Chapter 2
PIP Kinases from the Cell Membrane to the Nucleus

Mark Schramp, Andrew Hedman, Weimin Li, Xiaojun Tan and Richard Anderson

Abstract Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a membrane bound lipid molecule with capabilities to affect a wide array of signaling pathways to regulate very different cellular processes. PIP_2 is used as a precursor to generate the second messengers PIP_3, DAG and IP_3, indispensable molecules for signaling events generated by membrane receptors. However, PIP_2 can also directly regulate a vast array of proteins and is emerging as a crucial messenger with the potential to distinctly modulate biological processes critical for both normal and pathogenic cell physiology. PIP_2 directly associates with effector proteins via unique phosphoinositide binding domains, altering their localization and/or enzymatic activity. The spatial and temporal generation of PIP_2 synthesized by the phosphatidylinositol phosphate kinases (PIPKs) tightly regulates the activation of receptor signaling pathways, endocytosis and vesicle trafficking, cell polarity, focal adhesion dynamics, actin assembly and 3′ mRNA processing. Here we discuss our current understanding of PIPKs in the regulation of cellular processes from the plasma membrane to the nucleus.

Keywords Cell Migration · mRNA processing · Phosphatidylinositol phosphate kinase (PIPK) · Phosphatidylinositol 4,5-bisphosphate (PIP_2) · Vesicle trafficking

2.1 Introduction

Studies by Hokin and Hokin on exocrine tissue in the 1950s brought to light how changes in phospholipids could regulate cellular processes (Hokin and Hokin 1953). Later, discoveries in the 1980s advanced our understanding of how phosphatidyli-

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nositol 4,5-bisphosphate (PIP_2) can be utilized by phospholipase C to generate IP_3 (Streb et al. 1983), a molecule that mobilizes Ca^{2+} stores from the endoplasmic reticulum, and by PI3K to generate PIP_3, a signaling molecule initially discovered downstream of the Src oncoprotein critical for cell proliferation and transformation (Whitman et al. 1988). In 1985, Lassing and Lindberg discovered that PIP_2 could directly associate with profilactin and profilin (Lassing and Lindberg 1985). These studies provided the backbone of PIP_2 biology and illuminate the importance of PIP_2 as a signaling nexus that not only is modified to generate PIP_3, DAG and IP_3, but can also directly modulate the activities of an ever growing array of proteins to regulate virtually every cellular process. The past few years have seen a resurgent interest in the regulation of PIP_2 synthesis. Unlike PIP_3, PIP_2 levels are relatively high and undergo only small changes in total cellular content upon stimulation (Heck et al. 2007). However, the spatial and temporal targeting of phosphatidyl-inositol phosphate kinases (PIPKs), the molecules that generate the vast majority of cellular PIP_2, via the actions of its functional protein associates can result in a dramatic and localized surge in PIP_2 levels to coordinate the activation of its regulated signaling pathways (Doughman et al. 2003b; Honda et al. 1999; Kisseleva et al. 2005; Sasaki et al. 2005). The findings that PIPK activity is enhanced by Rho and Arf family GTPases provided regulatory mechanisms and clues to the processes that PIP_2 generation might affect (Krauss et al. 2003; Weernink et al. 2004). Perhaps even more surprising, roles for PIPKs and PIP_2 within the nucleus are defining how these molecules can regulate mRNA processing and gene expression (Mellman et al. 2008). Thus how PIPKs are regulated has emerged as crucial yet relatively unexplored frontier in molecular biology that could dramatically impact how we view normal and pathogenic behavior. This chapter will focus on the role that PIPKs and PIP_2 generation play in modulating signaling pathways and cellular process from the plasma membrane to the nucleus.

2.2 The Enzymes. Sequence, Structure and Enzymology

2.2.1 Sequence and Structure of Phosphatidylinositol Phosphate Kinases

There are three known families of PIP kinases, type I, II and III, which catalyze the formation of phosphatidylinositol bisphosphates. Type I PIP kinases contain three separate genes, α, β and γ, and primarily catalyze phosphorylation of PI4P to $PI4,5P_2$ (Anderson et al. 1999; Doughman et al. 2003a; Heck et al. 2007; Ishihara et al. 1996; Jenkins et al. 1991; Loijens et al. 1996; Schill and Anderson 2009b). Type II PIP kinases, also have three separate genes, α, β and γ, and generate $PI4,5P_2$ by phosphorylating the 4' OH position of PI5P (Boronenkov and Anderson 1995; Rameh et al. 1997). First identified in yeast for their function in vacuole morphology, the most recently discovered type III PIP kinases generate $PI3,5P_2$ by phosphorylating PI3P (Cooke et al. 1998; Gary et al. 1998; McEwen et al. 1999; Yamamoto et al. 1995). Within this family of kinases are the yeast Fab1p and the human PikFYVE

(Cabezas et al. 2006; Sbrissa et al. 1999). At ~2200 amino acids the type III PIP Kinases are much larger in size than type I and II kinases and contain an N-terminal FYVE domain that allows for association with endosomal compartments (Gary et al. 1998; Odorizzi et al. 1998; Sbrissa et al. 1999; Yamamoto et al. 1995). The conserved feature of all PIP kinases is ~280 amino acid kinase domain (Ishihara et al. 1996).

2.2.1.1 Sequences of PIP Kinases

There is little conserved sequence among PIPK subtypes, with 60% identity among PIPKI, 60–77% identity among PIPKII, while PikFYVE and Fab1p only share 20% identity. Compared to the yeast MSS4p, human type I PIPKs share only 20% identity even though they catalyze the same reaction and can complement Mss4p deficient cells (Homma et al. 1998). Little identity is conserved between PIPK subtypes, PIPKI and PIPKII share less than 30% identity, while type PIPKIII shares less than 20% identity with human PIPKI and PIPKII. In the C-terminal region of type I, II and III PIPKs is the kinase homology domain, which is responsible for catalytic activity and is the only region that shares significant homology between family members (Anderson et al. 1999; Boronenkov and Anderson 1995; Ishihara et al. 1998; Loijens et al. 1996). PIPKIα, β and γ are approximately 75–80% identical, PIPKIIα, β and γ are 66–78% identical, and 41% identity is shared between the PikFYVE and Fab1p kinase domains. There is ~30% shared identity in the kinase domain between family members.

While there is little overall sequence conservation among PIPKs, there are highly conserved regions within the kinase domain that are important for catalytic activity and substrate recognition. These essential components include the G-loop, catalytic residues and the activation loop. Deletion of regions N-terminal and C-terminal to the 280 amino acid kinase domain abrogate activity, indicating the importance of these regions in maintaining structural integrity (Ishihara et al. 1998). Starting from the N-terminus, PIPK kinase domains (highlighted in yellow) (Fig. 2.1) contain the consensus sequence of GxSGS in PIPKI and a conserved II**K**, corresponding to a region found in protein kinases known as the G-loop, which consists of a glycine patch followed by a downstream lysine that mediates nucleotide binding (Anderson et al. 1999; Hanks et al. 1988; Heck et al. 2007; Ishihara et al. 1998; Rao et al. 1998; Saraste et al. 1990). Mutations in this region can inhibit kinase activity. Mutation of the murine PIPKIα Glycine 124 in the GxSGS consensus to Valine reduced kinase activity by one third, while mutating the downstream Lysine, K138, to Alanine completely abolished kinase activity (Ishihara et al. 1998). This Lysine in the II**K** sequence corresponds with Lysine 72 in Protein Kinase A, which together with Mg^{2+} coordinates the α and β phosphates in ATP. Mutation of K72 also abolishes kinase activity of PKA and mutation of the corresponding residue abolishes activity of all PIP kinases (Anderson et al. 1999; Heck et al. 2007; Iyer et al. 2005; Knighton et al. 1991a, 1991b; Rao et al. 1998; Taylor et al. 1992, 1993).

C-terminal to the G-loop are two highly conserved regions responsible for PIPK catalytic activity (highlighted in magenta) (Fig. 2.1). These are the **D**LKGS and

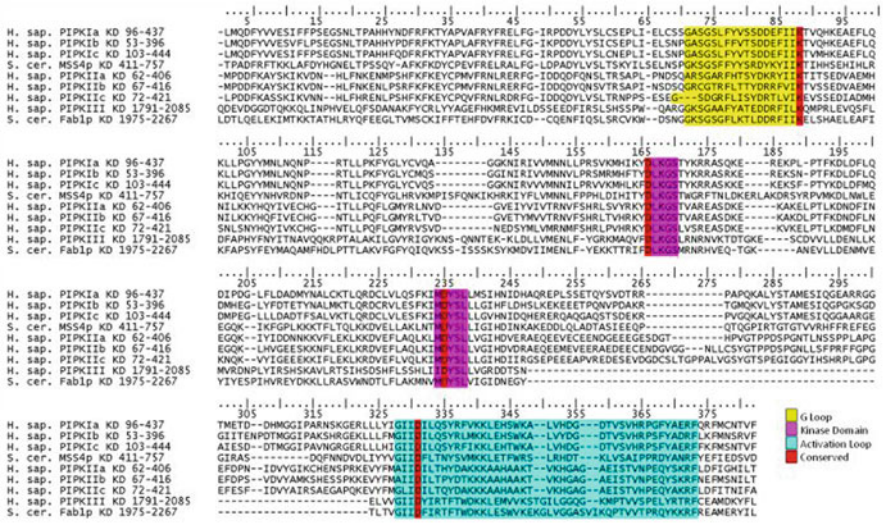

Fig. 2.1 ClustalW multiple sequence alignment for human and yeast PIPK kinase domains. Conserved regions are highlighted—G-loop (*yellow*) for ATP binding, catalytic kinase domain sequences (*Magenta*), activation loop (*cyan*). Conserved residues (*Red*) PIPKIIβ K150, D216, D278 and D369 are required for PIP kinase activity

M**D**YSL motifs. The **D**LKGS motif shows similarity to the HR**D**LK catalytic sequence in PKA, but is not equivalent based on structure (Anderson et al. 1999; Rao et al. 1998; Yamamoto et al. 1995). However the conserved Aspartate in the **D**LKGS motif has been shown to be required for kinase activity in PIPKIβ and PIPKIγ (Anderson et al. 1999; Narkis et al. 2007). Furthermore, human D253 N mutations in PIPKIγ that result in an **N**LKGS sequence lead to lethal contractural syndrome type 3 disease (Narkis et al. 2007). The PKA Aspartate 166 in the HR**D**LK motif functions as a catalytic base, corresponding to Aspartate 278 within the M**D**YSL motif in PIPKIIβ that superimposes on its structure (Krupa et al. 2004; Rao et al. 1998). Additionally, the catalytic Aspartate 369 in PIPKIIβ, within the (G/A)II**D**IL motif, corresponds to Aspartate 184 in PKA found within a conserved **D**FG motif. This region coordinates Mg^{2+} ions and is required for kinase activity (Heck et al. 2007; Rao et al. 1998; Zheng et al. 1991). These four conserved residues (K138, D216, D278 and D369 in PIPKIIβ) in PIPKs are essential for kinase activity.

Finally, at the C-terminus of the kinase homology domain is a region of 20–30 amino acids (highlighted in cyan) (Fig. 2.1), known as the activation loop, responsible for substrate specificity and PIPK intracellular targeting (Anderson et al. 1999; Burden et al. 1999; Kunz et al. 2000, 2002; Rao et al. 1998). The activation loop is common amongst protein kinases (Cox and Taylor 1994; Hanks and Hunter 1995; Johnson et al. 1996; Rao et al. 1998) and its modification is one means to regulate these proteins. The activation loop of PIP kinases begins with the consensus GII**D**IL with the aspartate conserved among other protein kinases, and ending with

an RF that is conserved in PIP kinases. In experiments where the activation loop of PIPKIβ and PIPKIIβ were swapped, the specific activity of these chimeric PIP kinases matched its activation loop, for example, PIPKIβ containing the IIβ activation loop phosphorylated PI5P, while chimeric PIPKIIβ containing the Iβ activation loop phosphorylated PI4P (Kunz et al. 2000). Additionally, these domain swaps altered PIPKI targeting (Kunz et al. 2000). Furthermore, specific residues conserved within PIPKI and PIPKII are essential for specificity. PIPKIIβ A381 to E point mutations alter substrate specificity from PI5P to PI4P, while the reverse mutation, E362A, in PIPKIβ allowed for PIP_2 synthesis from PI4P and PI5P, demonstrating the importance of this Glutamate in PI4P specificity (Kunz et al. 2002). The role of the activation loop was further shown through complementation studies in yeast deficient in Mss4p. Yeast cells lacking Mss4p can be rescued by the PIPKIIβ chimera containing the Iβ specificity loop, thereby restoring type I activity, but not the PIPKIβ chimera with the IIβ loop (Kunz et al. 2000). There is also a conserved KK motif in the activation loop found among PIPKs. Mutation of this KK to NN reduced substrate affinity, while a conservative KK to RR mutation did not alter function (Kunz et al. 2000). This suggests that this KK motif is important for lipid substrate interaction, but not for recognition of specific PIP isomers.

2.2.1.2 PIP Kinase Structure

Structures have been solved for human PIPKIIβ (PDB ID: 1BO1) and PIPKIIγ (PDB ID: 2GK9), with publications focusing on PIPKIIβ (Burden et al. 1999; Kunz et al. 2000; Rao et al. 1998). The PIPKIIβ structure reveals a homodimer, with two PIPKIIβ monomers flush against one another. A set of antiparallel beta sheets and a single alpha helix form a flat face that allows for membrane association (Burden et al. 1999). Ten basic residues result in a net charge of + 14 on this face; mutation of three lysines on this face to glutamates (K72/76/78) inhibited lipid binding and partially inhibited phosphoinositide binding, suggesting the basic face plays a role in membrane association but is not the only requirement for substrate binding (Burden et al. 1999). Figure 2.2a, b show that the planar face that interacts with the plasma membrane consists primarily of a beta sheet and a single helix. Individual monomers are shown in blue and green, with the basic residues found on the planar face, shown in red. Figure 2.2c shows the molecules from the side, with the planar face that interacts with the membrane facing downwards.

The conserved regions of the kinase domain described above are highlighted in Fig. 2.3. The G-loop (yellow) links two beta strands. The kinase catalytic DLKGS and MDYSL motifs (shown in magenta) are also in this pocket and the activation loop (shown in cyan) faces the membrane. The conserved catalytic residues within PIPKIIβ (K150, D216, D278 and D369) are highlighted in red. The structures of ATP and the PIPKIIβ substrate, PI5P, were modeled onto the structure (Fig. 2.3b) (Rao et al. 1998). This reveals a pocket, where the γ phosphate of ATP is oriented such that it faces the membrane, while PI5P fits such that the four hydroxyl of the myo-inositol ring faces this phosphate, consistent with PIPKIIβ function (Heck et al.

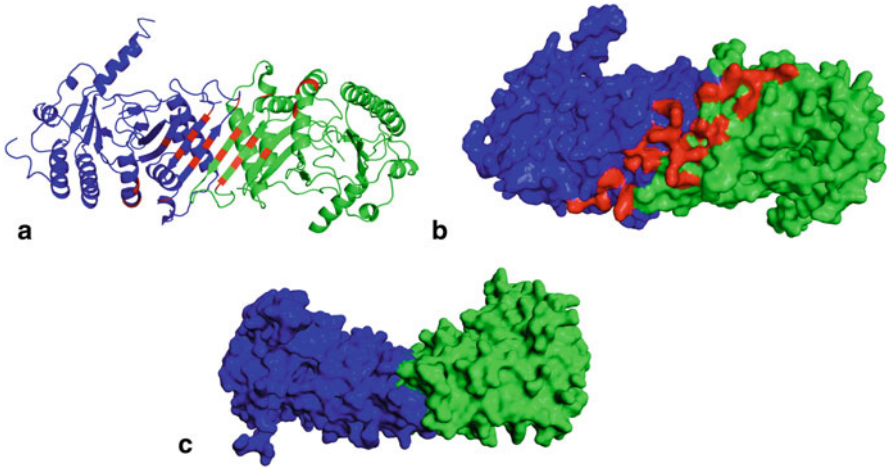

Fig. 2.2 PIPKIIβ structure. **a**, **b** and **c** Pymol rendering of the PIPKIIβ structure (PDB: 1BO1). PIPKIIβ forms a dimer (individual monomers in *blue* and *green*). a and b show the planar face that binds to the membrane, with key basic residues highlighted in *red*. c shows the side view of PIPKIIβ dimer, with membrane binding plane shown facing down

2007; Rao et al. 1998). Proposed residues necessary for ATP (133G, 136G, 139F, 148V, 150K, 201T, 203N, 204V, 205F, 278D, 282L, 368I, 369D) (Blue) and PI5P binding (134R, 218K, 224R, 239K, 278D, 372T) (Red) in the model are shown in Fig. 2.3b (Rao et al. 1998). The planar face of the dimer allows for the pocket to interact with the phosphoinositide head group and allow for catalytic activity without interfering with membrane structure.

2.2.2 Enzymology of PIPKs

The basic reaction catalyzed by PIP kinases is the ATP-dependent phosphorylation of phosphatidylinositol monophosphates to produce phosphatidylinositol bisphosphates (Heck et al. 2007). The substrate specificity of PIPKI, II and III was discovered through kinase activity on purified phosphatidylinositol phosphates from biological sources and synthetic phosphoinositides. These studies identified the primary substrate activity of PI4P for PIPKI, PI5P for PIPKII and PI3P for PIPKIII (Bazenet et al. 1990; Cabezas et al. 2006; Rameh et al. 1997; Sbrissa et al. 1999; Zhang et al. 1997). However, the PIP kinases can also utilize other PIP substrates with lower activity. For example, PIPKIs can phosphorylate PI3P to produce PI3,4P_2, and subsequently PI3,4,5P_3, or PI3,5P_2 (Tolias et al. 1998; Zhang et al. 1997). While the PIPKI affinity for PI3P is similar to PI4P, the reaction rate is higher for PI4P (Zhang et al. 1997). Similarly, PIPKII can also catalyze the phosphorylation of PI3P to PI3,4P_2 but is not the preferred substrate (Rameh et al. 1997). Finally, PIPKIII has been reported to generate PI5P from PI (Sbrissa et al. 1999; Shisheva et al. 1999). Though the enzymes can make multiple products, the *in vivo* importance of enzyme specificity can be observed through yeast complementation studies. Yeast deficient

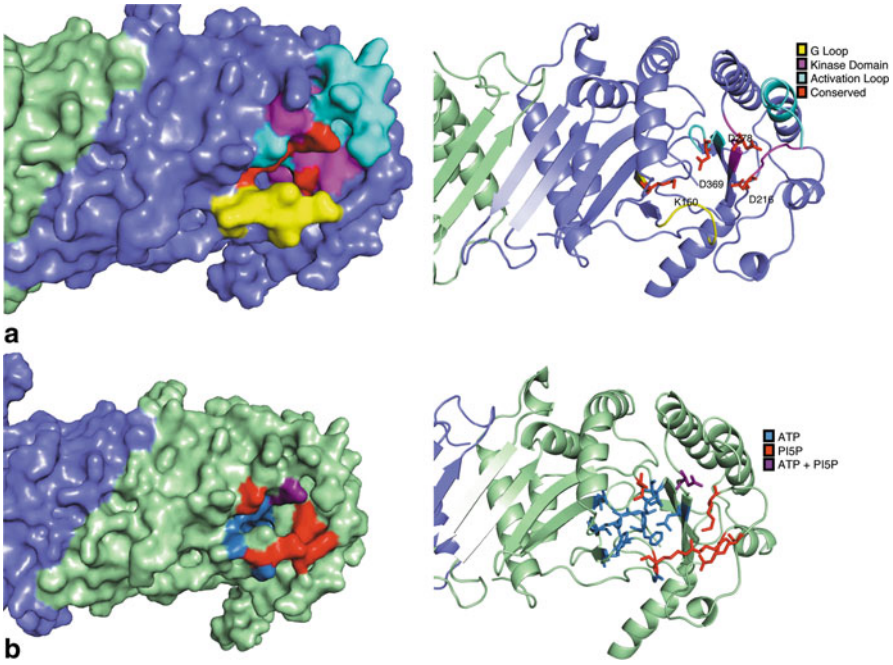

Fig. 2.3 PIPKIIβ structure. **a** and **b** Pymol rendering of PIPKIIβ highlighting conserved regions—G loop (*yellow*), catalytic kinase domain sequences (*magenta*) and activation loop (*cyan*). Conserved residues required for PIP kinase activity are shown in *red* (PIPKIIβ K150, D216, D278 and D369). Highlighting residues believed to mediate ATP (*blue*) and PI5P (*red*) binding on PIPKIIβ structure

in MSS4p can be rescued using human PIPKI but not PIPKII (Homma et al. 1998), while yeast deficient in Fab1p can only be rescued by human PIPKIII (McEwen et al. 1999). However, the function of substrate diversity for each kinase *in vivo* has not been examined closely.

The activity of PIP kinases is regulated by a variety of stimuli, including lipids, proteins and post-translational modifications. Following their initial purification, phosphatidic acid was shown to enhance PIPKI kinase activity (Jenkins et al. 1994; Moritz et al. 1992a, b). More recently, phosphorylation has been shown to regulate PIPKs. Activation of Protein Kinase A (PKA) resulted in phosphorylation of PIPKIα on Serine 214 in the kinase domain, reducing activity, and activation of PKC via LPA treatment reduced PIPKIα phosphorylation and enhanced kinase activity (Park et al. 2001). Also, PKD can phosphorylate the activation loop of PIPKIIα at Thr 376, and mutation of this site reduced kinase activity (Hinchliffe and Irvine 2006). Phosphorylation of PIPKs does not always affect kinase activity as Casein Kinase (CK) II phosphorylation of PIPKIIα at Ser304 affects localization, but not kinase activity (Hinchliffe et al. 1999a, b). PIPKI and PIPKIII also have protein kinase activity, as these enzymes can auto-phosphorylate, reducing enzyme activity (Itoh et al. 2000; Sbrissa et al. 2000). Protein-protein interactions are also critical regulators

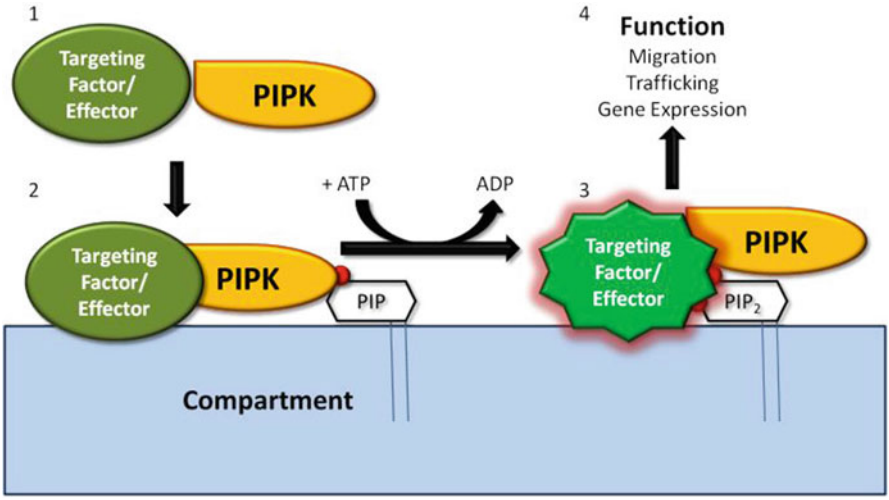

Fig. 2.4 PIPKs are recruited to intracellular compartments by PIP_2-effector proteins. *1* PIPKs interact with proteins that mediate their localization within the cell. *2* Once at a specific site, PIPKs generate PIP_2. *3* The interacting proteins that targeted PIPKs to specific sites are then modulated by the newly synthesized PIP_2. *4* The activated PIPK effectors regulate various cellular processes

of PIP kinase activity. Interactions with GTPases and specific proteins such as Talin and adaptor protein (AP) complexes have been shown to enhance PIPK activity (Di Paolo et al. 2002; Krauss et al. 2006; Weernink et al. 2004). The roles of phosphorylation and protein-protein interactors in regulating PIPK activity will be described in detail later.

The unique sequences in PIPKs allow for specific interactions with proteins that serve to target PIPKs to specific compartments. Often, these same interacting proteins are PIP_2 effectors (Heck et al. 2007). These protein-protein interactions target PIP kinases to specific compartments to generate a local pool of PIP_2, which can then regulate the activity of its effectors. This hypothesis is illustrated in Fig. 2.4. Multiple examples of regulation of PIP kinase targeted PIP_2 production have been demonstrated with diverse roles in cell-adhesion, migration, protein trafficking and nuclear signaling.

2.3 Membrane Associated PIPKs Drive Cell Migration and Vesicle Trafficking

2.3.1 PIPKs Help Regulate Directional Migration

Cell migration is a critical process for normal development, wound healing, cell survival and immunological responses, but can also have deleterious effects as evidenced by its role seen in metastatic tumor formation. Cell migration requires a tight

coordination of many molecular biological processes, including the establishment of cell polarity, the organized formation and turnover of adhesive structures and a regulated control of dynamic cytoskeletal rearrangements (Fig. 2.5a). Regulation of these processes produces the well-characterized migration pattern defined by a continuous cycling between membrane protrusions at the leading cell front, anchoring of the newly formed front, the rolling of the cell body forward, and the release of posterior adhesions to retract the trailing cell membrane. Many signal transduction pathways can influence these processes in large part due to their ability to regulate the spatio-temporal generation of the key lipid messenger, PI4,5P_2, herein referred to as PIP_2.

2.3.1.1 PIPKs Regulate PIP_2 Synthesis at the Leading Edge to Drive Membrane Protrusion

In 1985 Lassing and Lindberg discovered that PIP_2 specifically interacts with profilin, thus starting a long and multifaceted look at the role of phosphoinositides in migration and invasion (Lassing and Lindberg 1985). Since then, PIP_2 has been shown to directly bind many actin-associated proteins that both directly and indirectly regulate the cellular cytoskeletal machinery (Yin and Janmey 2003). Increased PIP_2 levels promote actin polymerization, whereas a decrease results in actin disassembly. PIP_2 controls actin polymerization at many levels, including inactivating proteins involved in actin severing or depolymerization as well as enhancing actin polymerization, branching and bundling (Sechi and Wehland 2000; Takenawa and Itoh 2001). During cell migration, rampant F-actin synthesis at the migrating front pushes the cell membrane forward (Fig. 2.5) resulting in the formation of subcellular structures including membrane ruffles, lamellipodia, microvilli, motile actin comets, filopodia, microspikes and dorsal ruffles (Janmey and Lindberg 2004; Nicholson-Dykstra et al. 2005). All of these membranous protrusions have been observed in different cell types overexpressing type I PIPKs, a family of kinases responsible for the vast majority of PIP_2 produced in a cell (Honda et al. 1999; Matsui et al. 1999; Rozelle et al. 2000; Shibasaki et al. 1997; Yamamoto et al. 2001). Using GFP-tagged PH domains or antibodies that specifically recognize PIP_2, this lipid messenger was found to concentrate in dynamic, actin-rich regions of the cell (Tall et al. 2000).

PIP_2 plays a multifaceted role in regulating F-actin dynamics at these protrusive cell fronts. PIP_2 directly binds gelsolin, a capping protein found at the barbed end of F-actin filaments, blocking its association with F-actin thereby inducing a rapid and local actin polymerization (Niggli 2005). The association of PIP_2 with profilin frees up actin monomers so that they can be incorporated into the growing filaments (Lambrechts et al. 2002; Skare and Karlsson 2002). Using *in vitro* actin co-sedimentation assays, PIP_2 was shown to attenuate the association between F-actin and cofilin, an actin-severing and depolymerizing factor (Gorbatyuk et al. 2006). However, cofilin activity enhances migration (Sidani et al. 2007). The hydrolysis of PIP_2 by Phospholipase C (PLC) releases and activates a distinct membrane-bound pool of cofilin, triggering the formation of membrane protrusions and the establishment of a leading edge (van Rheenen et al. 2007). A localized activation of cofilin enhances the

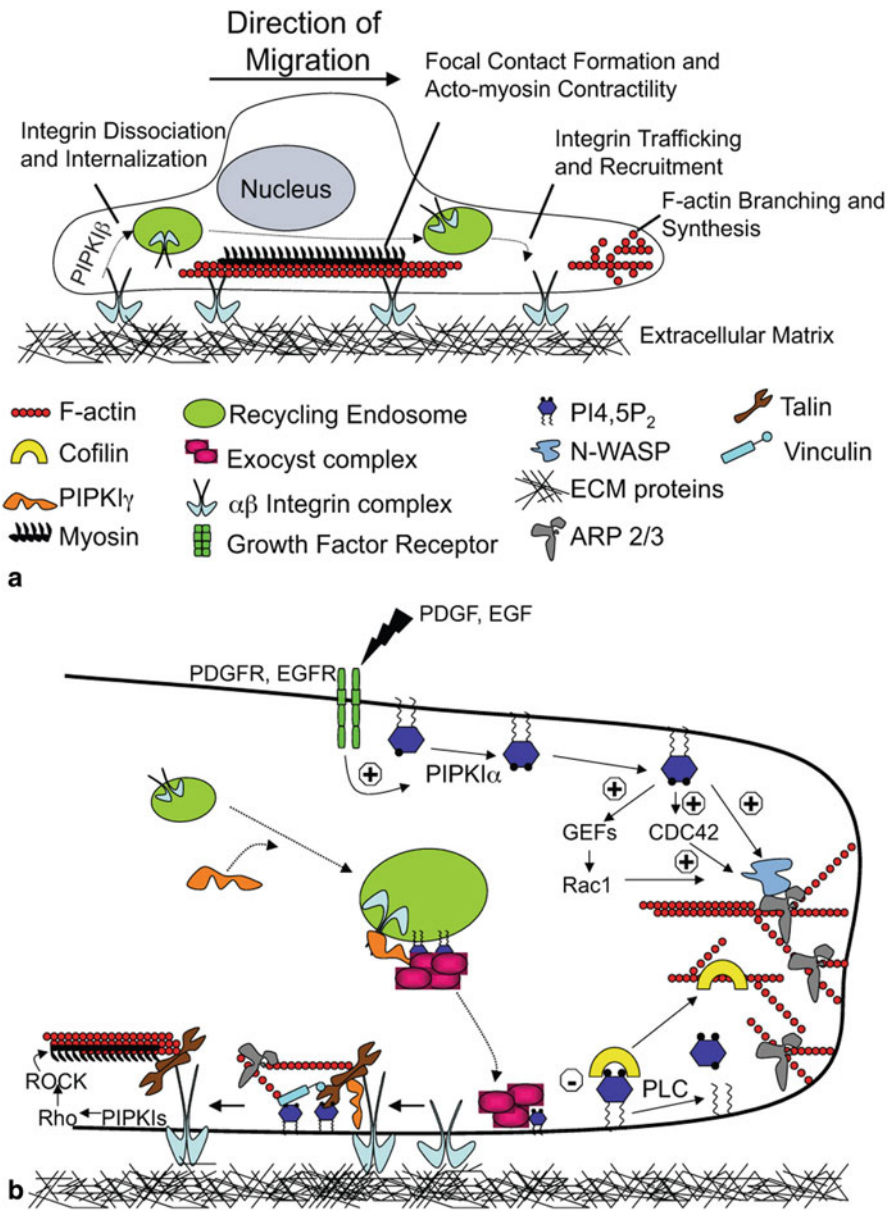

Fig. 2.5 PIPKs are involved in multiple processes associated with cell migration. **a** PIPKs are integral components at each step in the migration process. Cell migration involves a continuous cycle of membrane protusion, adhesion formation and maturation at the leading edge, adhesion disassembly (mediated by PIPKIβ) at the trailing edge and contractile forces to pull the cell body forward. **b** At the leading edge, PIPKIα regulates Rac and Cdc42 to initiate F-actin synthesis. PIPKIγ isoforms are involved in the trafficking of proteins to the leading edge, the assembly of proteins that mediate adhesions and the maturation of these adhesions to induce acto-myosin contractions

number of F-actin barbed ends, setting a site for membrane protrusion, however, curtailing that activity is also required to promote enhanced filament formation and actin bundling required to push the membrane forward. The dynamic interplay between actin severing and F-actin synthesis is further observed in the association of PIP_2 with villin. PIP_2 binding to villin differentially regulates the protein's activities, inhibiting its actin severing and capping activity while enhancing its ability to bundle actin (Kumar and Khurana 2004; Kumar et al. 2004).

While uncovering roles for PIP_2 in regulating actin-associated proteins has been widely successful, defining the specific proteins involved in its synthesis during cell migration has been less concrete. PIPKIα is recruited to membrane ruffles and increases PIP_2 levels in response to EGF and PDGF (Fig. 2.5b) (Doughman et al. 2003b; Honda et al. 1999). Depletion of PIPKIα in mouse embryo fibroblasts blocks cell migration, and PIPKIα-deficient mast cells display atypical actin organization (Kisseleva et al. 2005; Sasaki et al. 2005). Both PIPKIα and PIPKIβ have been shown to directly bind Rac1, a member of the Rho family of small GTPases that help regulate actin-cytoskeletal dynamics (Hall and Nobes 2000), and are thus thought to be Rac1 effectors (Weernink et al. 2004). PIPKIα is targeted to lamellipodia and its presence within signaling cascades that include Rho-GTPases is widely known (Doughman et al. 2003b). In addition, the uncapping of actin filaments in cells expressing a constitutively active mutant of Rac1 could be blocked by the overexpression of a dominant negative mutant of PIPKIβ but not a kinase-dead mutant of PIPKIα (Tolias et al. 1998, 2000). However, conflicting evidence fails to define whether PIPKIα is upstream or downstream of these proteins. Mutations in the PIPKIα binding region on Rac1 prevents translocation to the plasma membrane and fails to activate downstream signaling responses to integrin-mediated adhesion (Del Pozo et al. 2002). A more recent report shows that PIPKIα regulates Rac1 activation and downstream F-actin assembly, focal adhesion formation and directed migration (Chao et al.). Other reports identify PIPKIα as an effector of Rho-GTPases downstream of G protein-coupled and growth factor receptor activation (Chatah and Abrams 2001). GTPase activation is facilitated by guanine-nucleotide exchange factors (GEFs) that are often activated by phosphoinositides including PIP_2 (Di Paolo and De Camilli 2006). However, effectors of GTPases are often the PIPKs that generate phosphoinositides (Di Paolo and De Camilli 2006). This positive feedback loop results in the generation of membrane domains enriched for specific phosphoinosites. Oude Weernink et al. have shown that all type I PIPK isoforms can associate with RhoA and Rac1 but not Cdc42, another Rho family GTPase (Weernink et al. 2004). However, Cdc42 can stimulate PIP5K activity (Weernink et al. 2004). The concomitant stimulation of neuronal Wiskott-Aldrich syndrome protein (N-WASP) by Cdc42 and PIP_2 increases actin filament nucleation and F-actin synthesis via activation of the actin-related protein 2/3 complex (Arp2/3) (Prehoda et al. 2000).

2.3.1.2 PIPKIγ Regulates the Formation and Maturation of Integrin-mediated Contacts

Following membrane protrusion at the migrating front, the cell anchors this region to the underlying matrix via the formation of specialized adhesive structures, including

focal adhesions, focal complexes, podosomes, invadopodia and hemidesmosomes (Fig. 2.5) (Broussard et al. 2008; Webb et al. 2002). Cell migration requires a tight coordination of the formation/turnover of these structures (Webb et al. 2002). Focal adhesions are large, multi-protein complexes that physically link the cellular cytoskeleton to the extra-cellular matrix (ECM). Their formation generally involves integrin molecules and is dependent on PIP_2 (Ling et al. 2002). Many of the proteins required for focal contact formation, such as α-actinin, vinculin, talin and ezrin, are positively regulated by PIP_2 (Ling et al. 2006). PIPKIγi2 is required for EGF-stimulated directional migration and is phosphorylated by the EGFR and Src within its C-terminus (Sun et al. 2007). PIPKIγi2 phosphorylation modulates an association with talin, facilitating its assembly into dynamic adhesive complexes at the leading edge (Ling et al. 2002, 2003). Talin is a scaffolding protein that can bind both β integrin, resulting in its activation and engagement to the ECM, and F-actin or vinculin at nascent adhesions (Nayal et al. 2004). Furthermore, talin binds PIP_2 and this interaction is required for targeting talin to nascent contacts and enhancing its association with β integrin (Ling et al. 2002; Martel et al. 2001). Expression of a PIP_2-specific PH domain blocked the membrane localization of talin essential for focal adhesion maintenance (Martel et al. 2001). In addition, a local enrichment of PIP_2 enhances vinculin assembly into newly formed focal contacts (Chandrasekar et al. 2005). PIP_2 binding to vinculin disrupts its auto-inhibition, exposing protein binding domains that mediate actin and talin association (Bakolitsa et al. 2004). Additionally, PIP_2 binding to vinculin exposes a proline rich domain resulting in a vinculin/VASP association that recruits profilin/G-actin or Arp2/3, facilitating their assembly into nascent contacts and enhancing F-actin nucleation and synthesis (DeMali et al. 2002; Millard et al. 2004). This suggests that PIPKIγi2 is recruited to newly forming contacts and the generation of PIP_2 aides their maturation. The result is a rigid adhesive complex that strengthens the cell infrastructure, a critical step in migration as contractile forces use these anchors to pull a cell in the direction of migration.

2.3.1.3 Trafficking of Integrin-containing Vesicles to the Leading Edge Is Mediated by PIPKIγ and PIP_2

Coordinated cell migration requires the trafficking of newly synthesized and endocytosed proteins to the leading edge (Caswell and Norman 2006; Prigozhina and Waterman-Storer 2004; Schmoranzer et al. 2003). Impairment of the endocytosis, trafficking and exocytosis of integrins drastically impairs the establishment of polarity and directionality of cell migration (Tayeb et al. 2005; Zovein et al.). Furthermore, PIP_2 generation is required for vesicular trafficking (Wenk and De Camilli 2004).

PIPKIγ knockout studies have revealed vesicle trafficking defects both at the plasma membrane and in endosome-like structures in neuronal cells (Di Paolo et al. 2004; Wenk et al. 2001). Furthermore, several studies define a pivotal role for the exocyst protein complex in the polarized trafficking of transmembrane proteins during cell polarization and migration (Letinic et al. 2009). The exocyst complex consists of eight subunits and facilitates the exocytosis of post-Golgi and endocytic recycling

endosomes (including integrin-containing vesicles) to regions of rapid membrane expansion, such as occurs in migrating cells (Yeaman et al. 2001). Depletion of exocyst complex components inhibits wound healing and migration (Rosse et al. 2006). Rab11 and Arf6, two small GTPases that regulate PIPKs, also regulate the exocyst complex and integrin trafficking (Oztan et al. 2007). Furthermore, at least two exocyst subunits, Sec3 and Exo70, bind PIP_2 via conserved basic residues in their C-termini (Liu et al. 2007). PIPKIγi2 is able to associate with the exocyst complex and regulate the trafficking of integrin molecules in migrating cells (unpublished data). PIPKIγ could play a role in the assembly of the exocyst complex onto vesicles (Fig. 2.5). In epithelial cells, PIPKIγi2 is required for the basolateral sorting and endocytosis of E-cadherin via the AP1B and AP2 adaptor protein (AP) complexes respectively (Ling et al. 2007). This occurs by a novel mechanism where PIPKIγ interacts directly with E-cadherin at regions within the PIP kinase domain and then recruits specific AP complex subunits through interactions within its unique C-terminus. This may be a mechanism for regulation of both cell adhesion and migration. In addition, a localized generation of PIP_2 may be important for vesicle fusion at the plasma membrane (Fig. 2.5). PIP_2 and type PIPKI activity have been shown to be critical during Ca^{2+}-activated secretion of large dense-core vesicles (Hay et al. 1995). A critical player in this process, synaptotagmin, binds PIP_2 via C2 domains and mediates membrane fusion (Bai et al. 2004). Furthermore, Gong et al. showed that chromaffin cells taken from the adrenal gland of PIPKIγ –/– mice displayed a defect in the readily releasable pool of vesicles containing catecholamine and a delay in fusion pore expansion (Gong et al. 2005). Both Sec3 and Exo70 were shown to bind PIP_2 at the plasma membrane in yeast, a critical interaction for normal exocytosis and maintenance of cell morphology (He et al. 2007; Zhang et al. 2008).

2.3.1.4 Rear Retraction of the Cell Requires the Dissociation and Internalization of Integrin and Acto-myosin Contractility

During cell migration, new adhesions are formed along the protruding edge. Their maturation develops a focal point used to generate of forces required to push the membrane in the direction of migration. In addition, the maturation of focal adhesions stabilizes forces used to pull the rear of the cell forward. Overexpression of type I PIPKs has been shown to induce the formation of F-actin stress fibers, though the formation of actin-based protrusions is inhibited at the cell rear (Shibasaki et al. 1997; Yamamoto et al. 2001). Instead, rear retraction occurs at least in part through RhoA-regulated acto-myosin contractility (Yoshinaga-Ohara et al. 2002). In fibroblasts, integrin-mediated adhesion to fibronectin results in a rapid increase in PIP_2 synthesis and previous work has shown that PIPKIγ interacts with RhoA resulting in enhanced PIP_2 production (Fig. 2.5) (Weernink et al. 2004). Lokuta et al. show that PIPKIγi2 is enriched in the uropod of directionally migrating neutrophils (Lokuta et al. 2007). Overexpression of a kinase-inactive mutant of PIPKIγi2 compromised uropod formation and retraction of the cell rear (Lokuta et al. 2007). When bound to GTP, RhoA can signal through ROCK to initiate acto-myosin contractility via its ability to enhance the phosphorylation of the myosin light chain both directly and

indirectly through MLCK (Schramp et al. 2008). ROCK also plays a critical role in the RhoA-mediated activation of PIP5 K to synthesize PIP_2, a signaling pathway utilized during neurite remodeling (van Horck et al. 2002; Yamazaki et al. 2002).

In addition, focal contacts at the rear of the cell need to be disassembled. A prevailing theory of cell migration adheres to the idea that disassembled adhesions are internalized via endocytosis and recycled to the protruding edge where they once again are utilized in the formation of nascent adhesions (Pierini et al. 2000). The protease calpain, FAK, phosphatases and kinases that regulate FAK, microtubules and dynamin 2 have all been shown to regulate focal adhesion disassembly (Broussard et al. 2008; Burridge et al. 2006; Ezratty et al. 2005; Franco et al. 2004). All three isoforms of type I PIPKs have been implicated in clathrin-mediated endocytosis (Franco and Huttenlocher 2005). Recently, Chao et al. showed that PIPKIβ is required for b1 integrin uptake and adhesion turnover during cell migration (Chao et al.). Localized PIPK recruitment and PIP_2 formation can recruit molecules that directly regulate endocytosis to the plasma membrane and modulate proteins involved in the inactivation of focal adhesions (Fig. 2.5). PIP_2 can directly bind many endocytic clathrin adaptors, including AP-2, AP180 and epsin (Qualmann et al. 2000). Clathrin adaptor components and dynamin become enriched at focal adhesion sites prior to internalization (Chao and Kunz 2009; Ezratty et al. 2005). Loss of PIPKIβ blocked the assembly of clathrin adaptors at adhesion plaques and prevented an association between dynamin and FAK, an interaction required for endocytosis (Chao et al.). Interestingly, in addition to its role in promoting F-actin synthesis at nascent contacts, a role for vinculin in promoting focal adhesion disassembly is also emerging. The introduction of a vinculin mutant unable to bind PIP_2 into mouse melanoma cells repressed its translocation from the membrane to the cytosol and blocked the disassembly of focal adhesions, cell spreading and migration (Chandrasekar et al. 2005). Calpain is another PIP_2 regulated protein that directly mediates focal adhesion disassembly (Franco and Huttenlocher 2005; Franco et al. 2004). Calpain is a protease that can cleave adhesion proteins including talin, vinculin, paxillin and β integrin and is required for focal adhesion turnover and cell migration (Franco and Huttenlocher 2005; Franco et al. 2004). However, the activation cues to signal these processes remain to be fully uncovered.

PIPKs are proving to be integral components in helping regulate the many processes required for cell migration. The existence of multiple PIPK isoforms may enable cells to coordinate PIP_2 synthesis at specific sites to regulate the forces that induce membrane protrusion, the trafficking events that localize proteins at the leading edge, the assembly, maturation and endocytosis of adhesions and the forces generated to retract the trailing rear of the cell.

2.3.2 PIP Kinases in Adaptor Protein Complex Assembly and Protein Trafficking

In the past decade, considerable evidence has demonstrated that PIPKs and their lipid biproducts, PIP_2, are actively involved in various protein trafficking processes.

Table 2.1 Adaptor complexes consist of different subunits

AP complex	Subunits
AP-1A	β1, γ, σ1, μ1A
AP-1B*	β1, γ, σ1, μ1B
AP-2	β2, α, σ2, μ2
AP-3A	β3A, δ, σ3, μ3A
AP-3B*	β3B, δ, σ3, μ3B
AP-4	β4, ε, σ4, μ4

*Tissue specific AP complexes

Among these, the most widely characterized is the role of PIPKs in clathrin-mediated endocytosis and endosomal recycling, both of which are dependent on AP complexes. PIPKs directly bind AP complexes and their ability to synthesize a localized pool of PIP_2 results in AP complex assembly and activation. Here we will summarize how PIPKs regulate different AP complexes to modulate distinct protein trafficking processes.

2.3.2.1 AP Complexes

AP complexes are indispensible components of clathrin-coated vesicles in the endocytic and post-Golgi trafficking pathways. AP complexes not only link clathrin to the membrane, but also determine the specificity of cargo selection at various membrane compartments (Nakatsu and Ohno 2003; Ohno 2006). To date, four ubiquitously expressed AP complexes (AP-1A, AP-2, AP-3A, and AP-4) have been identified (Dell'Angelica et al. 1999; Nakatsu and Ohno 2003), with two additional complexes, AP-1B and AP-3B, found specifically in epithelial and neuronal cells respectively (Fölsch et al. 1999; Nakatsu and Ohno 2003; Ohno 2006). Each AP complex consists of two large adaptin subunits, one derived from the β-class (β1-4) and one of γ, α, δ or ε, together with one medium subunit (μ1-4) and one small subunit (σ1-4) (Table 2.1) (Jackson 1998; Ohno 2006). In each AP complex, the large adaptin subunits are responsible for membrane association through PIP_2 (Gaidarov et al. 1999; Ohno 2006; Rohde et al. 2002). The μ subunit determines specific cargo selection, while the β subunit is required for clathrin recruitment (Ohno 2006). Therefore, different AP complexes direct distinct cargo proteins at the membrane surface of vesicles (Fig. 2.6).

2.3.2.2 PIP Kinases in Regulation of AP Complex Assembly

Recent studies have demonstrated that PIP kinases are involved in clathrin-mediated vesicle trafficking at the plasma membrane. PIPKs have been found to directly bind AP complexes, regulating AP complex assembly and the formation of clathrin-coated pits. Here we will discuss how PIPKs regulate protein trafficking at the plasma membrane through specific interactions with AP complexes.

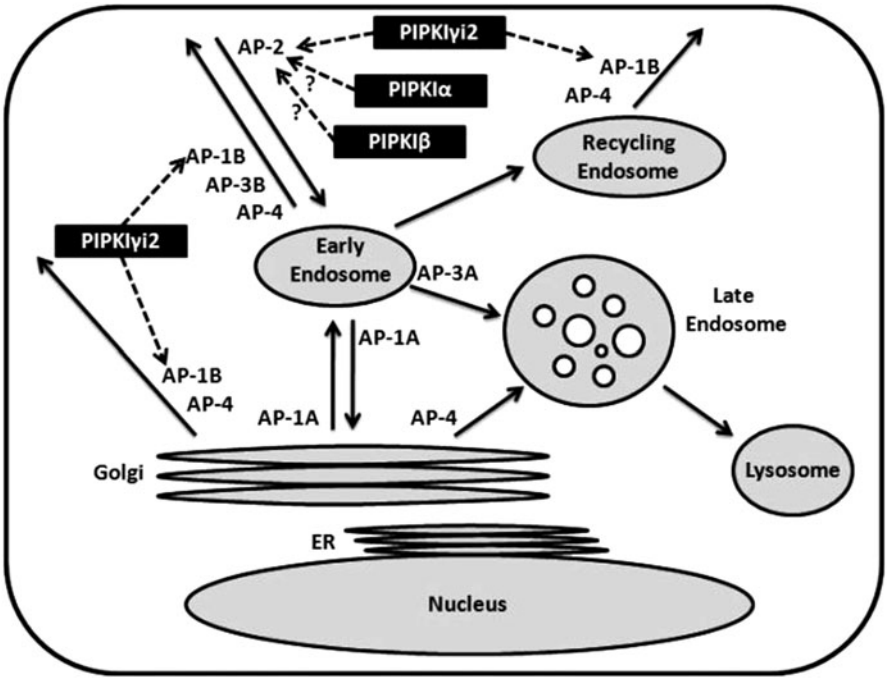

Fig. 2.6 AP complexes mediate endocytic and post-Golgi trafficking. Different AP complexes are involved in clathrin-mediated protein trafficking between different compartments. PIPKIγi2 interacts with AP-1B and AP-2 complexes, modulating basolateral transport and endocytosis

E-cadherin maintains epithelial cell morphology by forming adherens junctions (AJs) with adjacent cells. The amount of E-cadherin on the plasma membrane directly determines the strength of AJs (Yap et al. 2007). In addition to E-cadherin gene expression, post-translational regulations such as exocytosis, endocytosis, recycling and lysosomal degradation have also been implicated as important factors affecting the stability of AJs (Schill and Anderson 2009a). Furthermore, PIP_2 has been shown to be an essential regulator of different E-cadherin trafficking processes (Schill and Anderson 2009a). PIPKIγ directly binds all type I classical cadherins and colocalizes with E-cadherin in epithelial cells (Akiyama et al. 2005; Ling et al. 2007). Specifically, PIPKIγi2 has been found to regulate AJ assembly by modulating E-cadherin trafficking between endosomes and the plasma membrane (Ling et al. 2007). Expression of wild-type PIPKIγi2 promotes both internalization and recycling of E-cadherin, while a kinase dead mutant of PIPKIγi2 inhibits both of these processes (Ling et al. 2007), indicating that phosphoinositide generation is required for E-cadherin trafficking.

PIPKIγi2 can directly associate with both E-cadherin and the μ1B subunit of AP-1B (Ling et al. 2007), suggesting that it could act as a scaffold to link these proteins (Fig. 2.7). Within the unique PIPKIγi2 C-terminus is the sequence YSPL, Yxxϕ tyrosine-sorting motif (x represents any and ϕ represents a bulky hydrophobic amino

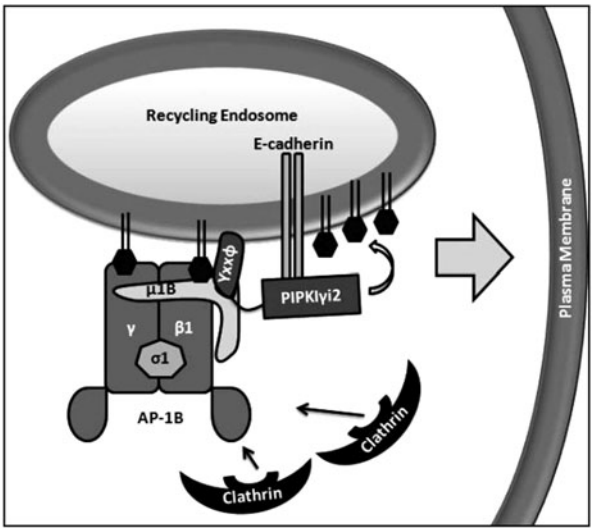

Fig. 2.7 PIPKIγi2/AP-1B interaction regulates E-cadherin trafficking. PIPKIγi2 interacts with the μ1B subunit of AP-1B through the Yxxϕ motif in the C-terminus and recruits E-cadherin via the kinase domain. Additionally, PIPKIγi2-generated PIP_2 assembles PIP_2-interacting proteins of the AP-1B and clathrin vesicle complexes at specific sites

acid) recognized by the μ1B subunit of AP-1B (Bairstow et al. 2006; Bonifacino and Traub 2003; Ling et al. 2007; Sugimoto et al. 2002). It has been demonstrated that the PIPKIγi2 YSPL sequence is responsible for the μ1B interaction, as a mutation of tyrosine to a phenylalanine disrupted their interaction and subsequently decreased the association between E-cadherin and μ1B (Ling et al. 2007). Interestingly, PIPKIγi2 also binds to the β2 subunit of AP-2 (Nakano-Kobayashi et al. 2007). Whether the PIPKIγi2/AP-2 interaction is also involved in E-cadherin trafficking is not clear.

Current evidence supports a model where PIPKIγi2 acts as both a scaffold and direct regulator of complex assembly through the generation of PIP_2 during E-cadherin trafficking to the plasma membrane (Fig. 2.7). The μ subunit of the AP complex is the predominate molecule mediating selection through a direct interaction with cargo proteins (Ohno 2006; Sugimoto et al. 2002). However, PIPKIγi2 could function as a scaffold protein that links E-cadherin to the μ1B subunit of the AP-1B complex. At the same time, PIPKIγi2 also functions by temporally and specifically producing PIP_2, which in turn recruits and regulates AP complex proteins and other components of the clathrin-coated vesicle. Certain patients suffering from hereditary diffuse gastric cancer have been found to contain a mutation within the PIPKIγi2-binding site in the C terminus of E-cadherin (Yabuta et al. 2002; Ling et al. 2007). The mutated E-cadherin shows inhibited interaction with PIPKIγi2, and localizes mostly to cytoplasmic compartments rather than the plasma membrane, losing the ability to form AJs (Ling et al. 2007; Suriano et al. 2003; Yabuta et al. 2002). These results underscore the role of PIPKIγi2 in E-cadherin trafficking to the plasma membrane.

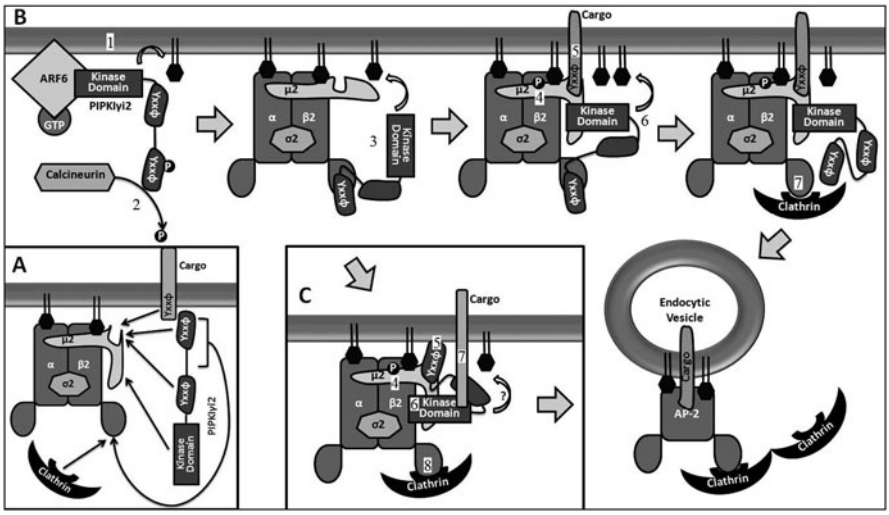

Fig. 2.8 PIPKIγi2/AP2 interaction modulates endocytosis. **a** PIPKIγi2 directly interacts with the AP-2 complex through multiple sites. **b** PIPKIγi2 facilitates clathrin-AP-2-mediated endocytosis of Yxxϕ-motif-containing cargos. Activated Arf6 recruits and activates PIPKIγi2, leading to $PI4,5P_2$ generation, which in turn recruits AP-2 (*1*). Dephosphorylation of PIPKIγi2 by calcineurin or another upstream molecule (*2*) induces PIPKIγi2-binding to the Ear domain of the β2 subunit of AP-2, further activating PIPKIγi2 (*3*). Conformational change in μ2 caused by Thr156 phosphorylation (*4*) promotes cargo binding (*5*) to μ2 through Yxxϕ motif with the help of $PI4,5P_2$. This results in μ2 binding to and activating the PIPKIγi2 kinase domain (*6*). Clathrin-engagement to AP-2 disrupts PIPKIγi2 association with the β2 subunit (*7*). $PI4,5P_2$ accumulation in step 1, 3, and 6 recruits proteins involved in clathrin vesicle formation. PIPKIγi2 is removed from AP-2 by phosphorylation at Ser645 or other unidentified mechanisms before maturation of the clathrin-coated vesicle (*8*). **c** PIPKIγi2 may enhance clathrin-AP-2-mediated endocytosis of Yxxϕ-independent cargos. Conformational change in μ2 caused by Thr156 phosphorylation (*4*) promotes PIPKIγi2 association with μ2 through one of the two Yxxϕ motifs in the C-terminus of PIPKIγi2 (*5*), which may in turn enhance the interaction between PIPKIγi2 kinase domain and the μ2 subunit (*6*). Yxxϕ engagement to μ2 may allow cargo selection through Yxxϕ-independent mechanisms (*7*). Clathrin-binding to β2 (*8*) might facilitate these processes

During clathrin-mediated endocytosis, PIPKIγi2 regulates AP-2 complex assembly by directly binding multiple subunits in the AP-2 complex (Fig. 2.8a). The kinase domain of PIPKIγi2 binds to the μ2 subunit, an association that does not block cargo engagement to μ2 (Krauss et al. 2006). In this situation, cargo proteins bind to μ2 through tyrosine or dileucine sorting motifs. This interaction can then potentially activate PIPKIγi2 activity (Kahlfeldt et al. 2010; Krauss et al. 2006). In addition to this, the tyrosine-based sorting motif (^{641}SWVYSPL^{647}) within the PIPKIγi2 specific C-terminal tail has also been found to directly bind to the μ2 subunit of AP-2 (Bairstow et al. 2006). A recent study discovered another tyrosine-based sorting motif (^{495}RSYPTLED^{502}) within the C terminus of PIPKIγi2 that can bind to the μ2 subunit (Kahlfeldt et al. 2010). Moreover, the C-terminal tail of PIPKIγi2 can also interact with the β2 subunit of AP-2, enhancing PIPKIγi2 activity (Kahlfeldt et al. 2010;

Nakano-Kobayashi et al. 2007; Thieman et al. 2009). Where PIPKIγi2 interacts with the AP-2 complex is believed to mediate the formation of different clathrin-coated vesicles that regulate the trafficking of various cargo proteins (Kahlfeldt et al. 2010; Kwiatkowska 2010).

The PIPKIγi2/AP-2 interaction is subject to regulation by phosphorylation of the PIPKIγi2 C-terminus. Src-mediated phosphorylation of PIPKIγi2 at Tyr644 (Tyr 649 in the human isoform) inhibits its interaction with the μ2 subunit (Bairstow et al. 2006; Ling et al. 2003) and based on crystallographic data could impair PIPKIγi2 binding to the β2 subunit (Kahlfeldt et al. 2010). In addition, phosphorylation of PIPKIγi2 at Ser645 (Ser650 in the human isoform) by cyclin-dependent kinase-5 (Cdk5) diminishes PIPKIγi2 interaction with the β2 subunit (Nakano-Kobayashi et al. 2007; Thieman et al. 2009), while dephosphorylation of PIPKIγi2, possibly by calcineurin upon plasma membrane depolarization, enhances this binding affinity in clathrin-mediated endocytosis at the presynapse (Nakano-Kobayashi et al. 2007). Interestingly, it has been shown that phosphorylation of either Tyr644 or Ser645 inhibits the phosphorylation of the other (Lee et al. 2005). Therefore, dephosphorylation of both Tyr644 and Ser645 promotes PIPKIγi2 interaction with both β2 and μ2, while Tyr644 phosphorylation alone inhibits this interaction and Ser645 phosphorylation alone might increase PIPKIγi2 binding to the μ2 subunit. As clathrin competes with PIPKIγi2 for the same binding site in the β2 subunit (Thieman et al. 2009), a PIPKIγi2 binding switch from β2 to μ2 via phosphorylation changes of Tyr644 and Ser645 might occur during AP-2 complex assembly (Kwiatkowska 2010).

PIPKIγi2-mediated PIP_2 generation recruits AP-2 complex components (Fig. 2.8b) (Höning et al. 2005). Dephosphorylation of PIPKIγi2 by upstream signals triggers its binding to the Ear domain of the β2 subunit, which activates PIPKIγi2 to produce PIP_2. In the presence of PIP_2, phosphorylation of μ2 subunit at Thr156 causes a conformational change that favors cargo engagement through tyrosine sorting motifs (Höning et al. 2005; Olusanya et al. 2001). Although cargo association with μ2 subunit via acidic dileucine motifs is not enhanced by the Thr156-phosphorylation-mediated conformational change, the μ2 subunit has the highest affinity towards both cargo signals when PIP_2 is present (Höning et al. 2005). Cargo engagement induces μ2 to interact with the kinase core domain of PIPKIγi2 enhancing its activity. Dynamic PIP_2 production recruits other proteins, such as AP180, epsin and dynamin-2, to facilitate the formation of the clathrin-coated vesicles during endocytosis. It is noteworthy that although PIPKIγi2 plays important roles in clathrin-AP-2 complex assembly, it is not enriched in clathrin-coated vesicles (Thieman et al. 2009; Wenk et al. 2001), suggesting that PIPKIγi2 functions in the dynamic assembly of AP-2 complex and clathrin-coated pits, but it is not a component of mature vesicles. Clathrin binding to the β2 subunit has been found to eliminate PIPKIγi2 association with β2 through the C-terminus (Thieman et al. 2009), yet whether this removes PIPKIγi2 from the AP complex or only causes a binding switch of PIPKIγi2 from β2 to μ2 still needs to be determined.

An alternative hypothesis is that PIPKIγi2 Yxxϕ motifs associates with μ2 like a cargo protein (Kahlfeldt et al. 2010; Kwiatkowska 2010) (Fig. 2.8c). This alternative Yxxϕ/μ2 interaction might provide a mechanism for Yxxϕ sorting motif-independent cargo selections. Yxxϕ/μ2 interactions have been suggested to facilitate μ2 interactions with the kinase core domain of PIPKIγi2 and activate PIPKIγi2 (Kahlfeldt et al.

2010; Kwiatkowska 2010). Kahlfeldt et al. showed that PIPKIγi2 activity could be increased when purified μ2 and the C-tail of PIPKIγi2 were incubated with cell lysates containing overexpressed PIPKIγi2. However, it is possible that the PIPKIγi2 C-terminus mimicks the effects of a cargo protein like EGFR, as they did not see any increase in PIPKIγi2 activity when the lysates were incubated with μ2 alone (Kahlfeldt et al. 2010). Although evidence shows that PIPKIγi2 can interact with μ2 through either the kinase core domain or through one of the two Yxxϕ motifs (Bairstow et al. 2006; Kahlfeldt et al. 2010), whether the μ2-PIPKIγi2 complex alone can lead to PIP kinase activation *in vivo* or whether inhibiting the μ2 association with the PIPKIγi2 C-terminus impairs PIPKIγi2 functions in AP-2 complex assembly is not clear. Future studies are needed to understand how the interaction between μ2 and the Yxxϕ motifs in the C-terminus of PIPKIγi2 correlates with Yxxϕ sorting motif-independent cargo selections.

2.4 Nuclear Localized PIPKs Regulate Gene Expression and mRNA Processing

2.4.1 Nuclear PIP Kinases and Phosphoinositides

Much as how the discovery of the phosphatidylinositol (PI) cycle became fundamental to our understanding of signal transduction and overall cell biology (Gurr et al. 1963; Hokin and Hokin 1953; Kleinig 1970), the discovery of nuclear phospholipid signaling has revolutionized our view of the processes regulated by phospholipids (Cocco et al. 1987, 1988). Still, nuclear phospholipid signaling is fulfilled by lipid second messengers and requires an array of coordinated activities from lipid kinases, phosphatases, and effectors. However, the lipid kinases within the nucleus appear to be more selective, in that not all identical lipid kinases exist in the nucleus and the phosphoinositides they generate differ both spatially and temporally. Interestingly, a substantial pool of nuclear phosphoinositides does not appear to be associated with the nuclear membrane (Payrastre et al. 1992). These observations are helping to unravel another facet of phospholipid signaling found in cells.

2.4.1.1 PIPKs and PIP_2 at the Nuclear Envelope

Continuous with the ER, the nuclear envelope is a bilayered membranous structure. Pioneering studies on isolated rat liver nuclei revealed the existence of PI and PIP pools in the nuclear envelope (Smith and Wells 1983a). A recent study in sea urchin sperm identified an atypical composition of polyphosphoinositides in the nuclear membrane, as much as 51% of the total phospholipids, an enrichment conserved in mammalian sperm (Garnier-Lhomme et al. 2009). Incubation of isolated nuclear envelopes with [γ–^{32}P]ATP resulted in rapid labeling of phopholipid

products, later identified as phosphatidylinositol 4-phosphate (PI4P), phosphotidylinositol 4, 5-bisphosphate (PI4,5P_2), and phosphatidic acid (PA), suggesting nuclear envelopes contain PIK, PIPK, and diacylglycerol kinase (DGK) activity (Smith and Wells 1983b). Additionally, substantial evidence demonstrates the requirement of PIP_2, diacylglycerol (DAG), and phospholipase C (PLCγ) in the membrane fusion events leading to nuclear envelope formation during mitosis (Dumas et al. 2010). In conjunction with this, PKC βII was identified as a mitotic lamin kinase and its phosphorylation of lamin B is critical for nuclear envelope disassembly, providing evidence that phosphoinositde signaling extended from the nuclear membrane into the nucleus (Goss et al. 1994; Thompson and Fields 1996). The hydrolysis of PIP by phosphomonoesterases and a PIP-dependant ATPase are also associated with the nuclear envelope (Smith and Wells 1984a, b). Besides the canonical PIPKs, phosphatidylinositol 3-kinase (PI3K) may also produce PI3,4P_2 at the nuclear surface and therefore act as a "PIPK" (Yokogawa et al. 2000). These studies suggest that a crucial phosphotidylinositide metabolic cycle is present at the nuclear membrane and is required for normal cellular processes. Interestingly, PIPKs and PIP_2 were not found to be associated with the invaginations of the nuclear envelope (Boronenkov et al. 1998), indicating that PIPK activity and PIP_2 production are temporally and spatially regulated. At present, it is unclear which phosphoinositides (other than PI4P, PI3,4P_2, and PI4,5P_2) and which PIPKs are involved in the synthesis of phosphoinositides at the nuclear envelope and even less is known about how nuclear PIPKs are regulated.

2.4.1.2 The Intra-nuclear PIPKs and PIP_2

Studies by Cocco et al. in the late 1980s revealed that not all PIPKs in the nucleus appeared to be associated with the nuclear membrane. Using highly purified nuclei from mouse erythroleukemia (MEL) and Swiss 3T3 cells, nuclear membranes were stripped away using detergents and the remaining matrix was able to produce both PI4P and PIP_2 (Cocco et al. 1987, 1988). The existence of phosphoinositides in non-membranous structures within the nucleus raised the question of where these second messengers and the enzymes that generate them localize. Plausible hypotheses include the nuclear matrix/nucleoskeleton, chromatin, and protein complexes that are associated with these nuclear microenvironments (Fig. 2.9). Payrastre and colleagues showed nuclear matrix-associated PI4 K, PIPKIs, DGK, and PLC in mouse NIH 3T3-fibroblasts and rat liver cells (Payrastre et al. 1992). Using laser scanning confocal microscopy and immunofluorescence staining, PIP, PIPKIα (PIPKIβ in mouse), PIPKIIα (PIP4Kα), PIPKIIβ (PIP4Kβ), PI(4,5)P_2, and PI(3,4)P_2 were identified at nuclear speckles (interchromatin granule clusters) (Boronenkov et al. 1998). However, the amount of PI4P is about 20-fold higher than that of PI5P in nucleus, and [γ–^{32}P]ATP incubation with isolated rat liver nuclei determined the relative labeling ratio for PI4,5P_2 generation at the 5 vs. 4 OH position was ~ 1.8, suggesting that PIPKIs are the major kinase for PIP_2 synthesis within the nucleus (Keune et al. 2010; Vann et al. 1997).

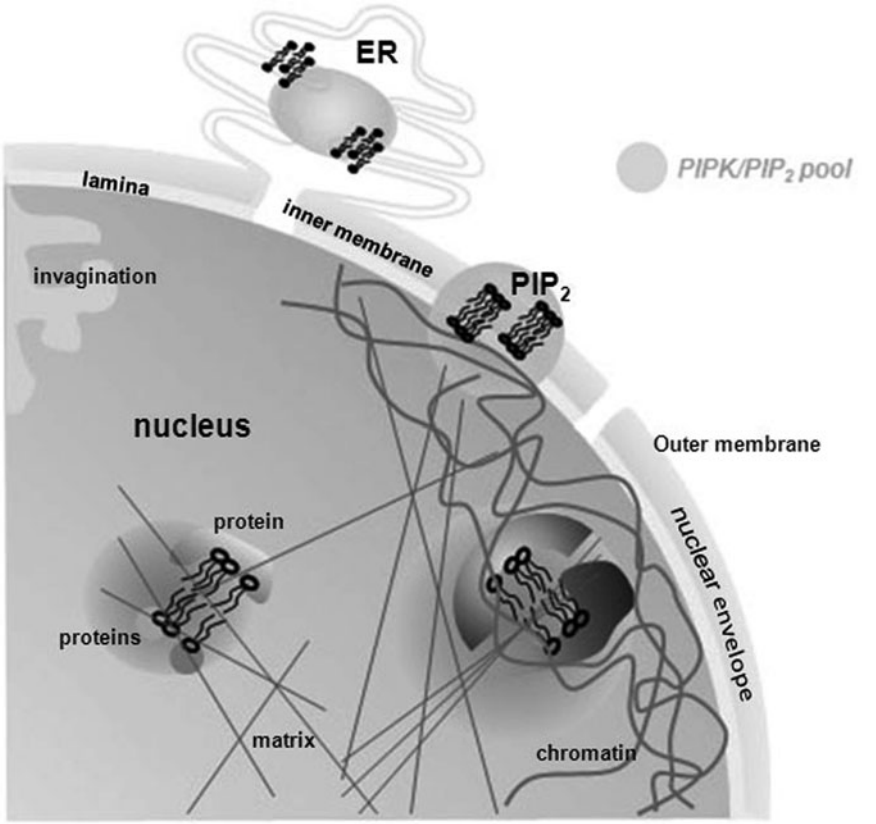

Fig. 2.9 The nuclear PIPKs and PIP_2 pools. The nuclear envelope continues from the ER and contains PIP_2 as well as associated PIPK activities in both outer and inner bilayers. The interchromatin granule clusters (nuclear speckles) nest another PIPK and PIP_2 pool. Some protein complexes docked on the nuclear matrix may also serve to recruit PIPK and PIP_2 or that the PIP_2/PIPK can regulate the assembly of such matrices

So far, nuclear PIPKIα has been identified in complex with a *s*peckle *t*argeted PIPKI*a*lpha *r*egulated-*p*oly(*A*) *p*olymerase (Star-PAP), a PIP_2-sensitive protein kinase, CKIα, and mRNA processing factors (Mellman et al. 2008). The yeast Mss4p (mammalian PIPK homologue) contains a functional nuclear localization signal (NLS) and can shuttle between the cytoplasm and the nucleus (Audhya and Emr 2003). However, it remains to be determined whether PIPKIα can shuttle between the cytoplasm and nucleus or is capable of generating the majority of the nuclear PIP_2. Recently, another PIPKI family member, PIPKIγi4, was identified in the nucleus (Schill and Anderson 2009b). PIPKIγi4 is the only PIPKIγ subfamily member to be detected in the nucleus though its function remains to be characterized. PIPKIIβ predominantly localizes in the nucleus in certain cell lines owing to a specific 17-amino-acid α-helix insertion (Ciruela et al. 2000). PIPKIIα also partially localizes

Table 2.2 The identified nuclear PIP kinases

PIPK	Substrate	Product	Localization	Reference
PIPKI α	PI4P	PIP_2	Nuclear speckle	Boronenkov et al. 1998; Mellman et al. 2008
PIPKIγi4	PI4P	PIP_2	Nucleus	Schill and Anderson 2009
PIPKII β/α	PI5P	PIP_2	Nucleus	Boronenkov et al. 1998; Ciruella et al. 2000; Bultsma et al. 2010

to the nucleus via PIPKIIβ an interaction that is indispensible for protecting PIPKIIβ from degradation via the nuclear ubiquitin ligase, Cul3-SPOP (cullin 3-speckle-type POZ domain protein) (Bultsma et al. 2010; Bunce et al. 2008). It appears that the principle role of nuclear PIPKIIβ is to regulate PI5P levels rather than generate PIP_2 (Bultsma et al. 2010; Keune et al. 2010). The different PIPK isoforms in the nucleus and potentially different localizations of these kinases (Table 2.2) suggest the production of PIP_2 is spatially and temporally regulated, which could result in differential control of the cellular signaling and biological functions.

2.4.2 Regulation and Functions of the Nuclear PIPKs

Compared to the vast amount of data detailing the functions of PIPK and PIP_2 in the cytoplasm, the nuclear functions of these molecules are less understood. Emerging data indicate that nuclear PIP_2 regulates diverse processes including stress response, cell cycle control and mitosis, transcription, mRNA processing and export, DNA repair, chromatin remodeling, and gene expression (Barlow et al. 2010; Bunce et al. 2006b; D'Santos et al. 1998; Gonzales and Anderson 2006; Irvine 2003; Keune et al. 2010). It is becoming clearer that specific nuclear PIPKs produce localized PIP_2 in response to different external and internal signals. Nuclear PIPKs could be associated with scaffolds containing other proteins including effector proteins or even phospholipids, which are organized into the nuclear matrix. The resources of PIPs can be supplied locally or delivered by phosphoinositide-carrier proteins. Upon PIPK stimulation and PIP_2 production, conformational/stoichiometric changes in effector proteins could lead to their activation and the induction of nuclear biological processes (Bunce et al. 2006a).

2.4.2.1 Regulation of the Nuclear PIPKIα

PIPKIα has been shown to be targeted to nuclear speckles where it complexes with the non-canonical poly(A) polymerase Star-PAP, CKIα, RNA polymerase II, and splicing factors (Gonzales et al. 2008; Mellman et al. 2008). The PIPKIα C-terminus directly interacts with Star-PAP. It regulates the expression of select genes induced by oxidative stress through the generation of PIP_2 and modulation of Star-PAP activities at the 3′-end of pre-mRNAs (Mellman et al. 2008). However, the upstream

regulator of this PIPKIα intra-nuclear activity has not yet been identified. CKIα is one of the potential site-specific regulators of PIPKIα, but it needs another protein kinase for priming phosphorylation of PIPKIα. Another possibility could be PIP_2 downstream signaling molecules, which can mediate PIPKIα activities by a feedback mechanism. PIPKIα also associates with pre-mRNA splicing factors at nuclear speckles (Boronenkov et al. 1998; Mellman et al. 2008). Speckles are storage sites for these factors whose structure dynamically changes during the cell cycle concomitantly with changes in nuclear PIP_2 levels (Clarke et al. 2001; Lamond and Spector 2003). Interestingly, PIP_2 and PI3K all assemble in nuclear speckles (Boronenkov et al. 1998; Didichenko and Thelen 2001; Osborne et al. 2001). These observations suggest multiple roles for nuclear PIPKIα and PIP_2 in addition to mediating Star-PAP activity.

The tumor suppressor retinoblastoma protein RB (pRB) is another PIPKIα regulator. pRb interacts with and highly activates PIPKIα in a large T antigen-regulated manner (Divecha et al. 2002). In line with this, PIP_2 is sufficient to target the chromatin remodeling complex BAF, which can then be recruited to gene transcription sites by pRB (Zhao et al. 1998), and may further promote an interaction between chromatin and the nuclear matrix via the BAF complex subunit BRG-1 (Rando et al. 2002). The results implicate a role of PIPKIα in transcriptional control and in the regulation of proteins via their ability to bind PIP_2. PIP_2 can also bind histones H1 and H3 alleviating the suppression of transcription (Yu et al. 1998). These studies suggest PIPKIα and its product PIP_2 are involved in the regulation of different phases of gene transcription.

2.4.2.2 Regulation of the Nuclear PIPKIIβ/IIα

PIPKIIβ in combination with PIPKIIα (as addressed formerly) potentially contribute to the generation of a second nuclear PIP_2 pool. Studies in MEL cells demonstrate that the function of PIPKII in the nucleus is mainly to regulate nuclear PI5P levels, because depletion of PIPKIIβ by RNAi increased PI5P levels while overexpression of PIPKIIβ decreased the nuclear PI5P levels (Jones et al. 2006). Like nuclear PIP_2, nuclear PI5P levels also change during the cell cycle (Clarke et al. 2001), implying a role of PIPKIIβ and PI5P in cell cycle regulation. PIPKIIβ can be activated by phosphorylation at Ser326 by p38-MAPK in response to UV irradiation resulting in nuclear accumulation of PI5P (Jones et al. 2006). The Cul3-SPOP ubiquitin ligase complex also regulates PIPKIIβ levels and PI5P production (Bunce et al. 2008). In concert with PIPKIIβ, the type I PI4,5P_2 4-phosphatase converts nuclear PIP_2 to PI5P and therefore modulates PI5P levels (Barlow et al. 2010; Bunce et al. 2008; Zou et al. 2007). PI5P can induce the activation of the p38-MAPK pathway and stimulate Cul3-SPOP activity for PIPKIIβ ubiquitylation (Bunce et al. 2008). As a PIPKIIβ interacting partner, PIPKIIα nuclear targeting seems to be required for suppressing the Cul3-SPOP-dependent PIPKIIβ ubiquitylation, because expression of the kinase-dead PIPKIIα and a nuclear targeting-defective PIPKIIα were not

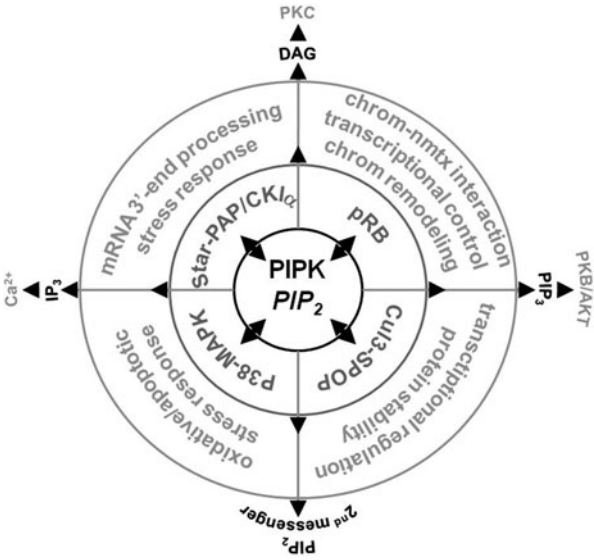

Fig. 2.10 PIPKs generate PIP_2 in the nucleus to control many signaling events. Nuclear PIPKs and PIP_2 regulate different yet specific cellular molecules and processes through interactions with select nuclear proteins or by producing other second messengers. chrom=chromatin; nmtx=nuclear matrix

able to suppress ubiquitylation of PIPKIIβ, whereas overexpression of the wild-type PIPKIIα did (Bultsma et al. 2010). An extension of PIPKIIβ regulated signaling lies in that PI5P can interact with inhibitor of growth protein 2 (ING2) and regulate the localization of ING2 to chromatin. ING2, in turn, modulates the acetylation of the tumor suppressor protein p53, linking PIPKIIβ activities to transcriptional regulation and gene expression (Bultsma et al. 2010; Jones et al. 2006).

2.4.3 Downstream Signaling of the Nuclear PIPK and PIP_2

As discussed above, PIP_2 can directly regulate interacting proteins or it can be processed to generate other second messengers including PIP_3 (Fig. 2.10). Class I and class II PI3Ks have been found in nucleus with the class IA PI3K being responsible for generating the majority of nuclear PIP_3 [reviewed in (D'Santos et al. 1998; Gonzales and Anderson 2006)]. Another bifurcated signaling pathway from nuclear PIP_2 is mediated by the nuclear phosphoinositide-specific phospholipase C (PI-PLC). PI-PLCs break down PIP_2 into DAG and inositol-1,4,5-triphosphate (IP_3). DAG is a direct activator of nuclear PKCs, which are known regulators of gene transcription, DNA synthesis, stress response, mitosis and cell cycle progression (D'Santos et al. 1998). IP_3 receptors have been identified on both the outer and inner nuclear membrane (Humbert et al. 1996). The nuclear IP_3 is recognized to be involved in Ca^{2+} import into nucleus and nuclear Ca^{2+} homeostasis, processes important for gene expression, DNA synthesis and repair, chromatin condensation, protein import, and apoptosis (Bading et al. 1997; D'Santos et al. 1998; Hardingham et al. 1997; Malviya and Rogue 1998) (Fig. 2.10).

The PIPK and PIP_2 downstream signaling cascades are far more intricate than we have thought so far. Identification of more PIPK regulators and effectors as well as

other roles for PIPKs, perhaps even independent of their kinase activity, will enhance our understanding of phosphoinositide signaling in the nucleus.

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