in the compact conformation. In the absence of this compact conformation, fibronectin fibrils could form intermolecular associations that could result in premature fibril assembly.

While little research has been conducted on the secretion of fibronectin, one study points to the possibility of a chaperone-binding site. Fibronectin is dimerized, partially glycosylated, and folded in the ER (Hynes and Yamada, 1982). Dimerization requires the variable domain on at least one fibronectin molecule in the dimer and deletion of this

domain inhibits fibronectin secretion. An 18 amino acid section of the variable domain was determined to contain the required residues for dimerization and secretion (Schwarzbauer *et al.*, 1989).. The binding of a molecular chaperone to the 18 amino acid residues of the variable domain of fibronectin may mediate proper folding, dimerization, and secretion of fibronectin as a compact molecule.

The next question that arises is how PLC-γ1 might regulate that chaperone? It is known that PLC-γ1 regulates PDGF-mediated expression of several immediate early genes and interleukin-2 through Ca^{2+} release and/or PKC activation (Nakano *et al.*, 1994; Liao *et al.*, 2001). It is also known that changes in the levels of Ca_i^{2+} regulate the transcription of certain molecular chaperones. For example, transcription of the molecular chaperone grp78/BiP can be stimulated under conditions in which there is a gradual depletion of intracellular calcium stores through the regulation of a calcium sensitive transcription factor NF-Y. At lower concentrations of calcium, association of NF-Y to the grp78 promoter is increased due to a slower dissociation rate leading to increased transcription of the molecular chaperone (Drummond *et al.*, 1987; Roy *et al.*, 1996). It is possible that PLC-γ1 could negatively regulate the expression of a molecular chaperone through the release of Ca^{2+} into the cytosol or the activation of PKC (Figure 20). Another possibility is that if the molecular chaperone modulates the activity of one of the many GTPases involved in vesicular traffic, PLC-γ1 could regulate the function of the molecular chaperone through Ca_i^{2+} release and/or PKC activation.

Experimental Model

PLCg1 Null and Null + mouse embryonic cell lines were used as the experimental model for the role of PLC-γ1 in fibronectin secretion and assembly. Below, the merits and the pitfalls of this cell system and other cell possible systems are compared and discussed. Because the project studied the effect of PLC-γ1 on fibronectin secretion and fibrillogenesis, it was important to use a cell system that produces and secretes significant levels of fibronectin. Therefore, the choice was essentially among fibroblast models. Transformation of cells has been shown to reduce the production of fibronectin as well as the expression of integrin α5β1 (Olden and Yamada, 1977; Akiyama *et al.*, 1990). This means that the use of a non-transformed cell system is preferable in order to maximize fibronectin secretion and assembly. In support of this, attempts to subject the human metastatic breast cancer cell line of epithelial origin, MDA-MB-231, to the hanging drop assay were not successful. MDA-MB-231 cells treated with PLC-γ1 siRNA or left untreated failed to form cell aggregates, most likely due to insufficient fibronectin production or integrin expression (data not shown).

Immortalized *Plcg1* Null and Null + Cells

In my experiments, I utilized PLC-γ1 Null mouse embryonic fibroblasts (MEFs). These cells lack all functional PLC-γ1 protein expression and are immortalized using the 3T3 method, making the continuous culture of these cells possible without the use of a transforming virus (Todaro and Green, 1963). The downside to using the PLC-γ1 MEFs is that these cells have undergone a crisis and have thus become hypotetraploid (Xu, 2005). This could cause changes in protein expression and thus change the signaling

pathways within the cells. In order to reduce the impact that the chromosomal rearrangements have on the experimental system, I have reintroduced PLC-γ1 into the immortalized Null cells to generate the Null + cells. These two cell lines theoretically have the same chromosomal arrangements and differ mainly in the presence or absence of PLC-γ1.

The Null + cells express approximately two-fold more PLC-γ1 as a population than wild-type cells do. I generated a pooled population of Null + cells because the Null cells were also derived in this manner. If I had generated the Null + cell line from single clones, then the possibility for artifacts increases due to the variance that likely exists between individual Null clones. One caveat of this system is the possibility that the results obtained with the Null + cells could be due to an overexpression of PLC-γ1. The variation of PLC-γ1 expression level in individual Null + cells is unknown. The vector used to generate the Null + cells contains PLC-γ1 followed by an IRES sequence and GFP. Due to the IRES sequence, it is unlikely that the GFP expression correlates with the PLC-γ1 expression. A GFP-tagged PLC-γ1 might be useful to generate an add-back cell line as GFP-expression would correlate with PLC-γ1 expression. However, it is not desirable to make an add-back cell line using a GFP-tagged PLC-γ1, as the possibility always exists for the GFP tag to block some protein interaction. I could determine the polymorphic distribution of PLC-γ1 in the Null + cells by fixing the cells, staining them for PLC-γ1, and performing fluorescence activated cell sorting,

In the past, wild-type immortalized MEFs have also been used as a control for immortalized knockout cell lines(Ji *et al.*, 1998; Wang *et al.*, 2001). The positive aspect

of using the wild-type cells is that these cells would contain two copies of the *Plcg1* gene and therefore represent normal PLC-γ1 expression. On the negative side, the wild-type cells must be isolated from a separate animal and undergo a separate immortalization process, resulting in different chromosomal rearrangements with different changes in cell signaling or integrin expression. Therefore, I have decided to compare *Plcg1* -/- (Null) cells to the reconstituted Null + cells.

Primary *Plcg1* Null and Wild-Type Cells

Another alternate model system could be the use of primary fibroblasts isolated from *Plcg1* null and wild type mice. The benefits of using this model system are that diploid *Plcg1* Null and wild-type cells could be compared. The pitfalls of this system are availability of the mice. At present the embryos are frozen and would have to be re-implanted.

Human Foreskin Fibroblasts

Another available experimental system is to use a primary human cell line in which PLC-γ1 is knocked down using small interfering RNA or inhibited using the U73122 compound or the Z-fragment of PLC-γ1(Homma *et al.*, 1992; Thomas *et al.*, 2003). Human foreskin fibroblasts (HFF) are a common fibroblastic cell line used to study fibrillogenesis(Pankov *et al.*, 2000). Use of this cell line is beneficial because cells have not undergone crisis and the subsequent chromosomal rearrangements that the immortalized mouse embryonic fibroblasts have. Diploid human cells, such as the HFF can be cultured for a limited number of passages prior to senescing. In addition, HFF are difficult to transfect meaning that obtaining a consistent level of knockdown may be

difficult. This could potentially affect the reproducibility of the experiments. That said, the use of this cell system to confirm either the results obtained in the hanging drop assay or the assembly assay would be very useful to demonstrate that the results obtained in the Null and Null + MEFs can be extended to other cell types. At present, I have been unable to obtain a sufficient knockdown of PLC-γ1 using these cells.

One might argue that the U73122 compound or Z-fragment, which contains the SH2 and SH3 domains of PLC-γ1, might be used to inhibit PLC-γ1 function in the HFF. However, these reagents do not specifically inhibit PLC-γ1(Homma *et al.*, 1992; Homma and Takenawa, 1992; McNamara *et al.*, 1993; Mogami *et al.*, 1997; Wilsher *et al.*, 2007). The Z-fragment of PLC-γ1 consists of the two SH2 domains and the SH3 domain of PLC-γ1. The idea behind this is that the two SH2 domains would bind to the phosphorylated tyrosines on receptor tyrosine kinases such as the EGFR or PDGFR or tyrosine kinases such as Src. The cDNA for the fragment must be transfected into cells and is probably over-expressed, meaning that the possibility of non-specific interactions is increased. In support of this, evidence shows that the Z-fragment is not specific for PLC-γ (Homma and Takenawa, 1992). There may be several reasons for this. Although PLC-γ1 activation is inhibited by blocking association of the enzyme with the receptor tyrosine kinase, the SH2 domains of the Z-fragment also blocks the binding of other proteins to that same site on the receptor tyrosine kinase, thus preventing non-PLCγ1-associated receptor tyrosine kinase signaling. The Z-domain of PLC also contains a PLC inhibitory domain that has been shown to inhibit multiple PLC isozymes(Homma and Takenawa, 1992).

The compound U73122 has often been used as a PLC inhibitor as it prevents the turnover of phosphoinositides and thus prevents the formation of the second messengers IP_3 and DAG (Vickers, 1993; Thomas *et al.*, 2003). It us unclear how this compound prevents hydrolysis and it has come under increased scrutiny for the numerous effects the compound has on cells. There is an inactive isomer, U73343, which is commonly used as a control. Both compounds have been show to have varying effects on cell signaling by activating nuclear estrogen receptors (Cenni and Picard, 1999), acting as a protonophore in rabbit parietal cells, and activating ion channels (Mogami *et al.*, 1997). One other study showed that U73122 spontaneously formed conjugates with components of the cell culture medium (Wilsher *et al.*, 2007). In summary, while the compound may prevent PIP_2 hydrolysis, we do not know what the mechanism of action is within the cell and what other effects the compounds may have on cell function. Because of this, U73122 and the control U73343 are not ideal reagents to examine the effects of PLC-γ1 inhibition.

Physiological Significance

I propose that because of its biologic importance that there are multiple points of control in fibronectin biogenesis and that PLC-γ1 regulates one step in this process. In this model, PLC-γ1 functions to prevent the secretion of excess fibronectin matrix. Excess secretion of matrix translates into increased assembly of matrix, which may regulate a variety of cell behaviors discussed in this section.

The biologic importance of fibronectin is exemplified by several examples. First, fibronectin provides a surface to which multiple integrins can bind, thus facilitating cell

adhesion and migration (Plow *et al.*, 2000). Second, fibronectin matrix mediates the deposition and/or activation of other matrix components and growth factors such as collagens I and III, fibulin-1, VEGF, and TGF-β (Park *et al.*, 1993; Fontana *et al.*, 2005; Dallas *et al.*, 2006; Kadler *et al.*, 2008). Third, fibronectin activation of integrins stimulates intracellular signaling pathways that result in cell survival, adhesion, migration, and growth depending on the cell type (Huang and Ingber, 2005; Reddig and Juliano, 2005; Cheng-Chin Hsu, 2007). Because of its importance, it would make sense for a cell to control fibril formation at multiple levels, especially at the secretion phase.

Relation to Development

Fibronectin assembly and cell aggregation are important components of development. The developing embryo undergoes a process of compaction that can be related to the aggregation observed in the Null and Null + cells in that it involves cell-fibronectin interactions. Fibronectin-mediated compaction is also observed in limb development and parallels the formation of the leg bud, possibly by providing a matrix by which migrating cells can adhere. When fibronectin assembly is inhibited both by the use of peptides or in a knockout mouse, compaction of the cells is affected and somites fail to properly form (Lash *et al.*, 1984; Takahashi *et al.*, 2007). But how could an increase in fibronectin affect development? Fibronectin assembly levels induce a bimodal effect on cell migration(Palecek *et al.*, 1997; Hocking and Chang, 2003; Li *et al.*, 2005). At very low and very high concentrations of fibronectin matrix, migration is inhibited. It is feasible to speculate that a PLC-γ1 mediated decrease in fibronectin secretion and assembly might increase the migration of cells.

Increased fibronectin secretion and assembly could also affect the thickness of the extracellular matrix. One organ that could possibly be affected is the kidney. Increased matrix deposition by mesangial cells and fibroblasts is implicated in renal fibrosis (Liu, 2006). The increased deposition of matrix affects the structure and function of the glomeruli and thus seriously affects kidney function. This has been observed in the integrin α1 knockout mouse, which produces excess amounts of collagen (Gardner *et al.*, 1999). Upon renal injury, excess matrix accumulation results in renal fibrosis and glomerulosclerosis(Chen *et al.*, 2004).

PLC-γ1 knockout mice die a embryonic day 8.5 with a complete absence of vasculogenesis (Liao *et al.*, 2002). Fibronectin null mice die at about the same age depending on genetic background with defects in somite formation and vasculogenesis (George *et al.*, 1993a). In the absence of fibronectin, the vessels that form are leaky due to unknown reasons. This may indicate that PLC-γ1 impacts vasculogenesis at a point prior to fibronectin. While it is possible that PLC-γ1 might have a function in fibronectin-mediated development, more research would be needed to identify a possible link.

Relation to Cancer

A hallmark of transformation is a decrease in secretion of fibronectin and the transformed phenotype can be suppressed by the over expression of fibronectin (Akamatsu *et al.*, 1996). Increases in fibronectin matrix result in the increased adhesion and spreading of the cancer cell, thus making it more difficult for the cell to detach and migrate from the primary tumor. Since PLC-γ1 negatively regulates the secretion of fibronectin and positively regulates cell migration and tumor metastasis, there is a very

real possibility that PLC-γ1 modulates migration and metastasis though regulation of fibronectin secretion(Turner *et al.*, 1997; Kassis *et al.*, 1999; Wells and Grandis, 2003; Jones *et al.*, 2005; Tvorogov *et al.*, 2005; Shepard *et al.*, 2006; Wang *et al.*, 2007).

Future Directions

PLC-γ1 Regulation of Secretion

The data presented in this dissertation indicate that PLC-γ1 negatively regulates the secretion of fibronectin. The next step would be to determine the mechanism by which PLC-γ1 regulates fibronectin secretion. I hypothesize that PLC-γ1 may regulate the expression of a molecular chaperone, which may in turn facilitate the secretion of fibronectin. If equal amounts of fibronectin are translated in the Null and Null + cells, and more fibronectin is secreted in the Null cells, then the fibronectin that is not secreted in the Null + cells must aggregate somewhere in the secretory pathway prior to being degraded.(Schwarzbauer *et al.*, 1989)(Schwarzbauer *et al.*, 1989)(Schwarzbauer *et al.*, 1989)(Schwarzbauer *et al.*, 1989)(Schwarzbauer *et al.*, 1989)(Schwarzbauer *et al.*, 1989)(Schwarzbauer *et al.*, 1989)(Schwarzbauer *et al.*, 1989)

To further characterize the biogenesis of fibronectin I would perform a pulse chase experiment on the Null and Null + cells and determine a timeline for when the difference in intracellular secretion arises. For example, after a 10-minute pulse and no chase, the data shows that the production of fibronectin protein is equivalent. This experiment would give some indication as to where in the secretory pathway the

difference in secretion originates. This would give an indication as to where PLC-γ1 exerts its effect in the secretory pathway, as it is known that fibronectin biogenesis takes about 30 minutes (Choi and Hynes, 1979; McDonald *et al.*, 1987). A difference at 10 minutes of chase suggests that fibronectin is being degraded by an unfolded protein response or ERAD, whereas a difference that is observed at 30 minutes of chase may indicate a role for PLC-γ1 in a later stage of secretion.

To confirm the results of the pulse chase experiment, I would isolate the ER and Golgi organelles from the cells subjected to pulse-chase experiments and examine the levels of labeled fibronectin within these organelles. This would allow for a quantitative comparison of organelle-associated fibronectin. Retention of fibronectin in the ER or Golgi of the Null + cells could provide more evidence leading to the identification of where secretion is affected by PLC-γ1.

In order to identify possible molecular chaperones for fibronectin, I would immunoprecipitate fibronectin from the intracellular pools. I would then subject the immunoprecipitates to mass spectrometry to identify putative molecular chaperones. Once candidate chaperones were identified I would assess whether PLC-γ1 regulates the expression of the chaperone or even possibly the activity of the chaperone by comparing the expression and association with fibronectin in Null and Null + cells.

Regulation of Migration and Metastasis

Previous studies show that 1) PLC-γ1 is required for migration and metastasis, and 2) that increases in fibronectin assembly reduce cell migration and metastasis (Wells and Grandis, 2003; Tvorogov *et al.*, 2005). This leads to a logical question of whether PLC-γ1 regulation of fibronectin secretion affects migration and metastasis. In order to examine this, I would knock down PLC-γ1 in fibronectin-null cells and subject them to a transwell migration assay towards fibronectin. I could also knock down fibronectin in Null and Null + cells and subject them to the transwell assay. If increased fibronectin secretion and assembly account for the decrease in migration, then in the absence of fibronectin secretion migration would be equal in the Null and Null + cells.

To investigate whether PLC-γ1 mediated regulation of secretion affects metastasis, both PLC-γ1 and fibronectin would have to be knocked down by siRNA in tumor cells. Cells would then be implanted into the mammary fat pad of nude mice and metastasis to the lungs would be compared. Cells transfected with fibronectin siRNA only, Fibronectin + PLC-γ1 siRNA, PLC-γ1 siRNA only, or scrambled siRNA would be compared for the number of metastases in the lungs. One would expect to see a difference in metastasis in PLC-γ1 siRNA and control treated cells. If differences in fibronectin secretion can account for the effect of PLC-γ1 on metastasis, then knocking down fibronectin should remove any difference in metastasis in PLC-γ1 positive and negative cells.

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