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Kateřina Apolínová

The role of PIP5K family kinases in plasma membrane remodeling

Úloha kináz PIP5K rodiny v remodelaci plazmatické membrány

Bachelor's thesis

Supervisor: Mgr. Marie Macůrková, Ph.D.

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Poděkování:

Především bych chtěla poděkovat vedoucí své práce, paní doktorce Marii Macůrkové, nejen za její odbornou pomoc, ale i za její trpělivost a laskavost.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

Abstrakt:

Fosfatidylinositol 4-fosfát 5-kináza (PIP5K) je enzym, který je zodpovědný za tvorbu fosfatidylinositol 4,5-bisfosfátu (PI(4,5)P2). Je již dlouho známo, že PI(4,5)P2 slouží jako prekurzor dvou velmi důležitých druhých poslů, diacylglycerolu a inositol trisfosfátu. PI(4,5)P2 ovšem také sám funguje jako druhý posel a reguluje mnoho procesů probíhajících na plasmatické membráně, jako například endo- a exocytózu, remodelaci aktinového cytoskeletu a tvorbu mezibuněčných spojů. Aktivita PIP5K musí být v buňce pečlivě časově i prostorově regulována, aby mohlo dojít k lokální tvorbě membránových mikrodomén bohatých na PI(4,5)P2. Tyto mikrodomény jsou zásadní pro regulaci mnoha různých buněčných dějů, kterých se PIP5K účastní. Tato bakalářská práce se zaměřuje na popis regulačních mechanismů, které ovlivňují aktivitu PIP5K *in vivo*, a na fyziologické funkce PIP5K na plasmatické membráně.

Klíčová slova:

Fosfatidylinositol 4-fosfát 5-kináza; fosfatidylinositol 4,5-bisfosfát; fosfoinositidy; plasmatická membrána; druhý posel; aktinový cytoskelet

Abstract:

Phosphatidylinositol 4-phosphate 5-kinase (PIP5K) is the enzyme responsible for the production of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), which has long been known as a precursor of two important second messengers, diacylglycerol and inositol trisphosphate. However, PI(4,5)P2 also acts as a second messenger in its own right and regulates many processes occurring on the plasma membrane such as endo- and exocytosis, actin cytoskeleton remodeling, and the formation of cell-cell contacts. The action of PIP5K is carefully spatially and temporally regulated in order to form localized pools of PI(4,5)P2 crucial for its many roles in a wide variety of cell processes. This bachelor's thesis focuses on the description of regulatory mechanisms that control PIP5K activity *in vivo* and on its physiological functions at the plasma membrane.

Key words:

Phosphatidylinositol 4-phosphate 5-kinase; phosphatidylinositol 4,5-bisphosphate; phosphoinositides; plasma membrane; second messenger; actin cytoskeleton

List of Abbreviations:

ANTH domain	AP180 N-terminal homology domain
AP1	Adaptor protein 1
AP2	Adaptor protein 2
Arf	ADP-ribosylation factor
Arp2/3 complex	Actin related protein 2/3 complex
ATP	Adenosine triphosphate
c-Src	Cellular Src
cAMP	Cyclic adenosine monophosphate
CAPS	Ca^{2+}-dependent activator protein for secretion
DAG	Diacylglycerol
DCV	Dense core vesicle
E-cadherin	Epithelial cadherin
ENTH domain	Epsin N-terminal homology domain
ERM proteins	Ezrin-radixin-moesin proteins
FAK	Focal adhesion-associated kinase
FERM domain	Four-point-one, ezrin, radixin, moesin domain
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GIRK channel	G-protein coupled inward-rectifier K+ channel
GTP	Guanosine triphosphate
IP3	Inositol trisphosphate
LPA	Lysophosphatic acid
N-WASP	Neural Wiskott-Aldrich syndrome protein
NCX1	Cardiac Na^{+}-Ca^{2+} exchanger
PA	Phosphatidic acid
PH domain	Pleckstrin-homology domain
PI	Phosphatidylinositol
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PI(4)P	Phosphatidylinositol 4-phosphate
PI(5)P	Phosphatidylinositol 5-phosphate
PI4K	Phosphatidylinositol 4-kinase
PIP4K	Phosphatidylinositol 5-phosphate 4-kinase
PIP5K	Phosphatidylinositol 4-phosphate 5-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PP1	Protein phosphatase-1
ROCK	Rho-associated protein kinase
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SV	Synaptic vesicle
TRP channel	Transient receptor potential channel
WASP	Wiskott-Aldrich syndrome protein

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1 INTRODUCTION

Phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks, type I PIPKs) are enzymes that play an important role in the modulation of membrane lipid composition, namely in the regulation of lipids from the phosphoinositide family. These membrane lipids, referred to as phosphoinositides, are derivatives of phosphatidylinositol (PI). PI consists of two non-polar fatty acids on a glycerol backbone, which is phosphate-bound to inositol, a cyclic polyalcohol (Figure 1). Derivatives of PI are formed by variable phosphorylation of the inositol ring in positions 3, 4, and 5, which gives rise to one phosphatidylinositol trisphosphate (PI(3,4,5)P3), three PI bisphosphates (PI(3,5)P2, PI(3,4)P2, PI(4,5)P2), and three PI monophosphates (PI(3)P, PI(4)P, PI(5)P) (Di Paolo and De Camilli 2006*). The production of these molecules is spatially and temporally regulated in the phosphatidylinositol cycle, which is a metabolic pathway in which each phosphoinositide species can be created from another species through the action of various phosphatases and kinases (Figure 2) (Vicinanza et al. 2008).

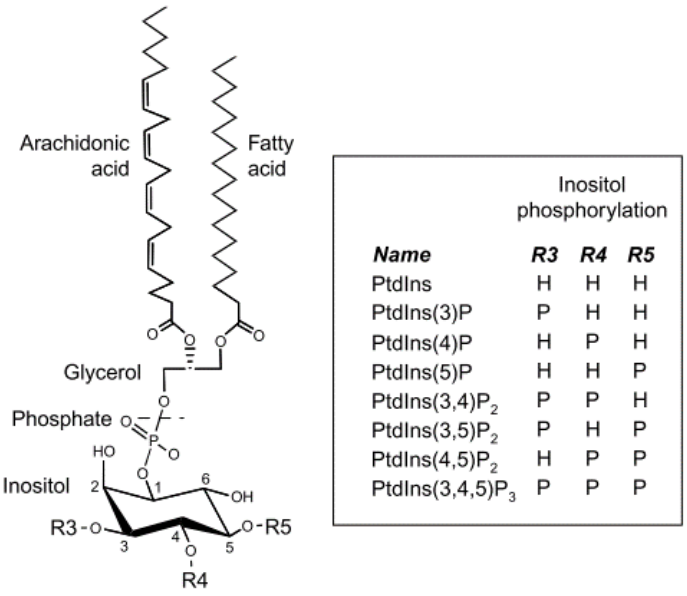

Figure 1. The structure of phosphoinositides and the phosphorylated positions of the inositol ring in each phosphatidylinositol derivative. Taken from Suh and Hille 2005*, Figure 1 a, edited.*

PIP5K catalyzes the production of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), an important signaling lipid molecule, from phosphatidylinositol 4-phosphate (PI(4)P) through phosphorylation in the 5-position of the inositol head. Another class of enzymes, phosphatidylinositol 5-phosphate 4-kinases (PIP4Ks, type II PIPKs), are also involved in the production of PI(4,5)P2 through the phosphorylation in the 4-position of PI(5)P. PIP5K is thought to be the major producer of PI(4,5)P2 in the cell, as cellular levels of PI(4)P are about 10 times higher than those of PI(5)P (van den Bout and Divecha 2009*). However, recent findings suggests that majority of PI(4)P in the plasma membrane, which was initially thought to serve only as a precursor for PI(4,5)P2 synthesis, possesses a distinct signaling function and does not contribute to the generation of PI(4,5)P2 (Hammond et al. 2012). Instead, most PI(4,5)P2 used for rapid signaling is probably created by a sequential action of

phosphatidylinositol 4-kinase (PI4K) and PIP5K on membrane PI (Qin et al. 2009). The substrate specificity of type I and II PIPKs is given by a varying key amino acid in the activation loop, the major determinant of subcellular localization and substrate specificity in PIPKs (Liu et al. 2016). This allows for a differential localization of PIPK subfamilies and isoforms and a specific spatial and temporal regulation of distinct PI(4,5)P2 pools on various membranous structures in the cell.

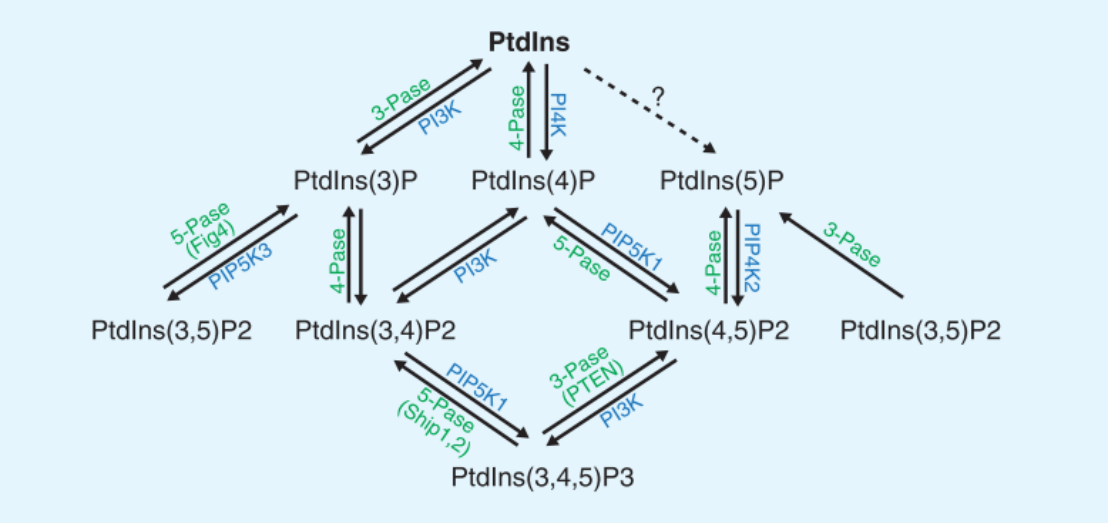

Figure 2. The seven phosphoinositide species are interconvertible and their levels are carefully regulated by kinases (blue) and phosphatases (green). Taken from Vicinanza et al. 2008*, Figure 1 a.*

The PIP5K enzyme family comprises of three isoforms in mammals, named PIP5Kα, PIP5Kβ, and PIP5Kγ, which are coded for by PIP5K1A, PIP5K1B, and PIP5K1C genes in H. sapiens, respectively ("Gene: PIP5K1A (ENSG00000143398) - Summary - Homo Sapiens - Ensembl Genome Browser 92" 2018; "Gene: PIP5K1B (ENSG00000107242) - Summary - Homo Sapiens - Ensembl Genome Browser 92" 2018; "Gene: PIP5K1C (ENSG00000186111) - Summary - Homo Sapiens - Ensembl Genome Browser 92" 2018). The isoform nomenclature is somewhat confusing, since it is not consistent throughout different mammalian species. Most experiments have been executed in mice, but unfortunately the murine isoform α corresponds to human isoform β, and vice versa. In this thesis, the human nomenclature will be strictly used. In most tissues, all three isoforms are expressed, but their levels vary significantly; the PIP5Kα isoform is strongly expressed in skeletal muscles, PIP5Kβ in the heart, and PIP5Kγ shows a strong abundance in nerve tissue, especially the brain (Kwiatkowska 2010*; Volpicelli-Daley et al. 2010).

The three isoforms serve distinct functions in the cell by creating distinct PI(4,5)P2 pools. Even higher variability is created through the process of alternative splicing. So far, 17 α, 8 β, and 8 γ human splice variants have been identified; in mice, there are currently 5 α, 7 β, and 10 γ known variants ("Gene: Pip5k1a (ENSMUSG00000028126) - Summary - Mus Musculus - Ensembl Genome Browser 92" 2018; "Gene: Pip5k1b (ENSMUSG00000024867) - Summary - Mus Musculus - Ensembl Genome Browser 92" 2018; "Gene: Pip5k1c (ENSMUSG00000034902) - Summary - Mus Musculus - Ensembl Genome Browser 92" 2018).

The isoforms and splice variants are differentially localized in the cell and their subcellular localization also determines their variability in function. While the PIP5Kα isoform localizes predominantly to the plasma membrane and the Golgi complex (van den Bout and Divecha 2009*), and interestingly also to nuclear speckles (Mellman et al. 2008), areas in the nucleus that are enriched in splicing factors, the PIP5Kβ isoform has been found in the plasma membrane and perinuclear vesicles (van den Bout and Divecha 2009*). The localization of the γ isoform may be the most interesting, as it depends on the splice variant. The full-length protein, referred to as PIP5Kγ661 or PIP5Kγ90 (based on the amino acid length or molecular mass, respectively) is targeted to focal adhesions and adherens junctions through its C-terminus (Ling et al. 2002, 2007; Di Paolo et al. 2002; Legate et al. 2011). However, the shorter splice variant PIP5Kγ635 (PIP5Kγ87), which lacks the C-terminal 26 amino acids, fails to localize to focal adhesion sites and remains free in the cytoplasm and only associates with the plasma membrane upon cell-cell contact (Akiyama et al. 2005).

PI(4,5)P2, the product of PIP5K, is an incredibly versatile signaling lipid. It localized mainly to the inner leaflet of the plasma membrane and is predicted to be selectively enriched in cholesterol-rich membrane rafts (Johnson, Chichili, and Rodgers 2008). It has long been known that the digestion of PI(4,5)P2 by phospholipase C (PLC) generates important second messengers diacylglycerol (DAG) and inositol trisphosphate (IP3), which are involved in protein kinase C (PKC) and Ca^{2+} release from cellular deposits, respectively (Nishizuka 1984; Berridge and Irvine 1989). However, it can act as a messenger on its own through the interaction with a number of proteins involved in various crucial cell processes including clathrin-mediated endocytosis, phagocytosis, vesicular trafficking, cell-cell adhesion, and cell motility through actin cytoskeleton remodeling and the formation of focal contacts (van den Bout and Divecha 2009*; Kanaho, Kobayashi-Nakano, and Yokozeki 2007*). The recruitment of proteins to specific subcellular destinations is mediated by the association between phosphoinositides and PI-binding motifs present in these proteins. The modules known to bind PI(4,5)P2 include the pleckstrin-homology (PH) domain, polybasic domains, and four-point-one, ezrin, radixin, moesin (FERM) domain (Lemmon 2008*). This targeting of proteins to specific subcellular locations enables them to interact with other molecules involved in signal transduction pathways.

The activity of PIPK5s is carefully spatially and temporally regulated; the distinct subcellular localization enables them to create strictly regulated pools of PI(4,5)P2, which can then be used for targeting various cell components to the desired destination (Sun et al. 2013*; Hammond 2016*). All phosphoinositide-metabolizing enzymes are spatially restricted to specific locations, which enables the selective enrichment of specific phosphoinositides in particular membranous structures. This in turn helps define dynamically regulated organelle identity, as the various phosphoinositides can be rapidly converted to another PI species (Di Paolo and De Camilli 2006*). There are many mechanisms of PIP5K regulation, which include direct or indirect interactions with monomeric G-proteins from the Ras, Rho, and Arf family, stimulation by phospholipase D (PLD) product phosphatidic acid (PA), and regulation by phosphoinositide substrate or target proteins such as talin, ajuba, or AP2 (van den Bout and Divecha 2009*; Kwiatkowska 2010*; Sun et al. 2013*).

2 REGULATION OF LOCALIZATION AND ACTIVITY

2.1 REGULATION BY PHOSPHATIDIC ACID

All PIP5K isoforms have been found to be activated by phosphatidic acid (PA) (Jenkins, Fisette, and Anderson 1994). PA is a phospholipid consisting of a glycerol backbone carrying two fatty acids and a phosphate, which is created by the cleavage of phosphatidylcholine by phospholipase D (PLD). PA acts as a precursor of many other lipids such as DAG and has also been discovered to play a role as a second messenger (Testerink and Munnik 2005*). PA is the only phospholipid that has a profound effect on PIP5K activity (Jenkins, Fisette, and Anderson 1994). Apart from enhancing PIP5K enzyme activity, PA also regulates its affinity to the substrate, phosphatidylinositol 4-phosphate (PI(4)P) (Jarquin-Pardo et al. 2007).

The PA producing enzyme, PLD, has been shown to colocalize with PIP5K (Divecha et al. 2000). The targeted continuous activation of PIP5K by PA is essential in many processes in which PIP5K is involved, and in turn, the presence of PI(4,5)P2 is required for the activation of PLD, which contains a phosphoinositide-binding PH domain (Hodgkin et al. 2000). For example, the production of PA by PLD is essential for PIP5K activity in membrane ruffle formation, which is further confirmed by the finding that both PIP5K and PLD are stimulated by the small monomeric GTPase Arf6 (O'Luanaigh et al. 2002). This common activator may trigger the mutual positive feedback loop between PLD and PIP5K by their respective products to specifically enhance their activity in membrane ruffles.

Interestingly, PA stimulates both the PI(4,5)P2-generating enzyme PIP5K and the PI(4,5)P2-digesting enzyme PLC (G. Jones and Carpenter 1993), which possibly couples the production of PI(4,5)P2 to its cleavage by PLC and the generation of second messengers IP3 and DAG (Jenkins, Fisette, and Anderson 1994),

2.2 REGULATION BY MONOMERIC GTP-BINDING PROTEINS

Monomeric GTP-binding proteins are small enzymes capable of hydrolysis of GTP to GDP, thus acting as GTPases. These so-called small GTPases are involved in the regulation of many cellular processes dependent on signal transduction such as ligand-dependent cell division, actin cytoskeleton remodeling, and vesicle trafficking (Bourne, Sanders, and McCormick 1991*). These proteins can act as binary molecular switches, as they exist in an inactive (GDP-bound) and an active (GTP-bound) state, latter of which is capable of activating many downstream effectors.

The activation of small GTP-binding proteins is carefully spatially and temporally regulated by the action of GTPase regulators, which can be divided into three groups: GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and guanine nucleotide dissociation inhibitors (GDIs). GAPs promote the transition of GTPases from the active, GTP-bound state to the inactive, GDP-bound state by stimulating the rate of GTP hydrolysis, which is normally extremely slow. On the other hand, GEFs activate the small GTPases by facilitating the dissociation of GDP, as GDP is tightly bound to the GTPase and does not dissociate spontaneously. Finally, some GTPases are only functionally relevant

while localized to the plasma membrane, and they can be effectively inhibited by GDIs, which enforce such conformational changes to retain GTPases in the cytoplasm, preventing them from reaching the site of action (Cherfils and Zeghouf 2013*).

Small GTPases can be divided into five subfamilies: Ras, Rho, Arf, Rab, and Ran. Members of two of these subfamilies (Rho and Arf) play a crucial role in PIP5K regulation and thus will be further discussed in detail.

2.2.1 Regulation by members of the Rho subfamily

The Rho subfamily of small GTPases features several key regulators of the actin cytoskeleton (Etienne-Manneville and Hall 2002*). This suggests a potential link between PI(4,5)P2 and Rho GTPases, as PI(4,5)P2 also regulates actin cytoskeleton dynamics (Sechi and Wehland 2000*). Accordingly, three small GTPases belonging to the Rho subfamily have been identified as PIP5K regulators in various cellular processes involving actin cytoskeleton remodeling and membrane trafficking processes: RhoA, Cdc42, and Rac1.

RhoA has been found to play a significant role in PIP5K regulation during the formation of actin stress fibers and focal adhesion complexes (Ridley and Hall 1992). RhoA positively regulates and directly binds to all PIP5K isoforms independently on its nucleotide-binding state; however, the direct RhoA-PIP5K interaction is presumed not to enhance the kinase function of PIP5K, but to somehow modulate its recruitment to specific cellular compartments instead (Weernink et al. 2004). The suggested mechanism of RhoA regulation of PIP5K is through RhoA downstream effector, the Rho-associated protein kinase (ROCK), which mediates Rho signaling in processes involving actin rearrangement (Amano 1997). ROCK is a serine-threonine kinase related to protein kinase A (PKA) and protein kinase C (PKC). Regulation of PIP5K activity through the action of PKA and PKC has been described in detail and will be discussed below, but the specific molecular processes mediating the activation of PIP5K by ROCK still remains to be fully elucidated.

Rac1 also directly associates with PIP5K independently of the GDP/GTP-binding state and stimulates PI(4,5)2 synthesis, and has been proposed to work independently of the Rho/ROCK pathway (Weernink et al. 2004). Rac1 has been identified as a regulator in processes involving growth factor-induced actin polymerization such as membrane ruffling or the formation of actin stress fibers (Ridley et al. 1992), which are processes where PIP5K is also involved due to its ability to promote actin branching through the activation of Neural Wiskott-Aldrich syndrome protein (N-WASP) (Mao et al. 2009). Wiskott-Aldrich syndrome protein (WASP), which is only expressed by hematopoietic cells, and N-WASP, which is more ubiquitously expressed, promote actin polymerization by stimulating the actin related protein 2/3 (Arp2/3) complex, which serves as a nucleation site for new actin filaments (Mullins, Heuser, and Pollard 1998). Rac1 exclusively stimulates N-WASP and works in tandem with PIP5K and its product PI(4,5)P2 to regulate actin polymerization through the N-WASP/Arp2/3 pathway (Tomasevic et al. 2007).

Another member of the Rho subfamily, Cdc42, is also involved in actin polymerization through a direct interaction with WASP, and to a lesser extent, N-WASP (Rohatgi et al. 1999; Tomasevic et al. 2007) in processes that require precisely controlled actin branching, for

example during regulation of lamellipodial motility (Golub and Caroni 2005). Cdc42 is also a PIP5K activator, but unlike RhoA or Rac1, it does not directly bind to PIP5K, and research suggests that Cdc42 acts independently of the RhoA/ROCK pathway (Weernink et al. 2004). However, the exact mechanism of PIP5K activation by Cdc42 remains to be elucidated.

2.2.2 Regulation by members of the Arf subfamily

Another small GTPase family, ADP-ribosylation factors (Arf), has also been observed to activate PIP5K. Members of this family are divided into three classes: class I consists of Arf1, 2, and 3; class II of Arf4 and 5; and the sole member of class III is Arf6. GTPases belonging to classes I and II localize primarily to intracellular membranous structures such as the Golgi complex and endoplasmic reticulum, while the class III localizes to the plasma membrane (Funakoshi, Hasegawa, and Kanaho 2011). Arfs generally associate with membranes through myristoylation, and are involved in vesicular trafficking and endocytosis (Haun et al. 1993; Funakoshi, Hasegawa, and Kanaho 2011). Members of all classes, namely Arf1, Arf5, and Arf6, were observed to activate PIP5Kβ in vitro in the presence of PA (Honda et al. 1999).

Arf1, which localizes to the Golgi complex and regulates its structure and function, was observed to stimulate both the PI(4)P-forming enzyme PI4K and PIP5K by recruiting them to Golgi membranes (Godi et al. 1999; Jones et al. 2000). As PI4K synthesizes PIP5K substrate, PI(4)P, and Arf1 recruits both of these enzymes, PI(4,5)P2 is most probably created by the sequential activity of these enzymes on phosphatidylinositol. The localized production of PI(4,5)P2 in the Golgi complex is presumed to play a role in actin cytoskeleton rearrangement during vesicular transport (Vicinanza et al. 2008).

The only member of Arf class III, Arf6, localizes to the plasma membrane and endosomes and regulates traffic between these two compartments together with actin cytoskeleton remodeling, endocytosis, and exocytosis (Aikawa and Martin 2003; Funakoshi, Hasegawa, and Kanaho 2011*). PIP5K is suggested as the Arf6 downstream effector that exhibits these functions, and more precise mechanisms of Arf6-induced PIP5K activation have been shown in membrane ruffling (Honda et al. 1999), axonal elongation and arborization (Hernández-Deviez et al. 2004), synaptic vesicle endocytosis (Nakano-Kobayashi et al. 2007), and Ca^{2+}-dependent exocytosis on synapses (Aikawa and Martin 2003). Arf6 enhances PIP5K function in concert with PA created by PLD, which is also activated by Arf6 (O'Luanaigh et al. 2002). In unstimulated cells, Arf6 has been shown to localize diffusely through cytoplasm and to the plasma membrane, and upon stimulation it concentrates at sites of actin cytoskeleton remodeling together with PIP5Kβ (Honda et al. 1999). PIP5Kβ also acts downstream of Arf6 in the neuronal growth cone leading edge, where it modulates growth cone cytoskeleton during axonal elongation and branching in a process regulated by Arf6 (Hernández-Deviez et al. 2004). The constitutive activation of Arf6 led to an accumulation of intracellular membranous structures in the endosomal compartment that was probably caused by a defect in recycling endosomes trafficking; PIP5K was translocated by Arf6 to these vesicles from the plasma membrane and persistently activated, and the resulting absence of the plasma membrane PI(4,5)P2 pool lead to an inhibition of Ca^{2+}-mediated exocytosis in these cells

(Aikawa and Martin 2003). These observations suggest that Arf6 modulates PIP5K during processes involving vesicular transport.

2.3 REGULATION BY PIP5K PHOSPHORYLATION AND DEPHOSPHORYLATION

An important way of regulating the kinase activity of PIP5K *in vivo* is its phosphorylation by kinases and dephosphorylation by phosphatases in specific sites. This either changes the ability of the enzyme to bind its partners, or changes its conformation in such a way that its activity is altered (van den Bout and Divecha 2009*).

2.3.1 Regulation by autophosphorylation

PIP5K is not only a lipid kinase; it also possesses the activity of a protein kinase, and all isoforms have the ability to autophosphorylate themselves. The lipid kinase inactive mutants of PIP5K also lose the protein kinase autophosphorylating activity, which suggests that the lipid and protein kinase activities are executed by the same mechanism (Itoh et al. 2000). Contrary to the lipid kinase activity, which is stimulated by the presence of phosphatidic acid, the protein kinase activity is strongly and specifically stimulated by PI, and all isoforms exhibit a PI-dependent protein serine-threonine kinase activity (Itoh et al. 2000). The resulting effect of autophosphorylation is a significant decrease in PIP5K lipid kinase activity *in vitro*; however, the exact importance of this auto-inhibiting feature in the context of living cells is currently not known (Itoh et al. 2000; Mao and Yin 2007*). It is presumed that PIP5K is constitutively phosphorylated in resting cells at Ser^{257} in PIP5Kα, which corresponds to Ser^{214} in PIP5Kβ and Ser^{264} in PIP5Kγ, and is dephosphorylated upon stimulation by a PKC- and Ca^{2+}-dependent pathway described below (Park, Itoh, and Takenawa 2001; Mao and Yin 2007*).

2.3.2 Regulation by PKA and PP1

The cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) is able to phosphorylate all PIP5K isoforms both *in vitro* and *in vivo* on the same Ser residue in the catalytic domain which is also acted upon by PIP5K autophosphorylating activity (Park, Itoh, and Takenawa 2001). Again, the phosphorylation at this residue keeps PIP5K in an inactive, resting state, probably because it is unable to bind its activators in this conformation, and is thought to regulate the function of PIP5K in resting cells (Park, Itoh, and Takenawa 2001; Aikawa and Martin 2003). Upon the addition of extracellular stimuli, e.g. lysophosphatic acid (LPA), PIP5K activity is enhanced through its dephosphorylation by protein phosphatase-1 (PP1) at this Ser residue (Park, Itoh, and Takenawa 2001).

2.3.3 Regulation by PKC and Ca^{2+}

The serine-threonine Ca^{2+}-dependent protein kinase C (PKC) is activated by second messengers generated by PI(4,5)P2 hydrolysis by PLC. DAG, one of the products of this reaction, is involved in the recruitment of PKC to the plasma membrane. The second product of PLC-induced PI(4,5)P2 hydrolysis, IP3, provides the elevation of cytoplasmic Ca^{2+} levels through the binding to its receptor on the surface of endoplasmic or sarcoplasmic reticulum and the subsequent opening of the ligand-gated Ca^{2+} channel, which releases the stored Ca^{2+} in the cytoplasm (Berridge and Irvine 1989*).

The influx of Ca^{2+} into the cytoplasm was reported to stimulate PIP5K dephosphorylation (Wenk et al. 2001) probably through the resulting PKC activation through the elevation of cytoplasmic Ca^{2+} levels. The activation of PKC by elevated cytoplasmic Ca^{2+} levels and DAG contributes to PIP5K activation by regulating PP1 activity (Aikawa and Martin 2003*). PKC has been found to be able to indirectly activate PP1 through the phosphorylation of PP1-specific inhibitor proteins (Eto 2009*). These findings suggest that PIP5K is regulated by the Ca^{2+}- and DAG-activated kinase PKC not by a direct interaction, but through the phosphorylation and resulting inactivation of PP1 inhibitors. PP1 can then activate PIP5K by dephosphorylation at the residue Ser^{257} in PIP5Kα, Ser^{214} in PIP5Kβ, and Ser^{264} in PIP5Kγ. Therefore, PIP5K is proposed to be a Ca^{2+}-dependent effector which is activated through a series of events relying on the influx of Ca^{2+} in the cytoplasm, and its activation and generation of PI(4,5)P2 at the plasma membrane plays an important role in promoting Ca^{2+}-triggered endocytosis and exocytosis (Park, Itoh, and Takenawa 2001).

2.3.4 Regulation by Src and FAK

The tyrosine kinase family Src is a major group of signal transducers in the cell that regulate many vital cellular processes such as cell migration and proliferation, and contains potent proto-oncogenes such as cellular Src (c-Src) (Ma et al. 2000*). c-Src has been proposed to function as an activator specific for the longer PIP5Kγ splice variant, PIP5Kγ661, in focal adhesions; this process is mediated through another tyrosine kinase, the focal adhesion-associated kinase (FAK) (Ling et al. 2003).

Originally, FAK was thought to directly phosphorylate PIP5Kγ661 and thus facilitate the association with its partners necessary for the formation of stable focal adhesion sites, e.g. talin (Di Paolo et al. 2002; Ling et al. 2002). The regulation of PIP5Kγ661 binding of its partners and activators does indeed depend on its phosphorylation state, namely at residues Tyr^{649} and Ser^{650} (murine residues Tyr^{644} and Ser^{645}) in the γ661 splice variant-specific C-terminal region (Sang et al. 2005). However, the kinase responsible for the phosphorylation at Tyr^{649} is not FAK, but c-Src; FAK does not directly activate PIP5Kγ661, but it stimulates PIP5Kγ661 phosphorylation through an interaction with c-Src (Ling et al. 2003). The proposed mechanism of FAK- and c-Src-dependent activation of PIP5Kγ661 in focal adhesions is as follows: the phosphorylation of Tyr^{649} by c-Src is crucial for the activation of PIP5Kγ661 not directly by modulating its activity, but rather indirectly by sterically inhibiting phosphorylation at the residue Ser^{650}, which leads to an increase in activity (Sang et al. 2005). Thus, the phosphorylation of PIP5Kγ661 by FAK-activated c-Src consequently activates PIP5Kγ661 by enabling it to bind its activators in focal adhesions, e.g. talin.

3 PIP5K ACTIVITIES AT THE PLASMA MEMBRANE

3.1 REGULATION OF INTRINSIC PLASMA MEMBRANE PROTEINS

The product of PIP5K, PI(4,5)P2, has been observed to regulate many diverse structures in the eukaryotic cell, including a growing number of intrinsic plasma membrane proteins such as ion channels, transporters, and receptors (Huang 2007*). The first of the plethora of ion transport proteins that was observed to be regulated by phosphoinositides, and specifically by PI(4,5)P2, is the erythrocyte and rat brain plasma membrane Ca^{2+} ATPase pump (Choquette et al. 1984). Many other membrane proteins regulated by PI(4,5)P2 in a direct or indirect fashion have been discovered since then, including Ca^{2+}, K^+ and Cl^- ion channels, G protein–coupled receptors, and transporters such as the Na^+/Ca^{2+} exchanger (Suh and Hille 2005*).

3.1.1 Regulation through an interaction with PI(4,5)P2

The first discovered mechanism of ion channel regulation by PI(4,5)P2 is through the direct binding of PI(4,5)P2, whose head groups interact laterally with the channels and transporters in the inner leaflet of the plasma membrane (Huang, Feng, and Hilgemann 1998). These interactions partially activate or inactivate the channels by stabilizing them in a certain conformational state and thus changing their opening probability (Chu and Stefani 1991; Huang 2007*).

In inward-rectifier K^+ (Kir) channels, such an interaction is made possible by clusters of positively charged amino acids near the C termini of the membrane proteins, which are able to cooperatively bind the phosphate groups of PI(4,5)P2 (Huang, Feng, and Hilgemann 1998). The interaction between G-protein coupled inward-rectifier K^+ (GIRK) channels and PI(4,5)P2 is modulated by $G_{\beta\gamma}$ subunits of G-coupled receptors, which also directly bind to GIRK channels and activate them. $G_{\beta\gamma}$ were observed to stabilize the interaction between GIRK and PI(4,5)P2, and this interaction results in an enhancement of channel opening probability through the stabilization of its open conformation (Huang, Feng, and Hilgemann 1998).

Another type of K^+ channels, the voltage-gated K^+ channel, is also PI(4,5)P2-sensitive (Zhang et al. 2003). Crucial members of this K^+ channel family are the neuronal M channels, which are responsible for the generation of M current, a slowly developing K^+ current whose modulation has dramatic effects on the action potential threshold (Marrion 1997*). M current is suppressed by the activation of a large number of neuronal metabotropic receptors, and a marked delay has been observed between the activation of such receptors and the suppression of M current, which led to the conclusion that a diffusible second messenger is responsible for the coupling of receptor activation and M current suppression (Marrion 1997*). The second messenger has been identified as PI(4,5)P2, whose synthesis is required for M channel function (Zhang et al. 2003). Suppression of M current is caused by the receptor-induced hydrolysis of PI(4,5)P2 by PLC to form IP3 and DAG, and the C-terminal region of M channel subunits contains a stretch of conserved positively charged amino acid residues, which are presumed to electrostatically interact with PI(4,5)P2 (Zhang et al. 2003).

PI(4,5)P2 also has a remarkable effect on some voltage-gated K^+ channels' ball and chain inactivation, in which the N-terminal region of the channel forms a ball which physically blocks the channel pore from the cytoplasmic side to inactivate it. PI(4,5)P2 immobilizes the basic inactivation N-terminal domain by electrostatically capturing it in the cytoplasmic leaflet of the plasma membrane, and thereby preventing it from accessing the pore (Oliver et al. 2004).

Another channel that is activated by PI(4,5)P2 is the ryanodine-sensitive Ca^{2+} release channel localized to skeletal muscle sarcoplasmic reticulum terminal cisternal membranes (Chu and Stefani 1991). The terminal cisternae store Ca^{2+} which is rapidly released in the muscle cell cytoplasm upon stimulation, eliciting muscle contraction. PI(4,5)P2 increases the Ca^{2+} release channel opening probability by enforcing a conformation that favors longer and more frequent openings, and reduces the proportion of long closures (Chu and Stefani 1991). Ryanodine-sensitive Ca^{2+} release channels contain a PH domain which could be responsible for the interaction with PI(4,5)P2 ("Domain Architectures: Pleckstrin Homology Domain (IPR001849) < InterPro < EMBL-EBI" 2018).

3.1.2 Regulation by involvement in membrane protein trafficking

PI(4,5)P2 plays essential roles in the regulation of exocytic insertion of vesicles in the plasma membrane, and in their endocytosis (Martin 2001*). Such processes are used for the insertion and removal of proteins from the plasma membrane, and thus for the regulation of their activity on the cell surface. The precise mechanisms regarding the involvement of PIP5K in vesicle movement and regulated endo- and exocytosis will be discussed later.

In epithelial cells, RhoA signaling increases the activity of epithelial Na^+ channel through its downstream effector ROCK. ROCK activates PIP5K, which generates a localized pool of PI(4,5)P2; this process results in an elevation in plasma membrane levels of the channel, presumably due to an increase in channel insertion rate (Pochynyuk et al. 2006). A very similar mechanism has been observed for the activation of a member of transient receptor potential (TRP) family of ion channels, TRPC5 (Bezzerides et al. 2004). TRP channels are cation-nonselective and their opening mostly results in an influx of Ca^{2+} in the cytoplasm. The activation of Rac1 results in a stimulation of PIP5Kβ, whose product PI(4,5)P2 mediates the rapid translocation of TRCP5 to the plasma membrane from readily available vesicles through a currently unknown mechanism (Bezzerides et al. 2004).

Cardiac Na^+-Ca^{2+} exchanger NCX1 is an antiporter membrane protein responsible for Ca^{2+} efflux important for cardiac muscle relaxation (Herrmann et al. 2013). Overexpression of PIP5Kβ results in a decrease of the NCX1 fraction on the cell surface through its internalization, which suggests a mechanism where PI(4,5)P2 promotes the removal of NCX1 from the membrane by mediating endocytosis (Shen et al. 2007). Moreover, the availability of PI(4,5)P2 seems to be a prerequisite for the internalization of a large group of G protein–coupled receptors, namely phospholipase C-coupled and adenylyl cyclase-linked receptors (Sorensen et al. 1998, 1999). This suggests that the requirement of PI(4,5)P2 could be common for all receptors regulated by internalization, which limits the duration of their activation (Sorensen et al. 1999).

3.2 REGULATION OF THE ACTIN CYTOSKELETON AND RELATED FUNCTIONS

One of the most prominent functions of PIP5K is the transmission of signals from the plasma membrane to the actin cytoskeleton (Sechi and Wehland 2000*). Actin cytoskeleton is formed of actin filaments, also termed microfilaments, and is involved in many cell processes such as cell motility and changes of cell shape, endocytosis, exocytosis, and phagocytosis. Actin networks are reshaped by the action of many proteins, which include capping proteins, severing proteins, and nucleating proteins. Capping proteins capture fast-growing (barbed) ends of actin filaments, disabling their further elongation (Millard, Sharp, and Machesky 2004*) Severing proteins such as gelsolin break the actin filaments in two, creating more available barbed ends (Sun et al. 1999*). And lastly, the branching of actin filaments is mediated by proteins able of *de novo* actin filament nucleation, such as the Arp2/3 complex (Millard, Sharp, and Machesky 2004*). PI(4,5)P2 associates with many of these proteins, regulating their localization and activity in various processes regarding the actin cytoskeleton remodeling, functionally connecting the plasma membrane to the actin cytoskeleton.

3.2.1 Interactions with actin remodeling proteins

PI(4,5)P2 is a potent regulator of many proteins involved in actin remodeling processes. The first discovered PI(4,5)P2-binding protein that plays a role in actin regulation is profilin, an actin monomer-sequestering protein, which is inactivated by PI(4,5)P2 (Sechi and Wehland 2000*). Gelsolin, a potent severing and capping protein, is also regulated by PI(4,5)P2; gelsolin severs the actin filament, creating two barbed ends, and then stays attached to the barbed ends as a capping protein, disassembling the actin network (Sun et al. 1999*). Gelsolin is a downstream effector of Rac in membrane ruffling, and PIP5K is presumed to mediate this association through a local production of PI(4,5)P2, which inhibits actin severing by dissociating gelsolin from actin filaments (Sun et al. 1999*). Another important intermediate between the plasma membrane and the actin cytoskeleton is the ezrin-radixin-moesin (ERM) protein family, which contains a PI(4,5)P2-binding FERM domain (Sechi and Wehland 2000*; Mao and Yin 2007*). The binding of ERM proteins to PI(4,5)P2 enhances their targeting to the plasma membrane and activates them, possibly in a Rho-mediated manner (Sechi and Wehland 2000*).

Probably the most important process of actin regulation in which PIP5K is involved is actin polymerization driven by the Arp2/3 complex. Arp2/3 complex binds to the side of actin filaments and nucleates the formation of a nascent daughter filament, thus organizing actin filaments into networks capable of exerting physical force upon the membrane in processes of plasma membrane reorganization (Mullins, Heuser, and Pollard 1998). This is not the only effect Arp2/3 has on the actin cytoskeleton; it also serves as a slow-growing (pointed) end capping protein to prevent pointed ends from actin monomer dissociation or addition (Mullins, Heuser, and Pollard 1998). PI(4,5)P2 does not regulate Arp2/3 directly, but through the action of WASP and N-WASP. PI(4,5)P2 binds to the N-terminal domain of WASP proteins, and this interaction stabilizes them in an activated conformation, enabling their activation by small GTPases (Rohatgi et al. 1999, 2000; Millard, Sharp, and Machesky 2004*). N-WASP and WASP C-termini associate with Arp2/3 and activate it, promoting actin polymerization (Rohatgi et al. 1999). Actin cytoskeleton branching via the N-

WASP/Arp2/3 pathway is crucial for many cellular events, such as lamellipodia and membrane ruffle formation in cell motility.

3.2.2 Modulation of the actin cytoskeleton during phagocytosis

Many stages of phagocytosis are influenced by the actin cytoskeleton, including the formation of the phagocytic cup, its extension, particle ingestion, and the closure of the phagocytic cup (Swanson 2008*). PI(4,5)P2 levels increase in the phagocytic cup during its formation, which promotes actin nucleation essential for phagocytosis initiation; on the other hand, PI(4,5)P2 cleavage by PLC and conversion to PI(3,4,5)P3 in later stages of phagocytosis promotes closure of the cup (Yeung et al. 2006*). Interestingly, two PIP5K isoforms, namely PIP5Kα and PIP5Kγ, have been observed to be recruited to phagocytic cups, and exert seemingly opposite effects on actin branching (Mao et al. 2009). PIP5Kα and PIP5Kγ both engage in the process of phagocytosis, but through a distinct mechanism; PIP5Kα is responsible for actin nucleation at the nascent cup through the activation of the WASP/Arp2/3 pathway, while the PIP5Kγ isoform controls actin depolymerization essential for particle attachment (Mao et al. 2009). The opposite effects on actin cytoskeleton can be explained by their differential regulation by kinases, and by their different placement within the small GTPase pathway. This regulatory mechanism is especially interesting, as PIP5Kγ seems to act upstream of RhoA/Rac1, contrary to PIP5Kα, which acts downstream of Rac1/Cdc42 as described above (Mao et al. 2009).

3.2.3 Focal adhesion formation

Focal adhesions are distinct areas of the plasma membrane which serve as a mechanical linkage to the extracellular matrix. This generally involves β-integrin, a transmembrane protein whose extracellular domain binds to extracellular proteins like collagen. Other focal adhesion proteins, e.g. vinculin and talin, form a bridge between β-integrin and the actin cytoskeleton (Sechi and Wehland 2000*). Talin mediates focal adhesion forming by binding to the cytoplasmic tails of β-integrin and actin cytoskeleton associated proteins, such as vinculin (Moser et al. 2009*). Focal adhesions are regulated by small GTPases of the Rho family, and their formation depends on the lipid kinase activity of PIP5K (Ridley and Hall 1992; Legate et al. 2011), because its product PI(4,5)P2 interacts with several focal adhesion proteins including talin and vinculin and stabilizes them in an active conformation (Sechi and Wehland 2000*).

PIP5Kγ661 specifically contains a talin-binding site within its C-terminus, and is thus the only isoform to localize to focal adhesions (Di Paolo et al. 2002; Legate et al. 2011). Phosphorylation at the Ser^{650} residue is crucial for the interaction between PIP5Kγ661 and talin (Ling et al. 2003) in a process described earlier. Talin binds PIP5Kγ661 through its FERM domain, and this interaction stimulates PIP5K kinase activity to produce a localized pool of PI(4,5)P2 at the sites of focal adhesion (Di Paolo et al. 2002). The increased level of PI(4,5)P2 at focal adhesions is crucial for their forming, as it facilitates talin recruitment to the plasma membrane (Ling et al. 2002; Legate et al. 2011). Moreover, the β-integrin-binding activity of talin is regulated through interaction with PI(4,5)P2 as well, as the association presumably orientates talin on the plasma membrane in such a way that favors the interaction

with β-integrin tails (Legate et al. 2011). Thus, PIP5Kγ661 is essential for the proper anchoring of cells within the extracellular matrix.

3.2.4 Regulation of cell migration

During cell migration, a leading edge forms in the direction of migration, whose production relies on a regulated turnover of membrane ruffles and lamellipodia. A lamellipodium is a flat protrusion on the leading edge of the cell, which is formed by a rapid and extensive branching of the actin cytoskeleton. Lamellipodia need to be anchored to the extracellular matrix by the formation of focal adhesions; if their attachment becomes inefficient, the leading edge curls and forms the other characteristic feature of migrating cell, membrane ruffles (Borm et al. 2005). This process is crucial for cell migration, and relies on Rac-controlled assembly and disassembly of actin cytoskeleton. As discussed earlier, Rac is a potent PIP5K activator, and Rac-driven PI(4,5)P2 synthesis is reciprocally responsible for further Rac activation. Injection of Rac into cells causes an immediate accumulation of membrane ruffles (Ridley et al. 1992), and the local generation of PI(4,5)P2 is essential, but not sufficient for membrane ruffles formation (Honda et al. 1999).

Leading edge structures are enriched in proteins that regulate the actin cytoskeleton, including PIP5Kα, which is the specific isoform recruited to membrane ruffles upon migration stimulus (Doughman et al. 2003; Kisseleva et al. 2005). PIP5Kα works in tandem with Rac in formation of membrane ruffles through localized actin assembly driven by PI(4,5)P2-activated
N-WASP/Arp2/3 actin nucleating pathway (Doughman et al. 2003; Kisseleva et al. 2005). Ajuba, a cytosolic actin-interacting protein which regulates actin assembly in actin migration, is required for the targeting and activation of PIP5Kα at lamellipodia and sites of membrane ruffling (Kisseleva et al. 2005). Ajuba was shown to directly interact with PIP5Kα after migration stimulus, and probably regulates PI(4,5)P2 synthesis at membrane ruffles together with Rac GTPases; ajuba enhances Rac1 activity during cell migration, which may enable Rac1 to regulate membrane ruffling cooperatively with PIP5Kα (Kisseleva et al. 2005).

Microtubules, polymers of tubulin, form another component of the cytoskeleton. Microtubules are also involved in organized motility and cell polarity determination during cell migration, and a precise coordination between the actin and the microtubule cytoskeleton is needed. PI(4,5)P2 is a likely candidate for signal integration between those two cytoskeletal systems (Golub and Caroni 2005). Spatially defined regions of lamellipodial motility are formed by the stabilization and clustering of PI(4,5)P2-rich rafts on the plasma membrane. A local accumulation of such rafts into patches is induced by signals triggering lamellipodial motility; however, in the absence of microtubules, these rafts rapidly dissipate, impairing organized cell migration (Golub and Caroni 2005). Microtubule capture by raft patches is essential for a continuous production of PI(4,5)P2 at sites of lamellipodial motility, and these pools of PI(4,5)P2 may serve as cues for vesicle trafficking through microtubules, which brings proteins essential for motility regulation to the leading edge raft patches (Golub and Caroni 2005).

3.2.5 Adherens junctions modulation

An adherens junction is a type of cell-cell contact which involves the linkage of the neighboring cells' actin cytoskeleton. Adherens junctions facilitate the formation of a polarized epithelial monolayer by defining the basolateral side of the cell. Their formation is crucial not only for the determination of cell polarity, but also for tissue integrity and the enforcing of a non-migratory phenotype in cells (Ling et al. 2007). This has an important physiological consequence, as the hereditary impairment of adherens junctions formation correlates with the development of gastric cancers (Yabuta et al. 2002). The structure responsible for cell-cell adhesion and the connection of actin cytoskeletons is the transmembrane protein termed epithelial cadherin (E-cadherin), whose extracellular domain is capable of associating with the extracellular E-cadherin domains coming from another cell (Shapiro and Weis 2009). The intracellular tail of E-cadherin connects to the actin cytoskeleton via accessory proteins and is regulated by many other proteins, including PIP5K (Ling et al. 2007; Xiong et al. 2012)

Both the longer and the shorter splice variant of PIP5Kγ have been observed to bind E-cadherin, which suggests that the variable 26-amino acid C-terminus is not involved in the interaction (Ling et al. 2007). The association with PIP5Kγ and a localized production of PI(4,5)P2 is presumably crucial for E-cadherin function in adherens junctions formation, as the dysfunction of PIP5Kγ results in a dissociation of E-cadherin from the sites of adhesion at the plasma membrane and a shift towards more migratory phenotype, as opposed to a stabilized polarized monolayer (Ling et al. 2007). PIP5Kγ is speculated to regulate E-cadherin by a modulation of its trafficking to and from the plasma membrane in endo- and exocytic processes.

PIP5Kγ661 has been observed to interact with clathrin adaptor protein 1 (AP1), which mediates basolateral transport in epithelial cells, and this association is splice variant-specific. The AP1-PIP5Kγ661 complex associates with E-cadherin, and possibly enables its transport from the trans-Golgi network to the recycling endosomes towards the basolateral plasma membrane. The function of PIP5K is probably dual, as it serves both as a scaffolding protein to mediate the association between AP1 complex and E-cadherin, and as a signaling molecule to regulate this process (Ling et al. 2007). Moreover, PIP5Kγ and PI(4,5)P2 have also been observed to interact with the exocyst, a protein complex crucial for the targeting of vesicles to the plasma membrane (Xiong et al. 2012). PIP5Kγ mediates the association between E-cadherin and the exocyst by creating localized pools of PI(4,5)P2 at sites of E-cadherin clustering, which guide the exocyst towards adherens junctions (Xiong et al. 2012). Additionally, PIP5K may also play a role in inducing actin polymerization and actin cytoskeleton remodeling, as these processes are also crucial for the maturation of adherens junctions and PIP5K has been observed to regulate such processes (Akiyama et al. 2005; Xiong et al. 2012).

3.2.6 Delivery to the apical membrane

Polarized epithelial cells are able to target intracellular cargoes to the apical or basolateral membrane through a yet not completely understood mechanism. Initial sorting takes place at the trans-Golgi network, where newly synthesized proteins are targeted to the apical or

basolateral domain of the cell. The process of apical delivery, unlike basolateral delivery, is presumed to largely depend on the actin cytoskeleton, and has been observed to rely on the formation of actin comets, fast-polymerizing actin filaments able of propelling vesicles through the cytoplasm (Rozelle et al. 2000; Guerriero et al. 2006).

Apical delivery is thought to depend on preferential actin polymerization from membrane microdomains enriched in specific lipids such as sphingolipids, cholesterol, and PI(4,5)P2, which form lipid rafts (Rozelle et al. 2000; Schuck and Simons 2004*). The apical membrane is also very rich on lipid rafts whose components probably serve as targets for actin comets loaded with such Golgi-derived vesicles (Schuck and Simons 2004*). The overexpression of PIP5K and a subsequent elevation in PI(4,5)P2 levels leads to an increase in the production of actin comets (Guerriero et al. 2006), and this process is presumed to rely on actin polymerization via WASP and Arp2/3 (Rozelle et al. 2000).

3.3 ROLES IN VESICLE TRAFFICKING

Vesicles, small lipid bilayer-enclosed structures, are crucial for the intake of extracellular material into the cell, for transport of cellular cargo, and for secretion. Plasma membrane is an important player in vesicle trafficking, as it invaginates and forms the surface of endocytic vesicles during endocytosis, and fuses with the vesicles in exocytosis, bringing the phospholipids back to the plasma membrane. PI(4,5)P2, which is enriched at the plasma membrane, serves as a cue to target the intracellular vesicles towards the membrane, and directly affects many proteins involved in the distinct processes surrounding vesicle trafficking, namely endo- and exocytosis (Vicinanza et al. 2008*). Precise tuning of PI(4,5)P2 turnover is crucial for the proper execution of these processes (Sun et al. 2013*), which is highlighted by the importance of PIP5Kγ, the major PI(4,5)P2-producing enzyme at the synapse, whose absence is lethal in mice (Volpicelli-Daley et al. 2010) and in humans (Narkis et al. 2007).

3.3.1 Modulation of clathrin-mediated endocytosis

Clathrin-mediated endocytosis is one of the most important entry routes into cells. It relies on the formation of clathrin-coated invaginations on the plasma membrane, which become further deepened until they are only connected to the membrane with a narrow neck. The vesicle is then released by the action of the GTPase dynamin (Higgins and McMahon 2002*). Clathrin takes the form of a triskelion capable of forming a lattice of pentagons and hexagons on the plasma membrane. However, it is unable to bind directly to the plasma membrane or to cargo, so it relies on the assistance of adaptor proteins and accessory proteins (McMahon and Boucrot 2011*). These proteins, such as AP2 adaptor complex, AP180, and epsin, bind PI(4,5)P2 via their N-terminal Lys-rich domain, AP180 N-terminal homology (ANTH) domain, and Epsin N-terminal homology domain (ENTH) domain, respectively (Martin 2001*), and are recruited to the plasma membrane from the cytoplasm through this interaction.

AP180 is able to bind to PI(4,5)P2 and clathrin simultaneously, and its function is to recruit clathrin to the membrane and nucleate the formation of a flat clathrin lattice (Ford et al. 2001, 2002). The interaction with PI(4,5)P2 is mediated through the ANTH domain, which is

extremely similar to ENTH except for a ANTH-defining consensus sequence which contains a region rich in Lys residues that bind PI(4,5)P2 (Legendre-Guillemin et al. 2004*). After being anchored to the membrane via the N-terminus, the C-terminal domain remains available for coat components which interact with the putative AP2 and clathrin-binding sites (Ford et al. 2001).

The curvature essential for clathrin coated-pit formation is induced by other adaptor and accessory proteins, most important of which are AP2 and epsin. The AP2 adaptor complex is responsible for the formation of clathrin-coated pits and for the selection of cargo proteins, and it consists of 4 subunits: α adaptin, β_2 adaptin, μ_2 adaptin and σ_2 adaptin. The α and β_2 adaptins interact with clathrin, and the α adaptin also comprises a phosphoinositide-binding polybasic domain with a Lys^{55-57} triad crucial for the binding of PI(4,5)P2. A functional PI(4,5)P2-binding domain is necessary for the recruitment of AP2 to the sites of clathrin-coated pit formation (Gaidarov and Keen 1999). Moreover, AP2 also directly binds the PIP5Kγ661 splice variant specifically (Nakano-Kobayashi et al. 2007). PIP5Kγ661 interacts with μ_2 and β_2 adaptins through its catalytic core and the 26-amino-acid C-terminus specific for this splice variant (Nakano-Kobayashi et al. 2007; Kwiatkowska 2010*). This association enhances PIP5Kγ661 lipid kinase activity and leads to a generation of PI(4,5)P2 at endocytic hotspots, which in turn enhances the recruitment of the coat components to such sites. In synaptic vesicle endocytosis, the interaction of PIP5Kγ661 with β_2 adaptin is regulated by the dephosphorylation of PIP5Kγ661 presumably by the serine-threonine protein phosphatase calcineurin, and PIP5Kγ661 appears to act downstream of Arf6 in this pathway (Nakano-Kobayashi et al. 2007; Kwiatkowska 2010*).

The AP2 α adaptin binds another important player in the process of clathrin coated-pit formation, epsin. The ENTH domain of epsin is targeted to the plasma membrane via an interaction with PI(4,5)P2, and the C-terminus interacts with AP2 and clathrin (Legendre-Guillemin et al. 2004*). After its recruitment to endocytic hotspots, epsin modifies membrane curvature and induces the formation of clathrin-coated pits together with AP2 (Ford et al. 2002). The interaction with PI(4,5)P2 enforces a conformational change of the ENTH domain; an amphipathic α-helix is formed, and this so-called 'helix 0' induces membrane curvature by inserting into the outer leaflet of the plasma membrane with its hydrophobic outer surface (Ford et al. 2002).

After the further deformation of the pit, the vesicle remains connected to the inner side of the membrane by a neck that is constricted through the action of dynamin. Dynamin is a GTPase that undergoes drastic conformational change after the hydrolysis of bound GTP to GDP, and this change leads to membrane scission and the release of the vesicle into the cytoplasm by a currently unidentified mechanism (Higgins and McMahon 2002*). Dynamin is also recruited to the membrane by an interaction with PI(4,5)P2 through its PH domain, and the binding of PI(4,5)P2 is crucial for the function of dynamin, and, consequently, clathrin-mediated endocytosis (Achiriloaie, Barylko, and Albanesi 1999).

Once the vesicle detaches from the parent membrane, the clathrin coat is removed and its protein components are recycled to the cytoplasm. Removal of PI(4,5)P2 from the vesicle membrane by a phosphoinositide phosphatase synaptojanin is necessary for vesicle uncoating

and endosomal fusion, as the disruption of the gene coding for synaptojanin leads to an accumulation of clathrin-coated vesicles in a proximity of the plasma membrane (Cremona et al. 1999). Synaptojanin is recruited to vesicles by AP2 (McMahon and Boucrot 2011), where it dephosphorylates PI(4,5)P2 at the 5-position, creating PI(4)P; this weakens the association of coat components with the vesicle membrane, resulting in the dissociation of the clathrin coat from endocytic vesicles (Legendre-Guillemin et al. 2004*).

3.3.2 Modulation of exocytosis

Contrary to clathrin-mediated endocytosis, in which the role of PIP5K is quite defined, its involvement in exocytosis and membrane fusion is mechanistically less well understood. PI(4,5)P2 seems to be required for both constitutive and regulated vesicle exocytosis independently of whether it relies on an elevation of Ca^{2+} levels (Martin 2012*); however, its role in Ca^{2+}-independent exocytic fusion of vesicles with the plasma membrane in constitutive secretory pathways has not been extensively studied in mammalian cells yet. Studies performed on *S. cerevisiae* suggest that the yeast PIP5K homologue Mss4 regulates exocytic events through a localized production of PI(4,5)2 that controls the actin cytoskeleton remodeling essential for the formation of the exocyst (Yakir-Tamang and Gerst 2009a, 2009b). A direct association between plasma membrane PI(4,5)P2 and the exocyst has been observed in mammalian cells, and this interaction is crucial for the anchoring of the exocyst to the plasma membrane, and, consequently, for the docking of secretory vesicles in the constitutive secretory pathway (Liu et al. 2007).

As for Ca^{2+}-regulated exocytosis, the role of PIP5K has been partially elucidated particularly during the process of neurosecretion. Exocytosis is a crucial step of the transduction of the nerve impulse, as neurotransmitter release at the synapse is mediated by the exocytic fusion of synaptic vesicles (SV) and dense-core vesicles (DCV) with the plasma membrane (Rettig and Neher 2002*). Fast-acting neurotransmitters are secreted from small SVs, and neuropeptides and neuromodulators from DCVs (Robinson and Martin 1998*). Regulated DCV exocytosis has been particularly studied, and the general conclusion is that PI(4,5)P2 extensively modulates this process. The absence of PI(4,5)P2 in plasma membrane leads to an inhibition in DCV exocytosis (Aikawa and Martin 2003), and the increase of its levels by overexpression of PIP5K enhances DCV exocytic activity (Aoyagi et al. 2005). Specifically, PI(4,5)P2 is needed for the priming step of DCV exocytosis, in which the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes form (Martin 2012*). Some PI(4,5)P2-enriched microdomains in the plasma membrane were found to correspond to the sites of DCV docking, and colocalize with the priming protein CAPS (Ca^{2+}-dependent activator protein for secretion) (James et al. 2008). CAPS is required for vesicle exocytosis, and the interaction between its PH domain and PI(4,5)P2 seems to be essential for SNARE-dependent membrane fusion (James et al. 2008; Martin 2012*). CAPS binds SNARE proteins via its C-terminus, and it has been proposed to rely on PI(4,5)P2 for membrane anchoring required for SNARE complex nucleation (Martin 2012*).

Synaptotagmins, which serve as Ca^{2+} sensors in the regulation of neurosecretion, bind PI(4,5)P2 in the presence of Ca^{2+}; one of the synaptotagmin Ca^{2+}-sensing modules changes its orientation after binding Ca^{2+} in such manner that it inserts itself in the PI(4,5)P2-

containing plasma membrane patches, and this membrane penetration is thought to play a role in membrane fusion by bringing the two membranes to close proximity (Bai, Tucker, and Chapman 2004). The process of SV exocytosis is presumed to be very similar to DCVs regarding the involvement of PIP5K (Martin 2012*), but the requirement of PI(4,5)P2 has not been sufficiently demonstrated yet.

4 SUMMARY

PIP5K is a fascinating enzyme that regulates a plethora of crucial processes and structures in the cell including vesicular transport, ion channels and receptors, the proper assembly of actin cytoskeletal network, and cell-cell contact via adherens junctions (summarized in Figure 3). Its proper function relies on the carefully spatially regulated generation of PI(4,5)P2 pools, which serve as a cue to target various PI(4,5)P2-binding proteins to the plasma membrane. This is made possible by the existence of PIP5K isoforms and splice variants, which differentially associate with proteins crucial for various processes.

In clathrin-mediated endocytosis, PIP5Kγ661 associates with the clathrin adaptor protein 2 (AP2) and recruits it to endocytic hotspots, enabling clathrin-coated pit formation (Figure 3A). This also has an important effect on the regulation of intrinsic plasma membrane proteins such as the transporter protein NCX1, whose function is attenuated through its removal from the plasma membrane by clathrin-mediated endocytosis (Figure 4). The reverse mechanism, exocytosis, also relies on PIP5K, as it has been shown to target the exocyst and the SNARE complexes to the plasma membrane (Figure 3B). This process again regulates membrane protein activity through their exocytic insertion in the plasma membrane, such as in the case of TRP channels (Figure 4). PIP5K also has the ability to activate or inactivate ion channels through its product, PI(4,5)P2 (Figure 3C). PIP5K creates a localized pool of PI(4,5)P2 which directly binds to ion channels such as Kir channels and M channels and stabilizes them in a conformation that favors longer opening times (Figure 4).

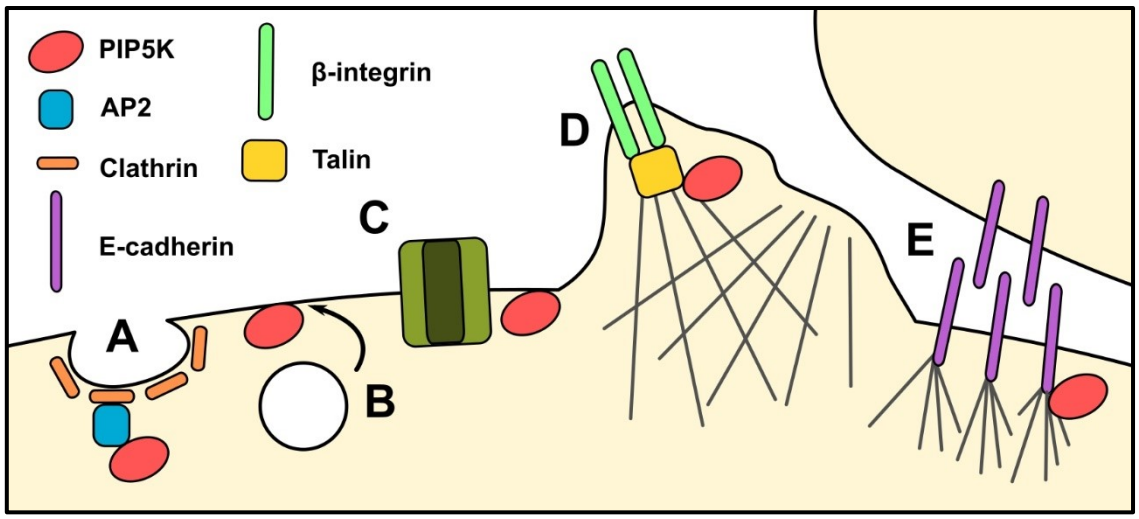

Figure 3. The role of PIP5K in various processes involving the plasma membrane. A endocytosis; B exocytosis; C ion channel regulation; D focal adhesion formation; E adherens junction formation.

PIP5K mediates cell-cell interactions through the regulation of adherens junctions, complexes that serve as mechanical linkage between cells' actin cytoskeletons (Figure 3E). PIP5Kγ binds the transmembrane protein responsible for the cell-cell contact, E-cadherin, and this association is crucial for the association of E-cadherin with the plasma membrane. Presumably, PIP5Kγ again regulates endocytic and exocytic events responsible for the assembly and disassembly of adherens juncitons.

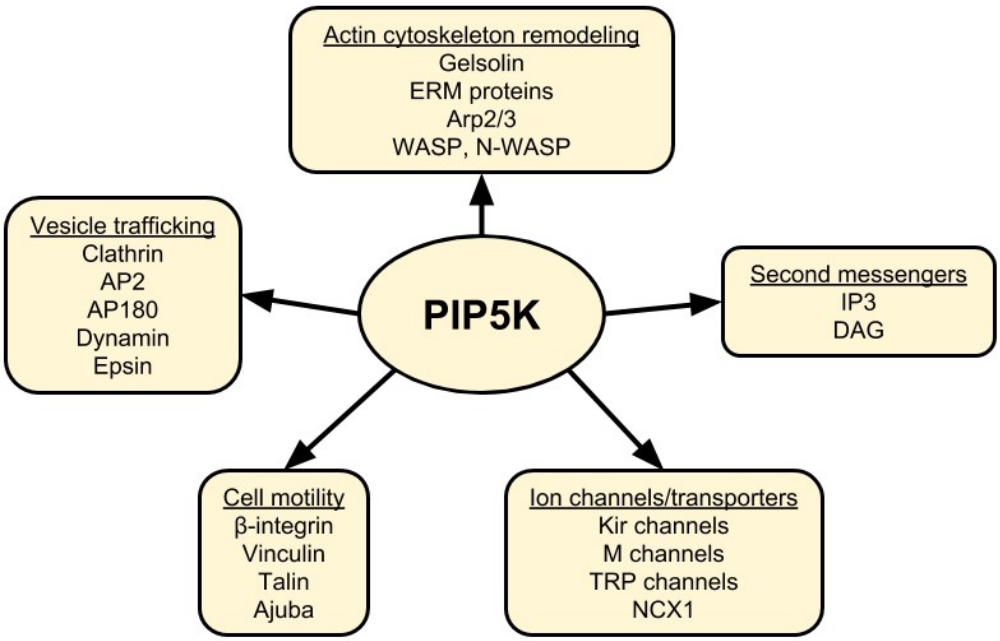

Figure 4. Chosen functions of PIP5K and its product PI(4,5)P2 in the context of a living cell. Apart from the most well-known function of PI(4,5)P2, the generation of second messengers IP3 and DAG, PI(4,5)P2 also serves as a second messenger on its own in processes such as vesicle trafficking, actin cytoskeleton remodeling, cell motility, and regulation of intrinsic membrane proteins such as ion channels and transporters.

Another crucial function of PIP5K is the regulation of actin cytoskeleton through the interaction with several actin remodeling proteins, e.g. gelsolin and ERM proteins. Perhaps the most important is the regulation of actin filament branching, which is stimulated by PIP5K through its activation of the N-WASP/Arp2/3 actin nucleating pathway (Figure 4). This process is especially important for the creation of cell motility related structures such as lamellipodia and focal adhesions (Figure 3D). The rapid PIP5K-driven assembly and disassembly of actin cytoskeleton is essential for the forming of leading edge structures, and their anchoring to the extracellular matrix via focal adhesions is also a PIP5K-dependent process. β-integrins, the transmembrane proteins responsible for the association with extracellular proteins, rely on PIP5K-mediated interaction with the actin cytoskeleton through talin. PIP5K associates with talin and targets it to the plasma membrane, enabling the formation of focal adhesions.

In conclusion, PIP5K is a very interesting target of further research as it regulates many processes connecting the plasma membrane to the actin cytoskeleton. More information needs to be gathered about the functions of the various isoforms and splice variants, especially in *H. sapiens*, and about their precise spatial and temporal regulation.

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