MOLECULAR AND FUNCTIONAL ANALYSIS OF PHOSPHATIDYLINOSITOL 4 KINASE TYPE II β

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Dedicated to My husband, Yohan Jin and My family

MOLECULAR AND FUNCTIONAL ANALYSIS OF PHOSPHATIDYLINOSITOL 4 KINASE TYPE IIβ

By

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DISSERTATION

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ABSTRACT

Phosphoinositides play fundamental roles in controlling membrane-based signaling events. Phosphatidylinositol 4-kinases (PI4Ks) catalyze the production of PI4P, a major precursor in phosphoinositide biosynthesis, and consist of two classes (type II and type III), each divided into two isoforms (α and β). PI4KIIα and β differ primarily in their distributions between cytosol and membranes: PI4KIIα is almost exclusively membrane-bound, by virtue of its palmitoylation in a cysteine-rich motif; although PI4KIIβ also contains a palmitoylatable cysteine-rich motif, this isoform is almost evenly distributed between membranes and cytosol, and only about half of the membrane-associated pool is palmitoylated.

My study focused on determining the functions of post-translational modifications and domains of PI4KIIβ and on identifying its binding partners, with the long-term goal of

understanding the roles and mechanisms of regulation of this kinase. Domain analysis shows that the C-terminal 160 amino acids of PI4KIIs determine the distinct membrane binding properties and activities of PI4KIIα and β. As expected, based on our previous work with PI4KIIα, palmitoylation of PI4KIIβ is important for its membrane binding and activity. Although PI4KIIβ is also phosphorylated in cells, this modification has no detectable effect on any examined property of the kinase.

Immunoprecipitation and mass spectrometry revealed a genuine binding partner for PI4KIIβ, Hsp90. The functional significance of the Hsp90-PI4KIIβ interaction was defined using geldanamycin, a specific Hsp90 inhibitor. Geldanamycin treatment disrupts the interaction and destabilizes PI4KIIβ, reducing its half-life by 40% and increasing its susceptibility to proteasomal degradation. Although full-length PI4KIIα does not bind Hsp90, and is not destabilized by geldanamycin treatment, a cytosolic PI4KIIα truncation mutant becomes sensitive to geldanamycin and binds to Hsp90. Thus, both PI4KII isoforms contain Hsp90 binding sites but only PI4KIIβ requires Hsp90 for stabilization, presumably because there is a substantial cytosolic pool of this isoform. Interestingly, brief exposure to geldanamycin causes a partial redistribution of PI4KIIβ from the cytosol to membranes, which results in increased PI4P synthesis in cells. Moreover, the growth factors EGF and PDGF also disrupt the interaction between Hsp90 and PI4KIIβ, suggesting that Hsp90 not only protects PI4KIIβ from degradation, but may also prolong its residency in the cytoplasm until extracellular signals release Hsp90 from the kinase.

Currently the precise roles of PI4KIIβ are unknown. Based on its partial redistribution to the plasma membrane upon cell treatment with growth factors, I speculated

that PI4KIIβ may somehow be involved in receptor-mediated endocytic trafficking. My results, employing siRNA-based knockdown strategies, indicate that depletion of PI4KIIβ enhances early steps of EGFR internalization and subsequent initiation of ERK activation in response to EGF treatment. The facilitated endocytosis that results from this depletion is likely due to an increase in endosomal fusion. Indeed, activities of the endosomal fusion facilitators EEA1 and Rab5 increase in PI4KIIβ depleted cells. These results suggest for the first time a role of PI4KIIβ in endocytic trafficking and signaling of the EGFR.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

AHR : aryl hydrocarbon receptor

AP-1 : adaptor protein-1

AP-2 : adaptor protein-2

AP-3 : adaptor protein-3

APT-1 : acyl protein thioesterase-1

BAR: Bin–Amphiphysin–Rvs

2-BP : 2-bromopalmitate

BSA: Bovine serum albumin

C2 domain: Ca2+-binding domain

CHX : cyclohexamide

CIN85 : Cbl-interacting protein of 85kDa

DAG : diacylglycerol

DMEM : Dulbecco's modified Eagle's medium

DMP : dimethyl pimelimidate

DMSO : dimethyl sulfoxide

EEA1: early endosomal antigen 1

EGFR: epidermal growth factor receptor

ENTH: Epsin N-terminal homology

ERK : extracellular signal-regulated kinase

Eps15 : Epidermal growth factor receptor pathway substrate 15

ESCRT : endosomal sorting complex required for transport

FAPP: four-phosphatase-adaptor protein

FBS : fetal bovine serum

FITC : Fluorescein isothiocyanate

FYVE: Fab1, YOTB, Vac1 and EEA1

Fwd: four wheel drive

GA : geldanamycin

GAP: GTPase activating protein

GEF: guanine nucleotide exchange factor

GFP: green fluorescent protein

GGA: golgi associated, gamma adaptin ear containing, ARF binding protein 1

GST: glutathione S-transferase

HA: haemagglutinin

HIF-1 : hypoxia inducible factor-1

Hsp70 : heat shock protein 70

Hsp90 : heat shock protein 90

INPP5: inositol polyphosphate-5-phosphatase

IP3 : inositol 1,4,5 phosphate

MVB : multi-vesicular body

OSBP: oxysterol binding protein

PBS : phosphate buffered saline

PH: Pleckstrin homology

PX: phox homology

PI: phosphoinositide or phosphatidylinositol

PI4KII: phosphatidylinositol 4 kinase type II

PI4KIII: phosphatidylinositol 4 kinase type III

PI3P: phosphatidylinositol 3- phosphate

PI4P: phosphatidylinositol 4- phosphate

$PI3,5P_2$: phosphatidylinositol 3,5-bisphosphate

PI4,5P$_2$ or **PIP$_2$**: phosphatidylinositol 4,5-bisphosphate

PI3,4,5P$_3$: phosphatidylinositol 3,4,5-triphosphate

PDK1: Pyruvate dehydrogenase kinase

PKC: protein kinase C

PLC: phospholipase C

PNS : post nuclear supernatant

PTEN: phosphatase and tensin homolog deleted on chromosome 10

R5BD : rab5 binding domain

RNAi: RNA interference

dsRNA: double stranded RNA

siRNA: small interfering RNA

SCAR: suppressor of cAMP receptor

SGK: Serum/glucocorticoid regulated kinase

SHIP: SH2-containing inositol-5'-phosphatase

SHR : steroid hormone receptors

SOS : Son of sevenless homolog

STAM : Signal transducing adaptor molecule

Stt4: staurosporine-sensitive mutation 4

Tf: transferrin

TGN: *trans*-Golgi network

TLC : thin layer chromatography

TPR : tetratricopeptide

Ub: ubiquitin

WASP: Wiskott-Aldrich-syndrome protein

WAVE: WASP family verprolin homology protein

CHAPTER 1

Overall Introduction

I. PHOSPHOINOSITIDES

Phosphoinositides (PIs) are polyphosphorylated derivatives of phosphatidylinositol, a phospholipid that resides almost exclusively on the cytosolic surface of cell membranes. Differential phosphorylation at the hydroxyl groups 3, 4 and 5 of the inositol ring in phosphatidylinositol generates seven distinct PIs, which are found in all higher eukaryotes and named according to their site(s) of phosphorylation. A dedicated system of phosphatidylinositol kinases and phosphatases rapidly interconverts one PI to another (Figure 1-1). (De Matteis et al., 2002)

In 1953, the Hokins reported that PIs turn over in response to extracellular signals, initiating research into PIs as signaling molecules (Hokin and Hokin, 1953). Subsequent studies demonstrated that hormone-stimulated hydrolysis of phosphatidylinositol 4, 5 bisphosphates (PI4,5P_2 or PIP_2) by phospholipase C (PLC) generates two second messengers, inositol 1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG), which transduce extracellular signals to elicit Ca^{2+} release and protein kinase C (PKC) activation. This well established and predominant step in PI metabolism is known as the "canonical pathway" (PI→PI4P→PI4,5P_2) (Hughes and Putney, 1988; Lee et al., 1995; Michell and Allan, 1975).

Accumulating evidence demonstrates that, besides generating second messengers, PIs act as specialized signals that aid in defining organelle identity and recruiting regulators (Krauss and Haucke, 2007). As organelle markers and recruiters, distinct sets of PIs exist in

different endomembranes and can be targeted for specific cellular activities (Figure 1-2). PIs fulfill their functional roles by associating with numerous coordinators, such as adaptors, protein kinases, and small GTPases that contain specialized PI binding domains (e.g. PH, FYVE, PX, ENTH, BAR, C2 domains). PIs control the direction and specificity of membrane traffic, cellular signal transduction, and cytoskeletal regulation, which are frequently combined with the additional modulation of protein-protein or protein-lipid interaction networks (Carlton and Cullen, 2005; De Matteis and Godi, 2004; Overduin et al., 2001). For example, PI4,5P_2 is especially enriched at the plasma membrane where it binds to Epsin (containing an ENTH domain) and the AP-2 complex for the formation of curved membranes into newly forming coated pits. PI3P is the major PI on early endosomes and recruits proteins containing FYVE domains, such as EEA1, Rabenosyn5 and PIKfyve. PI3,5P_2 is found on late endocytic organelles, and PI4P is predominantly found at the TGN and binds to the adaptors AP-1 and GGA. (Figure 1-2) (De Matteis and Godi, 2004; Roth, 2004; Wang et al., 2003).

An obvious mechanism for generating and maintaining distinct subcellular pools of PIs is the finely tuned balance of the enzymatic activities of phosphatidylinositol kinases and phosphatases. Multiple PI syntheses can be achieved by combination of different PI-modifying enzymes. Moreover, more than one kinase or phosphatase isoform catalyzes the same biosynthetic step, allowing differentially regulated local production of PIs. Because PIs have emerged as key regulators of cell function, interest in the regulation and cellular roles of the PI-metabolizing enzymes has grown accordingly.

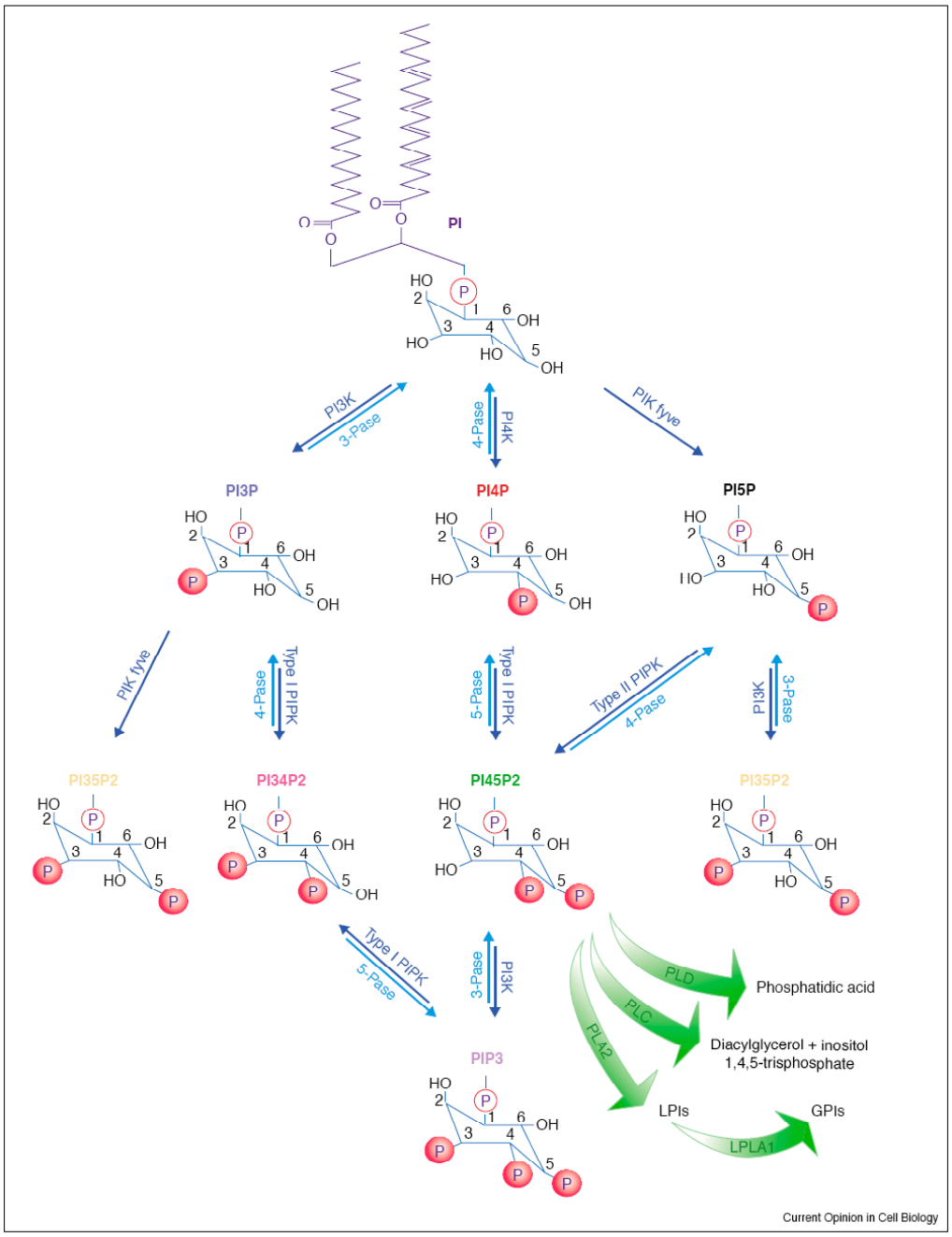


Figure 1-1. Pathways of PIs synthesis and degradation. Phosphatidylinositol undergoes phosphorylation and dephosphorylation catalyzed by kinases and phosphatases. The seven known PIs in eukaryotes are PI4P, PI5P, PI3P, PI4,5P_2, PI3,5P_2, PI3,4P_2 and PI3,4,5P_3. PI4Ks convert PI to PI4P which is subsequently modified to PI4,5P_2 and PI3,4,5P_3 by PIP 5-kinases and PI 3-kinases. PI4,5P_2 can be hydrolyzed by PLC to generate inositol 1,4,5trisphosphate (IP_3) and diacylglycerol(DAG). Figure is taken from *De Matteis,2002.*

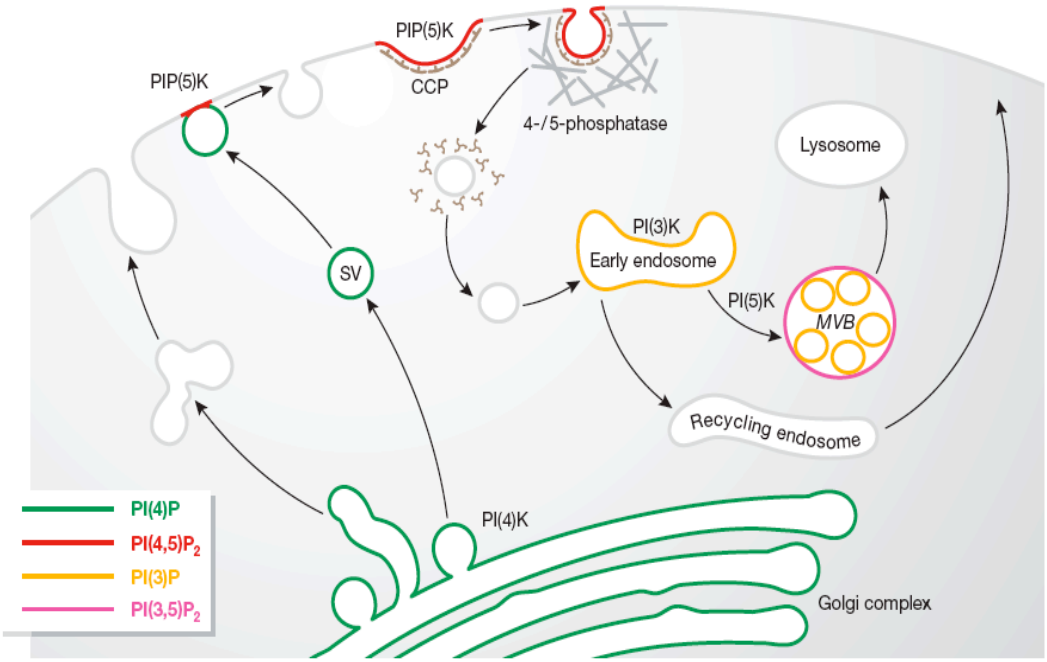


Figure 1-2. Compartmentalization of PIs and their metabolizing enzymes in membrane traffic. PIs and their metabolizing enzymes are concentrated at distinct sites in intracellular membrane traffic pathways. PI4P confers identity to the Golgi complex and is required for trafficking of secretory cargoes. PI3P on early endosomes regulates endosomal fusion and trafficking. PI3,5P_2 delineates sorting stations through multi-vesicular bodies (MVBs) to lysosomes. PI4,5P_2 is required for fusion of secretory vesicles (SVs), formation of endocytic clathrin-coated pits (CCPs) and changes in actin dynamics. Figure is taken from *Haucke, 2007.*

A. Phosphatidylinositol kinases.

Consistent with distinct PI localization, phosphatidylinositol kinases have been found to be in numerous different membrane structures including Golgi, endosomes, lysosomes, plasma membrane, and endoplasmic reticulum. Compartmentalized localization of enzymes partly accounts for their putative functions (Figure 1-2). Subsequent studies of these enzymes led to the categorization of kinases into two families; phosphatidylinositol kinases (PI-kinases) and phosphatidylinositol phosphate kinases (PIP-kinases). The PI-kinase family comprises PI 3-kinases and PI 4-kinases and the PIP kinase family consists of PI4P 5-kinases, PI5P 4-kinases and PI3P 5-kinases, fundamentally based on their substrate specificities. Based on amino acid sequence homology and similarity of biochemical properties, PI-kinases are subdivided into two families. The PI 3/PI 4-kinase family consists of the PI 3-kinases and Type III PI 4-kinases (Carpenter and Cantley, 1998). The other family, the Type II PI 4-kinases was identified within the last 6 years, when our group and that of Hsuan cloned and sequenced mammalian PI4KII and found that it was unrelated to any other known protein or lipid kinase (Figure 1-3, (Barylko et al., 2001; Minogue et al., 2001)).

Interestingly, PI 3-kinases have been extensively studied and shown to be altered in different diseases including cancer and diabetes. Many growth factors and cytokines stimulate the production of $PI3,4,5P_3$. $PI3,4,5P_3$ then activates a group of protein kinases including Akt, SGK, and PDK1, and small GTPases, mediating various cellular activities such as proliferation, differentiation, chemotaxis, survival, trafficking, and glucose homeostasis (Katso et al., 2001).

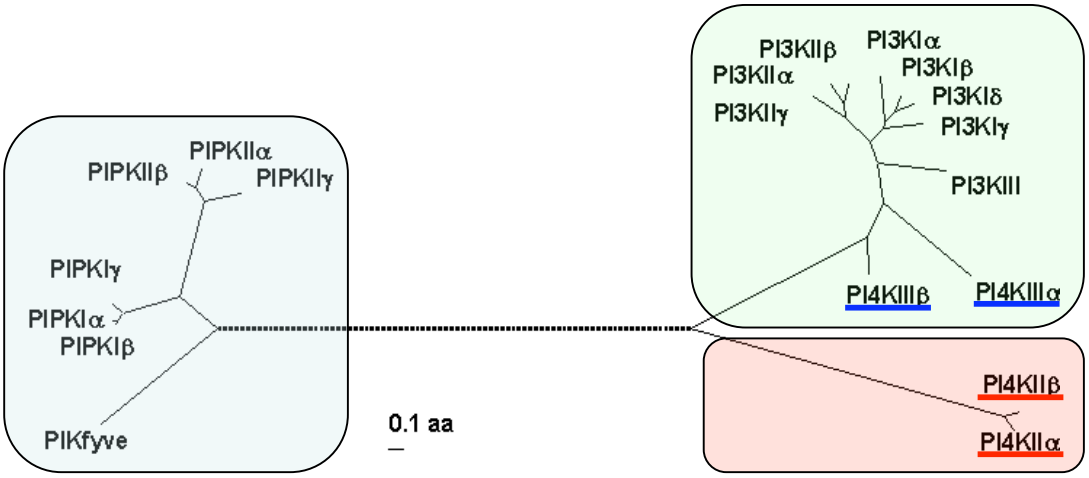


Figure 1-3. Phylogenetic tree of the three PI kinase families. Type II PI 4-kinases comprise a novel and distinct kinase family apart from PIP kinases and PI 3-kinases families. Type III PI 4-kinase α and β are classified with PI 3-kinases. Figure is modified from *Barylko,2002.*

B. Phosphatidylinositol phosphatases.

The family of PI phosphatases consist of 3-, 4-, 5- phosphatases based on substrate specificity. The best known lipid phosphatases are PTEN and SHIP which are 3-phosphatase and 5-phosphatase, respectively, and localize on the plasma membrane to attenuate PI 3-kinase dependent signaling pathways. The levels of PI4P and $PI4,5P_2$ are controlled by both synaptojanin and OCRL, 5-phosphatases which hydrolyze the 5-phosphate of $PI4,5P_2$, leading to a decrease in $PI4,5P_2$ and theoretically in the production of PI4P. Extensive studies have revealed that synaptojanin resides in clathrin coats (specifically those coating internalized synaptic vesicles in neuronal cells) and plays a key role in clathrin-mediated endocytosis of synaptic vesicles on the plasma membrane (Cremona et al., 1999). OCRL was found to be mutated in patients with Lowe syndromes (Zhang et al., 1998). Defects in the OCRL gene product result in elevated $PI4,5P_2$ levels and are believed to alter sorting processes at the TGN and endosomes (Krauss and Haucke, 2007; Lowe, 2005; Roth, 2004).

II. PHOSPHATIDYLINOSITOL 4 KINASES

PI 4-kinases catalyze the production of PI4P from phosphatidylinositol, the first step in the predominant, so-called "canonical" pathway toward biosynthesis of $PI4,5P_2$ and $PI3,4,5P_3$. In mammals, two Types of PI 4-kinases (II and III) have been identified and further subdivided into α and β isoforms for each type. The original Type I enzyme was subsequently identified as a PI 3-kinase. (Heilmeyer et al., 2003)

PI 4-kinases were first cloned and studied in yeast and named Stt4 and Pik1, corresponding to the mammalian Type III orthologs, PI4KIIIα and PI4KIIIβ, which were cloned shortly thereafter. These kinases showed high sequence similarity to the PI 3-kinases within the catalytic domain and their activities could be inhibited by the PI 3-kinase inhibitors, wortmannin (albeit at a higher concentration). It has been demonstrated that distinct PI 4-kinases mediate non-redundant functions by producing PI4P in specific cellular compartments; Stt4 localizes to the plasma membrane and is essential for cell wall integrity and proper actin organization and Pik1 localizes to the Golgi and nucleus and is important for integrity of Golgi structure, cytokinesis and vesicular trafficking in late stages of secretion (Balla and Balla, 2006; Gehrmann and Heilmeyer, 1998; Heilmeyer et al., 2003)

Mammalian PI4KIIIα (230kDa) and β (110kDa) localize predominantly to ER/Plasma membrane and Golgi complex, respectively. Production of the plasma membrane pool of PI4P is apparently mediated by PI4KIIIα (Balla et al., 2008), whereas PI4KIIIβ is believed to generate PI4P for Golgi to plasma membrane trafficking (Godi et al., 1999). PI4KIIIβ is one of the best-studied isoforms among the PI 4-kinases. It can be recruited to the Golgi by the small GTPase, Arf1, and in yeast, by the NCS-1 homolog frequenin (Godi et al., 2004; Godi et al., 1999; Haynes et al., 2005; Weisz et al., 2000). In turn, PI4KIIIβ is required to recruit the GTP-bound form of Rab11 to recycling endosomes, and thereby to regulate the recycling process (de Graaf et al., 2004). PI4KIIIβ is phosphorylated at multiple sites, which may regulate its localization on the Golgi or nucleus, as well as its kinase activity (Hausser et al., 2005; Suer et al., 2001; Szivak et al., 2006)

Although the Type III enzymes were first PI 4-kinases to be cloned and sequenced,

most of the early biochemical studies were carried out on the Type II enzymes, which accounts for >90 % of the PI4-kinase activity in cell extracts [Carpenter, 1990 #109]. However, until PI4KIIα was cloned in 2001, structural and functional analyses lagged behind that of the PI4KIIIs. Type II kinases were purified from a variety of sources in the late 1980s (Husebye et al., 1990; Kanoh et al., 1990; Li et al., 1989; Saltiel et al., 1987). They were found to migrate electrophoretically as 55kDa proteins and to behave as integral membrane proteins, requiring detergent for solubilization. As for their enzymatic properties, Type II PI4 kinases have lower K_ms for PI and ATP than Type III PI4 kinases (approximately 20 μM vs. 130 μM for PI, and 100 μM vs. 700 μM for ATP). Type II and III kinases also differ with respect to their sensitivities to inhibitors: PI4KIIs are highly sensitive to inhibition by adenosine but not by wortmannin; the reverse is true of the Type III kinases. (Table 1-1 and Figure 1-4) (Balla and Balla, 2006; Gehrmann and Heilmeyer, 1998; Heilmeyer et al., 2003; Pike, 1992).

Organism	Type III		Type II	
Yeast	Stt4	Pik1	Lsb6p (66kDa)	
Drosophila	PI4KIIIα	Fwd	dPI4KII (100kDa)	
Mammal	**PI4KIIIα**	**PI4KIIIβ**	**PI4KIIα**	**PI4KIIβ**
MW	230kDa	110kDa	55kDa	55kDa
Localization	ER, PM	Golgi	Golgi, En, SV	Golgi, En,PM
Distribution	Cytosol	Cytosol, Nucleus	Membrane	Cytosol/membrane
Km (ATP)	~700μM	~400μM	10-50μM	10-50μM
Km(PtdIns)	~100μM	~100μM	~20-60μM	~20-60μM
Wortmannin(K*i*)	~300nM	~300nM	insensitive	insensitive
Adenosine (K*i*)	Millimolar	Millimolar	10-70μM	10-70μM

Table 1-1. Distinctive features of the different forms of PI 4-kinase. Type II PI 4-kinase exists as a single form in yeast and *Drosophila*. In mammals, both Types of PI 4-kinases have two isoforms, α and β, which have distinct localizations and distributions. Type II kinases have lower Kms for ATP and PI than Type III kinases and are insensitive to wortmannin. Table is modified from *Balla, 2006*.

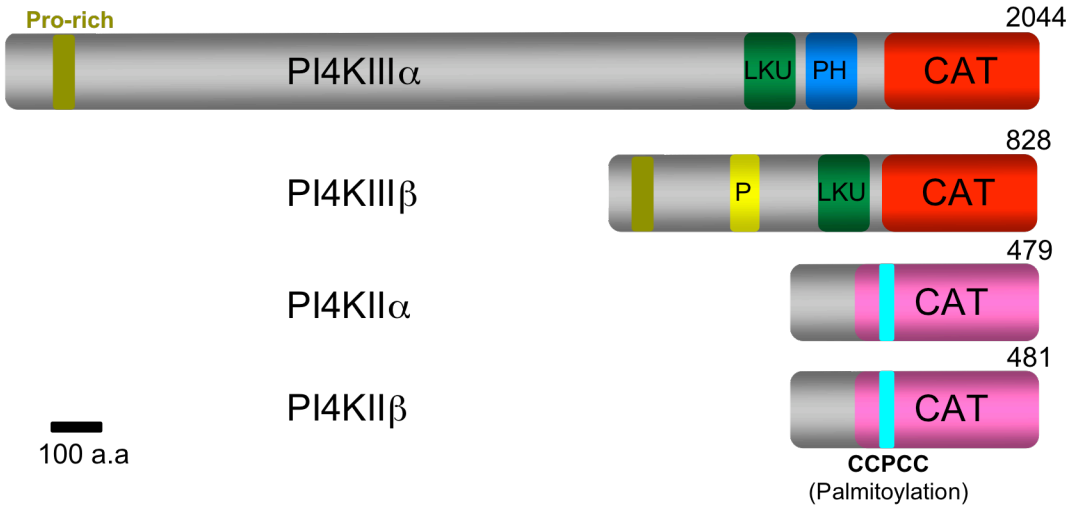


Figure 1-4. Schematic representation of mammalian PI 4-Kinases. Four isoforms of PI 4-kinase presented schematically to show domains and motifs. The numbers of amino acids refer to human isoforms. Bar represents 100 amino acids. CAT: kinase catalytic domain, LKU: lipid kinase unique domain, P: phosphorylation domain, PH: pleckstrin homology domain, Pro-rich: proline rich domain.

III. TYPE II PI 4-KINASES

A. Cloning and characterization of PI4KIIs.

Although the sequences of both Type III kinase isoforms (α and β) have been known for over a decade (Balla et al., 1997; Flanagan et al., 1993; Nakagawa et al., 1996a, b; Wong and Cantley, 1994), cloning of Type II PI 4-kinases had failed due to technical difficulties in handling the purified proteins, which have a tendency to aggregate in nonionic detergents. Moreover, as we now know that they share little sequence homologies to other lipid kinases, they proved impossible to identify in sequence database (Waugh et al., 2001). In 2001, our laboratory purified PI4KIIα from adrenal bovine chromaffin granule membranes and, based on partial sequence obtained from that protein, we cloned ratPI4KIIα and showed that the PI4KII enzymes are evolutionarily distant from other lipid kinases (Figure 1-3) (Barylko et al., 2001; Minogue et al., 2001). Database analyses revealed that mammalian cells have another Type II PI 4-kinase with a unique N-terminal 100 amino acids sequence. But it has a highly homologous downstream sequence that is conserved in that of PI4KIIα. This kinase, designated PI4KIIβ, was cloned in 2002 (Balla et al., 2002; Wei et al., 2002). Once the mammalian sequences were determined, homologous sequences were identified in *Drosophila, Arabidopsis, C. elegans, S. cerevisiae, Plasmodium falciparum*, and even in *Mycobacterium tuberculosis*. We have expressed homologues from *Drosophila* and yeast and found their kinetic properties to be consistent with Type II PI4-kinases (Barylko et al., 2002; Shelton et al., 2003). However, the *Arabidopsis* homologs proved to be protein kinases, and not lipid kinases (Galvao et al., 2008).

Limited proteolytic digestion revealed that the catalytic domain of rat PI4KIIα is located between residues 92 and 478 (the C-terminus) and that this stable 43 kDa core can be broken down into two inactive fragments of 17 kDa and 26 kDa. Thus the catalytic domain can be modeled as a bi-lobed structure consisting of a small N-terminal lobe and a large C-terminal lobe, a common feature of many protein kinases (Barylko et al., 2002; Taylor et al., 1999). Mutational analysis allowed identification of residues conserved among most protein and lipid kinases : K151: presumably involved in anchoring ATP phosphates; D307 involved in catalysis ; and N312 involved in Mg^{2+} coordination) (Figure 1-5) (Barylko et al., 2002).

B. Association of PI4KIIs with membranes.

As stated, Type II PI 4-kinases were long recognized to behave as integral membrane proteins. However, sequence analysis revealed no obvious transmembrane domain. Instead, PI4KIIα was found to be tethered to the bilayer by one or more palmitoyl groups covalently linked to a cysteine-rich segment ($_{173}$CCPCC$_{177}$ in rat PI4KIIα) in the center of the catalytic core. A deletion mutant lacking only these five residues behaves as a peripheral membrane protein, extractable at high pH in the absence of detergent (Barylko et al., 2001). Mutating all four cysteines to serines abrogates catalytic activity, suggesting that fatty acylation may be required to properly orient the kinase on membranes (manuscript submitted). Because palmitoylation is considered to be an important mechanism for the targeting of proteins to membrane subdomains, e.g. 'lipid rafts', this modification may explain the relative abundance of PI4KIIα on membrane raft subdomains (Barylko et al., 2002; Waugh et al., 1998). The presence of PI4KIIα in rafts has physiological relevance, as these domains have

been implicated as major sites of synthesis of agonist-sensitive PI pools (Liu et al., 1998; Pike and Casey, 1996; Pike and Miller, 1998).

The second mammalian isoform, PI4KIIβ, is approximately 480 residues in length and shows the greatest identity with PI4KIIα (68%) in its catalytic domain (residues 91-481). PI4KIIβ also contains the conserved cysteine-rich palmitoylation motif (CCPCC) and key active site residues within the catalytic domain (Figure 1-5B). Despite the presence of the CCPCC motif, at least 50% of PI4KIIβ is cytosolic and its kinase activity is 30% of that of PI4KIIα *in vitro* (Balla et al., 2002). Moreover, when assayed under identical conditions using PI/Triton X-100 mixed micelles as substrate, membrane-bound PI4KIIβ was found to be approximately 20-fold more active than its soluble counterpart (Wei et al., 2002).

C. Distribution of PI4KIIs.

PI4KIIs are expressed in most tissues. Expression of PI4KIIα appears highest in kidney, skeletal muscle, placenta and brain, whereas PI4KIIβ expresses highest in liver, kidney, skeletal muscle and cultured lymphocytes (Balla et al., 2002; Minogue et al., 2001). PI4KIIα localizes preferentially to perinuclear regions stained with βCOPI and TGN46, which are markers for the *cis*- and *trans*-Golgi network, respectively. It is also found on some other intracellular membranes, including early endosomes, synaptic vesicles, and AP-3-containing vesicles (Balla et al., 2002; Guo et al., 2003; Salazar et al., 2005; Wei et al., 2002). PI4KIIβ is also strongly concentrated in the perinuclear region but has a more diffuse cytosolic staining pattern than PI4KIIα. It is also more abundant on the plasma membrane than PI4KIIα (Wei et al., 2002). A significant pool of PI4KIIβ relocalizes to the plasma

membrane in response to growth factor receptor activation and RacV12 overexpression (Wei et al., 2002).

D. Functions of PI4KIIs.

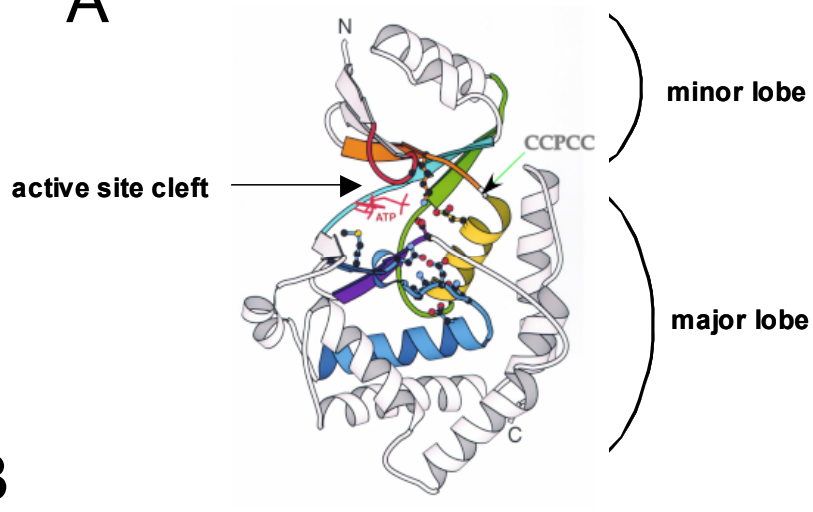
It is widely believed that distinct cellular pools of PI4P are utilized in various PI4P-dependent processes. In mammalian cells, PI4P is highly concentrated on the Golgi complex, as shown by localization of GFP-conjugated PH domains of PI4P binding proteins, FAPP or OSBP. Among the PI4-kinases, PI4KIIα and β and PI4KIIIβ are enriched in Golgi membranes, and RNAi-mediated depletion of PI4KIIα and PI4KIIIβ inhibits recruitment of several PI4P-dependent Golgi proteins (AP-1, GGAs, epsinR, FAPP1 and OSBP) (Balla et al., 2005; Wang et al., 2003). In contrast, PI4KIIβ RNAi does not generate detectable changes in the Golgi (Wang et al., 2003), although it alters endocytosis of the EGFR, as discussed in Chapter 4.

There is strong evidence that the pool of PI4P generated by PI4KIIα is involved in facilitating membrane trafficking in the secretory/endocytic pathway. For example, depletion of PI4KIIα inhibits constitutive secretion from the TGN, presumably by inhibiting recruitment of the clathrin adaptor protein AP-1 (Wang et al., 2003). More recently, PI4KIIα was shown to be important for late endosomal trafficking and lysosomal degradation of the EGFR (Minogue et al., 2006). This function is supported by another observation, showing that PI4KIIα localizes to AP-3 containing vesicles, forming complexes that may regulate endosome to lysosome transport (Craige et al., 2008). As mentioned, PI4KIIIβ also regulates intra Golgi and Golgi-to-cell surface transport (Bruns et al., 2002; Godi et al., 2004).

Another potential role for the PI 4-kinases is in cellular signaling. A wide variety of receptors stimulate PLC and the breakdown of $PI4,5P_2$, which must be replenished for continued signaling. Although Type II-like PI 4-kinase enzymatic activities have been detected in highly purified plasma membranes (Ekblad and Jergil, 2001; Payrastre et al., 1990) and in immunoprecipitates of activated EGF receptors (Cochet et al., 1991; Kauffmann-Zeh et al., 1994), T cell receptors (Fernandis and Subrahmanyam, 2000; O'Shea et al., 1986), and integrins (Berditchevski et al., 1997; Yauch and Hemler, 2000), there is no direct evidence that Type II PI 4-kinases function in the PLC pathway. On the other hand, PI4KIIIα RNAi decreases the level of PI4P on the plasma membrane, while apparently not affecting Golgi levels of PI4P (Balla et al., 2005). Furthermore, PI4KIIIα replenishes and maintains the PI4P pools involved in signaling, particularly on the plasma membrane (Balla et al., 2008).

Another potential role for PI4P generated by PI4Ks is as a precursor in the production of $PI4,5P_2$ and $PI3,4,5P_3$ utilized in regulation of the cortical actin cytoskeleton. These phosphoinositides bind to a wide array of cytoskeletal proteins (e.g., gelsolin, profilin, cofilin, vinculin, talin), regulators of actin nucleation (e.g., WASP, WAVE/SCAR), and GDP-GTP exchange factors (GEFs) for Rho family GTPases (Rac, Rho, Cdc42). There is currently no information concerning a role for any PI 4-kinases in actin reorganization.

A


N
CCPCC
ATP
C
minor lobe
active site cleft
major lobe

B

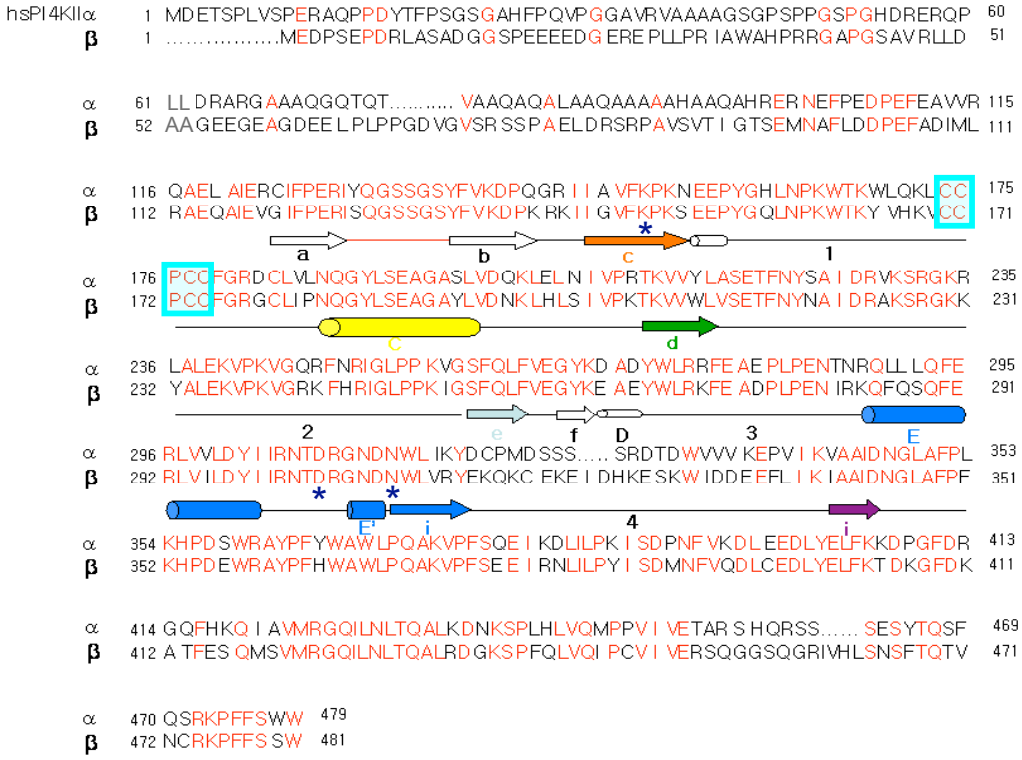

hsPI4KIIα 1 MDETSPLVSPERAQPPDYTFPSGSGAHFPQVPGGAVRVAAAAGSGPSPPGSPGHDRERQP 60
β 1 .....MEDPSEPDRLASADGGSPEEEEDGERELLPRIAWAHPRRGAPGSAVRLLD 51
α 61 LLDRARGAAAQGQTQT..... VAAQAQALAAQAAAAAHAAQAHRERNEFPEDPEFEAVVR 115
β 52 AAGEEGEAGDEELPLPPGDVGVSRSSPAELDRSRPAVSVTIGTSEMNAFLDDPEFADIML 111
α 116 QAELAIERCIFPERIYQGSSGSYFVKDPQGRIIAVFKPKNEEPYGHLNPKWTKWLQKLCC 175
β 112 RAEQAIEVGIFPERISQGSSGSYFVKDPKRKIIGVFKPKSEEPYGQLNPKWIKYVHKVCC 171
a b c 1
α 176 PCCFGRDCLVLNQGYLSEAGASLVDQKLELNIVPRTKVVYLASETFNYSAIDRVKSRGKR 235
β 172 PCCFGRGCLIPNQGYLSEAGAYLVDNKLHLSIVPKTKVVWLVSETFNYNAIDRAKSRGKK 231
C d
α 236 LALEKVPKVGQRFNRIGLPPKVGSFQLFVEGYKDADYWLRRFEAEPLPENTNRQLLLQFE 295
β 232 YALEKVPKVGRKFHRIGLPPKIGSFQLFVEGYKEAEYWLRKFEADPLPENIRKQFQSQFE 291
2 e f D 3 E
α 296 RLVVLDYIIRNTDRGNDNWLIKYDCPMDSSS..... SRDTDWVVVKEPVIKVAAIDNGLAFPL 353
β 292 RLVILDYIIRNTDRGNDNWLVRYFKQKCEKEIDHKESKWIDDEFFLIKIAAIDNGLAFPF 351
E' i i 4
α 354 KHPDSWRAYPFYWAWLPQAKVPFSQEIKDLILPKISDPNFVKDLEEDLYELFKKDPGFDR 413
β 352 KHPDEWRAYPFHWAWLPQAKVPFSEEIRNLILPYISDMNFVQDLCEDLYELFKTDKGFDK 411
α 414 GQFHKQIAVMRGQILNLTQALKDNKSPLHLVQMPPVIVETARSHQRSS..... SESYTQSF 469
β 412 ATFESQMSVMRGQILNLTQALRDGKSPFQLVQIPCVIVERSQGGSQGRIVHLSNSFTQTV 471
α 470 QSRKPFFSWW 479
β 472 NCRKPFFSSW 481

Figure 1-5. Structural model and alignment of PI4KIIs **A.** Predicted conformation of the PI4KII catalytic domain. Key residues involved in catalysis are shown as ball-and-stick figures. According to this model, the palmitoylation motif (designated CCPCC) is in close proximity to the nucleotide binding pocket. This model is based on the structure of actin/fragmin kinase, the most closely related enzyme with known structure. Figure is adapted from *Barylko. 2002*. **B.** Sequence alignment of human PI4KIIα and PI4KIIβ. Red indicates identity. The N-terminal domains (~ residues 1-100) are highly diverse. The catalytic domains extend approximately from residue 100 to the C-terminus. Within this catalytic domain is a core, extending from residue ~120-350 containing essential residues (marked as *) conserved among most kinases. CCPCC residues are boxed. Conformational predictions are shown beneath this catalytic core. Arrows represent α-helices; tubes represent β-strands, colors correspond to those in A.

IV. SUMMARY OF RESEARCH GOALS AND RESULTS

The major goals of my research project were to elucidate the regulation and functions of PI4KIIβ. Aside from its stimulus-dependent relocalization to the plasma membrane, mentioned above, essentially nothing was known about this kinase isoform prior to my work. Indeed, even the existence of a cytosolic pool of PI4KII, largely consisting of PI4KIIβ, was not established until 2001. Therefore, I focused on identifying regions in this kinase that determine its distribution between membrane and cytosol (Chapter 2); how this distribution is influenced by a direct interaction with Hsp90 (Chapter 3); and how PI4KIIβ affects trafficking and signaling of the EGFR (Chapter 4). In addition, I include some earlier studies involving characterization of the role Type II PI 4-kinase of *Drosophila* (Chapter 5).

CHAPTER 2

Identification and characterization of molecular determinants of activation and membrane targeting of PI4KIIβ

I. ABSTRACT

PI4KIIα is almost entirely palmitoylated and membrane-bound, suggesting that it is constitutively active in cells. In contrast, there is a large cytosolic, inactive pool of PI4KIIβ in resting cells, indicating that this isoform may be subject to regulation. One of my goals was to understand the basis for this regulation and to explain at a molecular level the different membrane-binding properties of the two isoforms. In this Chapter, I show that only the active, membrane-associated pool of PI4KIIβ is palmitoylated and phosphorylated. Palmitoylation, but not phosphorylation, was required for expression of kinase activity, at least *in vitro*. I further show that PI4KIIβ, and to a much lower extent PI4KIIα, relocalizes to cell-cell adhesion sites in cells that co-express a constitutively active Rac mutant, RacV12. The highly diverse N-terminal regions of the two kinases (~residues 1-90) are responsible for their differential recruitment to cell junctions. However, differences in their overall ability to bind to membranes are determined by the C-terminal 160 amino acids (residues 315-478 and 312-481 of PI4KIIα and β, respectively) of their conserved catalytic domains. Thus, distinct regions of the Type II PI4-kinases are responsible for membrane binding and specific subcellular targeting.

II. INTRODUCTION

PI4KIIα and β have conserved catalytic domains, extending approximately from residue 91 to the C-termini (residues 478 and 481 of rat PI4KIIα and human PI4KIIβ, respectively). However, their N-terminal 90 amino acids have almost no sequence similarity (Figure 2-1). Despite the absence of a transmembrane domain, more than 90% of PI4KIIα is membrane-bound and requires detergent for extraction. This tight membrane anchoring is due to palmitoylation in a cysteine-rich motif, CCPCC, located within the catalytic domain (Barylko et al., 2001). Protein palmitoylation is achieved by the activities of a newly discovered family of protein acyltransferases (PATs), which themselves are integral membrane protein (Bijlmakers and Marsh, 2003; Mitchell et al., 2006). Deletion of the CCPCC motif from PI4KIIα abrogates catalytic activity and converts the kinase into a tightly bound peripheral membrane protein, extractible by 0.1M Na_2CO_3 (pH 11), though not by 1M NaCl (Barylko et al., 2001). Although the CCPCC motif is also present in PI4KIIβ, this isoform is only 50% membrane associated, and at least half of the membrane-bound pool can be solubilized by 0.1M Na_2CO_3 (pH 11). Only membrane-bound PI4KIIβ expresses kinase activity (Wei et al., 2002).

At present, nothing is known about the regulation of Type II PI 4-kinases, aside from our own observations that palmitoylation of the kinase is essential for expression of catalytic activity. In this chapter, I will characterize the post-translational modifications of PI4KIIβ and identify the internal determinants that control the different distributions of the two isoforms.

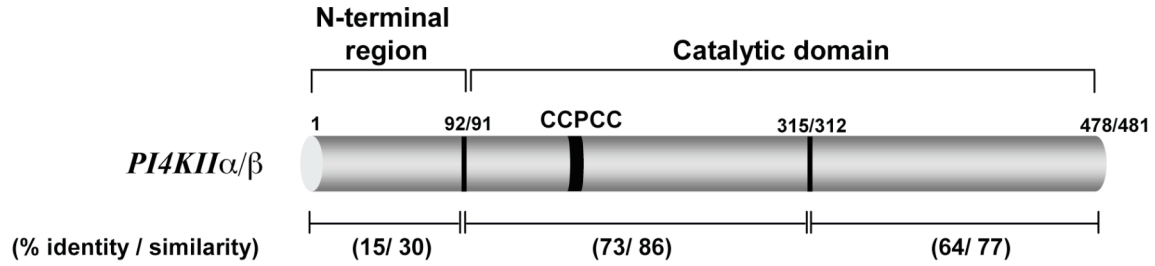


Figure 2-1. Scheme showing sequence identities and similarities of regions of PI4KIIα and β. The catalytic domain is divided into two segments corresponding to the hybrid molecules characterized in Table 2-1. CCPCC is the palmitoylation motif conserved in both isoforms.

III. EXPERIMENTAL PROCEDURES

Reagents- L-α-phosphatidylinositol was from Avanti Polar-Lipids, Inc. (Alabaster, AL). Primers were obtained from Sigma/Genosys and IDT (Coralville, IA). Cloning reagents and reagents for mutagenesis were from Stratagene. Triton X-100 and reagents for electrophoresis and immunoblotting were from Bio-Rad. Other reagents, including ATP, buffers, and protease inhibitors, were from Sigma. Monoclonal anti-Myc antibody 9E10 was obtained from the National Cell Culture Center (Minneapolis, MN). Polyclonal anti-Myc antibody was from Santa Cruz (Santa Cruz, CA). Monoclonal anti-HA antibody was from Covance (Berkeley, CA). Synthetic peptides corresponding to residues 2-17 of rat PI4KIIα and 2-15 of human PI4KIIβ conjugated to keyhole limpet hemocyanin via an extra N-terminal cysteine residues were used to immunize rabbits. The antibodies were affinity purified on SulfoLink pre-packed columns (Pierce) with cross-linked peptide according to the manufacturer's directions.

Cell culture and transfection - COS, HeLa, and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. The cells were plated one day prior to transfection. For transient expression of proteins, cells were transfected for 20 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

cDNA constructs and generation of mutants- Rat PI4KIIα (gi:16758553) and human PI4KIIβ (gi:20159767) cDNAs were cloned in pCMV5-Myc vectors as described previously

(Barylko et al., 2001; Wei et al., 2002). Cat α, the Myc-tagged ratPI4KIIα catalytic domain, was generated as described in (Barylko et al., 2001) wherein it was designated "92-478". Cat β, the Myc-tagged humanPI4KIIβ catalytic domain, was generated by PCR using primers: 5'-TGCACTGCAGACTATTGGTACTTCAGAGATGAATGCATTCTTGG-3' and 5'-GC-TCTAGACTACCAGGAGGAAAAAAATGGCTTCCTGC-3', which introduced the PstI and XbaI restriction sites, respectively. The palmitoylation motif of PI4KIIβ $_{170}$CCPCC$_{174,}$ was deleted by PCR using primers: 5'-GGACCAAATATGTCCATAAGGTC-TTTGGCCGAGGCTGCCTGATTCCTAATC-3' and 5'-GATTAGGAATCAGGCAGC-CTCGGCCAAAGACCTTATGGACATATTTGGTCC -3'.

To make chimeric cDNA constructs of full-length PI4KIIα and PI4KIIβ as shown in Table 2-1, residues 1-91 of ratPI4KIIα were replaced by residues 1-90 of human PI4KIIβ and vice versa. To generate the α/β chimera, two separate PCR products were generated using primers : A (5'-CCATCGATATGGACGAGACGAGCCCGCTAGTGTCC-3') and B (5'-CCAAGAATGCATTCATCTCTGAAGTACCAATCACCGCCACGGCCGCCTGGG-3') as well as primers C (5'-CCCAGGCGGCCGTGGCGGTGATTGGTACTTCAGAGA-TGAATGCATTCTTGG-3') and D (5'-GCTCTAGACTACCAGGAGGAAAAAAAT-GGCTTCCTGC-3'). The fragments were combined and overlap extended using primers, A and D. The final PCR product was subcloned into pCMV5-Myc vector using ClaI and XbaI. To generate β/α chimera, two PCR products were generated using primers: 1 (5'-GCGAATTCATGGAGGATCCCTCCGAGCCCGACC-3') and 2 (5'-CGCGGTGGGTC-TGAACGGCGTGAGTTACTGAAACCGCGGGGCGGC-3') as well as 3 (5'-GCCGCCC-CGCGGTTTCAGTAACTCACGCCGTTCAGACCCACCGCG-3') and 4 (5'-GCTCTA-

GACTACCACCATGAAAAGAAGGGCTTCCGACTCTGG-3') and combined for another round of PCR using primers 1 and 4. The PCR product was digested with EcoRI and XbaI and inserted into pCMV5-Myc vector.

The chimeric catalytic domains, Cat α/β and Cat β/α were generated by replacing residues 91-311 of human PI4KIIα with residues 92-314 of rat PI4KIIβ and vice versa. First and second round PCRs were performed as described above. The Cat α/β chimera was generated by using primers: 5'-CCATCGATCACGCCGTTCAGACCCACCGCGAGC-3' and 5'-CTGCTTTTCGTATCTGACCAACCAGTTGTCATTGCC-3' as well as 5'-GGCAATGACAACTGGTTGGTCAGATACGAAAAGCAG-3' and 5'-GCTCTAGACTA-CCAGGAGGAAAAAAATGGCTTCCTGC-3'. The extended product was ligated in pBluescript II SK vector and subcloned in pCMV5-Myc vector after digestion with KpnI and XbaI. The Cat β/α chimera was generated by using primers: 5'-CCATCGATACT-ATTGGTACTTCAGAGATGAATGC-3' and 5'- CGGATAGTCATATTTGATTAACCAA-TTATCATTGCC-3'as well as 5'-GGCAATGATAATTGGTTAATCAAATATGACTAT-CCG-3' and 5'-GCTCTAGACTACCACCATGAAAAGAAGGGCTTCCGACTCTGG-3'. The final PCR product was subcloned into pCMV5-Myc using ClaI and PstI.

Point mutations in pCMV5-Myc-PI4KIIβ cDNA constructs were performed using the QuickChange kit (Stratagene) according to the manufacturer's instructions. pCMV5-HA-Rac2V12 cDNA was kindly provided by Dr. Michael White (UT Southwestern Medical Center).

Preparation of cytosol and membranes- Cells were washed with PBS and scraped in a solution containing 0.25M sucrose, 20mM Tris-HCl (pH 7.5), 0.1M NaCl, 1mM EDTA,

0.2mM PMSF, protease inhibitor cocktail (10 μg/ml each of N^{α}-p-tosyl-L-lysine chloromethyl ester, N^{α}-p-tosyl-L-arginine methyl ester, N^{α}-p-tosyl-L-lysine chloromethyl ketone, leupeptin, and pepstatin A), and phosphatase inhibitors (50mM NaF, 50mM glycerophosphate, 1mM sodium orthovanadate). Cells were lysed by two freeze-thaw cycles and passage through a 27.5 gauge needle. Lysates were then centrifuged at 1,000 x g for 5 min to obtain post nuclear supernatants (PNS). The PNS were then centrifuged at 200,000 x g for 15 min to separate cytosol from membranes. The resulting membrane pellets were homogenized in one of the following solutions: 1). 0.1M Na_2CO_3 (pH 11) to extract peripheral proteins; or 2). 1% Triton X-100 (v/v), 20mM Tris-HCl (pH 7.5), and 1mM EDTA to extract integral proteins. After homogenization, samples were again centrifuged at 200,000 x g for 15 min at 4 °C to remove insoluble material. In some cases, the membrane fractions were prepared sequentially. In these cases, designated in the figure legends, pellets were first homogenized in 0.1M Na_2CO_3 (pH 11) to extract peripheral proteins, then in solutions containing 1% Triton X-100 to extract integral proteins.

Analysis of [^{3}H]palmitate and [^{32}P]P_i incorporation- For radiolabeling, transfected COS cells were incubated with phosphate-free or lipid-free medium containing 5% dialyzed FBS and either 0.25mCi/ml [^{32}P]P_i (PerkinElmer Life Sciences) or, as described by Linder et al (Linder et al., 1995), with [^{3}H]palmitate (0.3mCi/ml) (PerkinElmer Life Sciences) for 4 h. After three brief washes with PBS, labeled cells were scraped with buffer containing 50mM Tris-HCl (pH 8.0), 0.15M NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.05% SDS, 2mM EDTA, 0.2mM PMSF and protease and phosphatase inhibitors (RIPA buffer). The cell lysates were immunoprecipitated by 4 h incubation with anti-Myc antibody

that had been chemically cross-linked to protein G-sepharose beads (Zymed) using dimethyl pimelimidate (DMP) (Pierce). The immunoprecipitates were washed three times with RIPA buffer and eluted by boiling with 2 x SDS sample buffer and then electrophoresed. To analyze the incorporation of [^{32}P]P_i, dried gels were scanned using FLA-5100 (Fuji Photo film Co., Ltd) and radioactivity of bands was quantified using the Multi-Gauge V2.3 program (Fuji Photo film Co., Ltd). To analyze the incorporation of [^{3}H]palmitate, radioactive bands were exposed in X-ray film and those bands were quantified using Image J 1.34n (National Institutes Health, USA; http://rsb. info.nih.gov /ij/).

Identification of phosphorylation sites - To identify phosphorylation sites, HEK293 cells were transfected with pCMV5-Myc-PI4KIIβ for 20 h. Cell lysates were prepared in RIPA buffer containing phosphatase inhibitors. Expressed Myc-PI4KIIβ was immunoprecipitated with anti-Myc antibody and, after three washes with RIPA buffer, the beads were electrophoresed and visualized by Coomassie blue staining. The slowly migrated upper band was excised and subjected to mass spectrometric analysis. The lower kinase band was used for the control.

The excised Coomassie blue stained protein bands were subjected to in-gel digestion with trypsin essentially as described (Patterson and Aebersold, 1995; Shevchenko et al., 1996). The dried protein digests were dissolved in 5% formic acid and loaded onto a pulled capillary filled with POROS R2 resin. After washing three times with 5% formic acid, the peptides were eluted into a nanoelectrospray needle with 1 to 2 μL of nanoelectrospray sample solution (1% ammonium hydroxide, 60% methanol). All mass spectrometry (MS) analyses were performed on a QSTAR Pulsar-i quadrupole time-of-flight tandem mass

spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a nanoelectrospray ion source (MDS Proteomics, Odense, Denmark). For precursor ion scanning experiments, the instrument was set in negative ion mode, to detect the PO^{3-} fragment ion at m/z –79. The optimum collision energies were determined for each experiment by gradually increasing the collision energy in steps corresponding to one twentieth of the m/z value of the precursor ion. Phosphorylated peptides were detected by nanoelectrospray precursor ion scanning and the exact sites of phosphorylation were determined by manually interpretation of mass spec spectra in combination of result from site-specific mutagenesis. This was done with collaboration with Dr. Hongjun Shu. (Department of Molecular Oncogenesis, Genome Research Institute, University of Cincinnati).

Depalmitoylation - Prior to chemical or enzymatic deacylation, expressed PI4KIIβ was immunoprecipitated in RIPA buffer. Precipitated kinases were "renatured" by washing in a solution containing 0.1 % Triton X-100, 50mM Tris-HCl (pH 7.5), 0.1M NaCl, and 0.2mM PMSF. Chemical deacylation was carried out by incubation with 1M hydroxylamine (pH 7.0) for 16 h at 4°C. Control samples were incubated with 1M Tris-HCl (pH 7.0) under the same conditions. Enzymatic deacylation was performed by treating the immunoprecipitates with bacterially expressed acyl protein thioesterase 1 (APT1; clone provided by Dr. Susanne Mumby, UT Southwestern) for 2 h at 30°C.

Inhibition of palmitoylation with 2-bromopalmitate (2-BP) - To obtain unpalmitoylated kinase, COS cells expressing PI4KIIβ were treated with 2-BP according to a procedure

described by Webb et al. (Webb et al., 2000). Cells were incubated for 16 h in DMEM containing 2.5% dialyzed FBS and 0.25% defatted BSA, with or without 100μM 2-BP dissolved in ethanol. The cells were then labeled for 4 h with [^{3}H]palmitate (in the presence or absence of 2-BP), lysed in RIPA buffer, and the kinases were immunoprecipitated and renatured as above. Each immunoprecipitate was divided into aliquots in order to estimate the amount of kinase by immunoblotting, to measure incorporation of radioactive palmitate, and to assay for kinase activity.

Immunofluorescence microscopy - HeLa cells transfected with different constructs were grown on coverslips for 16 h. Cells were then washed three times with ice-cold PBS, fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. After two washes with PBS and blocking in solution containing 20mM Tris-HCl (pH7.5), 150mM NaCl, 0.1% Tween-20, and 1% BSA, cells were incubated with primary antibody followed by fluorescent-conjugated secondary antibody and FITC-phalloidin (Molecule Probes, Eugene, OR). All fluorescence microscopy was performed with a Ziess Axiovert 200M equipped with a Cooke SensicamQE 12 bit CCD camera. The 860002 version 2 JP4 filter set (Chroma Technology) was used for all image acquisition. Data collection and analysis were controlled by SLIDEBOOK 4.0 (Intelligent Imaging Innovations).

PI 4-kinase assays in vitro - Kinase activity was measured by phosphorylation of PI in PI/Triton X-100 mixed micelles (lipid/detergent molar ratio 1/10), using [γ-^{32}P]ATP (10mCi/ml) as radioactive phosphate donor as described previously (Barylko et al., 2001).

Phospholipids were extracted according to Bligh and Dyer (Bligh and Dyer, 1959) and separated by thin layer chromatography (TLC) in a solvent system consisting of *n*-propyl alcohol/H_2O/NH_4OH (65:20:15). Radioactive PI4P spots were detected by autoradiography using FLA-5100 (Fuji Photo film Co., Ltd) and radioactivity was quantified using the Multi-Gauge V2.3 program (Fuji Photo film Co., Ltd).

Other procedures - SDS-polyacrylamide gel electrophoresis and immunoblot analyses were carried out by the methods of Laemmli (Laemmli, 1970) and Towbin et al. (Towbin et al., 1979), respectively. To quantify PI4KII levels in various fractions, samples were electrophoresed and immunoblotted with anti-Myc antibody. The immunoblots were incubated with ^{125}I-labeled secondary antibody to estimate relative amounts of proteins. Alternatively, immunoblotted bands were quantified using Image J 1.34n. Protein concentrations were determined using the modified Lowry method (Lowry et al., 1951) according to Peterson (Peterson, 1979) with BSA as a standard.

IV. RESULTS

2.1. Roles of post-translational modifications of membrane-bound PI4KIIβ.

In cells, approximately 90% of PI4KIIα and 30% of PI4KIIβ behave as integral membrane proteins, requiring detergent for solubilization. The remaining 70% of PI4KIIβ is almost evenly divided between cytosolic and peripheral membrane pools (Figure 2-2A). This distribution pattern is similar for the recombinant, overexpressed kinase (Figure 2-2B). Our lab showed previously that only the membrane-associated pool of PI4KIIβ exhibits

enzymatic activity (Wei et al., 2002). The different catalytic activities of membrane-bound and cytosolic PI4KIIβ were likely to be due to differences in their post-translational modifications. Additionally, deletion of the palmitoylation motif ($_{173}$CCPCC$_{177}$) inactivated ratPI4KIIα (Barylko et al., 2002). Therefore, we first checked whether the two pools of PI4KIIβ are differentially acylated. As shown in Figure 2-3A, only membrane-bound PI4KIIβ incorporates radioactivity when expressed in [^{3}H]-palmitate labeled cells.

Cytosolic PI4KIIβ migrates on SDS-gels as a single polypeptide of approximately 55kDa, whereas the membrane-associated kinase contains a second, more slowly migrating band of variable intensity. Both bands of membrane-associated PI4KIIβ incorporate radioactive palmitate (Figure 2-3A). However, only the upper band is radioactive in cells labeled for 4 h with [^{32}P]P_i, indicating that a portion of membrane-bound PI4KIIβ is phosphorylated (Figure 2-3B). The upper electrophoretic band was lost upon alkaline phosphatase treatment, providing further evidence that phosphorylation is responsible for its slower migration on gels (not shown). As part of my project, I tested whether post-translational modifications, palmitoylation and phosphorylation, influence the membrane-binding properties and activity of PI4KIIβ.

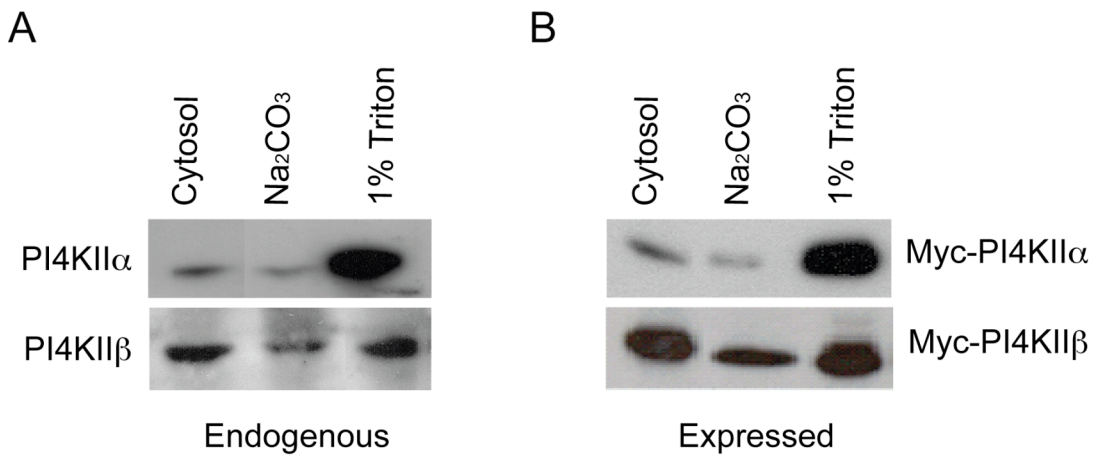


Figure 2-2. Distribution of PI4KIIα and β between membranes and cytosol. A. Hela cells were treated as described in Experimental Procedures to obtain cytosol and membranes. Membranes were extracted with 0.1M Na_2CO_3 (pH 11) to solubilize peripherally bound proteins, or 1% Triton X-100 to solubilize both peripheral and integral membrane proteins. Endogenous PI4KIIα and β were immunoblotted with anti-PI4KIIα and anti-PI4KIIβ antibodies, respectively. **B**. COS cells expressing Myc-PI4KIIα or Myc-PI4KIIβ were treated as described above. Expressed kinases were detected with anti-Myc antibodies.

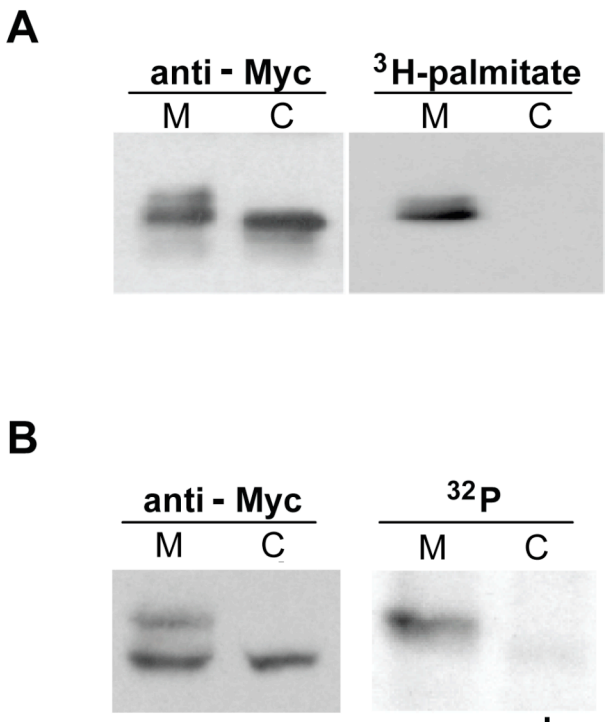


Figure 2-3. Palmitoylation and phosphorylation of membrane-bound PI4KIIβ. COS cells expressing Myc-PI4KIIβ were incubated for 4 h with [^{3}H]-palmitate or [^{32}P]P*i*. Kinase was then immunoprecipitated with anti-Myc antibodies from membranes (M) and cytosol (C) and subjected to SDS gel electrophoresis for subsequent analysis. Left panels are immunoblots of the precipitates; Right panels are autoradiograms to detect palmitoylation (A) or phosphorylation (B). **A.** [^{3}H]-palmitate incorporation into membrane-bound, but not cytosolic, PI4KIIβ. **B.** Incorporation of radioactive phosphate into the slowly migrating band of membrane-bound PI4KIIβ.

2.1.1. *Role of palmitoylation on membrane binding and activation.*

To investigate the role of palmitoylation on membrane binding and activity, we deleted the cysteine-rich motif within the catalytic domain, $_{170}$CCPCC$_{174}$, to eliminate palmitoylation (Figure 2-4A). The mutant was analyzed for its distribution between cytosol and membranes, and assayed for catalytic activity (Figure 2-4B). As shown in Figure 2-4C, this ΔCCPCC mutant is only slightly more soluble than wild-type PI4KIIβ, indicating that thioacylation is not essential for association of the kinase with biological membranes. However, deletion of the $_{170}$CCPCC$_{174}$ motif eliminates the pool of PI4KIIβ that requires detergent for solubilization (Figure 2-4D).

To confirm that palmitoylation is required for activity of wild-type PI4KIIβ, we subjected wild-type membrane-bound PI4KIIβ to chemical or enzymatic deacylation using hydroxylamine (NH_2OH) or bacterially expressed acyl protein thioesterase 1 (APT1), respectively. These interventions result in losses of activity that correspond closely to reductions in [^{3}H]palmitate incorporation (Figure 2-5). Treatment with 1M NH_2OH removes 95% of incorporated label and reduces enzymatic activity to a similar extent. APT1 treatment of membranes reduces both PI4KIIβ palmitoylation and activity by about 40%. A similar effect is observed when acylation was inhibited in cells by treatment with 2-BP (Figure 2-5), an agent that interferes with protein acyltransferase activity (Webb et al., 2000).

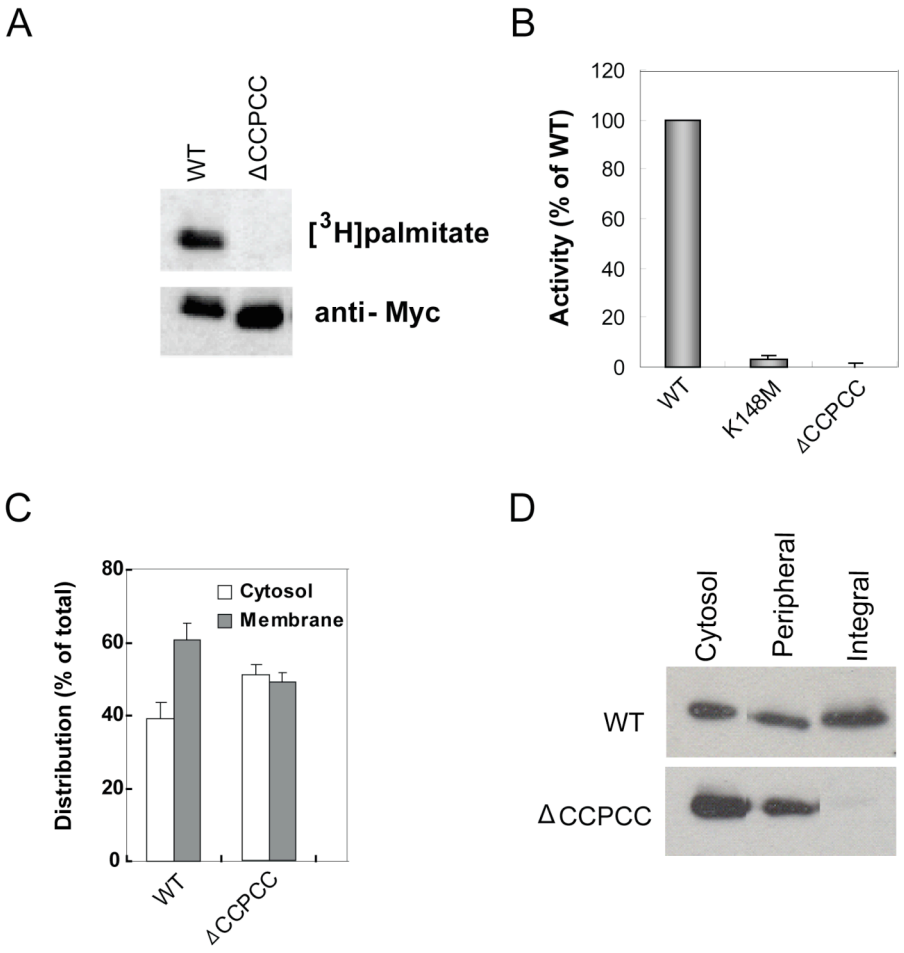


Figure 2-4. Palmitoylation of membrane-bound PI4KIIβ. A. Myc-PI4KIIβ (WT) and deletion mutant (ΔCCPCC) lacking the putative palmitoylation sites (residues 170-174) were expressed in COS cells. Following incubation of cells with [^{3}H]palmitate, both proteins were immunoprecipitated with an anti-Myc antibody. Upper panel, autoradiogram; lower panel, immunoblot. **B.** PI 4-kinase activities were assayed for 30 min at 20 °C using PI/Triton X-100 micelles (1/10 molar ratio) as substrate (See Experimental Procedures for details). An inactive mutant (K148M) served as a control. Bar graph shows the results of three independent experiments expressed as the means +/- S.D. **C.** Influence of palmitoylation on the distribution of PI4KIIβ between membranes and cytosol. Bar graph represents the results from four independent experiments. **D.** Sequential extraction of the membrane-bound pools of PI4KIIβ and ΔCCPCC. Peripheral membrane-bound proteins were extracted with 0.1M Na_2CO_3 (pH11) and, after centrifugation in 200,000 xg for 15 min, pellets were re-extracted with 1% Triton X-100.

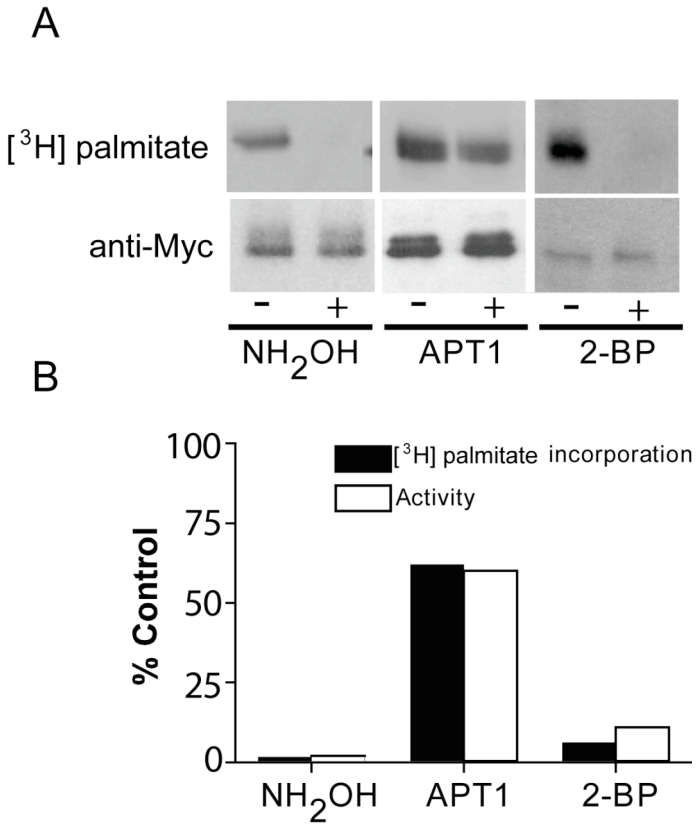


Figure 2-5. Correlation between catalytic activity and palmitoylation state of PI4KIIβ. A. Reduction of palmitoylation of PI4KIIβ. Myc-tagged PI4KIIβ was immunoprecipitated from [^{3}H]palmitate -labeled COS cells and chemically deacylated using 1M NH_2OH or enzymatically deacylated by incubation with acyl protein thioesterase 1 (APT 1) for 2 h at 30°C. In other experiments, COS cells were treated with 100 μM 2-bromopalmitate (2-BP) for 16 h prior to [^{3}H]palmitate labeling. Immunoprecipitates were electrophoresed to monitor [^{3}H]palmitate incorporation by autoradiography or assayed for kinase activity. **B.** Correlation between activity and [^{3}H]palmitate incorporation was normalized to the amount of kinase, which was estimated by immunoblotting with anti-Myc antibody followed by [^{125}I]-labelled secondary antibody. Bar graph represents the average of two measurements.

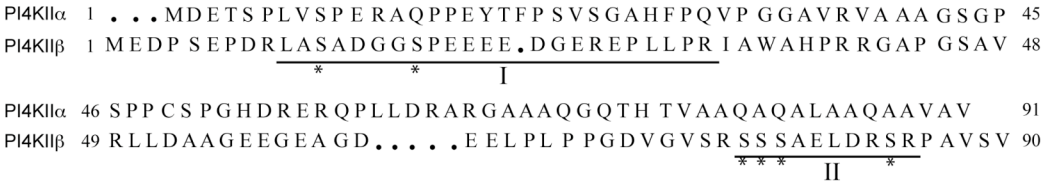
2.1.2. *Identification of the major phosphorylation site of PI4KIIβ.*

Because only the membrane-bound form of PI4KIIβ was phosphorylated, I asked whether this modification is important for its initial targeting to membranes or for its subsequent palmitoylation. To address this question I collaborated with Dr. Hongjun Shu to identify the major *in vivo* phosphorylation sites using mass spectrometry. Two modified peptides were present in the slowly migrating band of membrane-bound PI4KIIβ; both are located in the N-terminal region (Figure 2-6A). The two peptides contain six potential phosphorylation sites. Three of these, serines 12, 17, and 77, were predicted to be among the likeliest phosphorylation sites according to the NetPhos (CBS, University of Denmark) and Scansite 2.0 (MIT) programs. Based on these predictions we expressed four point mutants (S12A, S77A, S12,77A, and S12,17,77A) and measured their ability to incorporate radioactive phosphate relative to the wild-type kinase. Attempts to express the S17A mutant failed. Mutation of serine 12 does not diminish phosphate incorporation, whereas mutation of residue 77 reduces phosphate incorporation by 80%. Because the triple mutant, S12,17,77A, shows no further reduction in phosphorylation (top panel in Figure 2-6B), I concluded that serine 77 represents the major *in vivo* phosphorylated residue. The S77A mutant migrates on SDS gels as a single species; hence, phosphorylation of this residue is apparently responsible for the slowed elecrtrophoretic migration (bottom panel in Figure 2-6B). Neither the S77A nor the S12,17,77A mutant have impaired kinase activity (Figure 2-6C), consistent with the lack of inhibition observed upon alkaline phosphatase treatment (not shown). Moreover, both mutants are similar to wild-type PI4KIIβ with respect to their distribution between membranes and cytosol (Figure 2-6D).

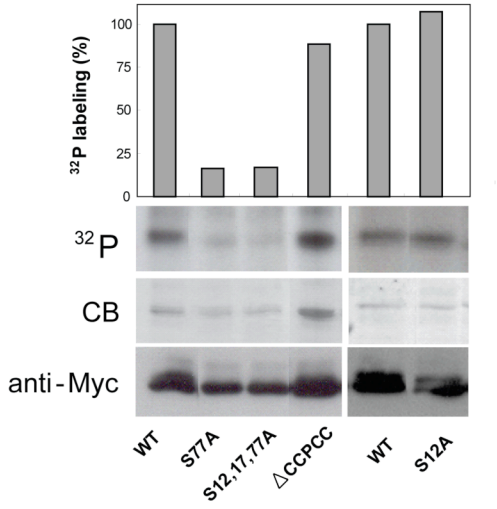
Next, I tested if there is a relationship between phosphorylation and palmitoylation of PI4KIIβ. To determine if palmitoylation is necessary for kinase phosphorylation, wild type PI4KIIβ and the deletion mutant lacking the palmitoylation motif (ΔCCPCC) were expressed in [^{32}P]Pi-labeled cells. Figure 2-6B shows that there is no difference in [^{32}P]Pi incorporation in the two proteins, indicating that palmitoylation is not a prerequisite for phosphorylation. To determine if kinase phosphorylation influences palmitoylation, I analyzed [^{3}H]-palmitate incorporation into wild type PI4KIIβ and the S77A and S12,17,77A mutants. Again, no difference in labeling between the wild type and mutant kinases is observed (Figure 2-6E).

Taken together, these results demonstrate that phosphorylation, at least in the N-terminal region, is not required for the interaction between PI4KIIβ and its yet unidentified protein acyltransferase. Moreover, PI4KIIβ does not have to be integrally bound to the membrane to undergo phosphorylation.

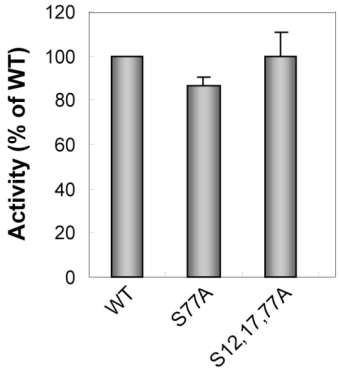
A


PI4KIIα 1 . . . MDETSPLVSPERAQPPEYTFPSVSGAHFPQVPGGAVRVAAAGSGP 45
PI4KIIβ 1 MEDPSEPDRLASADGGSPEEEE.DGEREPLLPRIAWAHPRRGAPGSAV 48
I
PI4KIIα 46 SPPCSPGHDRERQPLLDRARGAAAQGQTHTVAAQAQALAAQAAVAV 91
PI4KIIβ 49 RLLDAAGEEGEAGD. . . . . EELPLPPGDVGVSRSSSAELDRSRPAVSV 90
II

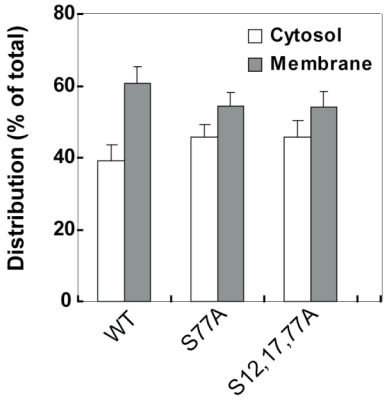
B


32P labeling (%)
100
75
50
25
0
32P
CB
anti-Myc
WT
S77A
S12,17,77A
ΔCCPCC
WT
S12A

C


Activity (% of WT)
120
100
80
60
40
20
0
WT
S77A
S12,17,77A

D


Distribution (% of total)
80
60
40
20
0
Cytosol
Membrane
WT
S77A
S12,17,77A

E

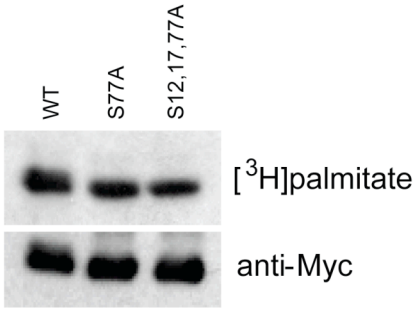

WT
S77A
S12,17,77A
[3H]palmitate
anti-Myc

Figure 2-6. Identification and characterization of the major phosphorylation site in PI4KIIβ. **A.** Alignment of the N-terminal segments of PI4KIIα and β, showing the phosphorylated peptides in PI4KIIβ identified by mass spectrometry. The upper band of electrophoresed PI4KIIβ was subjected to mass spectrometry as described in Experimental Procedures. Sequences of potentially modified tryptic peptides are underlined. Asterisks (*) designate the likeliest phosphorylation sites according to the Scansite program (MIT). **B**. Incorporation of [^{32}P]P_i into wild type PI4KIIβ and point mutants. COS cells expressing kinases were labeled for 4 h with [^{32}P]P_i as in Figure 2-3. Bar graph shows phosphate incorporation relative to wild-type PI4KIIβ. Top panel, ^{32}P incorporation; middle panel, Coomassie-blue staining of the precipitates; bottom panel, immunoblot of the precipitates. **C.** Effect of mutations on PI4KIIβ activity. Membranes were isolated from COS cells expressing wild-type or mutant forms of PI4KIIβ, extracted in Triton X-100, and assayed using PI/Triton X-100 mixed micelles as substrate. Activities were normalized to the amount of expressed kinase, quantified by anti-Myc immunoblotting. The results are shown as the means +/- S.D. from three separate experiments. **D.** Effect of mutations on the distribution of PI4KIIβ between membranes and cytosol. Membranes and cytosol were prepared from transfected cells as described in Materials and Methods and analyzed by anti-Myc immunoblotting to estimate relative levels of PI4KIIβ. **E.** Incorporation of [^{3}H]palmitate into wild-type and mutant PI4KIIβ. Transfected cells were labeled with [^{3}H]palmitate for 4 h as in Figure 2-3. Kinases were then immunoprecipitated with anti-Myc antibodies and subjected to electrophoresis and autoradiography.

2.2. Identification of domains that determine the differential distributions and membrane-binding properties of PI4KIIα and β.

Based on the sequence diversity of the N-terminal 90 amino acids of PI4KIIα and β (Figure 2-1), we predicted that these regions would determine the differences in distribution and palmitoylation level between the two isoforms. The N-terminal region of PI4KIIα has an isoelectric point of 6.9 and contains a long stretch of hydrophobic residues ($_{76}$VAAQAQ-ALAAQAAVAVHAVQ$_{95}$), whereas the N-terminal region of PI4KIIβ contains few hydrophobic residues, two stretches of charged residues ($_{19}$EEEEDGERE$_{28}$ and $_{55}$EEGEAGDEE$_{63}$), and has an isoelectric point of 4.2. Therefore, we speculated that the N-terminal region of PI4KIIα might enhance its interaction with membranes, whereas that of PI4KIIβ could interfere with membrane binding. However, this prediction proved to be incorrect, as the catalytic domains of the two kinases, designated Cat α and Cat β, behave similarly to the full-length proteins despite lacking the N-terminal 90 residues (Table 2-1). Indeed, Cat β is even more soluble than full-length PI4KIIβ (71% vs. 44%), indicating that the N-terminal region contributes somewhat to membrane binding. However, a Myc-tagged construct consisting of residues 1-90 of PI4KIIβ is exclusively cytosolic (not shown), suggesting that it is not sufficient for membrane binding, even though it contributes to binding in the context of the full-length protein. Consistent with these results an α/β chimera (residues 1-91 of PI4KIIα fused to residues 91-481 of PI4KIIβ) distributes similarly to PI4KIIβ. Likewise, a β/α chimera (residues 1-90 of PI4KIIβ fused to residues 91-478 of PI4KIIα) behaves like PI4KIIα (Table 2-1). The palmitoylation levels of the truncated kinases and chimeras are also determined by their catalytic domains: Cat α and β/α were

palmitoylated to the same extent as PI4KIIα; Cat β and α/β are palmitoylated to slightly lower extents than PI4KIIβ. The enzymatic activities of these mutant kinases correspond reasonably closely with their levels of palmitoylation.

Having established that the catalytic domains contain the major membrane binding and palmitoylation determinants of PI4KIIα and β, I next generated a pair of hybrid catalytic domains to more precisely localize these determinants within the two kinases. Because the C-terminal portions of the catalytic domains are slightly less similar than the N-terminal portions (77% vs. 86% similarity; Figure 2-1), I examined the distribution in cells of a chimera (termed Cat α/β) consisting of residues 91-314 of PI4KIIα fused to residues 312-481 of PI4KIIβ, and another chimera consisting of residues 91-311 of PI4KIIβ fused to residues 315-478 of PI4KIIα (Cat β/α). As shown in Table 2-1, Cat α/β is 85% cytosolic and, hence, more similar to PI4KIIβ with respect to solubility, whereas Cat β/α is, like PI4KIIα, almost exclusively bound integrally to membranes. Consistent with these observations, Cat α/β is a poorer substrate than Cat β/α for palmitate incorporation in cells, and expresses negligible kinase activity *in vitro*. These data demonstrate that the C-terminal ~160 amino acids are primarily responsible for the distinct membrane binding properties of PI4KIIα and β, and, more specifically, for the presence of a large pool of cytosolic PI4KIIβ which may be available for activation in response to cellular stimuli (Wei et al., 2002).

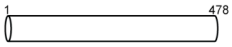
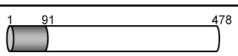
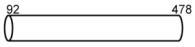
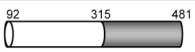
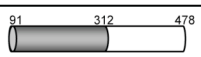
Constructs	Cytosolic (%)	Membrane-bound		Palmitoylation (% of IIα)	Activity (% of IIα)
		Peripheral (%)	Integral (%)		
α 	8	9	83	100	100
β 	44	27	29	30	30
α/β 	61	23	16	16	14
β/α 	6	10	84	85	124
Cat α 	3	17	80	118	107
Cat β 	71	14	15	13	9
Cat α/β 	85	5	10	11	1
Cat β/α 	2	8	90	56	35

TABLE 2-1. Membrane association, relative palmitoylation and activity of wild-type, truncated, and hybrid PI4KIIs. Wild-type, truncated, and hybrid kinases were expressed in COS cells. Cytosolic, peripheral and integral pools were prepared as described in Experimental Procedures. Values are the averages of at least two independent experiments for each construct. To estimate relative levels of palmitoylation, transfected COS cells were labeled for 4 h with [^{3}H]palmitate prior to immunoprecipitation and analysis of the kinase. Kinase assays were performed as described in Experimental Procedures and represent the average of results from three separate experiments, each carried out in triplicate.

2.3. Role of the N-terminal domain of PI4KIIβ in membrane targeting.

2.3.1. PI4KIIβ relocalizes to cell-cell adhesion sites in response to RacV12 expression.

In epithelial cells, overexpression of a constitutively active Rac mutant, RacV12, induces membrane ruffling and lamellopodial expansion, as well as enhancing the formation of cell-cell contacts (Braga et al., 1997; Takaishi et al., 1997). Here I show that PI4KIIβ also relocalizes to cell-cell contact sites when cells were grown to higher confluency (Figure 2-7). In contrast, PI4KIIα retains its predominant perinuclear distribution under this condition (Figure 2-7). As quantified in Figure 2-8, approximately 24% of RacV12-induced contacts display PI4KIIα staining whereas 70% of contacts display PI4KIIβ staining.

2.3.2. Role of the N-terminal domain in targeting PI4KIIβ to cell-cell contacts.

To determine which region of PI4KIIβ contains subcellular targeting information, I localized the truncated and hybrid mutants described above in control and RacV12-expressing cells. In control cells, the catalytic domains of PI4KIIα and β localize similarly to their parent molecules (Figure 2-8A). Cat α, like full-length PI4KIIα, is highly enriched in the perinuclear region. Cat β, though somewhat more diffusely distributes throughout the cytoplasm than full-length PI4KIIβ, is also found in the perinuclear region in control cells. However, both Cat α and Cat β show significantly reduced relocalization to cell-cell contact sites in response to RacV12 expression, with 9% and 13% of the contacts containing Cat α and Cat β, respectively (Figure 2-8B). The α/β hybrid consisting of the N-terminal 90 amino acids of PI4KIIα fused to the catalytic domain of PI4KIIβ relocalizes almost identically to wild-type PI4KIIα, with 20% of contacts displaying α/β staining. Likewise, the β/α hybrid

consisting of the N-terminal 90 amino acids of PI4KIIβ fused to the catalytic domain of PI4KIIα behaves similarly to wild-type PI4KIIβ, with 72% of contacts staining for the β/α construct.

Thus, whereas the N-terminal region does not influence the overall ability of PI4KIIβ to associate with membranes, it plays an essential role in stimulus-dependent relocalization of the kinase to the cell periphery.

Although the N-terminal region of PI4KIIβ is necessary for its targeting to cell-cell adhesions, it is apparently not sufficient. As mentioned above, when this region was expressed as a Myc-tagged fusion protein it was found exclusively in the cytosol. Co-expression of RacV12 did not alter this cytosolic distribution (not shown). To determine if palmitoylation or phosphorylation of the kinase is required for relocalization I expressed the ΔCCPCC or S77A mutants. Although ΔCCPCC mutant loses its preferential perinuclear distribution in control cells, it relocalizes as well as or better than, wild-type PI4KIIβ to cell-cell contacts in RacV12 expressing cells (Figure 2-9). The S77A mutant behaves similarly to wild type of PI4KIIβ. Thus, association of PI4KIIβ with these contact sites depends neither on its palmitoylation nor its phosphorylation.

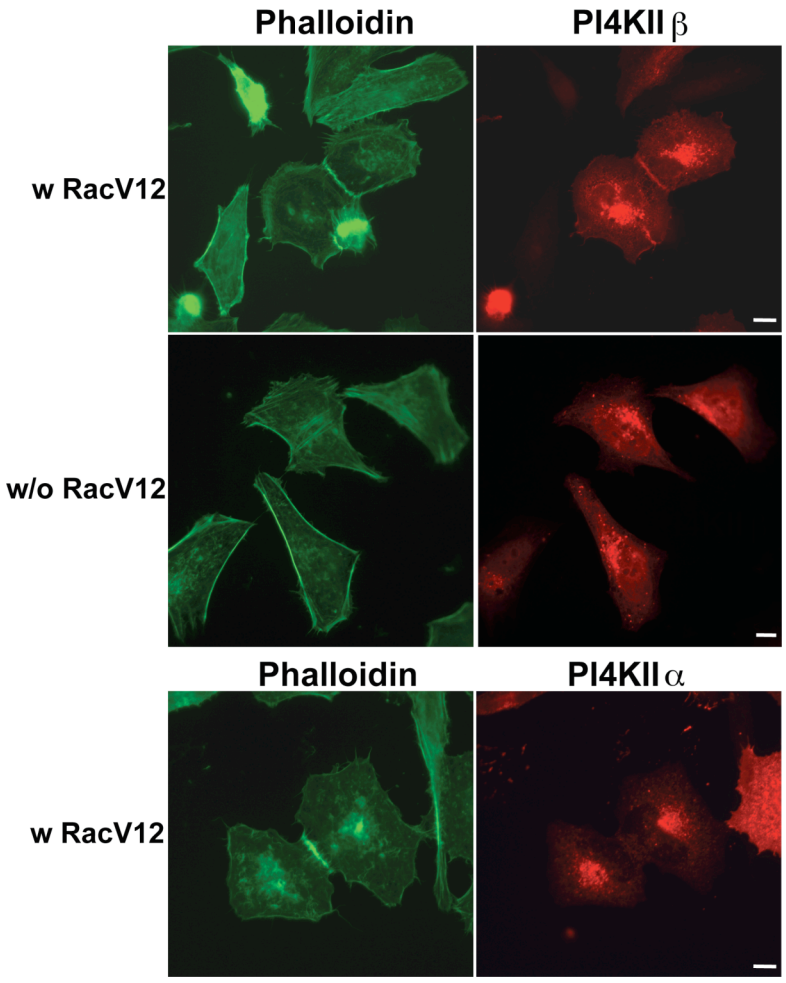


Figure 2-7. Relocalization of PI4KIIβ to cell-cell adhesions in response to RacV12 expression. HeLa cells expressing Myc-PI4KIIα or Myc-PI4KIIβ were co-transfected with HA-RacV12. Control cells were transfected with Myc-PI4KIIβ alone. PI4KIIs were visualized using anti-Myc primary antibodies and rhodamine-labeled secondary antibodies. Actin filaments were stained with FITC-phalloidin. Scale bars are 10μm. Results are quantified in Figure 2-8.

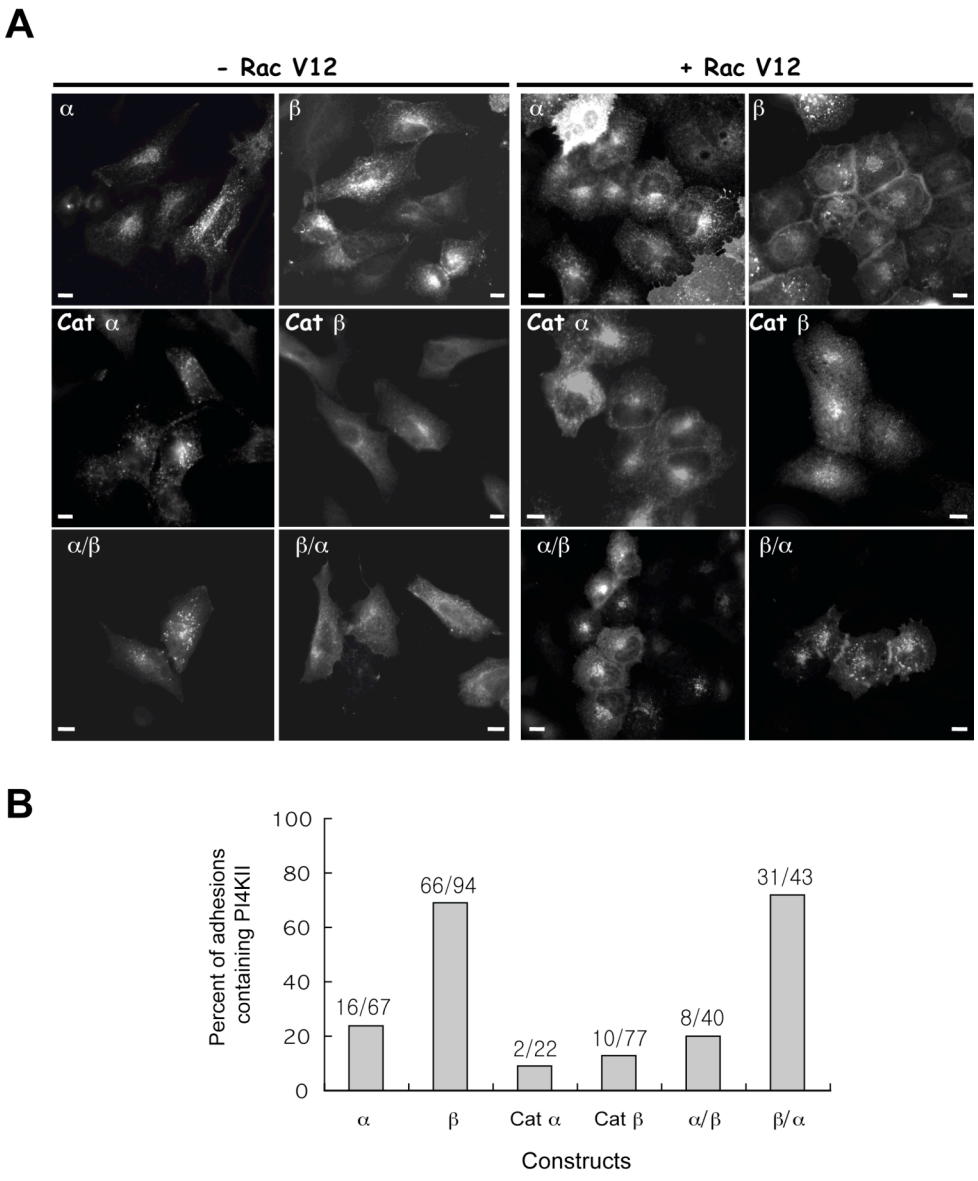


Figure 2-8. Redistribution of wild-type and mutant PI4KIIs in response to expression of activated RacV12. A. The constructs shown in Table 2-1 were expressed in HeLa cells and visualized using anti-Myc antibodies. **B.** The bar graph in the bottom shows the proportion of adhesions that display evident anti-Myc staining. Total adhesion sites were counted based on phalloidin staining in kinase-expressing cells. These values shown were obtained from 2 independent experiments which gave similar results. Scale bars represent 10μm.

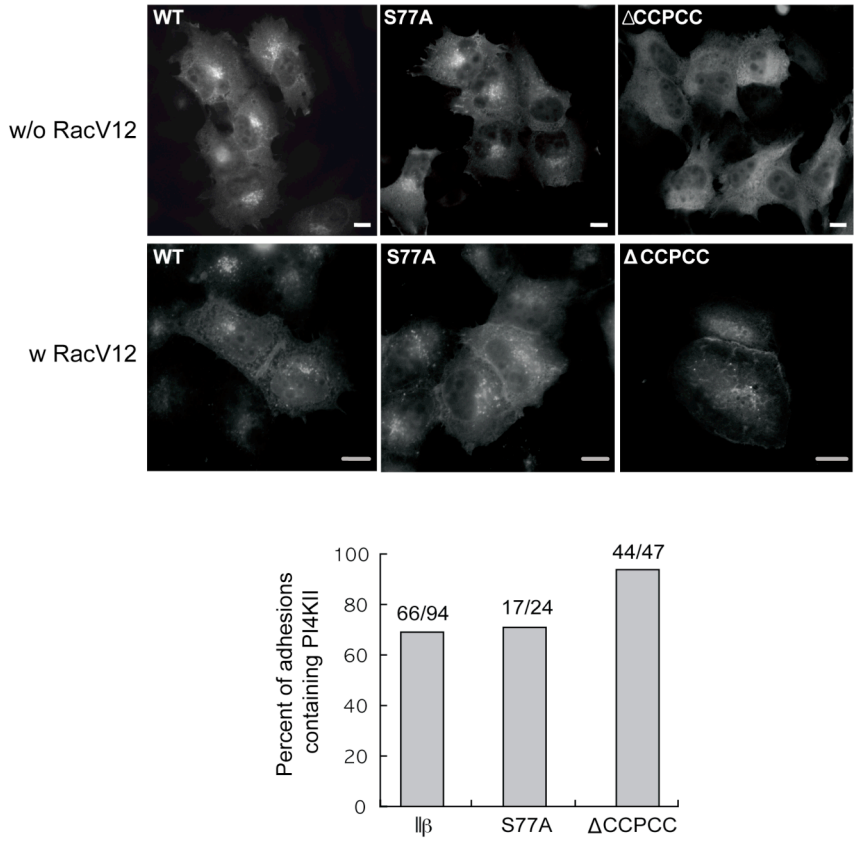


Figure 2-9. Relocalization of PI4KIIβ mutants (ΔCCPCC and S77A) in response to RacV12 expression. Wild-type PI4KIIβ and mutants lacking the palmitoylation motif (ΔCCPCC) or phosphorylation (S77A) were expressed in control cells or cells co-expressing RacV12. Tagged kinases were visualized with anti-Myc antibodies. Bar graph shows proportion of adhesions displaying anti-Myc-staining, as in Figure 2-8B. Scale bars are 10μm.

V. DISCUSSION

This study supports our previous report (Wei et al., 2002) and Balla's work (Balla et al., 2002) showing that PI4KIIβ is more soluble and less active than PI4KIIα, and that a portion of PI4KIIβ translocates to membrane ruffles in response to PDGF treatment or overexpression of RacV12. In an effort to explain these observations, I examined the effects of two post-translational modifications of PI4KIIβ, palmitoylation and phosphorylation, and identified a region in PI4KIIβ that is responsible for determining its association with cellular membranes.

PI4KIIα and β differ significantly in their extent of palmitoylation, with PI4KIIβ incorporating only 30% of the amount of [^{3}H]palmitate as PI4KIIα. Because palmitoylation is essential for expression of catalytic activity, at least *in vitro* (Figure 2-5), this result suggests that cells contain a large pool of inactive PI4KIIβ which may be mobilized and activated in a stimulus-dependent manner. It is interesting that Balla et al. (Balla et al., 2002) reported that the specific activity of PI4KIIβ is 30% that of PI4KIIα, corresponding exactly to the differences in palmitoylation state determined here (Table 2-1).

We also observed that only membrane-associated PI4KIIβ is phosphorylated, and that this modification accounts for the more slowly migrating electrophoretic band seen in SDS gels of membrane-bound, but not cytosolic, PI4KIIβ. The major phosphorylation site was identified by mass spectrometry and mutational analysis as serine 77, located in the N-terminal segment, which is most diverse between the two PI4KII isoforms. There is no effect on catalytic activity, palmitoylation state, or subcellular distribution when PI4KIIβ phosphorylation was reduced by alkaline phosphatase treatment or by mutation of serine 77

to alanine, or to the potentially phosphomimetic residue, glutamate (not shown). However, it remains possible that phosphorylation of the kinase regulates specific protein-protein interactions which are yet to be identified.

In view of the sequence diversity of the N-terminal segments of PI4KIIα and β (Figure 2-1), we expected that these regions would account for the differences in acylation and distribution between the two isoforms. However, the differences are maintained by the isolated catalytic domains, which are relatively conserved in sequence (64% identity, 77% similarity). The C-terminal ~160 residues of these catalytic domains are most responsible for the greater solubility and lower level of palmitate incorporation of PI4KIIβ than PI4KIIα. Within the C-terminal segments of the kinase there are two short stretches having essentially no sequence similarity. In future studies, it will be interesting to examine the importance of these two stretches in determining the different properties of PI4KIIα and β.

Although the different solubilities of PI4KIIα and β are determined primarily by their C-terminal ~160 residues, the unique N-terminal segment of PI4KIIβ nevertheless contributes to its affinity for cellular membranes. Deletion of this highly acidic region, residues 1-90, resulted in the redistribution to the cytosol of approximately half of the hitherto membrane-associated portion of the intact kinase, shifting from 44% to 71% cytosolic. This is consistent with the immunofluorescence microscopy data of Balla et al. (Balla et al., 2002), who showed that a truncated version of PI4KIIβ lacking the N-terminal 96 amino acids is predominantly cytosolic.

Our observations highlight the role of the C-terminal portions of the Type II kinases in membrane targeting. However, it is possible that the unique N-terminal regions are important in mediating specific protein-protein interaction within membranes. For example, Xu et al. (Xu et al., 2006) recently showed that the N-terminal region of PI4KIIα is essential to target this isoform to a specific subset of glucose transporter 4 (GLUT)-containing vesicles in HEK293 cells, i.e. those which also contain cellugyrin.

How do these C-terminal ~160 amino acids control the distribution of the two kinases? Two possibilities are evident, and they are not mutually exclusive: 1). The C-terminal portion of PI4KIIα may bind to a membrane-associated partner, allowing the kinase to undergo rapid, perhaps stable, palmitoylation; and/or 2). The C-terminal portion of PI4KIIβ may bind to a soluble protein which sequesters it to the cytosol until an appropriate stimulus causes the complex to dissociate. I present evidence in the next Chapter that PI4KIIb may be sequestered to the cytosol by interacting directly with Hsp90.

Although the N-terminal regions of PI4KIIα and β do not influence overall membrane binding, they are important for targeting PI4KIIβ to cell-cell adhesions in response to RacV12 expression. The significance of this targeting is unclear, though it raises the possibility that PI4KIIβ generates PI pools used in the formation and/or maintenance of adherens junctions (Betson et al., 2002; Kovacs et al., 2002). In this context, it is interesting that a Type II-like PI4-kinase activity has been detected in immunoprecipitates of α3β1 integrins, which localize to adherens junctions (Schoenenberger et al., 1994; Yanez-Mo et al., 2001), and that a PI4P 5-kinase has also been identified in these junctions (Akiyama et al., 2005). Moreover, I detected an interaction between PI4KIIβ and CD151, a tetraspanin

that associates directly with several laminin-binding integrins (particularly α3β1). It is possible that the PI4KIIβ-CD151 interaction recruits the kinase to integrin signaling complexes at cell-matrix and cell-cell adhesions. Because the isolated N-terminal region of PI4KIIβ is exclusively cytosolic, I suspect that its interaction with a putative binding partner at the cell-cell junction is relatively weak. Presumably, additional interactions involving the PI4KII catalytic domain are required for stable association with membranes.

CHAPTER 3

Modulation of phosphatidylinositol 4 kinase Type IIβ by interaction with Hsp90

I. ABSTRACT

As described in Chapter 2, slight differences in the C-terminal 160 residues of PI4KIIα and β result in their distinct distributions between membranes and cytosol. As a result, cytosolic, inactive PI4KIIβ is subject to distinct regulatory mechanisms from that of PI4KIIα, ensuring its translocation and subsequent activation. In this study, I identify Hsp90 as a binding protein for PI4KIIβ, but not for PI4KIIα. To define the functional significance of the Hsp90-PI4KIIβ interaction, I disrupt it using geldanamycin (GA), a specific Hsp90 inhibitor. GA treatment destabilizes PI4KIIβ, reducing its half-life by 40% and increasing its susceptibility to proteasomal degradation. Although endogenous PI4KIIα is not sensitive to GA treatment, a predominantly cytosolic truncation mutant of PI4KIIα displays GA-dependent degradation. This suggests that PI4KIIβ requires stabilization by Hsp90 because a large portion of this isoform is cytosolic. Shortly after exposure to GA there is a partial redistribution of PI4KIIβ from the cytosol to membranes, with a corresponding slight increase in enzymatic activity. Thus, it appears that Hsp90 not only protects PI4KIIβ from degradation, but may also prolong its residency in the cytoplasm. Stimuli such as PDGF and EGF tend to disrupt the association between PI4KIIβ and Hsp90. These results indicate that both PI4KII isoforms contain Hsp90 interaction sites, but that the interaction occurs predominantly with the cytosolic, inactive pool of PI4KIIβ. This interaction with Hsp90 may help to maintain the kinase in its inactive state until an extracellular stimulus, such as PDGF or EGF, triggers its translocation to the membrane.

II. INTRODUCTION

A. Hsp90 as a signaling chaperone.

Cells have developed a dedicated system of molecular chaperones to assist in folding and maturation of newly synthesized polypeptides and unfolded proteins. Nascent polypeptides or unfolded proteins have a strong tendency to aggregate due to exposure of extensive hydrophobic surfaces. To counteract these tendencies, molecular chaperones capture the unstable proteins and assist their proper folding. Among the chaperones, the 70-kDa heat shock protein Hsp70 binds nascent polypeptides and is thought to help in their co-translational folding. On the other hand, the 90-kDa heat shock protein, Hsp90 binds a subset of proteins to complete their folding in the late stages of the process (reviewed in (Young et al., 2004)). This defined set of proteins, termed Hsp90 "clients", require Hsp90 for conformational maturation, functional stability, and translocation. Currently, more than 100 proteins have been identified as clients of Hsp90 and most client proteins, such as steroid hormone receptors, transcription factors, and protein kinases, participate in key signal transduction processes. The specific association of Hsp90 with clients proved to be important for their activity as signal transducers. However, the specific effects that Hsp90 exerts on some of these proteins remains unknown. (reviewed in (Caplan et al., 2007; Mayer and Bukau, 1999; Pratt and Toft, 2003; Young et al., 2004)). As shown in Figure 3-1, the ATP–driven chaperone cycle of Hsp90 is accomplished by coordinated assistance of a range of co-chaperones (Terasawa et al., 2005; Young et al., 2004). Often, various co-factors link the different clients to the Hsp90 chaperone complex. Hsp90 was initially identified as a multiprotein complex with v-Src or with steroid hormone receptors (SHRs) in the 1980s

(Brugge et al., 1981; Joab et al., 1984). In the case of SHRs, Hsp90 as well as Hsp70, Hop and other immunophilins are essential factors for steroid binding, translocation to the nucleus, and other aspects of their activity (Richter and Buchner, 2001). In the case of the Hsp90-protein kinase complexes, (e.g. v-Src , Raf-1, Akt and Cdk4), Cdc37 serves as a co-chaperone. The Hsp90 machinery supports kinase stabilization and activity (Caplan et al., 2007; Pearl, 2005; Riggs et al., 2004).

B. Geldanamycin (GA) as a cancer therapeutic agent.

Because Hsp90 stabilizes mutant proteins such as v-Src, p53, and Bcr-Abl that appear during cell transformation, Hsp90 has been implicated in cancer (Blagosklonny et al., 1995; Gorre et al., 2002; Whitesell et al., 1994). Specific inhibitors of the Hsp90 ATPase reaction, such as GA and its derivatives, have promising effects in clinical trials (Neckers, 2006; Whitesell and Lindquist, 2005). These inhibitors were first studied as kinase-specific anti-tumor agents (Fukazawa et al., 1991). Surprisingly, instead of inhibiting kinase activity directly, GA was found to bind to Hsp90 and to disrupt the complex between the kinase and its chaperone. Disruption of the complexes was also found to promote proteolytic degradation of substrate proteins (Whitesell et al., 1994). In addition to its potential clinical role, GA is often utilized to test the functional significance of Hsp90-client interactions (reviewed in (Whitesell and Lindquist, 2005)).

C. Other Roles of Hsp90

Besides its common function in the stabilization of a subset of clients, Hsp90 has been proposed to have specific roles for certain clients. The selectivity of Hsp90 for its targets is so high that even closely related enzymes, such as c-Src and v-Src, or the cyclin dependent

kinases (CDKs), differ significantly in their requirement for Hsp90 (Terasawa et al., 2006; Xu et al., 1999). Hsp90 clients share certain characteristics: they are often intrinsically labile, or they have complex folding pathways, or they need to be kept in a 'competent' conformation that allows insertion of a cofactor, binding of a ligand, or covalent modification.

Transporter Activity: The Hsp90-based chaperone machinery assists the folding of transcription factors such as glucocorticoid receptor(GR) and p53. In addition, it assists in their rapid movement from cytoplasm to nucleus along microtubular tracks. Immunophilins, cofactors containing tetratricopeptide (TPR) domain (e.g. FKBP52 and CyP-40) are found in the Hsp90/GR complexes and link these complexes to cytoplasmic dynein, a motor protein (Czar et al., 1994; Galigniana et al., 2004; Pratt et al., 2004).

Repressor Function: ErbB2 is maintained as an inactive homodimer by Hsp90. Binding to the ligand induces dissociation of Hsp90 and allows receptor heterodimerization for full activation. A comparable effect is observed upon GA treatment (Citri et al., 2004). Furthermore, a recent paper by Xu et al.(2007) reports that Src phosphorylates ErbB2 and subsequently increases ErbB2 activity, provided that Hsp90 has dissociated from ErbB2. It is possible that Hsp90 inhibits ErbB2 activation both by restricting its activation by either heterodimerization or its binding to other activators.

Another client, hypoxia-inducible factor-1 (HIF-1) responds to Hsp90 inhibitors in a dose-dependent manner. Low-dose treatment with Hsp90 inhibitors increased HIF-dependent reporter gene activity, whereas higher doses resulted in a reduction of hypoxia-induced HIF-1 activity (Ibrahim et al., 2005). This bimodal effect on HIF-1 also applies to Src kinase and

dsRNA-dependent kinase PKR. In both cases, the transient treatment with Hsp90 inhibitors increases the activities of Src and PKR by dissociating Hsp90 from them whereas longer treatments destabilize clients by accelerating their proteasomal degradation (Donze et al., 2001; Koga et al., 2006).

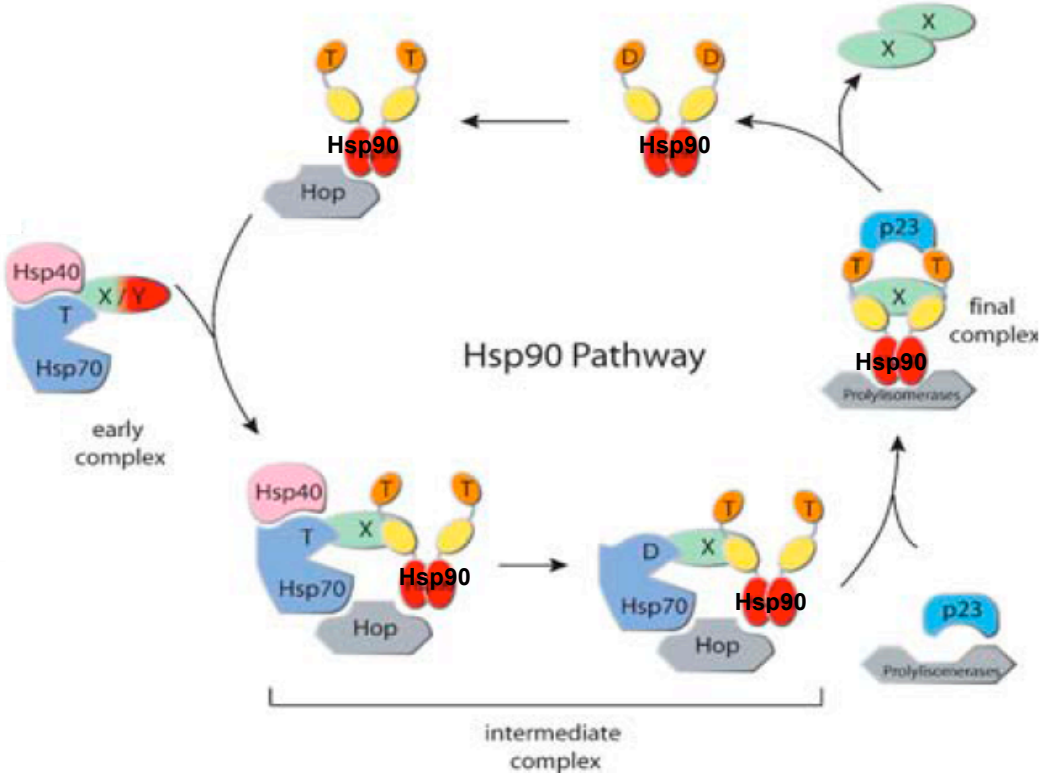


Figure 3-1. Hsp90 chaperoning cycle. Newly synthesized and/or partially unfolded proteins (X/Y) are delivered to the Hsp70 complex via Hsp40. In most cases, Hsp70 is able to process the substrate (Y) on its own. Other substrates (X), often nuclear receptors or kinases, require association with Hsp90 for proper folding or activation. In these cases, the scaffold protein Hop connects elements of the Hsp70 and Hsp90 machineries to form an "intermediate complex." The Hsp70 component dissociates and, at the same time, p23 and cofactors enter the complex (Cdc37 for kinases and immunophilins for steroid receptors). ATP-bound Hsp90 is in a closed state with its C-terminal domains being constitutively dimerized. Hydrolysis of ATP stimulated by Aha1 induces conformational changes and results in release of the substrates. After binding to Hop, Hsp90 is able to reenter the cycle. T: ATP-bound form; D: ADP-bound form. This figure is taken from *Wegele, 2004*.

III. ADDITIONAL EXPERIMENTAL PROCEDURES

Reagents - Hsp90 antibody (H-114) was from Santa Cruz. Hsp70 antibody was bought from Cell Signaling. Geldanamycin was obtained from InvivoGen (San Diego, CA). MG132 (proteasome inhibitor) was a gift from the Dr. Melanie Cobb (Dept. of Pharmacology, UT Southwestern Medical Center).

cDNA constructs and generation of mutants –Cat $\alpha_{92\text{-}431}$ was generated as described in (Barylko et al., 2002). PI4KIIα (SSPSS) was generated using Quick Change Mutagenesis (Stratagene). Hsp90α was amplified from mouse cDNA library with primers KpnI : 5'-CGGGGTACCCATGCCTGAGGAAACCCAGACCCAAGACC-3' and XbaI : 5'-GCTCTAGATTAGTCTACTTCTTCCATGCGTGATGTGTCG-3' and cloned into p3XFLAG-CMV-7.1 expression vector (Sigma).

In-gel digestion and protein identification using mass spectrometry - Silver-stained protein bands were excised from 1D SDS gels and cut further into small cubes (ca. 1x1 mm). After in-gel reduction, alkylation and destaining, proteins were digested overnight with trypsin (12 ng/µl). The tryptic peptides were extracted from gels dried in a vacuum centrifuge, and analyzed by electrospray liquid chromatography-tandem mass spectrometry using a nanoscale C18 column coupled in-line with an ion trap mass spectrometer (LCQ Deca, Thermo Finnigan). The instrument was set in a data-dependent acquisition mode, cycling between one full MS scan and MS/MS scans of the three most abundant ions. The MS and MS/MS data were used to search the non-redundant NCBI protein database using MASCOT search engine. This was done with collaboration with Dr. Hongjun Shu. (Department of

Molecular Oncogenesis, Genome Research Institute, University of Cincinnati).

Immunoprecipitation - Untransfected or transfected cells (for 20 h) were washed with PBS and lysed in Nonidet P-40 (NP-40) lysis buffer consisting of 20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 30mM NaF, 2mM $Na_4P_2O_3$, 1mM Na_2VO_4, and 1% NP-40. Cell lysates were obtained after centrifugation at 1000 x g for 5 min and were pre-cleared of nonspecific binding proteins by incubation with rec-Protein G-Sepharose 4B conjugate (Zymed) for 30 min. Myc-tagged proteins were immunoprecipitated by 4 h incubation with anti-Myc antibody cross-linked to protein G-Sepharose using the crosslinker, DMP (Pierce).

Analysis of [^{32}P]Pi incorporation into lipids- For radiolabeling, transfected COS cells were incubated with phosphate-free medium containing 5% dialyzed FBS and 25μCi/ml [^{32}P]P_i (PerkinElmer Life Sciences) for 4 h. After three brief washes with PBS, labeled cells were scraped into methanol: HCl (10:1) and lipids were extracted with chloroform. The organic phase was collected and washed with an equal volume of methanol and HCl (1:1). Aliquots were spotted onto TLC plates and separated in a solvent system consisting of *n*-propyl alcohol/H_2O/NH_4OH (65:20:15). Radioactive PI4P spots were scanned using FLA-5100 (Fuji Photo film Co., Ltd) and the radioactivity of bands were quantified using the Multi-Gauge V2.3 program (Fuji Photo film Co., Ltd).

Geldanamycin Treatment of Cells - Geldanamycin was solubilized in demethyl sulfoxide (DMSO) and added to cell culture medium to the various final concentrations indicated in the text and figure legends. Equal volumes of DMSO without GA were used in controls.

IV. RESULTS

3.1. Identification of Hsp90 as a binding partner of PI4KIIβ.

To better understand the regulation of PI4KIIβ, I attempted to identify proteins that preferentially interact with this isoform over its close relative PI4KIIα. Myc-tagged versions of each kinase were expressed in HEK293 cells and immunoprecipitated using anti-Myc antibodies. One electrophoretic band of 90 kDa is consistently enriched in immunoprecipitates of PI4KIIβ compared with those of PI4KIIα, or of an irrelevant expressed protein, green fluorescent protein (GFP) (Figure 3-2A). This band was extracted from gels and subsequently identified as Hsp90α by mass spectrometry (not shown). Apart from several other bands which proved to be fragments of PI4KIIβ, the only other band that selectively co-immunoprecipitated with PI4KIIβ was identified by mass spectrometry as peroxiredoxin I (double star in Figure 3-2A).

The selective binding of Hsp90 to PI4KIIβ was confirmed by immunoblotting PI4KIIα or PI4KIIβ immunoprecipitates with an anti-Hsp90 antibody (Figure 3-2B) and by demonstrating co-precipitation of expressed FLAG-tagged Hsp90 with Myc-PI4KIIβ, but not with Myc-PI4KIIα (Figure 3-2C).

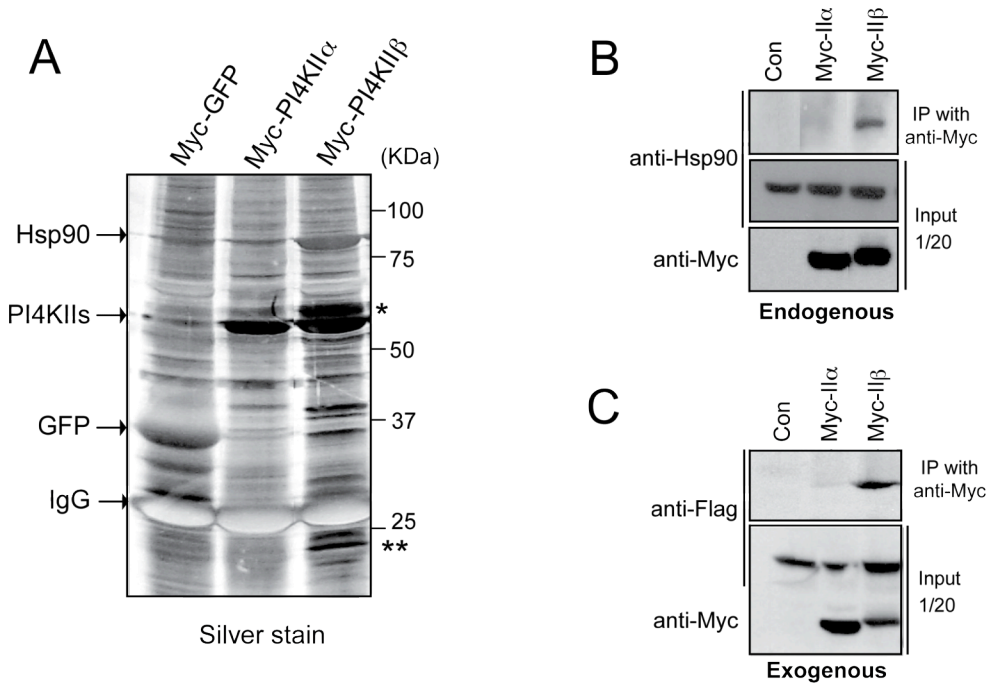


Figure 3-2. **Hsp90 co-immunoprecipitates with PI4KIIβ. A.** Silver-stained gel showing increased intensity of a 90 kDa band in precipitates of Myc-PI4KIIβ compared to those of Myc-PI4KIIα or an irrelevant protein, Myc-GFP. Myc-tagged proteins were expressed in HEK 293 cells, which were then lysed and subjected to immunoprecipitation with anti-Myc antibodies. The 90kDa band was extracted and identified as Hsp90 by mass spectrometry. The asterisk designates phospho-PI4KIIβ and the double asterisk designates peroxiredoxin I, another PI4KIIβ-binding protein identified in this study. Other unique bands in the precipitates were identified by mass spectrometry as fragments of the full-length kinases. **B and C.** Specific association between endogenous and exogenous Hsp90s and PI4KIIβ. Myc-PI4KIIα or Myc-PI4KIIβ were expressed in Hela cells without (**B**) or with (**C**) Flag-Hsp90α. Cell lysates were immunoprecipitated with anti-Myc antibody and blotted with anti-Hsp90 or anti-Flag to show the specific association of Hsp90 with PI4KIIβ, but not PI4KIIα.

3.2. Inhibition of the PI4KIIβ-Hsp90 interaction by geldanamycin results in destabilization of PI4KIIβ.

To examine the significance of Hsp90 binding to PI4KIIβ, I disrupted the interaction using GA, an agent which binds directly to the ATP-binding site of Hsp90 and blocks its association with many client proteins (Grenert et al., 1997; Whitesell et al., 1994). Treatment of cells with 2μM GA for 2 h significantly reduces the amount Hsp90 that co-immunoprecipitates with Myc-PI4KIIβ, whereas similar amounts of Hsp70 are present in immunoprecipitates from GA-treated and untreated cells (Figure 3-3A). Hsp70 serves as a general chaperone for newly synthesized proteins engaged in co-translational folding and for unfolded proteins. It is also a component of the Hsp90 chaperone complex, participating in the Hsp90 assisted folding cycle (reviewed in (Terasawa et al., 2005; Young et al., 2004)), but GA apparently does not disrupt the association of Hsp70 and its substrates (Shay et al., 2005).

In many cases, interfering with the interaction of Hsp90 with its client proteins reduces the stability of those clients. To test whether this also applies to PI4KIIβ, I analyzed the effect of GA treatment on the cellular levels of PI4KIIβ in a dose-dependent (Figure 3-3B) and time-dependent (Figure 3-3C) manner.

After cell exposure to GA, I observed reduction of cellular levels of endogenous PI4KIIβ but not of endogenous PI4KIIα. PI4KIIβ levels are reduced approximately by half following 4 h treatment with 1μM GA (Figure 3-3B). Reduction of total PI4KIIβ is evident after a 1 h exposure to 2 μM GA (Figure 3-3C). As expected, PI4KIIα levels are unaffected by GA. I also tested the sensitivity of PI4KIIIβ to GA, because Flanagan et al reported that

Hsp90 co-purifies with PI4KIIIβ in yeast (Flanagan and Thorner, 1992). Indeed, PI4KIIIβ has been classified as a binding protein of Hsp90 (Pratt and Toft, 2003; Wegele et al., 2004) although the function of this interaction was not investigated. Our results indicate that PI4KIIIβ is resistant to GA (Figure 3-3B and 3-5A).

Consistent with the above results, GA was found to shorten the half-life of PI4KIIβ, not that of PI4KIIα (Figure 3-4). HeLa cells expressing Myc-PI4KIIα or β were treated with the protein synthesis inhibitor, cycloheximide (CHX) in the presence or absence of 2μM GA. At various times thereafter, kinase levels were estimated by immunoblotting. The half-life of PI4KIIβ is reduced from 4 h to 1.5 h by GA treatment, whereas that of PI4KIIα is 4 h regardless of the presence of GA. This is the first reported measurement of the half-lives of Type II kinases and it demonstrates that, like many other signaling proteins, they exhibit relatively rapid turnover rates.

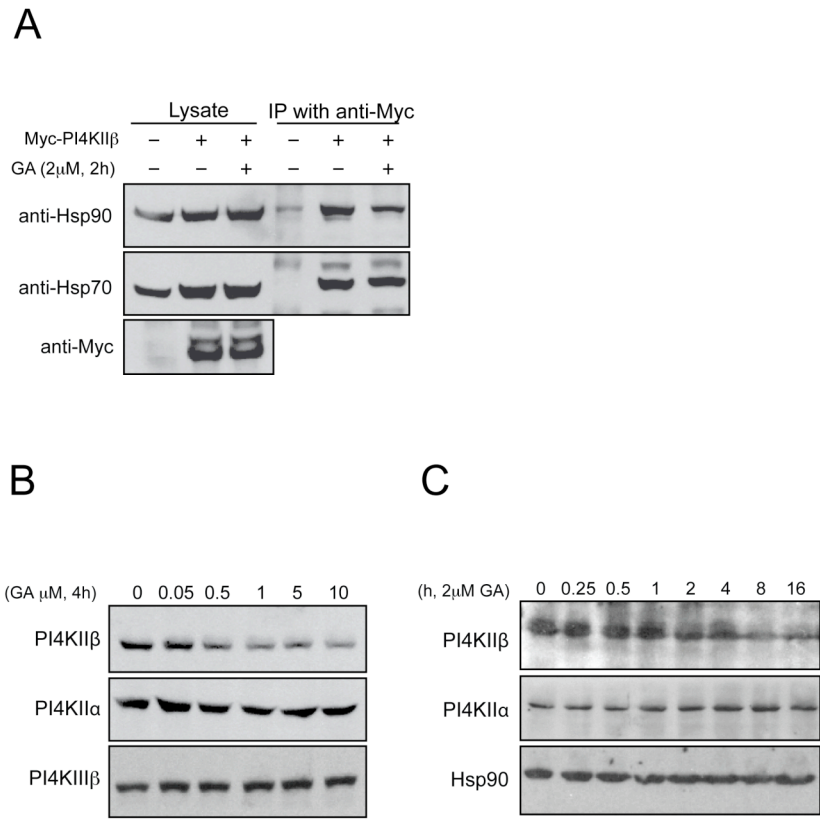


Figure 3-3. **Effects of the Hsp90 inhibitor, GA, on stability of PI4KIIβ. A** Disruption of the association between PI4KIIβ and Hsp90 by GA. Myc-PI4KIIβ was expressed and immunoprecipitated with anti-Myc antibodies from cells treated (or untreated) with 2μM GA for 2 h. Precipitates were electrophoresed and blotted with antibodies against Hsp90 and Hsp70. **B.** Dose-dependent loss of PI4KIIβ in response to GA treatment. HeLa cells were treated for 4 h with various doses of GA prior to isolation and electrophoresis of post-nuclear supernatants. Gels were blotted with antibodies against PI4KIIβ, PI4KIIα, and PI4KIIIβ to estimate changes in kinase levels in response to GA treatment. **C.** Time-dependent loss of PI4KIIβ in response to GA treatment. HeLa cells were exposed to 2μM GA for various times and prepared as in panel B.

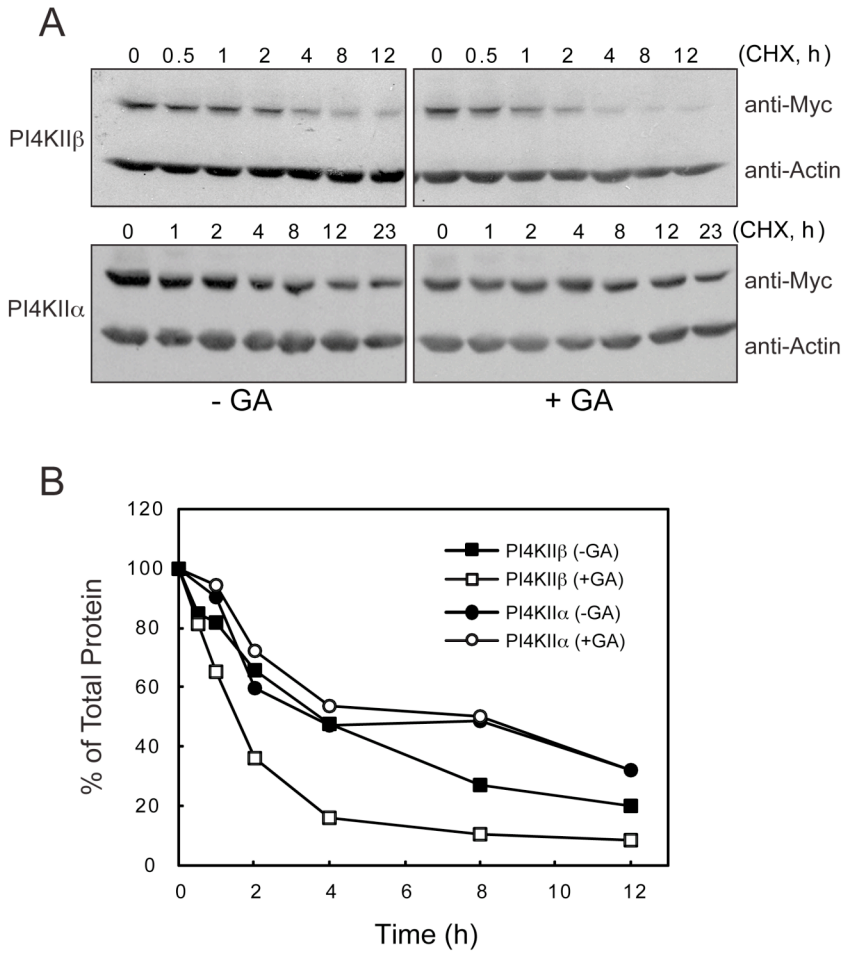


Figure 3-4. Effects of GA on the half-lives of PI4KIIα and β. Cells expressing Myc-PI4KIIα or Myc-PI4KIIβ were treated with cycloheximide (CHX) alone or CHX together with GA (2μM). At the indicated time points, cells were lysed and the lysates were electrophoresed and blotted with anti-Myc antibodies to detect kinases and anti-actin antibodies for normalization (**A**). The graph (**B**) shows the amount of each kinase as a percent of its initial amount, normalized to the amount of actin in each lane.

3.3. Hsp90 protects PI4KIIβ from degradation by the proteasome.

GA-mediated reductions in cellular levels of Hsp90 client proteins are likely due to their enhanced ubiquitylation and proteasomal degradation (Mimnaugh et al., 1996; Nomura et al., 2005; Schulte et al., 1997; Whitesell et al., 1997). To check whether this is also the case for PI4KIIβ, I examined the effect of treating cells expressing Myc-PI4KIIβ with both GA and the proteasome inhibitor, MG132. In the absence of these agents, the majority of Myc-PI4KIIβ is present in the soluble fraction of cells homogenized in buffer containing 1% NP-40. The remainder, presumably including aggregated kinase, is recovered in low-speed pellets (Figure 3-5A). GA treatment for 24 h reduces the amount of PI4KIIβ recovered either in the soluble or insoluble fractions. However, nearly the entire initial pool of kinase distributes to the pellet when cells are simultaneously treated with GA and MG132, indicating that PI4KIIβ is protected from proteasomal degradation, but accumulates in an aggregated state. Previously it was reported that PDK1, Raf1, and Src accumulate in the insoluble fraction on treatment with both GA and MG132 (An et al., 2000; Fujita et al., 2002; Schulte et al., 1997). In contrast to PI4KIIβ, the amounts of PI4KIIIβ (or of Hsp90) are not influenced either by GA or MG132.

I next verified that dissociation of the PI4KIIβ-Hsp90 complex by GA enhances ubiquitylation of the kinase. Cells were co-transfected with HA-ubiquitin (HA-Ub) and Myc-PI4KIIβ or Myc-PI4KIIα to allow detection of ubiquitylated kinase using anti-HA antibodies. In the absence of GA, inhibition of the proteasome with MG132 does not appreciably increase the amount of ubiquitylated kinases detected in anti-Myc immunoprecipitates (Figure 3-5B).

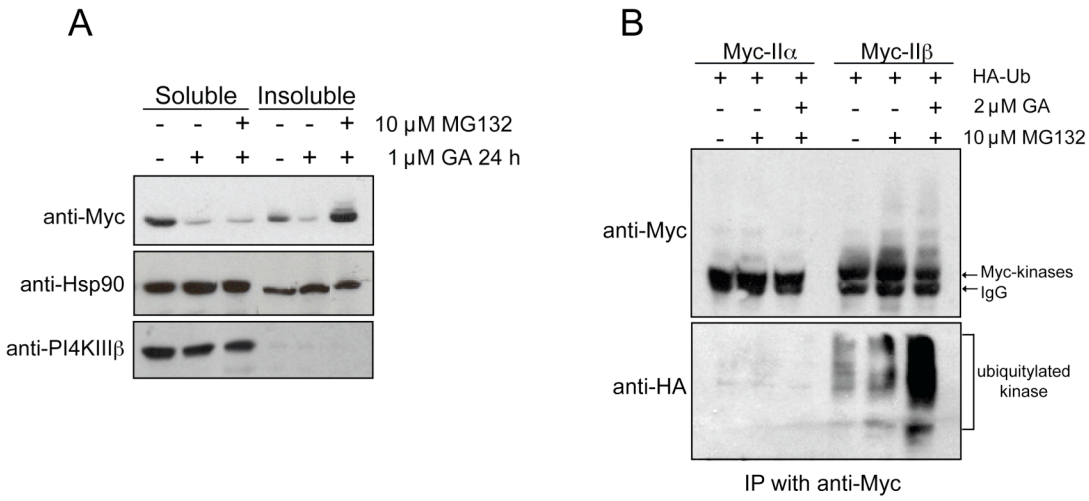


Figure 3-5. **Proteasome-dependent destabilization of PI4KIIβ. A.** Effect of MG132, a proteasome inhibitor, on PI4KIIβ stability. Cells expressing Myc-PI4KIIβ were incubated with 1μM GA with or without 10μM MG132. Soluble fractions were prepared in 1% NP-40 lysis buffer. Insoluble fractions (pellets) were mixed with SDS-sample buffer followed by electrophoresis and immunoblotting. Hsp90 and cytosolic PI4KIIIβ are shown as controls. **B**. Ubiquitylation of PI4KIIβ. Myc-PI4KIIα or Myc-PI4KIIβ was co-expressed with HA-tagged Ubiquitin (Ub) in HeLa cells. Prior to scraping in RIPA buffer, cells were incubated with 2μM GA and 10μM MG132 for 1.5 h. Ubiquitylated kinases, were detected by anti-HA antibody, appear as smeared bands.

However, co-treatment with GA for 1.5 h results in a significant accumulation of ubiquitylated kinase, whereas the ubiquitylation of PI4KIIα is unaffected either by MG132 alone, or in combination with GA.

3.4. Hsp90 selectively stabilizes the cytosolic pool of PI4KIIβ.

To explain the selective binding of Hsp90 to PI4KIIβ, I took advantage of the truncated and hybrid kinases described in Chapter 2. As shown in Figure 3-6, both catalytic domains alone (termed Cat α and Cat β consisting of residues ~90-480) display similar susceptibilities to GA as the full-length kinases, just as the catalytic domains distribute similarly as their parent molecules. Moreover, the hybrid, Cat α/β, containing residues 92-315 of PI4KIIα fused to residues 316-481 of PI4KIIβ, is >80% cytosolic and susceptible to GA. In contrast, Cat β/α, consisting of residues 91-315 of PI4KIIβ fused to residues 316-478 of PI4KIIα, is >80% integrally bound to membrane and is unaffected by GA treatment (Table 2-1 and Figure 3-6).

From these results, I conclude that the highly diverse N-terminal regions (1-90 residues) of PI4KIIα and β are not responsible for the different sensitivities of the two kinases to GA, but that instead the C-terminal 160 residues are the major determinants of Hsp90 binding and GA-dependent destabilization. Furthermore, solubilities of the constructs, which are determined by the C-terminal portions of the kinases, correlate well with the sensitivities to GA.

These results raise two hypotheses; 1) Hsp90 binds to the C-terminal 160 amino acids of PI4KIIβ but cannot bind to PI4KIIα, 2) the selective susceptibility of PI4KIIβ to GA-dependent degradation is due to its weaker association with membranes than PI4KIIα.

To test the first possibility, a series of C-terminally truncated mutants of

PI4KIIβ (PI4KIIβ$_{434,}$ PI4KIIβ$_{408}$, and PI4KIIβ$_{340}$) were generated. As shown in Figure 3-6, all three of the C-terminal deletion mutants bind to Hsp90 and are sensitive to GA. (Figure 3-6A and B). Therefore we exclude the first possibility and suggest that the Hsp90 binding region resides in the N-terminal portion (~90-315 residues) of PI4KII, catalytic domains, because both Cat α/β and PI4KIIβ$_{340}$ are still able to interact with Hsp90. Note that PI4KIIβ$_{434}$ becomes more cytosolic than wild-type (72% vs. 44%) and loses its ability to undergo palmitoylation on membranes. Additional deletions from the C-terminus of PI4KIIβ (PI4KIIβ$_{408}$ and PI4KIIβ$_{340}$) are also almost entirely cytosolic (not shown).

These results prompted me to consider the second hypothesis: that the differential requirements of PI4KIIα and β for stabilization by Hsp90 are due to their different distributions between membranes and cytosol. To test this hypothesis, I generated a truncated form of PI4KIIα, Cat α $_{91-431}$, which lacks both the N-terminal noncatalytic domain and the C-terminal 47 residues. Cat α $_{91-431}$ is >60% cytosolic, binds more strongly to Hsp90 than either PI4KIIα or Cat α, (Figure 3-7A and B), and is susceptible to proteolytic degradation in the presence of GA (Fiugre 3-7C). As expected, Cat α $_{91-431,}$ like PI4KIIβ, is hyperubiquitylated in the presence of GA (not shown). These results demonstrated that both PI4KIIα and β contain binding sites for Hsp90. However, PI4KIIα apparently does not interact with Hsp90 in cells, nor does it need the chaperone for stabilization, presumably because it is tightly bound to membranes.

The next question to address was whether palmitoylation helps to stabilize PI4KIIα. To answer this question I generated a mutant form of PI4KIIα in which all four cysteines in the palmitoylation motif, $_{174}$CCPCC$_{177}$, were mutated to serines. This mutant, termed PI4KIIα-

SSPSS, fails to incorporate [^{3}H]-palmitate in cells and is converted from an integral membrane protein to a tightly bound peripheral membrane protein, extractible by 0.1M sodium carbonate (pH11) but not by 1M NaCl (manuscript submitted). Despite its inability to undergo palmitoylation, PI4KIIα-SSPSS does not bind to Hsp90, nor is it destabilized by GA treatment (Figure 3-6).

In summary, I conclude that both PI4KIIαand β share the region that associates with Hsp90. This region resides in N-lobe of the kinase, which is 86% similar in sequence between the two isoforms. In this respect, PI4KIIs are similar to other Hsp90 binding protein kinases in which the structure in the N-lobe core have been shown to be essential for association with Hsp90 (Citri et al., 2006). PI4KIIα does not require stabilization by Hsp90 binding because it associates strongly with membranes, even in the absence of palmitoylation. In contrast, PI4KIIβ must bind to Hsp90, and is highly sensitive to its release, because a substantial portion of this isoform is cytosolic. Apparently, the inactive, cytosolic pool of PI4KIIβ is very unstable unless it associates with its chaperone.

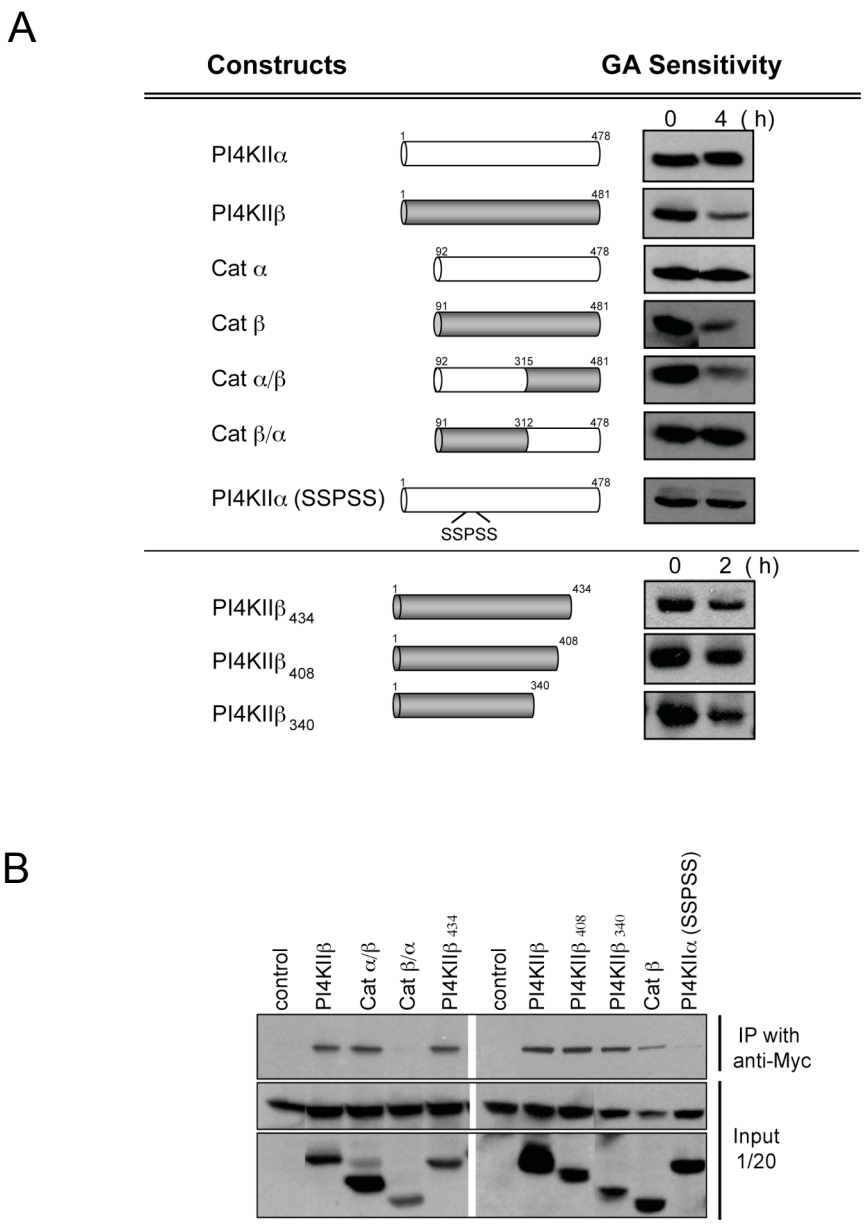
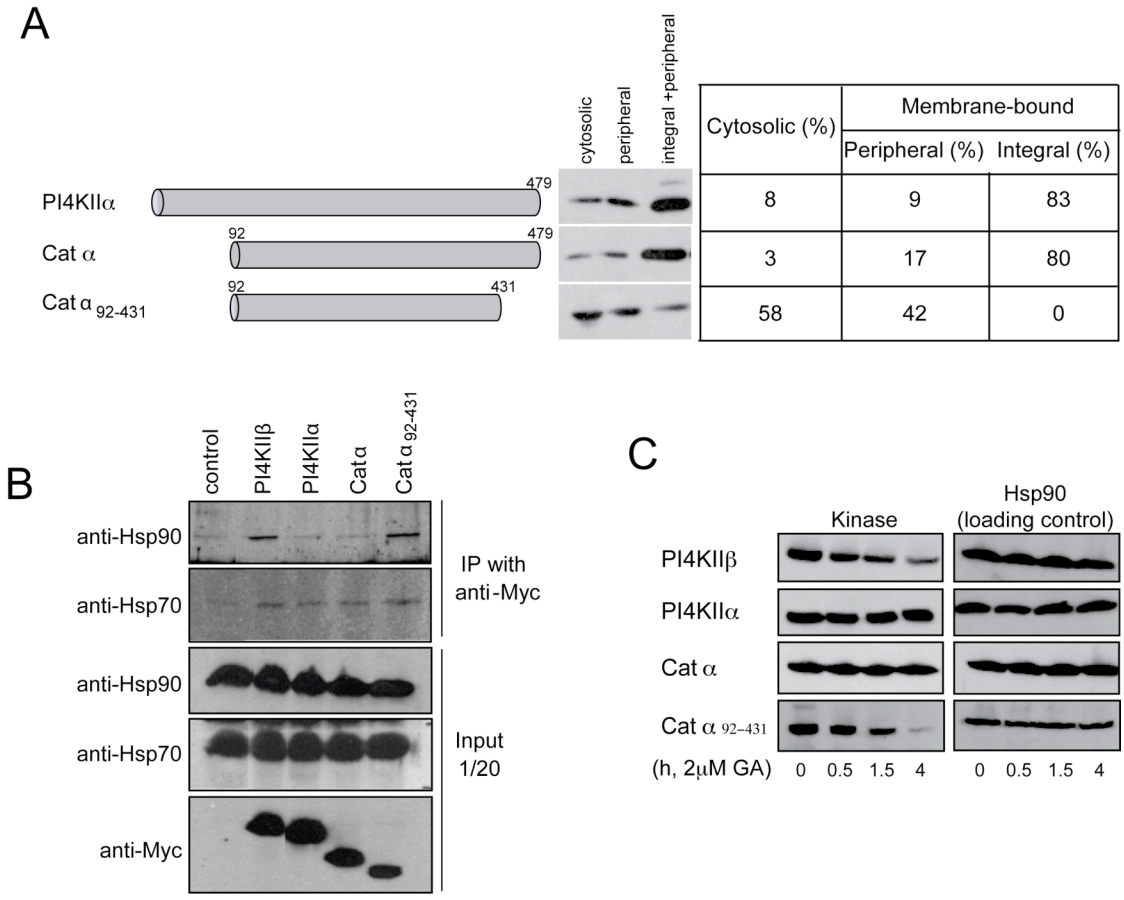


Figure 3-6. **GA sensitivity and Hsp90 binding of different PI4KII constructs.** **A.** The Myc-tagged constructs were expressed in cells which were treated with 2μM GA for 4 h or 2 h. The amount of each construct was detected by immunoblotting cell lysates with anti-Myc antibodies. **B.** The Myc-tagged constructs and Flag-Hsp90 were expressed and immunoprecipitated with anti-Myc-antibodies. The binding of Hsp90 was shown by immunoblotting with anti-Flag antibodies.



Cytosolic (%)	Membrane-bound	
	Peripheral (%)	Integral (%)
8	9	83
3	17	80
58	42	0

Figure 3-7. HSp90 binding and GA sensitivity of a cytosolic fragment of PI4KIIα. A. Distribution of full-length PI4KIIα, its full-length catalytic domain (Cat α), and a truncated catalytic domain (Cat $\alpha_{92\text{-}431}$) among cytosolic, peripheral membrane, and integral membrane pools. Fractions were prepared and analyzed as described in Experimental Procedures. **B.** Binding of Hsp90 to PI4KIIβ, PI4KIIα, and PI4KIIα fragments. All constructs were expressed as Myc-tagged proteins in HeLa cells and immunoprecipitated with Myc-tagged antibodies. Co-precipitating Hsp90 and Hsp70 were detected by immunoblotting. **C.** Effect of GA on the stability of PI4KIIβ, PI4KIIα, and PI4KIIα fragments. Cells expressing the Myc-tagged constructs were treated with 2μM GA for the designated times and their quantities in cell lysates were estimated by immunoblotting with anti-Myc antibodies. Anti-Hsp90 immunoblots were used as loading controls.

3.5. Dissociation of PI4KIIβ from Hsp90 by short (15min) exposure to GA results in transient translocation to membranes and increased kinase activity.

If only the cytosolic pool of PI4KIIβ must interact with Hsp90 in order to resist degradation, then it should diminish more rapidly than the membrane-bound pools (integral or peripheral) in response to GA. To test this possibility, cells expressing Myc-PI4KIIβ were treated with 2μM GA for various times (0, 0.25, 1, 3 h) before homogenization and separation into cytosolic, peripheral membrane, and integral membrane fractions. The amounts of PI4KIIβ in these fractions were estimated by immunoblotting with anti-Myc antibodies.

Longer (1 h and 3 h) incubations with GA result in an overall decrease in levels of PI4KIIβ, and the decreases are evident both in the cytosolic and membrane-associated pools. Significantly, the integral membrane-bound pool is the most stable, declining by only about 20% after a 3 h treatment with GA, compared to a decline of 60-70% for the cytosolic and peripherally membrane-associated pools. The loss of peripheral PI4KIIβ in response to GA suggests either that its presence on the membrane is readily reversible, or that it is susceptible to proteolytic degradation even when it is membrane-associated if its interaction with Hsp90 is disrupted, in contrast to PI4KIIα which is stably membrane-bound even in its unpalmitoylated state.

Interestingly, at 15 min incubation with GA, there appears to be a translocation of the kinase from cytosol to membranes (Figure 3-8A). Shorter time of GA exposure does not decrease the total amount of PI4KIIβ (Figure 3-8A) but disrupts the interaction between Hsp90 and PI4KIIβ (Figure 3-9), demonstrating that proteolytic degradation is not yet detectable even in the absence of Hsp90 binding. However, a greater proportion of PI4KIIβ

is present in the peripheral and integral pools following this brief GA treatment (1.2 fold and 1.5 fold, respectively).

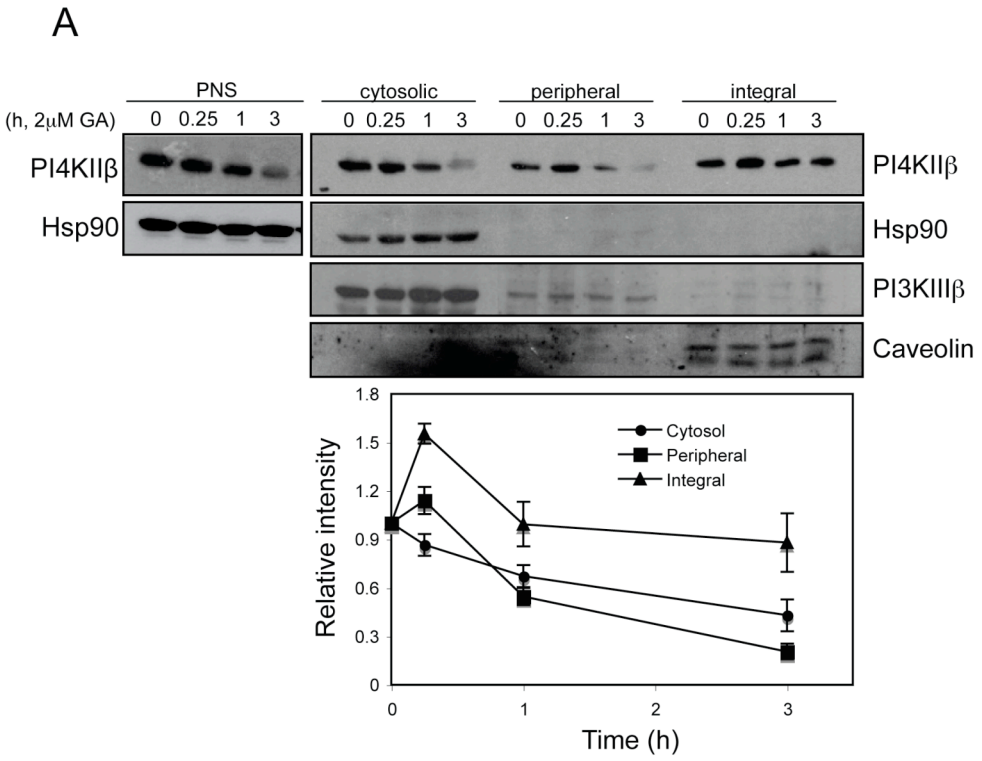
According to the current view, most Hsp90 client proteins are positively regulated by their interactions with the chaperone. However, GA was recently reported to rapidly but transiently activate dsRNA-dependent kinase (PKR) and Src kinase (Donze et al., 2001; Koga et al., 2006), suggesting that at least in some cases, interactions with Hsp90 can be inhibitory in the short term, though protective in the long term. (Koga et al., 2006; Yun and Matts, 2005).

As discussed in Chapter 2, membrane targeting is critical for expression of PI4KIIβ activity. We believe that only the integral membrane-bound kinase is palmitoylated and active. Therefore, it is plausible that translocation of PI4KIIβ from the cytosol to membranes by short-term (15min) GA treatment results in the transient activation of PI4KIIβ. I tested the catalytic activity by monitoring [^{32}P]Pi incorporation into cellular PI4P. A short disruption of the PI4KIIβ-Hsp90 interaction (15 min incubation with GA) elevates radioactive PI4P by 1.5 fold. It should be noted that the elevation is only ~1.2 fold upon even a 20-fold overexpression of PI4KIIβ. The increase in integral membrane-bound PI4KIIβ is approximately 1.5-fold over control, corresponding reasonably closely to the increase in [^{32}P]Pi incorporation into PI4P induced by GA treatment for 15min (Figure 3.8B).

As mentioned, certain stimuli (growth factor receptor activation or RacV12 overexpression) causes PI4KIIβ to partially redistribute to membranes (Wei et al., 2002). It would be interesting if this redistribution involves disruption of the PI4KIIβ-Hsp90 interaction, similarly to what occurs after short-term GA treatment. I tested this possibility by

co-transfecting COS cells with Myc-PI4KIIβ and FLAG-Hsp90, and measuring co-immunoprecipitation of the two binding partners in response to treatment with EGF, PDGF, or GA. In these experiments, the kinase was immunoprecipitated with anti-Myc antibodies, and the amount of co-pelleted Hsp90 was estimated by blotting the electrophoresed precipitates with anti-Hsp90 antibody. Both EGF (50ng/ml) and PDGF (100ng/ml) induce dissociation of the complex to a similar extent as GA (Figure 3-9).

Based on the above data, I proposed a model for the life cycle of PI4KIIβ shown in Figure 3-10. Cytosolic PI4KIIβ associates with Hsp90 for stabilization. An extracellular signal disrupts the interaction, allowing the free kinase to translocate to the membrane, where it may undergo palmitoylation and activation. Short exposure to GA may mimic the effect of growth factors. In contrast to PI4KIIβ, I suggest that PI4KIIα rapidly associates with Golgi membrane after its synthesis on cytosolic ribosomes, and then is stably palmitoylated and, hence, constitutively active.


A
PNS
cytosolic
peripheral
integral
(h, 2μM GA)
0 0.25 1 3
PI4KIIβ
Hsp90
PI3KIIIβ
Caveolin
Relative intensity
Cytosol
Peripheral
Integral
Time (h)

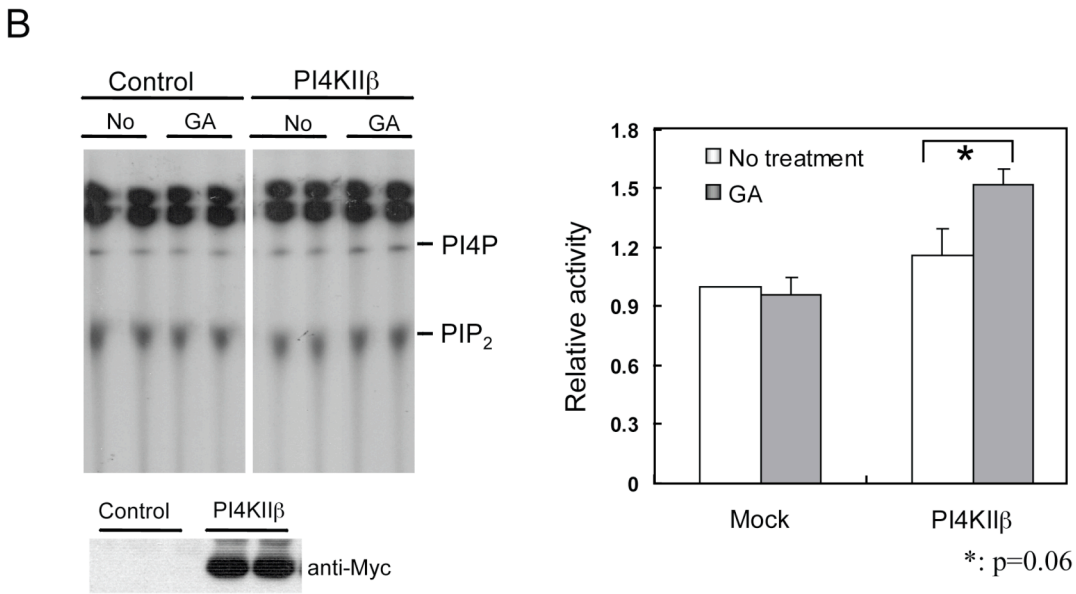

B
Control
PI4KIIβ
No
GA
PI4P
PIP2
anti-Myc
No treatment
GA
Relative activity
Mock
*: p=0.06

Figure 3-8. Effects of brief exposure of PI4KIIβ to GA. A. Effect of GA on the distribution of PI4KIIβ among cytosolic, peripheral membrane, and integral membrane pools. Cells transfected with Myc-PI4KIIβ were treated with 2μM GA for the designated times and fractionated as described in Experimental Procedures. Lysates were immunoblotted to estimate relative amounts of PI4KIIβ in each fraction, as shown in the graph. Hsp90, PI4KIIIβ, and caveolin were blotted as loading controls. The graph represents the mean +/-S.D from three independent experiments. **B.** PI4P production in cells briefly treated with GA. Cells transfected with vector (mock) or PI4KIIβ were labeled with [^{32}P]Pi for 4 h and incubated with GA for 15min. The cells were then scraped into methanol:HCl (10:1) to extract lipids, which were then quantified as described in Experimental Procedures. Phosphorylated lipids were shown in TLC. Graph shows the relative amounts of radioactive phosphate incorporated into PI4P. The average of three independent triplicate experiments are shown. Error bars designate standard deviation.

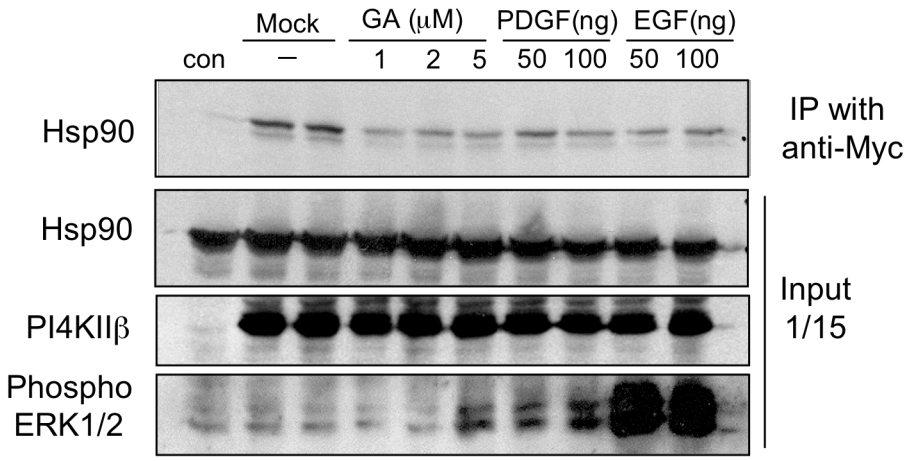


Figure 3-9. EGF and PDGF disrupt the interaction between Hsp90 and PI4KIIβ. COS cells were transfected with Flag-Hsp90 alone or together with Myc-PI4KIIβ and starved for 8 h to achieve quiescence. The cells were then treated with GA, PDGF, or EGF at different concentrations for 15 min. DMSO was added as a mock treatment. Cell lysates were prepared in 1% NP-40 buffer and immunoprecipitated with anti-Myc antibodies and blotted with anti-Hsp90 antibody. The inputs were immunoblotted with anti-Hsp90, anti-Myc and anti-phospho ERK1/2 antibodies (to demonstrate effectiveness of growth factor treatments). Note the slight increase in ERK1/2 phosphorylation at 5μM GA, as reported by (Donze et al., 2001) and (Koga et al., 2006). These data are representative of three independent experiments.

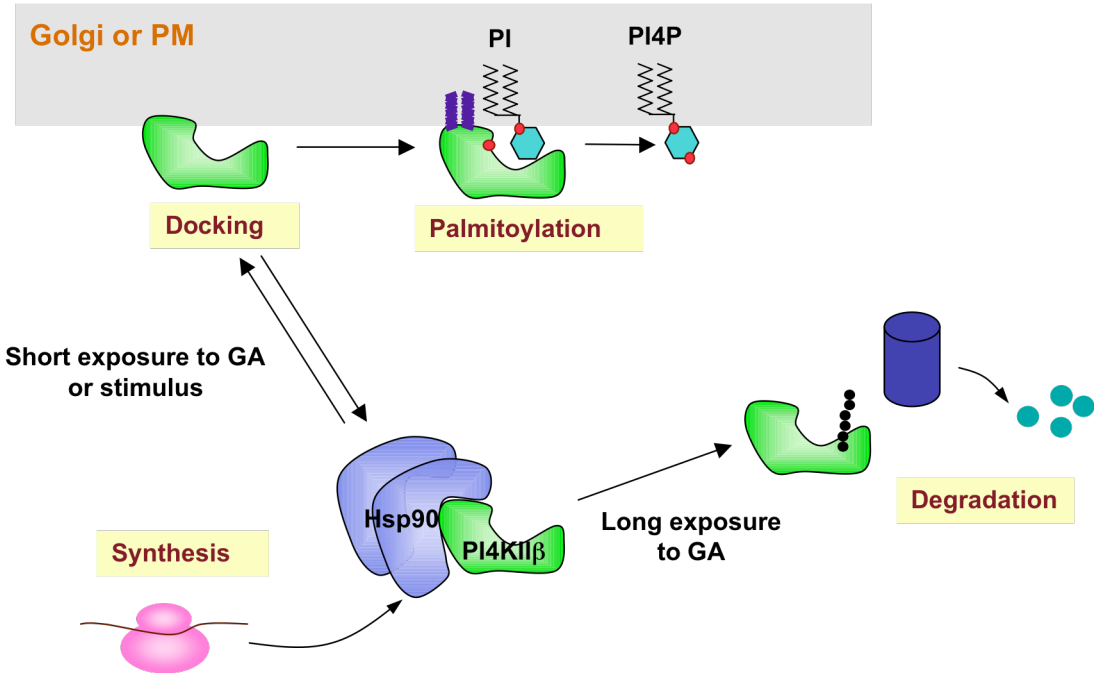


Figure 3-10. Proposed life cycle of PI4KIIβ. Hsp90 complex binds to cytosolic PI4KIIβ (green), either newly synthesized or dissociated from membranes. In response to stimuli, PI4KIIβ is released from the Hsp90 complex and then docks at the membrane through a yet unidentified docking protein. Acyltransferases on the membrane palmitoylate peripherally bound PI4KIIβ leading to expression of catalytic activity. A portion of membrane-bound PI4KIIβ, particularly that which is peripherally associated, recycles to the cytosol and is re-captured by Hsp90. GA disrupts the interaction between Hsp90 and PI4KIIβ. Short exposure to GA mimics the effect of other stimuli, whereas longer exposure to GA gives rise to proteasome-dependent degradation of PI4KIIβ.

3.6. Long-term treatment with GA induces PI4KIIβ, but not PI4KIIα, to relocalize to large punctate structures.

Treatment of cells expressing PI4KIIβ with 1μM or 5μM GA for 4 h not only reduces the overall amount of the kinase but causes at least a portion of the surviving kinase to relocalize from the Golgi to large cytoplasmic punctae (Figure 3-11A). In contrast, the location of PI4KIIα is unaffected by this treatment (Figure 3-11B). Because GA induces ubiquitylation of PI4KIIβ and its subsequent accumulation in NP-40-insoluble fractions (Figure 3-5), I co-transfected cells with Myc-PI4KIIβ and HA-Ub to determine if the larger punctae may contain ubiquitylated proteins (including ubiquitylated PI4KIIβ). In untreated cells, PI4KIIβ and ubiquitin are enriched at their normal locations, the perinuclear region and the nucleus, respectively (Figure 3-11C). However, incubation with GA for 1 h induces their co-localization into the large punctae. Interestingly the endosomal marker EEA1 is found to surround a sub-population of these punctae (Figure 3-11C, bottom panels). At present, we cannot state the identity of the GA-induced punctae, nor the mechanism that underlies the relocalization of PI4KIIβ to them.

V. DISCUSSION

In this chapter, I show that PI4KIIβ is protected from proteasomal degradation by Hsp90; disruption of the PI4KIIβ-Hsp90 interaction by GA treatment for > 1 h reduces the half-life of the kinase from ~ 4 h to 1.5 h. PI4KIIα does not bind to Hsp90, and its half-life (also ~ 4 h) is unaffected by GA. Because a cytosolic fragment of PI4KIIα is sensitive to GA treatment, we suggest that PI4KIIα is stable, even in the absence of Hsp90 binding, by virtue

of its strong association with membranes.

Incubation of cells with GA for 15 min or less is insufficient to elicit detectable PI4KIIβ degradation. However, in response to these brief GA treatments the kinase is partially redistributed form cytosol to membranes and, consequently, its catalytic activity is increased. Activation of EGF and PDGF receptors disrupts the PI4KIIβ-Hsp90 interaction and also promotes membrane relocalization and activation of PI4KIIβ. Based on these observations, I hypothesize that growth factor-dependent stimulation of PI4KIIβ is tightly and obligatorily linked to its release from Hsp90. There are other examples in the literature of Hsp90 serving as an inhibitor of kinase activity. For examples PKR is activated by dsRNA, which triggers its dissociation from Hsp90 (Donze et al., 2001). Also, Src is transiently activated upon its release from Hsp90 (Koga et al., 2006).

Another consequence of long-term GA treatment is relocalization of PI4KIIβ to large punctae which also stain for ubiquitin. In 2002, Lelouard and his colleagues identified structures in dendritic cells that transiently accumulate ubiquitylated proteins (Lelouard et al., 2002). These structures, termed “dendritic cell aggresome-like structures” (DALIS) are similar to, but distinct from, aggresomes or inclusion bodies. Subsequently, another group identified DALIS-like structures in several types of stressed cells and named them “aggresome-like induced structures” (ALIS) (Szeto et al., 2006). The punctae containing ubiquitylated PI4KIIβ in GA-treated cells resemble ALIS, but further work is required to establish a relationship between these two structures.

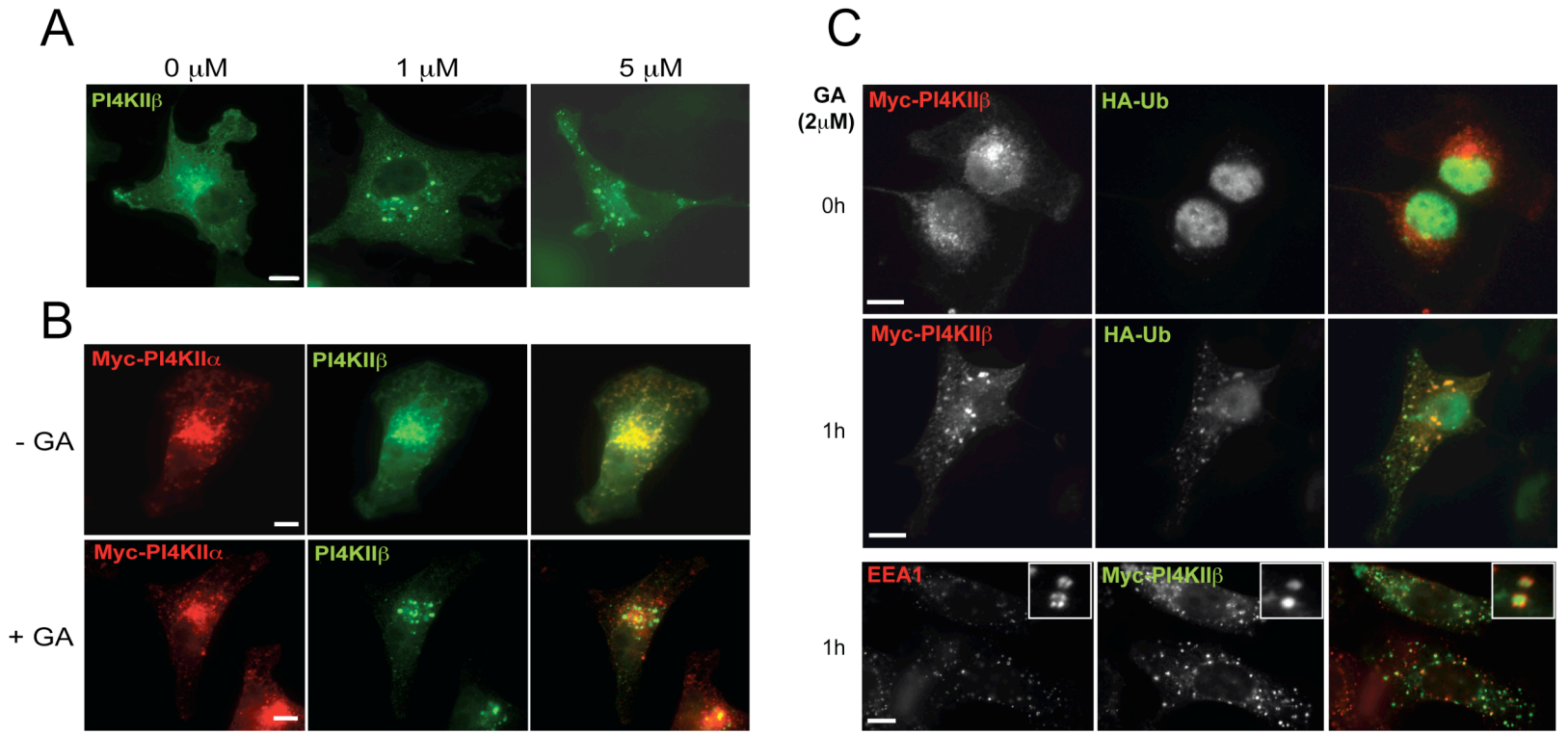

A
0 μM
1 μM
5 μM
PI4KIIβ
B
Myc-PI4KIIα
PI4KIIβ
- GA
+ GA
C
GA
(2μM)
Myc-PI4KIIβ
HA-Ub
0h
1h
EEA1

Figure 3-11. GA effect on the cellular localization of PI4KIIβ. A. Myc-PI4KIIβ was expressed in COS-7 cells. After 16 h, cells were incubated with GA (1μM or 5μM) or DMSO for 4 h. PI4KIIβ was localized using anti-Myc antibody and FITC conjugated secondary antibody. **B.** Constructs of Myc-PI4KIIα and untagged-PI4KIIβ were co-transfected into HeLa cells. PI4KIIα and PI4KIIβ were visualized by staining with anti-Myc and anti-PI4KIIβ antibodies, respectively. GA-treated cells were incubated with 2μM GA of the drug for 2 h. **C.** HeLa cells were cotransfected with Myc-PI4KIIβ and HA-Ub and treated with DMSO or 2μM GA for 1 h. Expressed proteins were visualized by anti-PI4KIIβ and anti-HA antibodies. The bottom row shows co-localization of EEA1 with PI4KIIβ in punctae generated in the presence of GA. Myc-PI4KIIβ and EEA1 were visualized using anti-PI4KIIβ and anti-EEA1 antibodies followed by FITC- and Alexa-596-conjugated secondary antibodies, respectively.

CHAPTER 4

Role of PI4KIIβ in endocytosis and signaling of the EGFR

I. ABSTRACT

PI4KIIα has been implicated in Golgi trafficking and in late stages of endocytosis. In contrast, the precise roles of PI4KIIβ are currently unknown. Based on its partial redistribution to the plasma membrane upon cell treatment with growth factors, we speculated that PI4KIIβ might somehow be involved in receptor-mediated endocytic trafficking and signaling. I consistently observed that depletion of PI4KIIβ accelerates EGF dependent-ERK phosphorylation and that ERK phosphorylation is sustained for longer periods than in control. In addition, I tracked the endocytic traffic of EGF/EGFR complexes using receptor antibodies and fluorescent conjugated EGF. Depletion of PI4KIIβ increased the accumulation of EGF/EGFR complexes beneath the plasma membrane following EGF treatment for 5 minutes. Inhibition of EGFR internalization by expression of dominant negative dynamin (K44A) abrogated the enhanced ERK phosphorylation that was caused by PI4KIIβ depletion. These results suggest that the stimulation of ERK activation due to RNAi-mediated knockdown of PI4KIIβ is somehow linked to the facilitated internalization of the EGFR.

As further evidence that PI4KIIβ slows certain steps in the endocytic process, depletion of the kinase accelerated EGF-dependent translocation to membranes of EEA1, a protein involved in early endosomal fusion. The activity of Rab5, the upstream regulator of EEA1, also increased slightly in RNAi-treated cells.

In this chapter, I present evidence that PI4KIIβ may influence an early stage of endocytosis, perhaps by regulating the activity of Rab5, a small GTP-binding protein that governs endosomal fusion as well as signaling from peripheral endosomes.

II. INTRODUCTION

A. General view of EGFR endocytosis

EGFR (epidermal growth factor receptor) is a transmembrane receptor tyrosine kinase (RTK) expressed on the cell surface. Upon activation by its cognate ligand, EGF, the EGFR undergoes a transition to homo-/hetero-dimers, which stimulates its intrinsic intracellular protein-tyrosine kinase activity. As a result, autophosphorylation of several tyrosine residues in the C-terminal domain of the EGFR elicits downstream activation and signaling by several other proteins that associate with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains. These downstream signaling proteins initiate several signal transduction cascades, principally the MAPK, PI3K/Akt and JNK pathways, leading to DNA synthesis, cell proliferation, and migration (Gaborik and Hunyady, 2004; Kirisits et al., 2007; Le Roy and Wrana, 2005).

Activation of the EGFR also leads to its rapid internalization via clathrin-mediated endocytosis, which occurs within minutes of ligand stimulation. Cbl, a RING-finger E3 ubiquitin ligase, persistently binds and promotes multiple monoubiquitylations of the activated EGFR, thereby mediating internalization of the EGFR. This multiubiquitylation targets the EGFR for subsequent sorting by interacting with adaptors bearing a Ub-binding domain. In addition, the coordinated activities of a variety of endocytic proteins, including

CIN85 (Cbl-interacting protein of 85kDa), dynamin, endophilins and Eps15 are critical for EGFR internalization and further trafficking. (Clague and Urbe, 2006; Kirisits et al., 2007)

The internalized receptors are first targeted to early sorting endosomes, located near the cell periphery. Subsequently, receptors can be sorted either to the recycling endosome and then back to plasma membrane, or together with Eps15 and ESCRT complexes to multivesicular body (MVB) and then to late endosomes and lysosomes, where both EGF and EGFR are degraded, resulting in signal termination as shown in Figure 4-1 (Kirisits et al., 2007; Le Roy and Wrana, 2005).

Substantial evidence indicates that many Rab GTPase proteins regulate the targeting/docking/fusion processes in distinct intracellular trafficking events. Therefore Rab cascades and conversions appear to confer directionality to membrane trafficking and couple each stage of traffic with the next along the pathway. To ensure the specificity of vesicle trafficking, Rab proteins have restricted distributions and are often used as organelle markers (Figure 4-2, reviewed in (Gaborik and Hunyady, 2004; Somsel Rodman and Wandinger-Ness, 2000)).

B. Early stage of endocytosis

Receptor internalization has classically been viewed as a mechanism for signal attenuation. However, recent studies have raised the possibility that intracellular trafficking of activated receptors may also have a role in maintaining, and even enhancing, certain signaling pathways. Early studies showed that a mutant EGFR defective for internalization could not transduce signals leading to mitosis and cell transformation (Wells et al., 1990). Vierira et al. confirmed this observation using K44A dynamin mutant to prevent EGFR internalization. In

these endocytosis-defective cells, a subset of signaling pathways could not be fully activated (Anderson, 2006; Kranenburg et al., 1999; Schmidlin et al., 2001; Vieira et al., 1996). Thus, endocytic trafficking of activated EGFRs plays a critical role not only in attenuating EGFR signaling but also in establishing and controlling specific signaling pathways.

Among the at least 60 Rab proteins, Rab5 has long been known to coordinate the initial trafficking steps of the endocytic pathway. Extensive studies have shown that Rab5 is required for the formation and dynamics of early sorting endosomes by regulating clathrin coated vesicle formation, fusion of incoming endocytic vesicles to early endosomes, homotypic fusion of these early endosomes, and their movement along microtubules. (Barbieri et al., 2004; Barbieri et al., 2000; Bucci et al., 1992; Rybin et al., 1996).

Rab5 has also been implicated in determining signal output, signal propagation or signal attenuation (Miaczynska et al., 2004b; von Zastrow and Sorkin, 2007; Zerial and McBride, 2001). A recent study found that the Rab5 effectors, APPL1/2 reside on peripheral endosomes and direct signaling pathway between endocytic membranes and the nucleus (Miaczynska et al., 2004a). Rab5 activity is functionally modulated by GTPase activating proteins (GAPs, e.g. RNtre and RabGAP5) (Haas et al., 2005; Lanzetti et al., 2000) and GTP exchange factors (GEFs, e.g. RINs and Rabaptin) (Grosshans et al., 2006; Schwartz et al., 2007; Stenmark et al., 1995).

C. Phosphoinositides in membrane trafficking

As discussed in Chapter 1, PIs are distributed among distinct subcellular organelles and play specific roles in membrane trafficking and signal transduction. The best studied PI, PI4,5P_2, is particularly abundant on the plasma membrane where it serves as a substrate for PLC in

the production of second messengers, as well as for $PI3,4,5P_3$ production which activates a set of protein kinases. Besides their roles in signaling, $PI4,5P_2$ and $PI3,4,5P_3$ are pivotal for cytoskeletal organization leading to cell migration and $PI4,5P_2$ has established roles in clathrin-mediated endocytosis and synaptic vesicle trafficking (reviewed in (Roth, 2004)). Another PI involved in membrane trafficking, PI3P, exists primarily in endosomes and recruits a variety of adaptors and signaling proteins that contain PI3P binding domains (e.g. FYVE). After rapidly losing their clathrin coats, incoming vesicles recognize PI3P-harboring early endosomes and fuse with them, resulting in directionality of trafficking (Gruenberg, 2003). PI4P recruits adaptors, including AP-1 and GGA for constitutive secretion from Golgi complex. Also, it is a precursor for $PI4,5P_2$ and $PI3,4,5P_3$ on plasma membrane and could be a target for regulation to produce $PI4,5P_2$ and $PI3,4,5P_3$.

D. Studies of PI4-kinases in membrane trafficking

Consistent with their endomembrane localization, evidence suggests that Type II PI 4-kinases regulate intracellular trafficking events in Golgi as well as other endosomal vesicles. Knockdown of PI4KIIα abolished the Golgi recruitment of AP-1 (Wang et al., 2003), and impairs the recruitment of AP-3 to endosomes (Craige et al., 2008). Minogue and Hsuan reported that elimination of PI4KIIα delays the late endosomal trafficking of EGFR, resulting in a reduction of its degradation in lysosome (Minogue et al., 2006). Type II PI 4-kinase activities have also been found to associate with several other membrane proteins, including the T cell receptor (Janes et al., 1999), the EGF receptor (Kauffmann-Zeh et al., 1994), and integrins (Claas et al., 2001).

Several experiments in yeast have also pointed to a role of PI-4 kinases in endocytic trafficking. The yeast PI4KII ortholog Lsb6p shows moderate PI 4-kinase activity and makes little contribution to the overall PI4P production of yeast cells under normal growth conditions (Buxeda et al., 1993; Shelton et al., 2003). Inactivation of Lsb6p causes only a mild alteration in the trafficking of the endocytosed mating factor receptor. This defect is rescued by a construct that does not contain the catalytic domain but requires regions that interact with Las17p, the yeast homolog of Wiskott–Aldrich syndrome protein, which is important for the regulation of actin polymerization. It is possible that Lsb6p acts as a scaffold and regulates the movements of vesicles, and its lipid kinase activity might not be crucial for at least some of its functions in yeast (Chang et al., 2005; Kim et al., 2006).

In contrast to PI4KIIα, which has been strongly implicated in Golgi budding and the late stages of endocytosis, no function has yet been ascribed to PI4KIIβ. In this chapter, I present evidence that PI4KIIβ may have a role in receptor trafficking and signaling that is distinct from that of PI4KIIα.

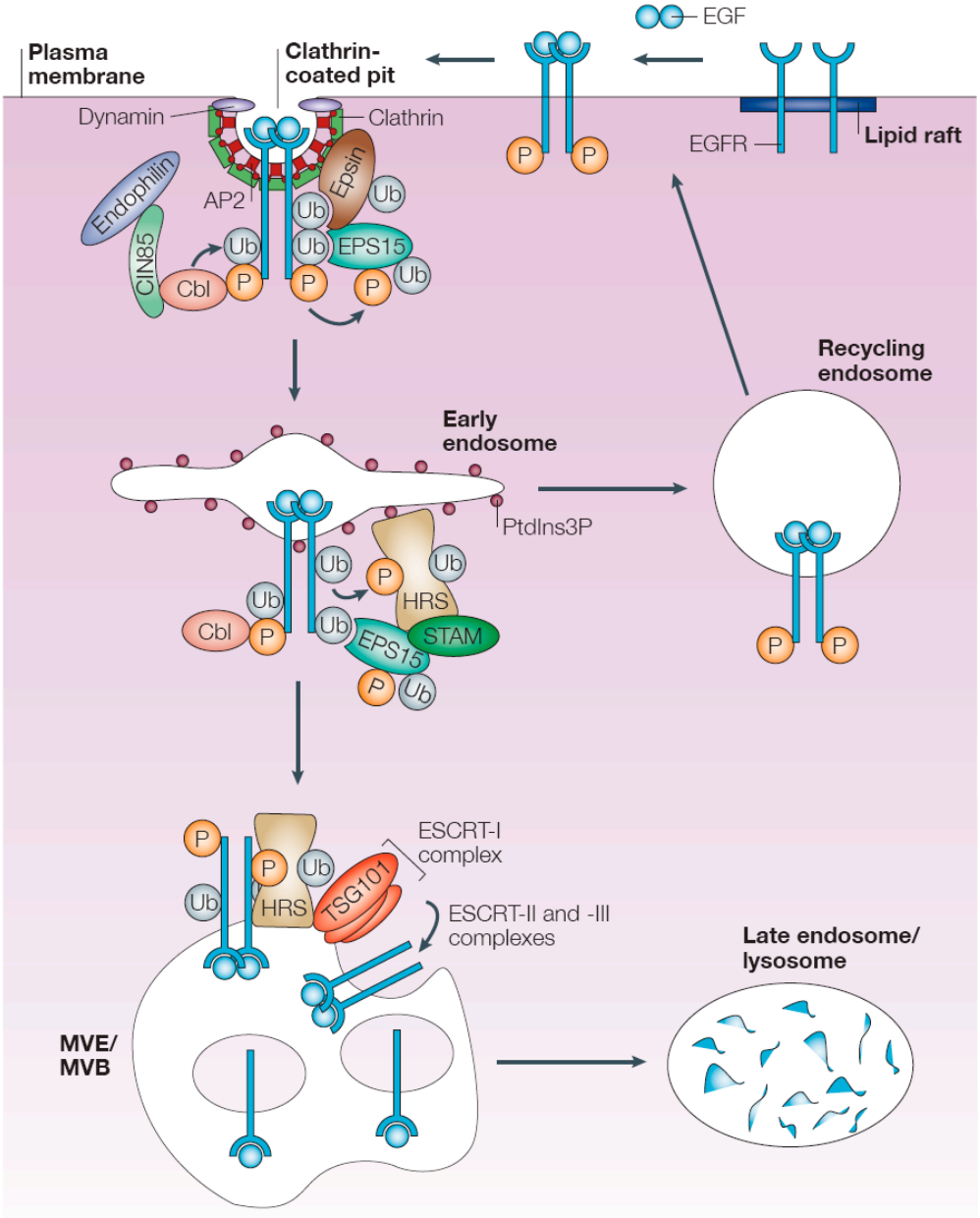

EGF
Plasma membrane
Clathrin-coated pit
Dynamin
Clathrin
Endophilin
CIN85
AP2
Epsin
Ub
EPS15
Cbl
P
EGFR
Lipid raft
Recycling endosome
Early endosome
PtdIns3P
HRS
STAM
ESCRT-I complex
TSG101
ESCRT-II and -III complexes
Late endosome/ lysosome
MVE/ MVB

Figure 4-1. Overview of EGFR endocytosis. Once EGF binds to the monomeric EGFR on the cell surface, EGFRs dimerize and activate trans-phosphorylations. Then c-Cbl, an E3 ubiquitin ligase, is recruited to the activated receptors and multi-monoubiquitylates them. Ubiquitylated EGFRs aggregate in clathrin coated pits and associates with CIN85 and endophilins. Epsin and Eps15 might bind to monoubiquitylated EGFRs through their ubiquitin-interacting motifs. Dynamin assists vesicle fission from the plasma membrane and Rab5 promotes fusion with endocytic vesicles to form early endosomes, respectively. In early endosomes, HRS binds to PI3P through its FYVE domain, and forms a ternary complex with signal transduction adaptor molecule (STAM) and Eps15 that interacts with EGFRs. From the early endosomes, most EGFRs are sorted towards the MVB. At the MVB,Hrs interacts with tumour susceptibility gene-101 (TSG101), a component of endosomal sorting complex required for transport-I (ESCRT-I). This interaction leads EGFRs to ESCRT-II and -III and to the intralumenal vesicles of MVEs/MVBs, and subsequently to late endosomes/lysosomes where they are degraded. P, phosphate; Ub, ubiquitin. Figure is taken from *Wrana, 2006.*

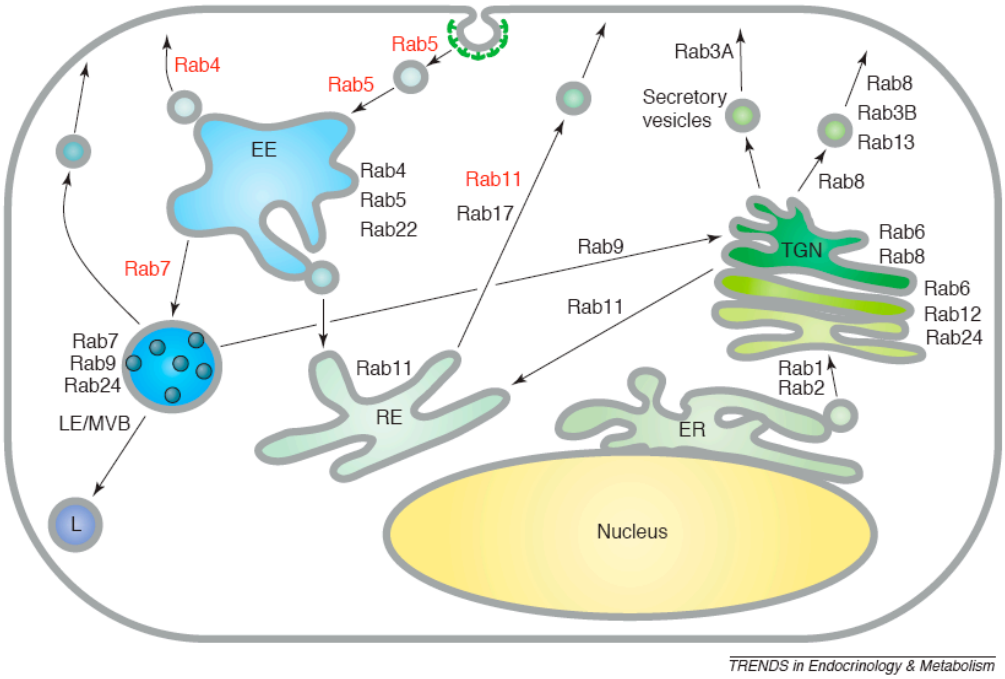


Figure 4-2. Distribution of Rab GTPases. Active Rab5 is important for endocytosis via clathrin-coated pits and subsequent fusion of vesicles with early endosomes. In addition to Rab5, early endosomes contain Rab5 effectors and regulator proteins, including early endosome antigen 1 (EEA1), rabaptin 5, rabenosyn-5, PI3P and partners. Endocytosed molecules can exit early endosomes via several pathways. Rab4 regulates a direct pathway for recycling receptors to the plasma membrane (fast recycling). Rab11 has been documented to have a role in late recycling of transferrin receptor through the recycling endosomes. Proteins destined for degradation are delivered to late endosomes and then to lysosomes. Rab7 is essential for transport from early to late endosomes. Rab proteins also participate in the vesicular transport mechanism between other organelles. This figure is taken from *Hunyady, 2004.*

III. ADDITIONAL EXPERIMENTAL PROCEDURES

Reagent - Rhodamine-EGF and FITC-transferrin were obtained from Molecular Probes (Invitrogen). EGFR antibody and Phospho ERK1/2 antibody were obtained from Cell Signaling (Denver, MA). Transferrin receptor antibody was from Zymed lab (San Francisco, CA). Dynamin I antibody and EEA1 antibody were from BD sciences (San Jose, CA). Total ERK antibody was a gift from the laboratory of Dr. Melanie Cobb (UT Southwestern Medical Center).

Constructs - Rab5a was amplified from a mouse cDNA library and cloned into pCMV5-myc and pCMV5-myc-GFP vectors using primers: forward 5'-CGGGGTACCATGGCTAATC-GAGGAGCAACAAGACC-3' and reverse 5'- CGCGGATCCTCAGTTACTACAACACT-GGCTTCTGG-3'. Mutants Rab5-Q79L and Rab5-S34N were generated using the Quick-change method using primers: 5'-GGGATACAGCTGGTCTAGAACGGTATCATAGC-3' and 5'-GCTATGATACCGTTCTAGACCAGCTGTATCCC-3' for Rab5-Q79L, 5'-GAGTCTGCTGTTGGCAAAAACAGCCTGGTTCTTCGCTTTGTG-3' and 5'-CACAAA-GCGAAGAACCAGGCTGTTTTTGCCACAGCAGACTC-3' for Rab5-S34N.

GST-R5BD was cloned in pGEX-KG using primers which were designed based on the Rabaptin sequence (739-862aa): For-R5BD-EcoRI, 5'-CCGGAATTCTAGCTTC-CATTTCTAGTCTAAAAGC-3' and Rev-R5BD-HindIII 5'- CCCAAGCTTTCATGTCT-CAGGGAGCTGGTTAATG-3'. Dynamin I K44A was generated using Quickchange Mutagenesis (Stratagene).

RNA interference (RNAi) of PI4KIIβ – The double stranded siRNAs for PI4KIIβ were designed and produced by Dharmacon (Chicago, IL) and UTSW core facility. siRNA-0 (346-365bp); 5'-AATGCATTCTTGGATGACCCA-3', siRNA-1(439-457bp); 5'-GGTTCAAGT-GGAAGTTACT-3', siRNA-2(678-696bp); 5'-GGTGGTTTGGCTTGTCAGT-3'. HeLa cells were grown in 60mm plates at 50 % confluence 1 day before RNAi. Treatment siRNAs (0.3 μM) were mixed with Lipofectamin2000 and transfected according to the manufactures instructions (Invitrogen). After 24 h, cells are replated on 35mm plates. The next day, cells were starved in DMEM w/o serum for at least 4 h and then stimulated with EGF (100ng/ml) for indicated times.

Rhodamine-EGF and FITC-transferrin uptake assay - EGF and transferrin uptake assays were performed as described in Haas et al. (Haas et al., 2005). RNAi-treated and untreated HeLa cells were grown on coverslips and starved for 15 h after washing with serum-free medium. Coverslips were washed three times in ice-cold PBS and placed on 40μl drops of uptake medium (DMEM, 2% BSA, 20mM HEPES-NaOH, (pH7.5), 2.5μg/ml rhodomine-EGF and 50μg/ml FITC-transferrin) on an ice-cold metal plate covered in parafilm for 30-60 min. Coverslips were then washed three times in ice-cold PBS to remove excess ligands and incubated in pre-warmed growth medium at 37°C. For the 0 min time point, coverslips were fixed without incubation at 37°C. At certain time points, cells were fixed for 15 min in 3.7% formaldehyde and mounted on slideglass. For EGF receptor tracking, RNAi-treated and untreated cells were stimulated with EGF (100ng/ml) after 4-6 h starvation and slides were prepared as described in the previous chapter.

Rab5 activity assay – A GST pull-down assay was used to estimate Rab5 activity, using Rab5-binding domain (R5BD, residues 739-862) of Rabaptin5 as described in Liu et al. (Liu et al., 2007). The R5BD construct was subcloned from a mouse cDNA library into pGEX-KG vector. The fusion protein, GST-R5BD was expressed in *E.coli* and affinity purified with glutathione-Sepharose 4B resins (GE healthcare, UK). Rab5 proteins (WT, Q79L, and S34N) were expressed in HeLa cells followed by lysis in buffer (25mM HEPES, pH 7.4, 100 mM NaCl, 5mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 1mM dithiothreitol, and protease inhibitors). Cell lysates were incubated with 20 µl of GST-R5BD bound to the glutathione-Sepharose 4B resin for 30 min at 4°C on rotator. After rinsing the resin with buffer, the resin was resuspended in SDS sample buffer followed by immunoblot analysis with the anti-Myc antibodies.

IV. RESULTS

4.1. Depletion of PI4KIIβ enhances EGF dependent ERK phosphorylation.

Over 80% of endogenous PI4KIIβ was knocked down using several different siRNAs to target different sequences of PI4KIIβ. Polyclonal antibodies against human PI4KIIβ were used to estimate the amount of PI4KIIβ. These antibodies also recognize a non-specific band above the kinase, the abundance of which varies in different preparations. After 4 h starvation, the basal level of activated, phosphorylated ERK in HeLa cells is undetectable, however upon addition of EGF (100ng/ml), ERK is phosphorylated within 5 min, as evident from immunoblotting in control cells. ERK phosphorylation is maximal after 15 min treatment with EGF but thereafter the response diminishes with time.

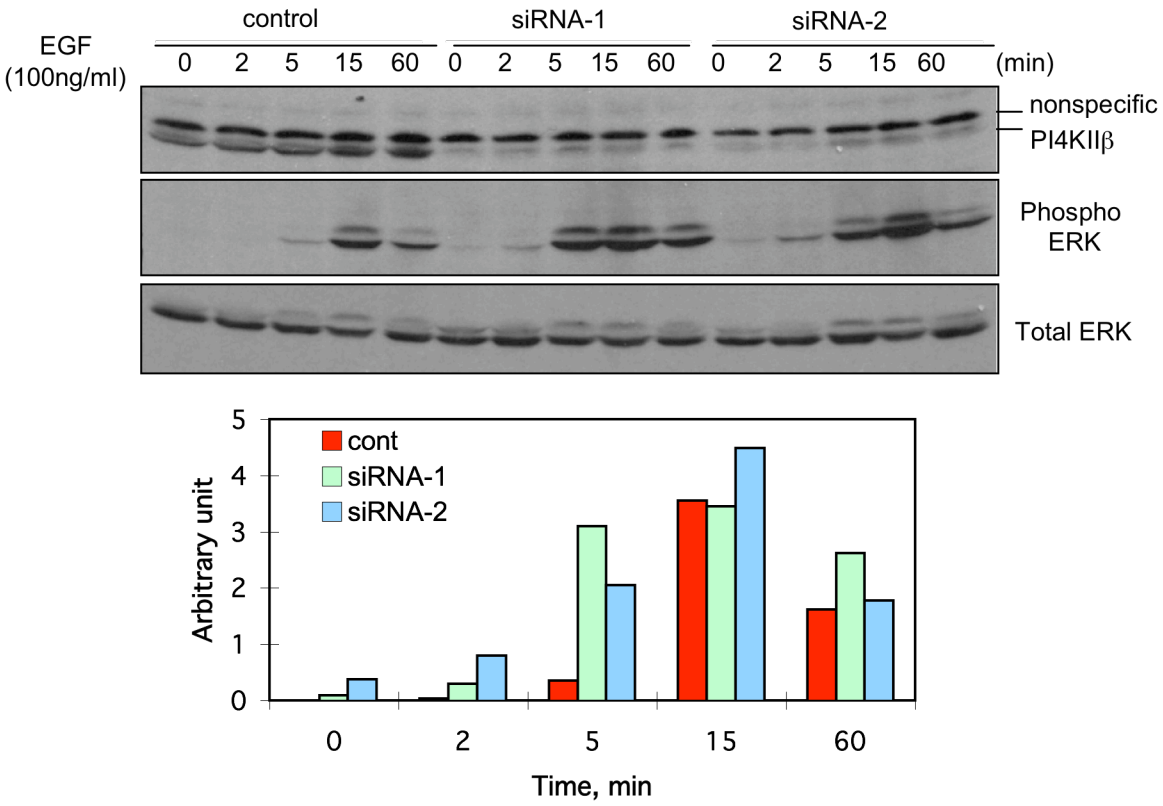


Figure 4-3. Depletion of PI4KIIβ accelerates EGF dependent-ERK phosphorylation. Control and two different siRNAs-treated cells were stimulated with EGF (100ng/ml) for the periods indicated after 4 h starvation. Depletion of PI4KIIβ and activation of ERK were analyzed by immunoblot of equal amounts of whole cell lysates using anti-PI4KIIβ and anti-phospho-ERK1/2 antibodies, respectively. Blot with anti-PI4KIIβ shows a nonspecific band above PI4KIIβ. Total amounts of ERK1/2 proteins are shown as loading control and the relative levels of phosphorylation of ERK1/2 were quantified and shown in the graph.

Surprisingly, elimination of PI4KIIβ accelerates EGF-dependent ERK phosphorylation at early time points (2 and 5 min) and its phosphorylation tends to persist slightly longer than in controls. The effect is obtained using two different oligos, siRNA-1 and siRNA-2 (Figure 4-3). The biggest difference between control and RNAi-treated cells occurs at 5 min after EGF treatment, as shown in the graph. From 3 independent experiments, the depletion of PI4KIIβ consistently increases the level and duration of ERK phosphorylation.

4.2. Depletion of PI4KIIβ accelerates an early stage of the EGF/EGFR endocytic pathway.

To investigate whether this signaling event is related to endocytic trafficking, I attempted to follow the endocytic pathway of EGF/EGFR by microscopy using two different approaches. First, at various time points during EGF stimulation, EGF receptors were localized using anti-EGFR antibodies. As an internal control in this experiment, I co-transfected double-stranded siRNA against PI4KIIβ with GFP to visualize the cells receiving the siRNAs. Control cells were transfected with single-stranded sense RNA oligo and GFP. Following starvation of cells for at least 4 h, most of the EGF receptors are located on the plasma membrane. Within 5 min, EGF stimulates EGFR clustering and induces small invaginations resulting in peripheral endosomes beneath the plasma membrane, which may correspond to clathrin-coated vesicles. I was able to see the formation of small endosomes and EGF receptors clustering into those small vesicles. At 15min in the presence of EGF, the small endosomes grow in size, presumably by fusion with early endosomes, and move toward the

center of the cell. These centrally located structures may represent a combination of early endosomes and late endosomes. At late time points, EGF/EGFR is presumably directed to MVBs and eventually to lysosomes for degradation. I infer the trafficking of EGF/EGFR in my experiments by virtue of the size and distribution of vesicles (Figure 4-4).

GFP expression itself doesn't affect the distribution and internalization of the EGFR over time in the control cells. In both control and RNAi-treated cells, receptors are normally stained on plasma membrane after 4 h starvation. Interestingly, at the 5 min time point, more intense EGFR signals are observed in enlarged vesicles in RNAi-treated cells compared with adjacent non-transfected cells. PI4KIIβ-depleted cells have apparently larger endocytic vesicles; the same size vesicles began to appear at later times in control cells. Thus, it appears that depletion of PI4KIIβ enhances the rate of formation of endosomes just beneath the plasma membrane. (Figure 4-4). The difference between control and RNAi-treated cells is most obvious at 2 to 5 min after EGF treatment in two independent experiments using different siRNAs. More intense signals from larger endosomes are also evident in PI4KIIβ-depleted cells after 15 min. But after 30 min when the receptors should be directed into late endosomes or lysosomes, there is hardly any difference between control and depleted cells. These effects are more easily discerned at higher magnification (Figure 4-4B). Consistent with EGF-dependent ERK phosphorylation, the major effect of elimination of PI4KIIβ is observed at the early stages of endocytosis.

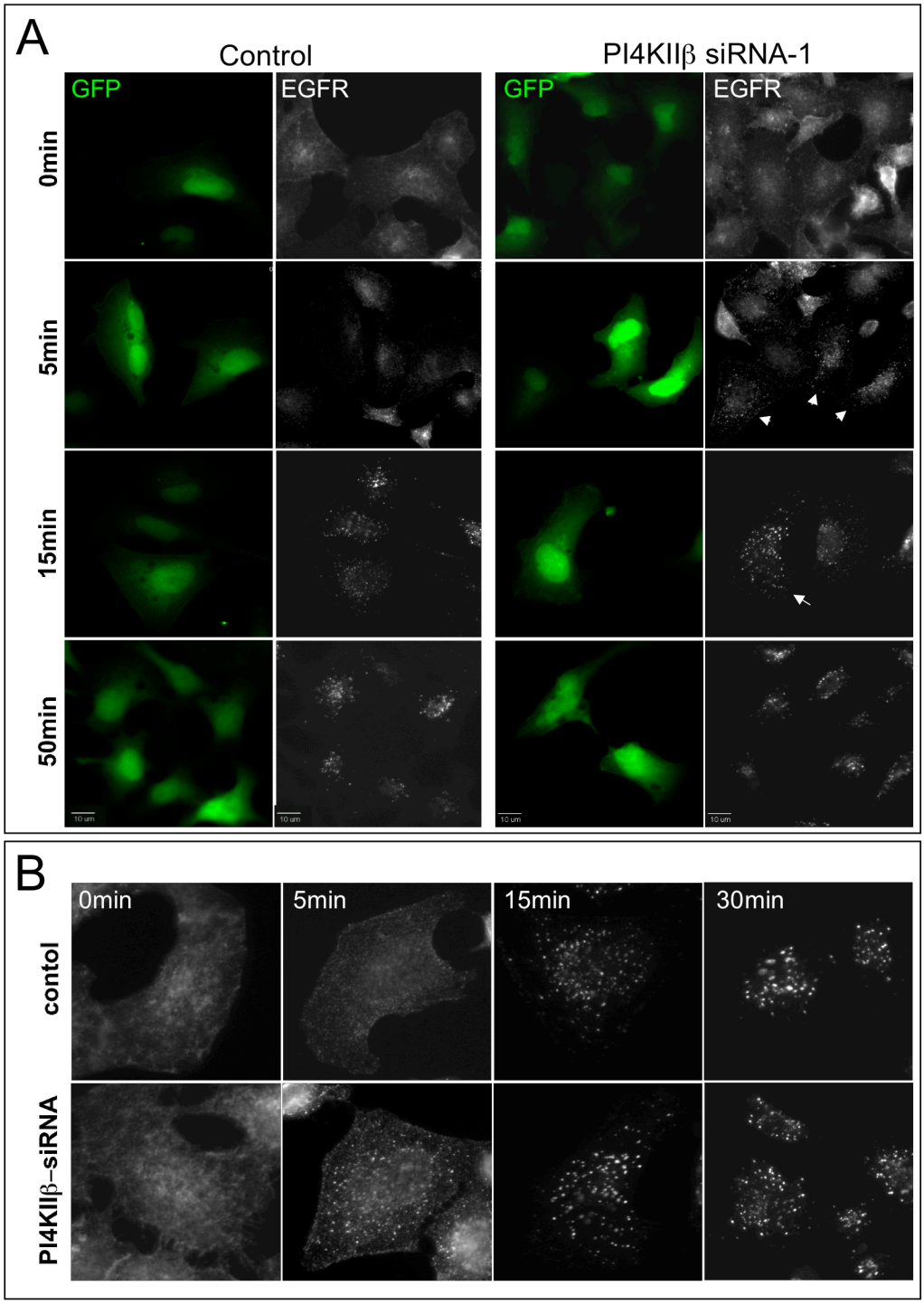


Figure 4-4. Depletion of PI4KIIβ accelerates early stages of the EGFR endocytic pathway. Contol and RNAi-treated cells were co-transfected with a GFP construct. After replating and starving for 4 h, the cells were stimulated with EGF (100ng/ml) for the indicated time points (**A**). Arrows show the GFP-transfected cells and larger EGFR staining vesicles. The magnified single cells present EGFR staining at each time points (**B**). Bars indicate 10 μm.

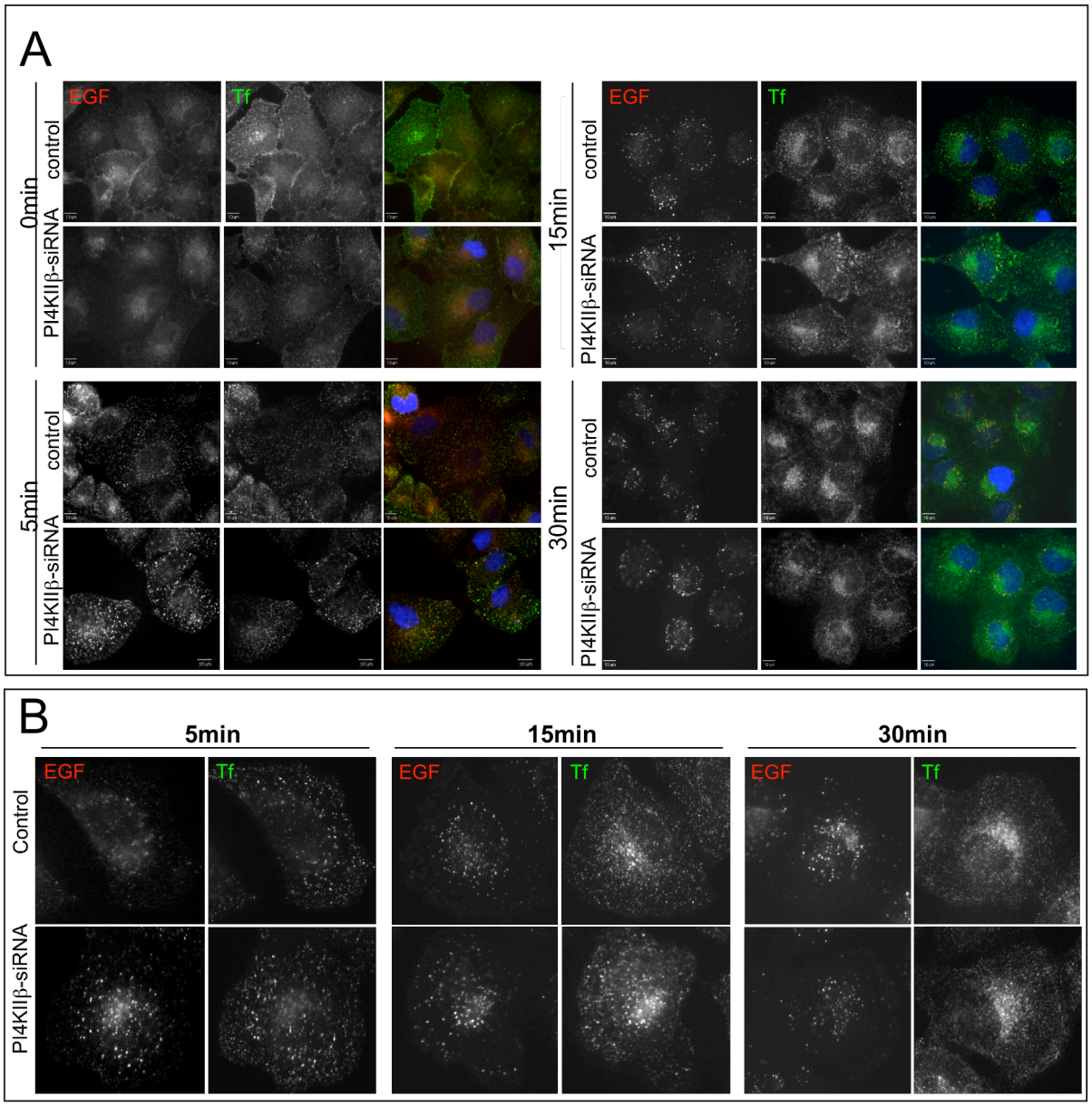


Figure 4-5 Depletion of PI4KIIβ accelerates an early stage of EGF uptake. Uptake of EGF (red) and Transferrin (green) by HeLa cells was followed by treatment with PI4KIIβ siRNA. After 0, 5, 15 and 30 min of uptake, cells were fixed and DNA was stained with DAPI (blue) in all panels. Bars indicate 10 μm. An enlargement of the images is shown in **B**.

To confirm this observation, I adapted an EGF uptake assay to follow the endocytic traffic of the EGF/EGFR complex. This assay allowed me to distinguish ligand-stimulated endocytosis from constitutive endocytosis using rhodamine-EGF and FITC-transferrin, respectively. In both cases, internalization of receptors is dependent on clathrin-coated vesicles (CCVs). However, the transferrin receptor is endocytosed and recycled continuously, employing a minimal set of internalization components (e.g. AP-2, clathrin, dynamin) (Gaborik and Hunyady, 2004; Haucke, 2006; Le Roy and Wrana, 2005). In contrast, EGFR internalization utilizes a more complex endocytic machinery, including factors, such as Epsin and Eps15, which assemble with the activated EGFR and are regulated by phosphorylation or ubiquitylation of the receptor as well as their own ubiquitylation (Le Roy and Wrana, 2005).

Comparable amounts of EGF and transferrin are bound to the surface of transfected and non-transfected cells at 4 °C after 15 h starvation (Fig4-5A left panel). The ligand/receptor complexes were allowed to enter cells by transferring them to 37°C. At the indicated time points, external ligands were washed away, to allow tracking of only the internalized ligands, and the cells were then fixed. Within 5 min, EGF and transferrin tend to accumulate in vesicle-like structures. Consistent with EGFR staining, a robust EGF signal is observed in larger endosomes in PI4KIIβ-depleted cells than in control cells. In contrast, transferrin uptake proceeds similarly in control and depleted cells. After 15 min of internalization, the two ligands apparently follow their expected pathways: EGF is directed to lysosomes for degradation, whereas transferrin begins to accumulate in the juxtanuclear region where recycling endosomes reside. At later time points I observe blurry FITC signals are seen around and even outside the cells, which may reflect recycling of transferrin to the

exterior (Figure 4-5). Although Balla's group proposed a role for PI4KIIs in transferrin recycling(Balla et al., 2002), we see no obvious differences in transferrin trafficking upon PI4KIIβ depletion. Taken together, these results raise the possibility that regulated endocytosis of the EGF receptor, but not constitutive endocytosis of transferrin, is a potential target for PI4KIIβ function.

4.3. Accelerated ERK phosphorylation by depletion of PI4KIIβ requires EGFR internalization.

Because endocytosis of RTKs has long been accepted as a mechanism for attenuating signals from RTKs, the observation that ERK signaling and endocytic trafficking are both enhanced in PI4KIIβ-depleted cells may seem contradictory to the current view. However, other lines of evidence suggest that RTKs achieve full activation only after downstream signaling. When receptor-mediated endocytosis is inhibited by a dominant-negative dynamin mutant (K44A) incapable of binding GTP, certain EGF-dependent signaling events (including ERK phosphorylation) are down-regulated in correspondence to a decrease in the formation of endosomes (Vieira et al., 1996). Additionally, EGF receptors on Rab5-containing endosomes initiate a nuclear signaling pathway through the action of a specific effector of Rab5, APPL1/2 (Miaczynska et al., 2004a). Indeed, endocytic trafficking of activated EGFRs plays such a critical role in controlling specific signaling pathways that it has been termed "endosomal signaling".

Based on this idea, I attempted to test whether the enhanced ERK-phosphorylation is derived from the facilitated internalization of EGFR. I expressed the K44A dynamin mutant

in control and RNAi-treated cells and examined whether the up-regulated phosphorylation caused by depletion of PI4KIIβ drops to the level of control. First, I confirmed the original observation that dynamin I K44A expression impairs both EGFR endocytosis and EGF-dependent ERK phosphorylation. At 10 min after EGF treatment, the K44A mutant prevents internalization of EGF receptors after they cluster on plasma membrane (Figure 4-6A). In addition, this mutant reduces ERK phosphorylation during EGF treatment by up to 50 %. In PI4KIIβ knockdown cells, which do not co-express K44A, more intense staining of EGFR appears in early endosomes, and up-regulated ERK phosphorylation is observed in 2 and 5 min time points, consistent with results described in Figure 4-3 and 4-4. However, these enhanced signals drop to control level in the presence of the K44A mutant, as shown in Figure 4-6B.

These results indicate a correlation between the accelerated endosome formation and the enhanced ERK phosphorylation resulting from depletion of PI4KIIβ. As stated, PI4KIIβ depletion promotes early steps of endocytosis without altering the late stage of endocytosis and degradation of EGF receptors. A previous study by Hsuan's group presented evidence that depletion of PI4KIIα causes defective degradation of the EGFR. To determine if the observed changes in endocytic traffic caused by PI4KIIβ depletion change the rate of degradation of the EGFR, degradation of the EGFR was monitored by immunoblotting. I found that siRNA-treated cells and control cells retain a similar amount of EGFR over the whole time course of EGF treatment (Figure 4-7), confirming that PI4KIIβ does not influence trafficking of the receptor to lysosomes.

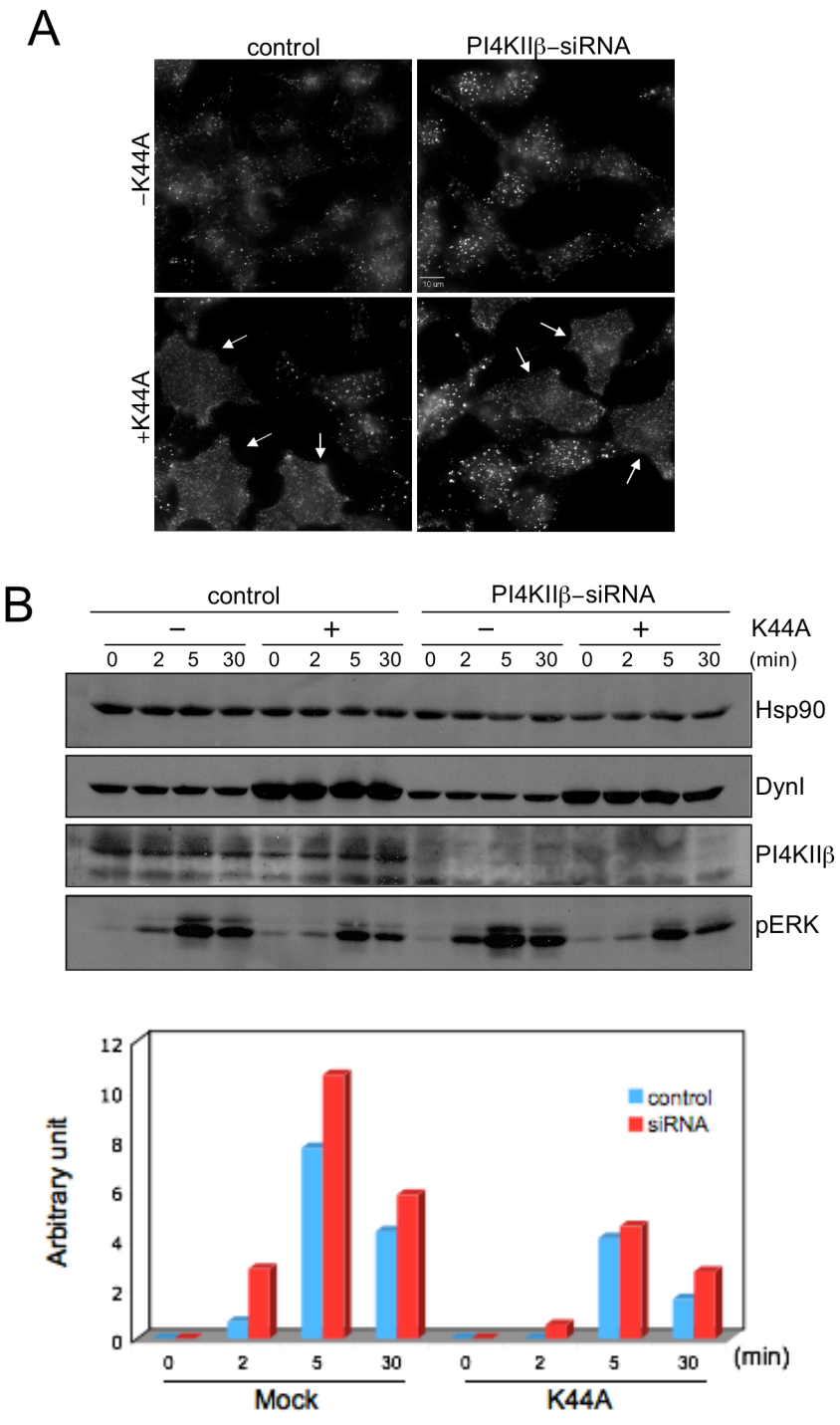

A
control
PI4KIIβ–siRNA
–K44A
+K44A
B
control
PI4KIIβ–siRNA
–
+
–
+
K44A
0 2 5 30 0 2 5 30 0 2 5 30 0 2 5 30
(min)
Hsp90
DynI
PI4KIIβ
pERK
Arbitrary unit
12
10
8
6
4
2
0
control
siRNA
0 2 5 30 0 2 5 30
(min)
Mock
K44A

Figure 4-6 Accelerated ERK phosphorylation by depletion of PI4KIIβ requires EGFR internalization. Control and PI4KIIβ-siRNA treated cells were transfected with vector control or K44A dynamin mutant followed by treatment with EGF (100ng/ml) after 8 h starvation. **A**. Following EGF treatment for 10 min, HeLa cells were fixed and strained with anti-EGFR antibodies. Arrows in the lower panel show the sustained presence of EGFRs on the plasma membrane in K44A expressing cells. **B**. Cells were treated with EGF and removed at 2, 5, and 30 min time points. Cell lysates were prepared and immunoblotted with anti-Dyn I, anti-PI4KIIβ, anti-Hsp90, and anti-phospho ERK1/2 antibodies. The relative levels of phosphorylation of ERK were quantified as shown in the graph.

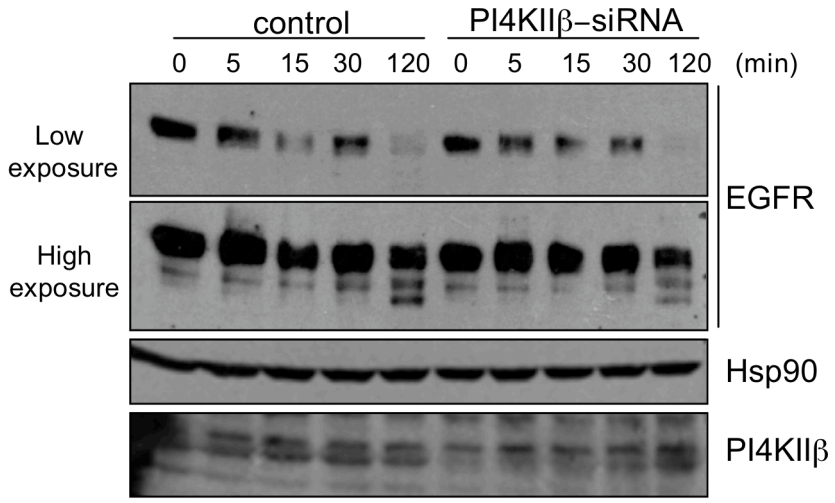


Figure 4-7 Effect of depletion of PI4KIIβ on degradation of the EGFR. Control and PI4KIIβ siRNA-treated cells were lysed in RIPA buffer after EGF treatment for the indicated periods. Total cell lysates were immunoblotted with anti-EGFR antibodies to show the remaining EGFR at low and high exposures. Hsp90 was used as a loading control.

4.4. Depletion of PI4KIIβ increases active EEA1, a Rab5 effector.

Based on my results, I postulated that depletion of PI4KIIβ facilitates an early stage of endocytosis, leading to the rapid formation of larger early endosomes. Thus I decided to examine the level of membrane-bound EEA1, a protein that plays a direct and critical role in endosome fusion by bringing early endosomes together. In quiescent cells, EEA1 has a cytoplasmic distribution but, in response to agonist, it targets to early endosomes via a FYVE domain that interacts specifically PI3P. Therefore, EEA1 activation is easily monitored by measuring the amount of membrane-bound EEA1. At 5 min after EGF treatment, the amount of EEA1 in membranes maximally increases, but subsequently decreases at later time points (Figure 4-8). In PI4KIIβ-depleted cells, a greater amount of EEA1 translocates to membranes after 5 min of EGF treatment than in control cells.

4.5. Rab5 activity is involved at an early stage of endocytic and signaling pathways

EEA1 is activated by Rab5 and recruited to endosomes where PI3P is enriched. Rab5 is a rate-limiting component of the machinery regulating the kinetics of membrane traffic in the early endocytic pathway (Bucci et al., 1992). It is activated by EGF stimulation through Rab5-GEFs (GTP exchange factors) and once active Rab5 provides a platform on early endosomes for a variety of effectors. Active Rab5 promotes the initial stages of endocytosis by regulating vesicle docking and fusion, as well as by coordinating downstream signaling events (Barbieri et al., 2004; Barbieri et al., 2000; Miaczynska et al., 2004a).

I hypothesized that PI4KIIβ modulates a rate-limiting step regulated by Rab5. To test this hypothesis, I first reproduced the published effects of Rab5 on EGFR endocytosis and

signaling using its catalytically inactive and constitutively active mutants, S34N and Q79L, respectively. Shortly after EGF stimulation, Rab5-WT co-localizes with the EGFR at the cell periphery, reflecting its association with nascent endosomes. In contrast, Rab5-S34N is predominantly cytosolic and its expression inhibits the initial internalization of the EGFR. Consistent with previous observations, Rab5-Q79L expression induces the formation of enlarged endosomes, and the EGFR begins to accumulate into these vacuole-like endosomes during stimulation with EGF (Figure 4-9A, (Stenmark et al., 1994)). Overexpression of Rab5-WT or Rab5-Q79L alters the steady-state localization of the EGFR. Disruptions in endocytic trafficking by Rab5 mutants correlate with ERK phosphorylation: Rab5-Q79L promotes EGFR internalization and leads to enhanced ERK phosphorylation, whereas Rab5-S34N inhibits EGFR translocating to early endosomes and dampens ERK phosphorylation, demonstrating that Rab5 activity is linked to EGFR signaling and trafficking, in a manner resembling PI4KIIβ (Figure 4-9B).

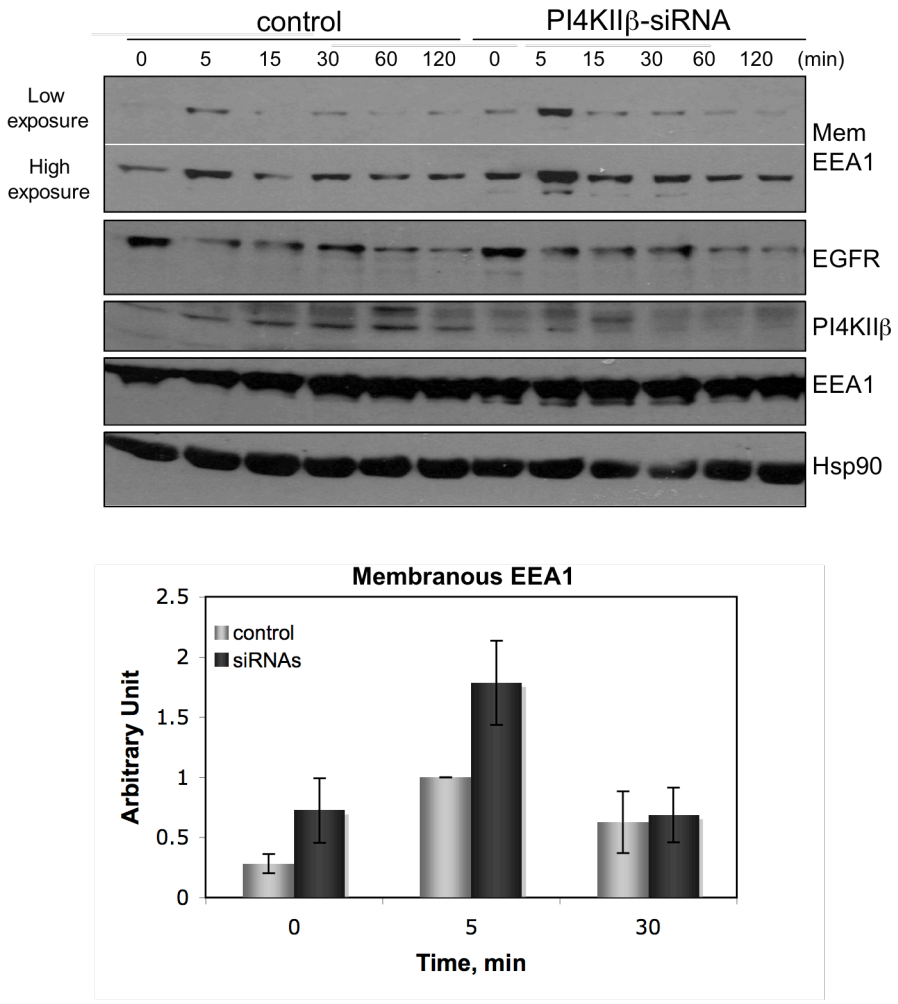


Figure 4-8. Depletion of PI4KIIβ increases active EEA1, a Rab5 effector. Membrane fractions from control and PI4KIIβ-siRNA treated cells were prepared in 1% Triton X-100 buffer during EGF treatment after 8 h starvation. The same amount of cell lysates were used for membrane fractionation as shown total EEA1 and Hsp90 blots. Membranous EEA1 was detected using anti-EEA1 antibodies at low or high exposures. The averages and error bars from 4 sets of experiments are shown in the graph.

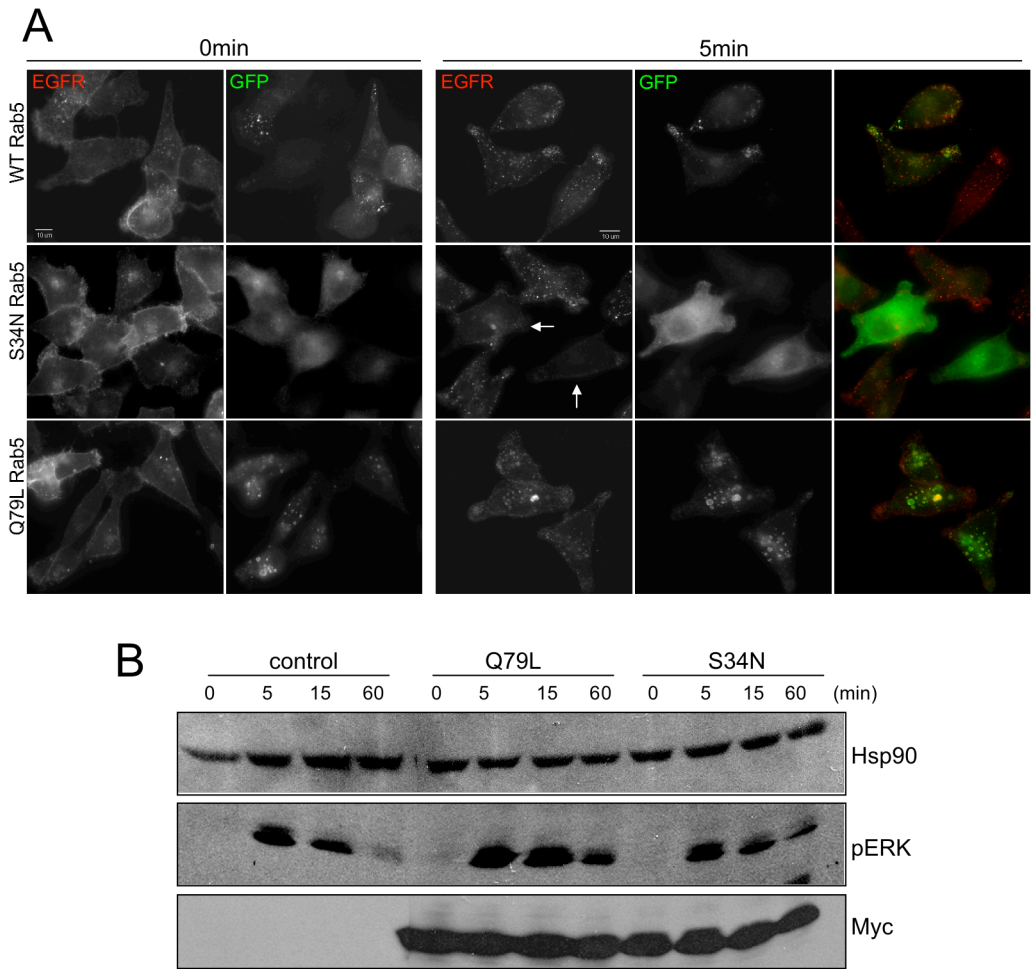


Figure 4-9. Rab5 controls initial endocytic trafficking and signaling of the EGFR. A. HeLa cells were transfected with GFP-Myc-Rab5-WT, GFP-Myc-Q79L and GFP-Myc-S34N. Following a 5 min EGF treatment, cells were fixed and stained with anti-EGFR antibodies. Arrows point to the cells expressing Rab5-S34N. Bar indicates 10 μm. **B.** Vector**,** Myc-Q79L, and Myc-S34N constructs were expressed in HeLa cells. The cells were incubated with EGF during the indicated time points. Phosphorylation of ERK was detected by anti-phospho-ERK antibodies.

4.6. Depletion of PI4KIIβ increases the activity of Rab5.

To extend the above results I checked if Rab5 activity is somehow regulated by PI4KIIβ. Rab5 activity in the cell is reflected by the ratio of GTP-Rab5 to GDP-Rab5. I utilized the method developed by Liu et al (Liu et al., 2007) to monitor Rab5 activation. The Rab5 binding domain (R5BD) in Rabaptin 5, a Rab5 GEF (Figure 4-10A) interacts specifically with GTP-bound Rab5. Consequently, R5BD conjugated to GST can be used to selectively pull down GTP-bound Rab5, which can then be quantified by immunoblotting. Wild type Rab5 and the two aforementioned mutants were expressed transiently; Rab5-Q79L is a constitutively GTP-bound form and inactive Rab5-S34N is a constitutively GDP-bound form. Without any stimulation, only Rab5-Q79L interacts strongly with GST-R5BD. However, after 5 min stimulation with EGF, Rab5-WT can also be pulled down with R5BD, indicating that EGF activates GTP loading into Rab5 (Figure 4-10B). To determine whether depletion of PI4KIIβ regulates the activity of Rab5, I performed this assay in RNAi-treated cells. In control cells, Rab5 activity increases to its maximal level 5min after EGF treatment, then decreases overtime. In PI4KIIβ-depleted cells, Rab5 activity is higher after 5 min EGF treatment than in control cells, and persists a bit longer, as shown in two separate experiments (Figure 4-10C). This differential activation of Rab5 correlates well with the effect on endocytosis that I observed upon depletion of PI4KIIβ.

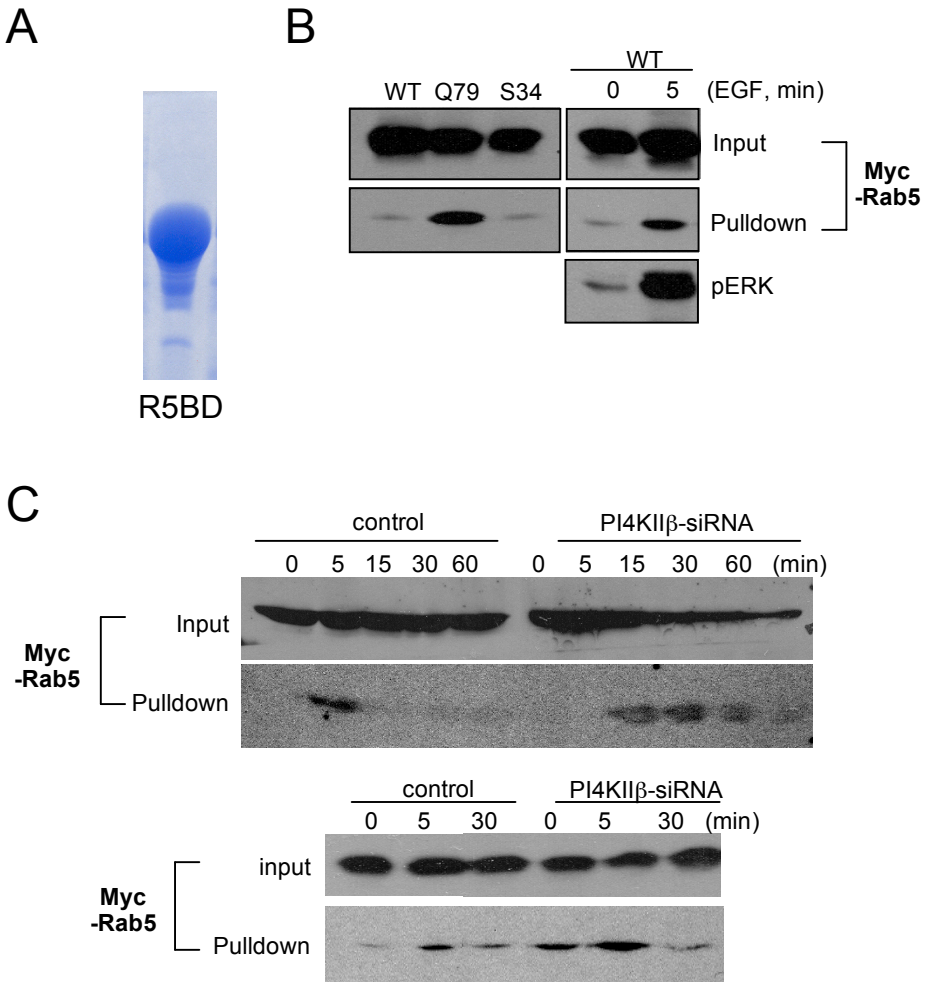


Figure 4-10. Effect of PI4KIIβ depletion on Rab5 activity. A. Rab5-GTP binding domain (R5BD) was cloned from a mouse cDNA library and expressed in *E.coli.* and purified by GST affinity chromatography as described in Experimental Procedures. **B**. Myc-Rab5-WT, Q79L, or S34N were expressed in HeLa cells and the cell lysates were incubated with GST-R5BD bound Sepharose beads to pull down GST-bound Rab5. Input cell lysates and pulldown Rab5 proteins were detected by anti-Myc antibodies (left panel). In the right panel, Myc-Rab5-WT expressing cells were starved for 4 h and then stimulated with EGF for 0 or 5min. Cell lysates were then incubated with GST-R5BD to pull down active Rab5. **C**. Control and siRNA treated cells were replated and stimulated at different time points and prepared in lysis buffer for Rab5 activity assay using GST-R5BD. Input and pulldown were immunoblotted with anti-Myc antibodies.

4.7. Colocalization of PI4KIIβ with other markers.

PI4KIIs are predominantly enriched in the junctanuclear region where Golgi-complex markers conventionally stain (Wang et al., 2003; Wei et al., 2002). In addition to the Golgi complex, PI4KIIα also localizes to endosomal compartments. Consistent with these localizations, PI4KIIα is reported to function in Golgi-based secretion, AP-3 –dependent transport, and late endosomal trafficking of the EGFR (Balla et al., 2002; Craige et al., 2008) (Guo et al., 2003; Minogue et al., 2006). Our group and Balla's showed that partly localizes to Golgi, plasma membrane, and EEA1-containing organelles (Balla et al., 2002; Wei et al., 2002). Here I further localized transiently expressed PI4KIIβ using several markers for endosomal compartments (Figure 4-11). At steady state, Myc-PI4KIIβ colocalizes with the transferrin receptor, with lesser amounts co-localizing with EEA1- and EGFR-containing vesicles. Interestingly, co-expressed GFP-Rab5 and Myc-PI4KIIβ show extensive colocalization on discrete punctae, both in the perinuclear region and on peripheral endosomal vesicles. Furthermore, Myc-PI4KIIβ collapses into enlarged endosomes that are induced by co-expression of Rab5-Q79L. Thus, PI4KIIβ is distributed on early endosomal compartments, as well as on the Golgi complex, further supporting the possibility that it has a role in early endocytosis.

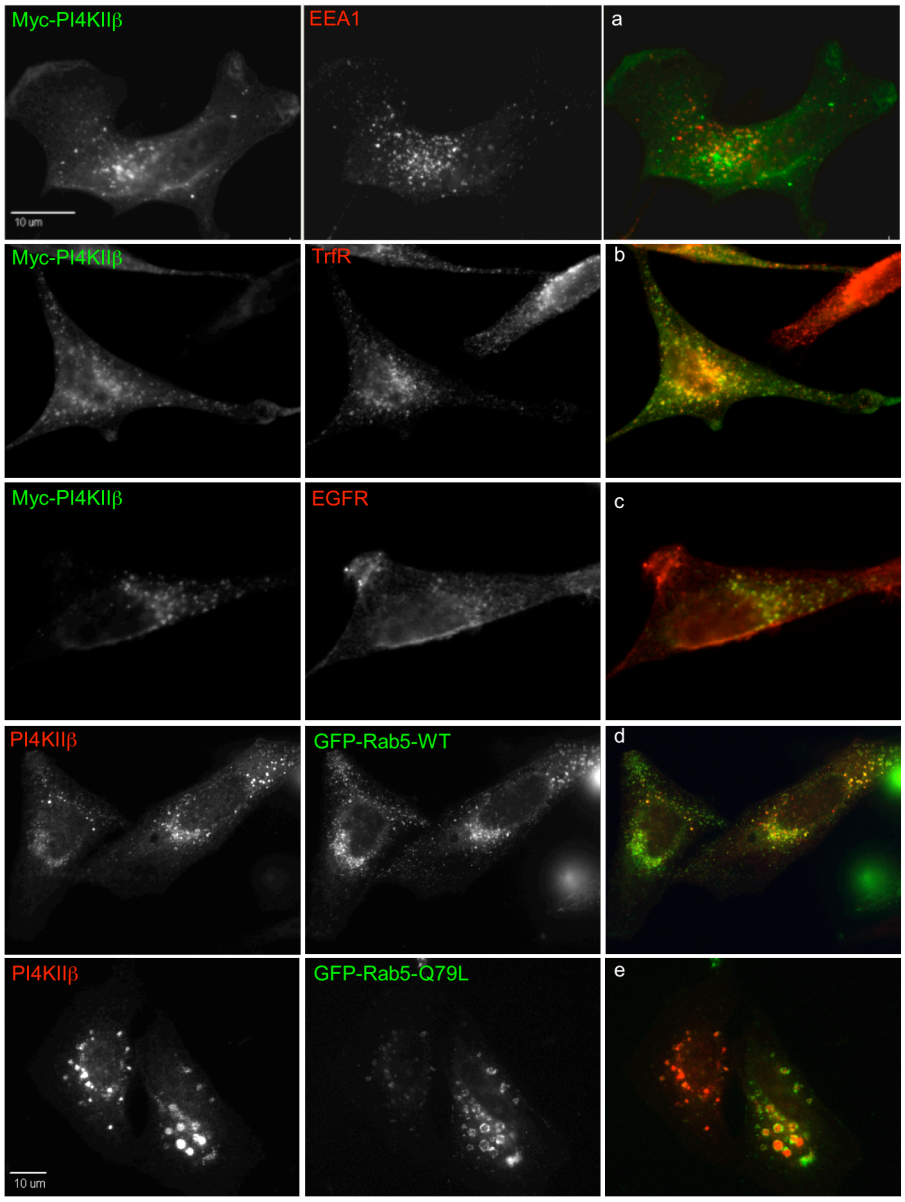


Figure 4-11. Localization of PI4KIIβ with trafficking markers. HeLa cells expressing Myc-PI4KIIβ were grown in media containing serum and fixed followed by staining with antibody against PI4KIIβ and EEA1, transferrin receptor (TrfR) or EGFR (a, b, c). Untagged PI4KIIβ and GFP-Myc-Rab5-WT or Q79L were co-transfected and visualized by anti-PI4KIIβ antibodies and GFP (d and e). Bars are 10μm

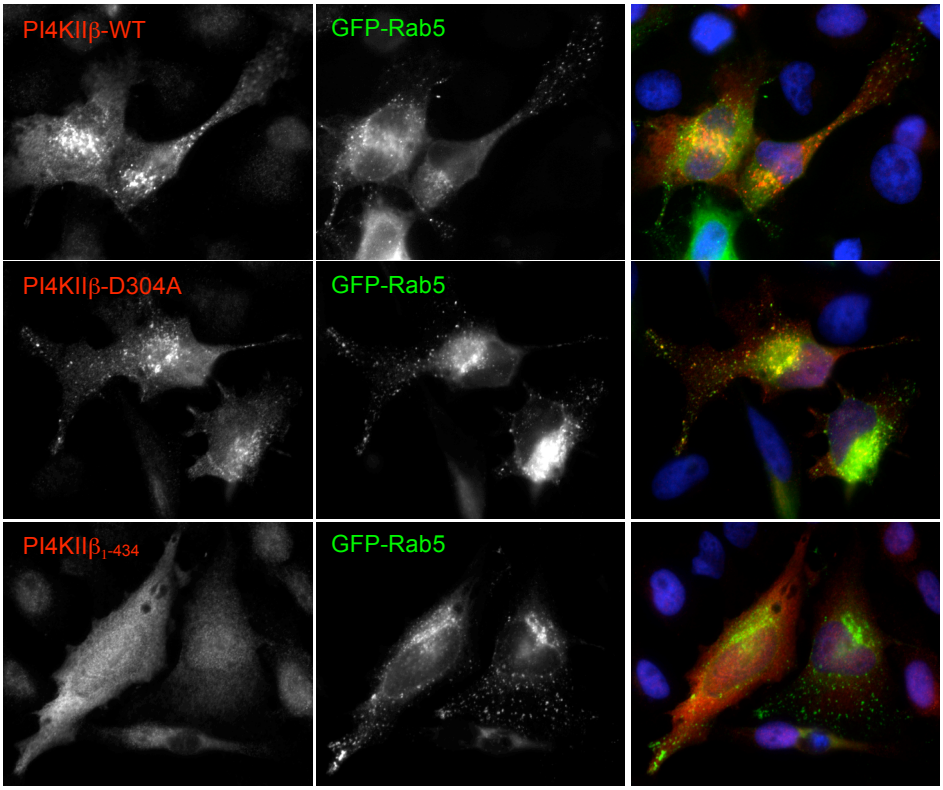


Figure 4-12. Localization of PI4KIIβ mutants with Rab5. Myc- PI4KIIβ (WT, D304A, and 1-434) and GFP-Myc-Rab5-WT were co-transfected and visualized by anti-PI4KIIβ antibodies and GFP. DAPI (blue) stains nucleus.

V. DISCUSSION

In this chapter, I explored the relevance of PI4KIIβ in receptor-mediated endocytosis using two different approaches to follow signaling and trafficking of the EGFR. Depletion of PI4KIIβ promotes the initial formation of endosomes beneath the plasma membrane, leading to accelerated endocytic trafficking of EGF/EGFR complex and enhanced ERK phosphorylation in an early stage of the endocytic pathway. The correlation between trafficking and signaling was confirmed by expressing the catalytically inactive dynamin mutant, K44A which inhibits internalization of the EGFR as well as ERK phosphorylation. The facilitated endocytosis observed in PI4KIIβ-depleted cells is due to excess of endosome fusion, which is caused by enhanced activations of Rab5 and subsequent EEA1. Therefore, my results point to an inhibitory role of PI4KIIβ in the endocytic trafficking and signaling of EGFR. The mechanism underlying this inhibition remains to be defined.

In contrast to the effects of PI4KIIβ depletion or the early endocytic steps, I did not observe an obvious difference in the rate of EGFR degradation between control and PI4KIIβ depleted cells. In HeLa cells, the EGFR is usually completely internalized within 5–10 min, and the half-life of the receptor is ~30 min (Vieira et al., 1996). Apparently small fluctuations in the initial rate of EGFR internalization may not affect the downstream stage of receptor degradation.

We demonstrated that depletion of PI4KIIβ causes the elevation of EEA1 and Rab5 activities in the very early steps of endocytosis, although the mechanism of regulation is still unknown. However, our preliminary data show that wild type and catalytically inactive (D304) PI4KIIβ both co-localize on Rab5-containing vesicles (Figure 4-12). Furthermore,

PI4KIIβ accumulates into the Rab5-Q79L induced larger endosomes, indicating that membrane associated PI4KIIβ co-localized with the active Rab5 containing vesicles and might function in modulation of Rab5 activity.

Several PI metabolizing enzymes have been shown to interact directly with Rab GTPases. Among these enzymes, PI 3-kinase is the best-known effector of Rab5 and generates PI3P on early endosomal membrane. This lipid is required for endosome fusion and for motility along microtubules (Christoforidis et al., 1999a; Christoforidis et al., 1999b). In addition, Type II inositol polyphosphate 5 phosphatase (INPP5), Type I INPP4, and OCRL1 proteins also were reported recently bind to several Rab GTPases (Fukuda et al., 2008; Hyvola et al., 2006; Shin et al., 2005). However, it still remains to be determined whether these interactions play roles in particular Rab regulating processes and how they may regulate membrane trafficking. There is also an example in which the lipid-modifying enzyme recruits the Rab to membranes, instead of the reverse. Graaf et al reported that Rab11 in its GTP-bound state functionally interacts with PI4KIIIβ to regulate vesicular transport from the Golgi complex to the plasma membrane. In this case, PI4KIIIβ acts as a docking factor for Rab11 on the Golgi complex (de Graaf et al., 2004; Giansanti et al., 2007). These links between Rabs and PI metabolizing enzymes provide new insights in the function of Rab5 and PI4KIIβ in membrane transport. For future experiments, it will be important to establish whether membrane recruitment of PI4KIIβ depends on Rab5 or vice versa using a series of mutants of PI4KIIβ or Rab5.

An interesting observation presented in this section is the specific effect of PI4KIIβ depletion on EGF (but not transferrin) uptake. Generally Rab5 is thought to regulate both

constitutive and RTK-stimulated internalization. At present it is unclear that PI4KIIβ depletion up-regulates Rab5 activity, but only results in stimulation of EGFR internalization.

In general, constitutive endocytosis (e.g. transferrin receptor) is dependent on short linear motifs (NPXY or YXXQ) of the receptors (Heilker et al., 1999). These motifs bind to the AP-2 complex and subsequently recruit into clathrin-coated pits. Disruption of this interaction abrogates TfR internalization (Nesterov et al., 1999). In contrast, mutational disruption of the interaction between EGFR and AP-2 minimally affects the rate of EGFR internalization (Nesterov et al., 1995), implying that it depends on additional endocytic machinery. Indeed, stimulated EGFRs enter the specialized coated pits with additional adaptors to link the receptor to clathrin, such as Eps15, Epsin and Hrs/Hgs via receptor phosphorylation or ubiquitylation (Di Fiore and De Camilli, 2001; Gaborik and Hunyady, 2004). In addition, active EGFR recruits specific downstream molecules such as Ras, PI3K, PLC, and Grb2 to transmit the external signals as well as to activate endocytic machinery (reviewed in (Anderson, 2006; Gaborik and Hunyady, 2004; Sorkin and Von Zastrow, 2002).

Rab5 activity can be stimulated by receptor activation via Ras-stimulated RIN1, a Rab5 GEF. Tall et al. demonstrated that binding of Ras-GTP to RIN1 promotes its GEF activity on Rab5. (Tall et al., 2001). On the other hand, Rab5 activity is negatively regulated by RN-tre, a Rab5 GAP, by active EGFRs (Lanzetti et al., 2000). Thus, signals emanating from active EGFR control both activation and deactivation of Rab5 and hence regulate receptor internalization (Teis and Huber, 2003). Perturbation of the function of RN-tre or RIN1 affect the internalization of the EGFR (a ligand-induced reaction), but not constitutive endocytosis of the transferrin receptor (Lanzetti et al., 2000; Tall et al., 2001). These data

support the idea that common components of the endocytic machinery such as Rab5, are controlled in a receptor-specific fashion.

A possible mechanism underlying the inhibition of early endosome fusion and endosomal signaling by PI4KIIβ is that PI4KIIβ shares a substrate, phosphatidylinositol, with PI 3-kinases on endosomes. In this mechanism, high PI4KIIβ activity depletes PI that would otherwise be used in the formation of PI3P, a lipid essential in early endocytic steps. PI4KIIβ depletion increases the amount of free PIs to be used by PI3K to increase PI3P production, resulting in more enhanced endocytosis of EGFR. Our preliminary data (not shown) indicate that overexpression of PI4KIIβ inhibits internalization of EGF/EGFR.

Beyond downregulation of the activated receptor, the delivery of the activated receptor to an endosomal compartment mediated by endocytosis is required for interaction with specific effectors that propagate downstream signaling events. RTKs can clearly signal from endosomes where signaling scaffolds nucleate around endocytosed/activated RTKs. For example p14, a protein that is localized to endosomal compartments, recruits the MP1 (MEK1 partner) MAPK scaffolding protein to endosomes and is important for robust EGF-dependent activation of ERK, but not of another MAP kinase, p38 (Anderson, 2006; Teis et al., 2002). Enhanced EGFR endocytosis by PI4KIIβ depletion is well correlated with the acceleration and prolongation of ERK phosphorylation indicating that PI4KIIβ negatively controls both receptor internalization and endosomal signaling.

CHAPTER 5

PI4KII in *Drosophila*

I. ABSTRACT

Unlike mammals, *Drosophila* has only a single gene for Type II PI 4-kinase (CG2929, termed dPI4KII). We previously cloned and expressed *Drosophila* PI 4-kinase (dPI4KII) and characterized its catalytic domain. Here we utilize an antibody directed against an N-terminal peptide of dPI4KII to localize the kinase in *Drosophila* S2 cells. Similar to mammalian PI4KIIs, dPI4KII colocalizes with Golgi and displays an unpolarized and scattered distribution characteristic of the Golgi apparatus in S2 cells. RNAi-mediated reduction of dPI4KII by 80% causes defects in S2 cell spreading and induces a stellate/serrate morphology on Concanavalin A treated coverslips. This morphology suggests that depletion of the kinase impairs organization of cortical actin filaments and that dPI4KII may regulate the pathway of actin assembly.

II. INTRODUCTION

Prior to the cloning and sequencing of rat PI4KIIα by our laboratory in 2001 (Barylko et al., 2001), no Type II PI 4-kinase had been identified in non-mammalian cells. However, we soon cloned sequenced, and expressed a *Drosophila* ortholog (dPI4KII) which contains a catalytic domain that is 54% identical in sequence to the catalytic domains of mammalian Type II kinases. There is no homology between the N-terminal noncatalytic regions of the mammalian and *Drosophila* enzymes (Barylko et al., 2002). In *Drosophila*, a single gene

(CG2929) produces three transcripts, with two major proteins of 710 and 563 amino acids being expressed. In *Drosophila,* dPI4KII is apparently enriched in the larval fat body and ovary (http://flyatlas.org). The 710 aa form of dPI4KII migrates on SDS gels as a protein of 97 kDa due to its highly acidic N-terminal domain (1-260aa). Like mammalian PI4KIIs, dPI4KII does not have an obvious transmembrane domain but has a cysteine rich motif ($_{333}$CCPCC$_{337}$) which presumably undergoes palmitoylation. Apparently 70% of endogenous dPI4KII behaves as an integral membrane protein in *Drosophila* S2 cell, and has similar biochemical properties to mammalian PI4KIIs(Barylko et al., 2002).

In addition to the single *dPI4KII* gene, *Drosophila* also expresses PI4KIIIα and PI4KIIIβ orthologs (Figure 5-1). Almost nothing is known about these kinases, but *Drosophila* PI4KIIIβ (also known as "four-wheel drive" or fwd) has a role in actin polymerization and in the formation of the intracellular bridge during meiosis (Brill et al., 2000). Moreover, fwd cooperates with Rab11 and Gio (phosphatidylinositol transfer protein; PITP) to control formation of new membranes during *Drosophila* cytokinesis (Giansanti et al., 2007).

One of my original goals was to determine at least some of the functions of PI4KIIs, particularly PI4KIIβ. To do this, I considered *Drosophila* as a valuable model system because : 1) *Drosophila* has only one isoform of PI4KII and lacks the genetic redundancy observed in mammals; and 2) RNAi techniques were well established in *Drosophila* S2 cells and the simple addition of dsRNA provided an efficient approach for loss of function phenotypic analyses.

My experiments involving depletion of dPI4KII from S2 cells focused on an examination of changes in cell morphology. Under routine culture conditions, S2 cells display a roughly spherical morphology with a diameter of ~10 μm. However, Rogers et al. discovered that S2 cells attach to a Concanavalin A (Con A) substratum, resulting in a flattened, discoid morphology (~50μm in diameter) within 30 min to 1 h. Spread cells have a radially symmetrical actin cytoskeleton (lammelae) and resemble a 'fried egg' with a central region containing the nucleus and the majority of organelles (Rogers et al., 2003). Lamella formation on Con A-treated coverslips can be monitored to identify components involved in regulation of cell morphology.

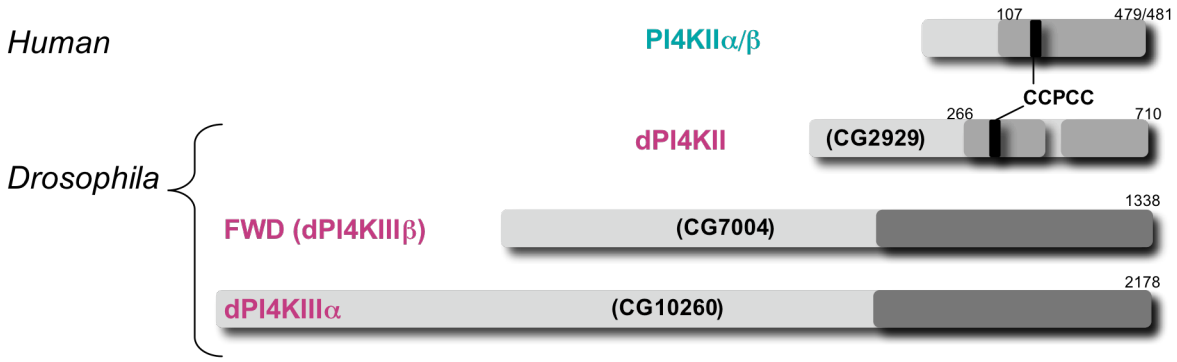


Figure 5-1. Schematic representation of *Drosophila* PI4-kinasess. *Drosophila* has a single gene for Type II PI4K and two isoforms of Type III PI4K, α and β. dPI4KII shares the catalytic domain with mammalian isoforms, α and β (65% similarity) and includes the CCPCC, palmitoylation motif. Locus tags were presented for each kinases and the size of kinases was shown as the number of amino acids.

III. ADDITIONAL EXPERIMENTAL PROCEDURES

Reagents - dPI4KII antibodies, provided by Dr. Rama Ranganathan, were generated against a peptide (MSGATDQTDPPLLQLEDEVD) representing residues 1-20 of the *Drosophila* PI4KII sequence. The antibodies were affinity purified on SulfoLink pre-packed columns (Pierce) with cross-linked peptide according to the manufacturer's directions. All media and reagents for RNAi were obtained from Invitrogen (Carlsbad, CA). ER-tracker, Mito-tracker, and $NBDC_6$-ceramide were from Molecular Probe (Carlsbad, CA). GFP-LAMP1-stable S2 cells and dmGolgi marker were kindly provided by Dr. Helmut Kramer (UT Southwestern Medical Center).

Cell Culture and Transfection - Schneider S2 cells were cultured in Schneider's *Drosophila* medium (GIBCO BRL) supplemented with 10% FCS (GIBCO BRL) at 27°C in a humidified incubator for all experiments. For transient transfection, Cellfectin® Reagent was used according to the manufacture's instructions (Invitrogen, Carlsbad, CA).

Knockdown of Protein Expression in S2 cells - RNAi in cultured *Drosophila* S2 cells was performed as described by Worby et al. (Worby et al., 2001). Cells were suspended in fresh medium at 1×10^6/ml and plated into 6-well plates (1 ml/well). Double-stranded RNA was added (15 μg/well) for 1 h. After that, medium (2 ml) was added to each plate, and cells were incubated for 7 days. RNAi-treated S2 cells were replated on Con A treated coverslips prepared by overnight incubation at R.T. Harvested cells were lysed in RIPA buffer for immunoblotting.

Target regions were amplified by PCR using a *Drosophila* cDNA library. PCR products were used to produce complementary double-stranded RNA using Ambion Megascript T7 kit

(Foster City, CA) according to the manufacturer's protocol. Synthesized double-stranded RNA was stored at -20 °C for use in all experiments. Primers were designed according to Barylko et al. for dPI4KII and Rogers et al. (Barylko et al., 2002; Rogers et al., 2003) for other constructs.

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
dPI4KII (760-1440)	TTAATACGACTCACTATAGGGGAT CACGTGGAGTTTGTATCG	TTAATACGACTCACTATAGGGGATCT TAGGGGCCACGTACCTG
CDC42 (530-1170)	CGAGATTACACACCATTGCC	ATAGAAACCGACCAAAATACCG
Profilin (546-997)	CTTCCGTGGTAGAGAAACTTGG	TTCTTAACTATTGATTGGGGCG
Myosin IA (2255-2857)	GATTTCCGTATCACCCACTACG	CACAATATGTGGGATCATTTCG

Immunofluorescence Microscopy - Cells attached to Con-A coverslips were incubated with ER-tracker (1.5 μM) or Mito-tracker (1 μM) for 1 h and washed 3 times with PBS. After fixation with 3.7% formaldehyde and permeabilization with 0.2% Triton X-100, cells were incubated with dPI4KII antibodies followed by fluorescent secondary antibodies. For $NBDC_6$-ceramide staining to visualize the Golgi complex, cells were incubated with $NBDC_6$-ceramide (5μM) for 30 min on ice prior to fixation. Cell morphology was visualized by FITC-phalloidin (Molecular Probes, Carlsbad, CA). Fluorescence microscopy was performed as described in Chapter 2.

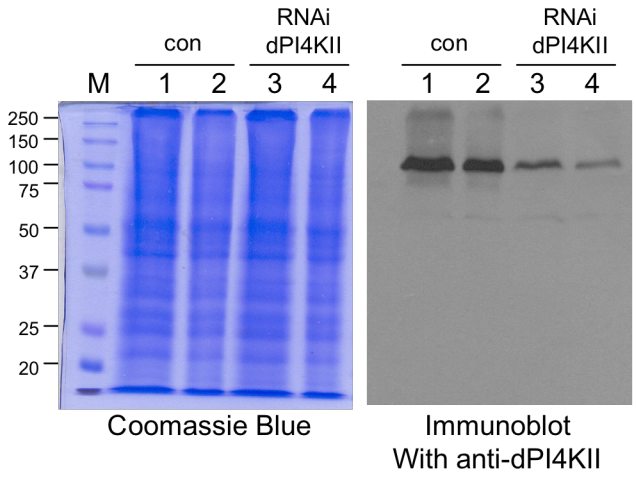
IV. RESULTS

5.1. Localization of PI4KII in S2 cells

To localize dPI4KII in S2 cells we utilized an anti-peptide antibody that recognizes a single band, migrating with an apparent mw of ~100 kDa, in immunoblots of S2 homogenates

(Figure 5-2A). We had previously reported that expressed dPI4KII exhibits an usually slow electrophoretic mobility, running as a 100 kDa protein despite its calculated MW of 80 kDa. This anomalous migration is apparently due to its highly negatively charged N-terminal domain (residues 1- 266, pI=4.34). Using this antibody, we localized dPI4KII in S2 cells grown on ConA-treated coverslips. The shape of these cells was visualized by FITC-phalloidin staining, which reveals thick actin filament bundles in the peripheral regions. dPI4KII distributes on punctae around the nucleus but is absent from the actin-rich cell periphery (Figure 5-2B). To identify the punctae we co-stained with markers for the endoplasmic reticulum (ER-Tracker), mitochondria (Mito-Tracker), lysosomes (GFP-LAMP1), and the Golgi apparatus ($NBDC_6$-ceramide). In addition, GFP-VSVG was transiently expressed to identify secretory vesicles. At high magnification, anti-dPI4KII antibodies surround ceramide-conatining vesicles but show no overlap with ER-Tracker or Mito-Tracker (Figure 5-2C). It should be noted that the Golgi apparatus of S2 cells is scattered among perinuclear vesicles and does not display the polarized stacks evident in the Golgi apparatus of mammalian cells. dPI4KII also colocalizes with GFP-VSVG, and very weakly with GFP-LAMP1. Thus, the *Drosophila* kinase is similar to mammalian PI4KIIα in distributing to the Golgi apparatus and Golgi-derived vesicles. This result was corroborated using S2R+ cells, which are larger, flat, and strongly adherent to plates even in the absence of the ConA substrate. In these cells, antibodies to a Drosophila Golgi protein strongly co-localize with those against dPI4KII (Figure 5-2D).

A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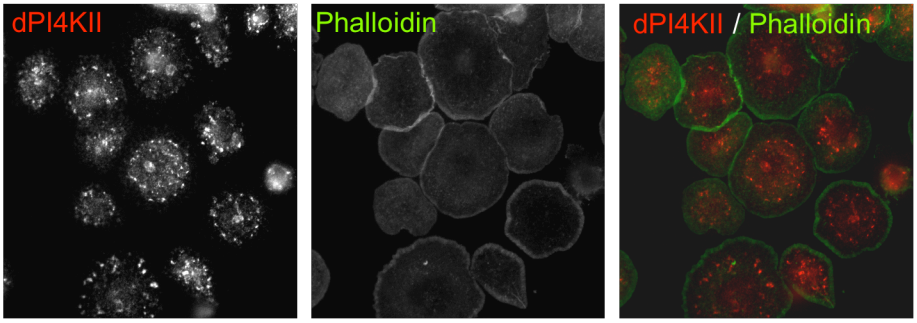
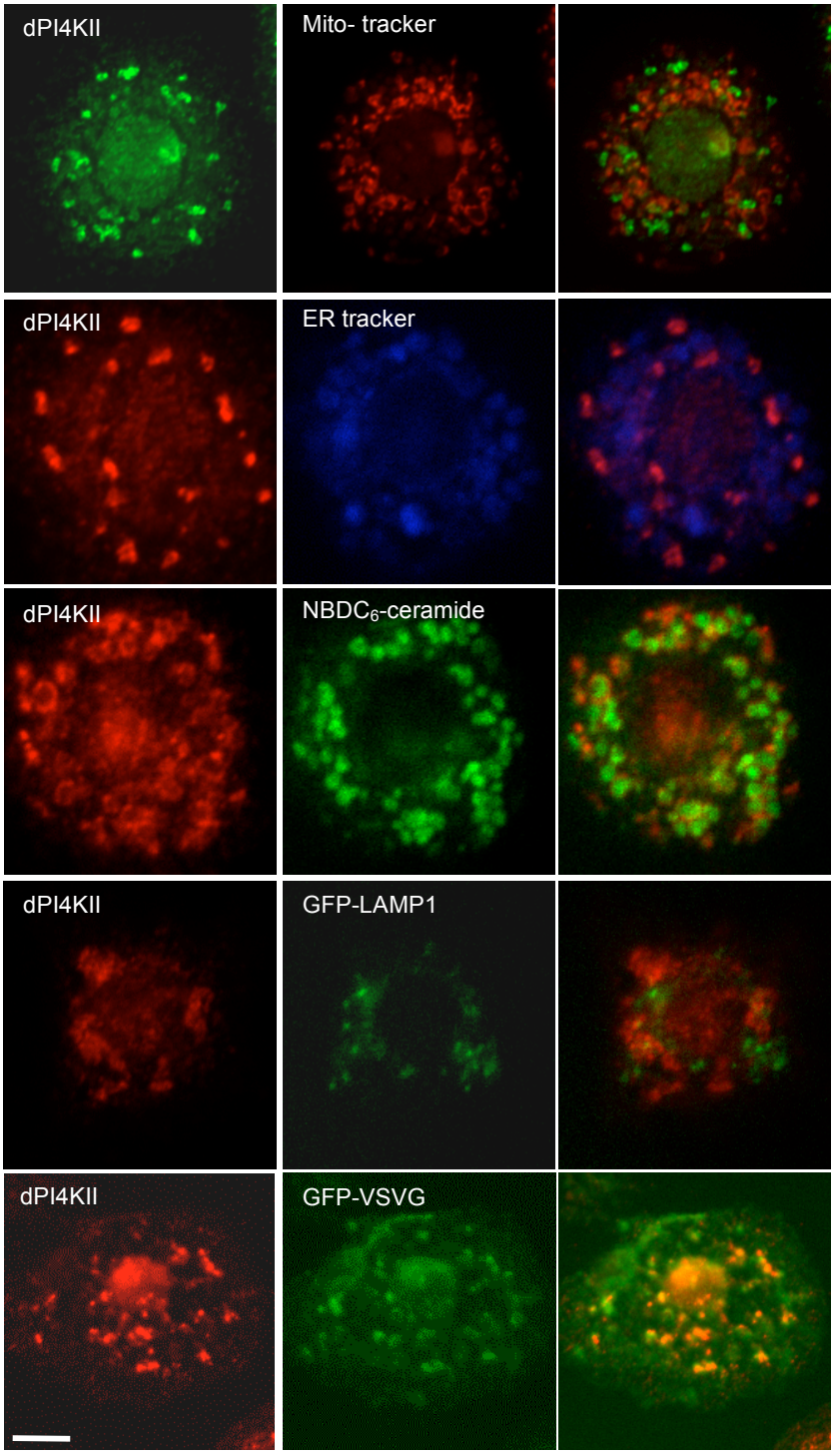
Immunoblot
With anti-dPI4KII

B


dPI4KII
Phalloidin
dPI4KII / Phalloidin

C


dPI4KII
Mito- tracker
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ER tracker
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NBDC6-ceramide
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GFP-LAMP1
dPI4KII
GFP-VSVG

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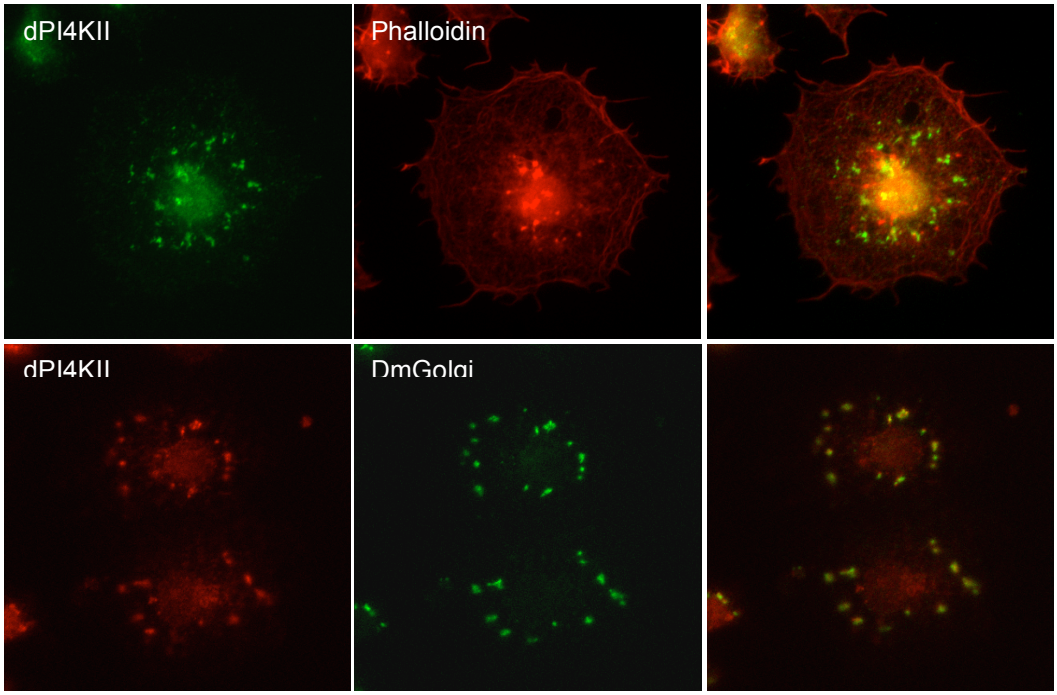
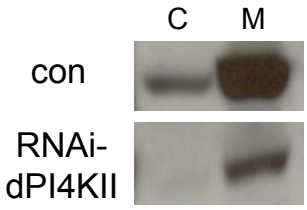


Figure 5-2. Localization of dPI4KII. A. Antibody against dPI4KII was generated and confirmed with whole cell lysates from control (lanes 1 and 2)and dPI4KII dsRNA (lanes 3 and 4) treated cells. Lanes 1 and 3 (40µl samples), Lanes 2 and (20µl samples). **B.** dPI4KII localization in S2 cells using anti-dPI4KII antibodies. FITC-phalloidin stained actin cytoskeleton showing round cell periphery. **C.** Colocalization of dPI4KII with markers. S2 cells were plated on Con A and endogenous dPI4KII was visualized by Rhodamine-conjugated secondary antibody (Red), except FITC for Mito-tracker costaining (Green). GFP-LAMP1 expressing stable S2 cell line was used as to detect lysosomes. Staining of Mito-tracker, ER-tracker and $NBDC_6$-ceramide show mitochondria, ER and Golgi complex, respectively. GFP-VSVG was transfected in S2 cells and used for tracking the secretory pathway. **D.** DmGolgi protein was colocalized with dPI4KII in S2R cells, which are modified S2 cells which grow on plates withouth Con A. The cell shape was shown with Phalloidin staining (Red).

5.2. Knockdown of dPI4KII in S2 cells.

Consistent with our previous observations that recombinant dPI4KII is a membrane-bound protein when expressed in mammalian cells (Barylko et al., 2002), the endogenous kinase distributes almost exclusively to S2 cell membranes (Figure 5-3A). Using dsRNA, I was able to deplete expression of PI4KII by more than 80%. I confirmed this selective depletion of dPI4KII using enzymatic assays, taking advantage of the observation that only the Type III PI 4-kinases are inhibited by wortmannin (Downing et al., 1996). Cytosolic and membrane fractions were prepared from control or dsRNA-treated S2 cells and kinase assays were performed in the presence or absence of 2.5μM wortmannin. As shown in Figure 3B, approximately 90% of the total PI 4-kinase activity is associated with membranes, and about 60% of this activity is lost upon dsRNA treatment. Of the membrane-associated kinase activity, approximately 80% is wortmannin-insensitive and, hence, can be ascribed to dPI4KII. dsRNA treatment reduces this dPI4KII-dependent activity by approximately 75%. Similar reductions of activity were observed in the cytosols of dsRNA-treated S2 cells (Figure 5-3B). Depletion of dPI4KII is also evident by light microscopys which show the disappearance of dot-like structures. Furthermore, knockdown of dPI4KII alters the morphology of S2 cell grown on Con A coverslips (Figure 5-3C).

A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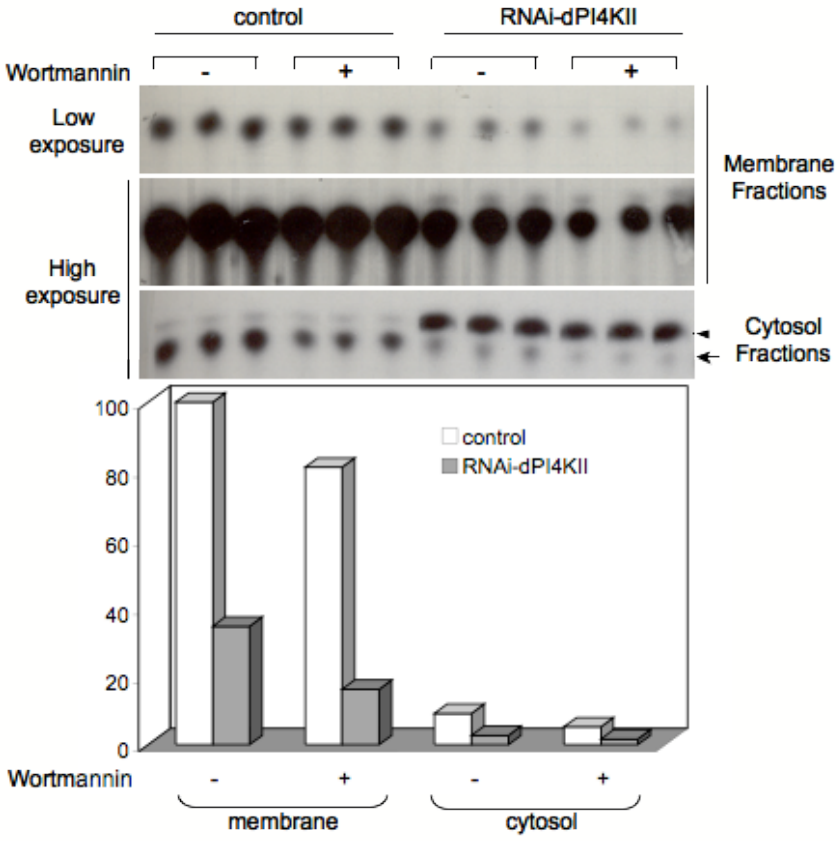
control
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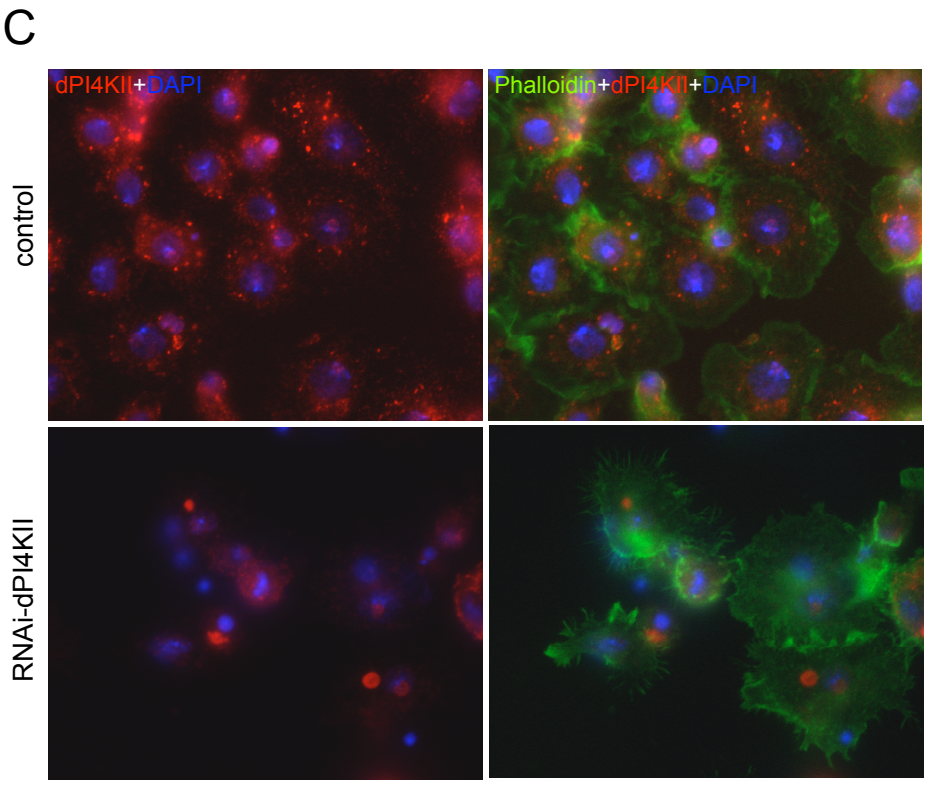

Figure 5.3 Effects of depletion of dPI4KII on S2 cells. Control and dPI4KII depleted cells were fractionated into cytosol and membranes. **A.** Immunoblot showing depletion of dPI4KII in both fractions. **B.** The fractions were assayed for kinase activity as described in Experimental Procedures. The production of PI4P was analyzed by thin layer chromatography. Top panel shows low exposure of kinase activity from membrane fractions. Botton two panels show the same level of exposure of cytosol and membrane. Arrow marks the level of PI4P and arrow head points out the unidentified upper band. The radioactivities from assay were quantified in the graph. **C.** Depletion of dPI4KII shown in Con A plated S2 cells using anti-dPI4KII antibodies. FITC-phalloidin stained actin and DAPI staining shows the nucleus.

5.3. Depletion of PI4KII impairs spreading of S2 cells on Con A coated coverslips.

In a large screen, Rogers et al categorized RNAi-induced morphological defects into several different subdivisions. One defect, resulting from knockdown of profiling, prevents cells from spreading on ConA but retaining their spherical shapes instead. In contrast, Cdc42-depleted cells display thin processes (Rogers et al., 2003). As positive controls for my experiments I reproduced the results obtained by depletion of profiling and Cdc42, and used MyoIA depletion as a negative control, because this knockdown was found not to alter cell morphology.

The target dsRNAs were amplified by PCR in ~650bp sizes and incubated in S2 cells for 7 days to deplete targeted proteins. The same set of S2 cells were replated on Con A treated coverslips for 1 h and the cells were visualized using FITC-phalloidin (Figure 5-4A). Similar to the previous report, depletion of profilin results in round and unspread cells of about ~10μm diameter (unspread, >90%) and the lack of Cdc42 induces mainly an unspread morphology (~60%) with thin processes and serrate phenotypes in S2 cells (~10%). In contrast, MyoIA depletion doesn't have much affect on cell spreading. Interestingly, S2 cells incubated with dsRNA for dPI4KII are defective in spreading and induce the stellate/serrate morphology (unspread, 31 % and stellate/serrate, 44 %). The graph shows the percentage of cells in unspread, stellate/serrate, and spread forms. Note that when S2 cells are grown on Con A, even a small percentage of control cells exist as unspread and stellate/serrate forms (12% and 5%, Figure 5-4B).

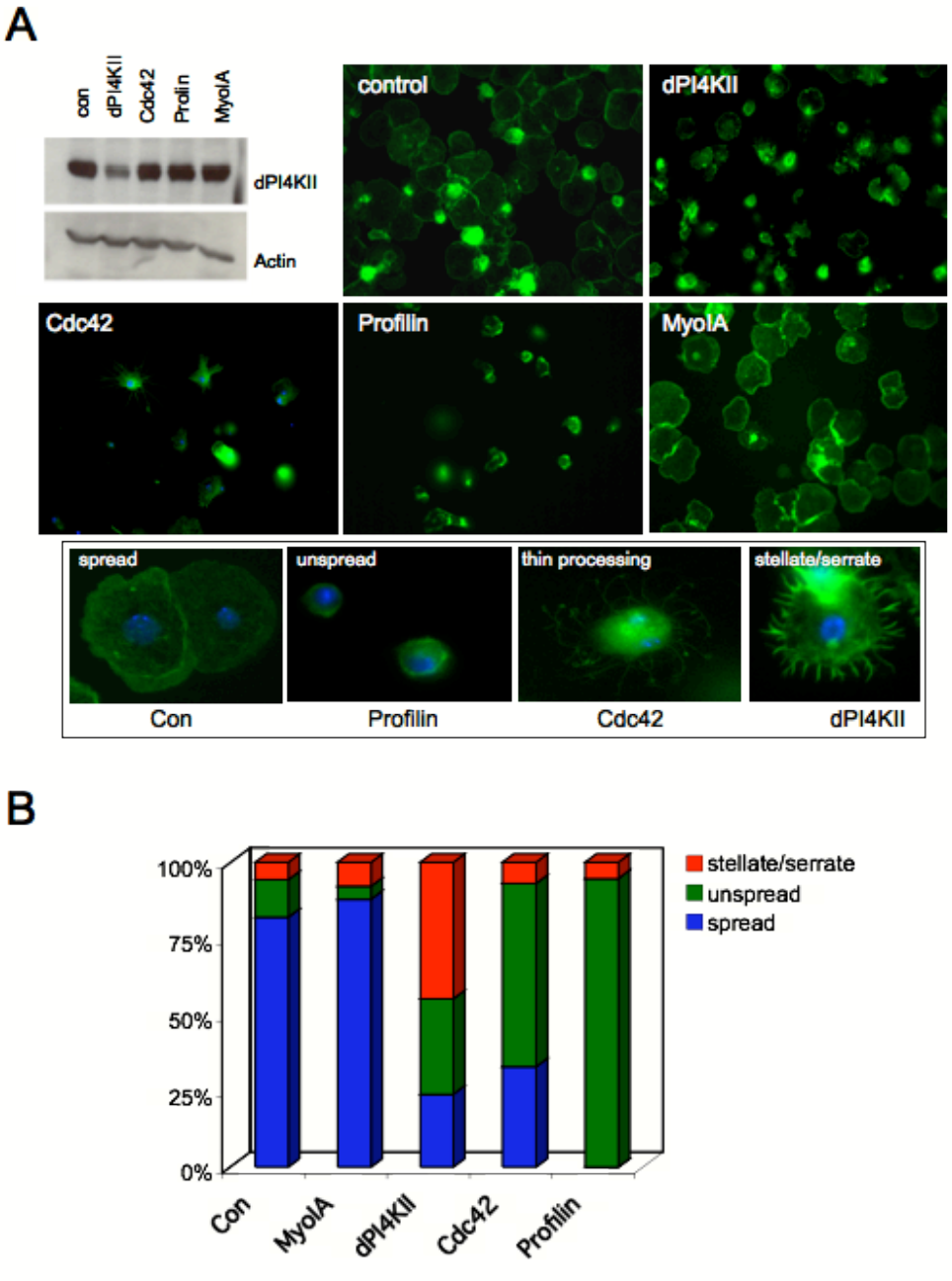

Figure 5.4 Effect of RNAi-mediated dPI4KII depletion on S2 cell morphology. A. Control and dPI4KII-depleted S2 cells were plated on Con A and stained with FITC-phalloidin to visualize actin. Other cells were treated with dsRNAs to reduce expression of Cdc42 and Profilin, two proteins known to be required for normal cell spreading. By contrast knockdown of the unconventional myosin, MyoIA, does not influence cell morphology. The enlarged cells at the bottom show examples of various spreading phenotypes from RNAi. **B.** The graph shows the quantification of morphological defects caused by inhibition of Profilin,, Cdc42, MyoIA, and dPI4KII. The box shows total number of cells in each phenotypes.

V. DISCUSSION AND FUTURE WORK

At present, very little is known about PI4P synthesis and function in *Drosophila*. Genome project databases indicate that *Drosophila* contain close homologs of both mammalian isoforms of Type III PI 4-kinase α and β. but only the PI4KIIIβ homolog (termed fwd for four-wheel drive) has been extensively characterized. There is evidence that fwd functions in in cytokinesis (Brill et al., 2000). A single ortholog of PI4KII from *Drosophila* was partially studied and its kinetic properties were almost identical to those of the mammalian enzymes (Barylko et al., 2002).

In this Chapter I showed the distribution of dPI4KII in S2 cells and studied the effects of its depletion on S2 cell morphology. Similarly to the distribution of mammalian PI4KIIs, dPI4KII is enriched in Golgi membranes and in some secretory vesicles. Depletion of dPI4KII reduces the production of PI4P (>60% of total) *in vitro* and impairs cell spreading on Con A, inducing a spiky, "stellate" morphology. I hypothesize that reduction of PI4P production causes a defect in actin polymerization by reducing $PI4,5P_2$, an important cytoskeletal regulator. The morphological defect observed upon depletion of dPI4KII is similar to the phenotype which occurs upon depletion of Arp2/3, Nck, SCAR, kette, Abi and Sra-1 and is categorized as a stellate morphology according to Roger's classification (Rogers et al., 2003). Interestingly all 6 proteins are in the same pathway leading to nucleation of cortical actin filaments. SCAR, (WAVE ortholog) activates Arp2/3, is itself activated by Nck, and, in non-adherent cells, is protected from proteolysis by forming a complex with Sra-1, Abi, and Kette. The connection could be studied whether PI4P or $PI4,5P_2$ directly regulates actin polymerization.

CHAPTER 6

Conclusions and Future Directions

Phosphoinositides (PIs) are important regulators of signaling pathways and also play critical role in membrane trafficking. PI4Ks are the kinases that initiate the biosynthetic pathway resulting in the generation of polyphosphoinositides. Among the PI4Ks, the recently cloned PI4KIIs are being actively studied. There is now strong evidence that PI4KIIα is important in Golgi and endosomal trafficking. My project focused on the regulation and function of the other PI4KII isoform, PI4KIIβ.

Interestingly, cells contain a large pool of cytosolic, inactive PI4KIIβ. Only membrane associated PI4KIIβ is constitutively active and undergoes palmitoylation and phosphorylation. In Chapter 2, I examined how post-translational modifications regulate the targeting and activity of PI4KIIβ. Palmitoylation is critical for expression of catalytic activity and 30% of PI4KIIβ incorporates [^{3}H]palmitate. I also identified the major phosphorylation site in membrane-associated PI4KIIβ by mass spectrometry (in collaboration with Dr. Hongjun Shu) and mutational analysis as serine 77. However, mutation of the phosphorylation site had no effects on catalytic activity, palmitoylation state, or subcellular distribution of the kinase. I next identified the regions in PI4KIIβ that determine its association with cellular membranes and which target it to distinct locations on the membrane. The N-terminal segment of PI4KIIβ is required for delivering the kinase to the cell-cell adhesion in response to RacV12, whereas the more highly conserved C-terminal 160

residues contribute to its the greater solubility and lower level of palmitate incorporation than PI4KIIα.

It remains possible that the unique N-terminal region, which contains the major phosphorylation site, cooperates with the C-terminal membrane targeting region to localize the kinase to specialized domain of membrane, perhaps by mediating specific protein-protein interaction within membranes.

In Chapter 3, I demonstrated the significance of Hsp90 binding to PI4KIIβ. I first identified Hsp90 as an authentic binding protein of PI4KIIβ. GA, the Hsp90 specific inhibitor, disrupts the Hsp90-PI4KIIβ interaction and renders PI4KIIβ highly susceptible to ubiquitylation and proteasomal degradation. Utilizing a series of hybrid and truncated mutants of PI4KIIs, I found that selective effects of GA on PI4KIIβ are due to its cytosolic distribution. Because a cytosolic fragment of PI4KIIα also binds to Hsp90, it is likely that cytosolic PI4KIIβ binds to Hsp90 via N-lobe of catalytic domain which is conserved in PI4KIIα and is stabilized by Hsp90. Short-term incubation (15min) in GA disrupts the association between Hsp90 and the cytosolic form of PI4KIIβ and redistributes the kinase transiently to membranes, leading to increase of PI4P production. Stimuli such as EGF and PDGF are also able to disrupt the interaction and this disruption may be essential for growth factor-dependent activation of PI4KIIβ. PI4KIIα does not require Hsp90 for stabilization. I speculate that this independence is due to the tight interaction of this isoform with membrane.

In Chapters 4 and Chapter 5, I began to explore some functional aspects of PI4KIIs, in particular PI4KIIβ. At an early stage of my project, I tried to utilize *Drosophila* S2 cells,

taking advantage of the observation that they express only one isoform of Type II PI 4-kinase. dPI4KII, the *Drosophila* PI4KII ortholog, localizes to the Golgi apparatus, as do the mammalian PI4KIIs. Depletion of dPI4KII by RNAi reduces by 60% the total PI 4-kinase activity of S2 cells. Knockdown of dPI4KII causes reduced spreading and induces stellate/serrate morphology on Con-A treated coverslips. This morphology also results from knockdown of cytoskeletal regulators such as Cdc42 and SCAR, and suggests that dPI4KII depletion causes defective regulation of actin polymerization. However, RNAi of PI4KIIα in mammalian cells has not been reported to cause defects in actin polymerization. Moreover, RNAi of PI4KIIβ also fails to alter cell morphology (my unpublished observations).

Based on previous results indicating that PI4KIIβ redistributes to the plasma membrane in response to growth factor receptor stimulation, I explored the potential effects of depletion and expression of PI4KIIβ on receptor-mediated endocytosis. Interestingly, depletion of PI4KIIβ not only promotes the initial formation of endosomes beneath the plasma membrane but also enhances ERK phosphorylation at an early stage of the endocytic pathway. Expression of a catalytically dead dynamin mutant, K44A which blocks internalization of EGFR, reduces the upregulated ERK phosphorylation by knockdown of PI4KIIβ to the level of control, indicating that depletion of PI4KIIβ enhances the internalization of active receptors followed by the downstream signaling. The facilitated endosome formation by PI4KIIβ depletion is likely due to enhancement of endosome fusion events. Indeed activities of EEA1 and Rab5 increase in PI4KIIβ-depleted cells. These results suggest for the first time that PI4KIIβ functions in endocytic trafficking and signaling of EGFR, although precise mechanisms remains unknown.

An important remaining question is how PI4KIIβ regulates Rab5 activity. Localization results show that PI4KIIβ colocalizes with Rab5-containing vesicles. Moreover, PI4KIIβ largely accumulates in Rab5-Q79L induced giant endosomes. In an attempt to identify a putative motif in PI4KIIβ that is responsible for its localization to Rab5-containing endosomes, I examined the sequence of PI4KIIβ and found a segment ($_{25}$EREPLL$_{30}$), similar to the dileucine motif ([DE]XXXL[LI]), which is found in several transmembrane proteins, such as the T cell receptor, LIMP-II, Ii and NPC1. These [DE]XXXL[LI] signals have been known to mediate rapid receptor internalization and to target proteins to the specialized endosome-lysosome compartment (Bonifacino and Traub, 2003). In future experiments, It will be interesting to analyze the effects of mutations in this $_{25}$EREPLL$_{30}$ motif on the location of PI4KIIβ and on its effects on endocytosis and signaling.

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