CHAPTER 1

General introduction
Phosphoinositides

This thesis is concerned with phosphoinositides, a group of phospholipids with diverse and often essential cellular functions. The basic building block for phosphoinositides is phosphatidylinositol (PtdIns), which comprises a hydrophilic inositol head group linked by a phospho-diester bond to two fatty acid chains. The most common fatty acid chains found in phosphoinositides are stearic acid (saturated) in the SN1 position and arachidonate (unsaturated with 4 double bonds) in the SN2 position (Figure 1A). Recent studies using mass spectroscopy have, however, established a complex variation in the presence of these fatty acid moieties (1-2). The inositol head group of phosphoinositides can be reversibly phosphorylated, which is important for their function as versatile second messengers. Mono-, bis- and trisphosphorylation at the 3, 4 and 5 positions of the inositol ring can all occur and to date, seven phosphorylated versions of PtdIns have been identified: PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,4)P2, PtdIns(3,5)P2, PtdIns(4,5)P2 and PtdIns(3,4,5)P3 (Figure 1B).

PtdIns have important roles in signalling and can serve as precursors for the generation of new second messengers. For example, PtdIns(4,5)P2 is a precursor of inositol trisphosphate (IP3), diacylglycerol (DAG) and PtdIns(3,4,5)P3. Additionally, PtdIns can recruit proteins and determine their localisation and downstream signalling through

Figure 1. Phosphoinositide metabolism. A. Structure of Phosphatidylinositol. The inositol headgroup with numbered positions, is connected to stearic acid in the SN1 position and arachidonate in the SN2 position. B. 7 phosphorylated versions of Phosphatidylinositol. The arrows represent possible phosphorylation and dephosphorylation reactions carried out by phosphatidylinositol kinases and -phosphatases. Depicted are the kinases (blue) and phosphatases (orange) involved in regulating the abundance of PtdIns(4,5)P2.
specific phosphoinositide-interacting domains (3). PtdIns are minor constituents of membranes and reside in a variety of cellular structures such as the plasma membrane, the Golgi, the Endoplasmatic Reticulum, exosomes (4), as well as the nucleus (5-6). In the nucleus, phosphoinositides may exist in the intranuclear membrane but also in non-membranous structures. Depending on their intracellular location, phosphoinositides regulate specific processes, such as gene transcription, cell migration, vesicular trafficking, cell survival, ion-channel function, endocytosis and exocytosis. They also contribute to the integrity of and dynamic changes in the cytoskeleton.

PtdIns can be phosphorylated by lipid kinases and phosphorylated PtdIns can be dephosphorylated by lipid phosphatases (Figure 1B). To date, more than 90 phosphoinositide regulators have been identified, including lipid kinases and phosphatases, lipases and phosphoinositide transfer proteins. PtdIns exist in all eukaryotes and many phosphoinositide regulators are conserved throughout evolution and can be found in animals (metazoa), fungi and plants (7). Regulation of the abundance of phosphoinositides is crucial for the existence and development of many eukaryotes and deregulation of phosphoinositides and their modifiers can lead to severe diseases such as cancer, myotubular myopathies and Lowe’s syndrome (8-11).

PtdIns5P and phosphatidylinositol-5-phosphate 4-kinases

This thesis focuses on PtdIns5P and the phosphatidylinositol-5-phosphate 4-kinases (PIP4Ks) that can regulate the abundance of this phosphoinositide. PtdIns5P is the latest addition to the phosphoinositide family, since its discovery by Rameh et al. in 1997. PtdIns5P exists in mammalian cells, plants, worms and flies (12-15). Although PtdIns5P has been suggested to function as a second messenger, it is not clear exactly how this occurs (12). The abundance of PtdIns5P is comparable to the level of PtdIns3P and relatively low under resting conditions compared to the level of PtdIns4P. However, its level increases upon several stimuli such as insulin (16), thrombin, (17) oxidative stress (18), dehydration and osmotic stress in mammalian cells (19) and in plant cells (15). PtdIns5P abundance also changes in the nucleus as cells progress through the cell cycle (14).

To understand signalling functions of PtdIns5P, we must know how and where the abundance of PtdIns5P is regulated. There are several ways in which cellular PtdIns5P can be generated or modulated: 1. PIP4Ks can phosphorylate PtdIns5P to generate PtdIns(4,5)P2 and thereby reduce PtdIns5P levels (12, 18) 2. PtdIns5P can be generated by members of the Myotubulin family of phosphatases that can dephosphorylate PtdIns(3,5)P2 (20-21). 3. Two PtdIns(4,5)P2 phosphatases can dephosphorylate PtdIns(4,5)P3 to generate PtdIns5P (22-23). 4. The lipid kinase PYKfyve phosphorylates PtdIns to generate PtdIns5P (24-25). Which route for PtdIns5P synthesis is more prevalent may be tissue-, cell-type and organelle dependent and reliant on the availability of the enzymes involved. A PtdIns-5-kinase dedicated to PtdIns5P generation has not been
identified in vivo, but PIP5Kinase and PYKfyve both phosphorylate PtdIns in vitro (24, 26). In vivo however, the data remain controversial. PYKfyve knockout MEFs show decreased levels of PtdIns5P (27) while studies in other cell types showed no decrease in PtdIns5P levels using inhibitors of PYKfyve and shRNA mediated knock down of the enzyme (28). Furthermore, measurement of the levels of PtdIns5P in PYKfyve knockout MEFs in our laboratory failed to confirm the decrease in PtdIns5P levels. Intracellular PtdIns5P can also be generated upon infection by Shigella flexneri (29-30) through the bacterial 4-phosphatase IpgD by dephosphorylation of PtdIns(4,5)P$_2$. Finally, exposure of cells to UV irradiation or hydrogen peroxide (H$_2$O$_2$) induces the synthesis of PtdIns5P in part by inducing the interaction between PIP4K and the prolyl isomerase Pin1 (31).

Several studies have indicated functions for PtdIns5P both in the cytosol and in the nucleus, although endogenous binding proteins and downstream targets are still ill-defined. Generation of PtdIns5P by the bacterial phosphatase IpgD results in EGFR activation and subsequent phosphorylation of Akt was shown to regulate vesicular trafficking (32). An increase in PtdIns5P was observed in cells transformed with the oncogenic tyrosine kinase NPM-ALK (nucleophosmin anaplastic lymphoma kinase), suggesting a possible role for PtdIns5P in NPM-ALK induced oncogenesis (33) and Oppelt et al. (34) recently implicated PtdIns5P in regulating cell migration. They suggested that MTMR3 and PYKfyve act in concert to regulate the levels of PtdIns5P via the generation and dephosphorylation of PtdIns(3,5)P$_2$ (Figure 2).

**Figure 2.** Inputs and processes that can regulate the abundance of PtdIns5P are presented in the top square. Known phosphoinositide kinases and phosphatases that can generate PtdIns5P are presented in the bottom square.
PtdIns5P in the nucleus

Since the discovery of the existence of a phosphoinositide cycle in the nucleus, much research has focused on specific nuclear functions for PtdIns including PtdIns5P. The abundance of nuclear PtdIns5P increases upon oxidative stress and UV treatment and also during the cell cycle (14, 18), but what are the implications of these increases? The discovery of two proteins that interact with PtdIns5P, Inhibitor of growth protein 2 (ING2) and Arabidopsis Trithorax-like protein (ATX1), has given insight into possible nuclear functions for PtdIns5P. Both ING2 and ATX1 can associate with PtdIns5P via their plant homeodomain (PHD) finger, a Cys\textsubscript{4}-His-Cys\textsubscript{3} motif, generally found in nuclear proteins. ING2 regulates acetylation of the tumour suppressor p53, which is increased by PtdIns5P to activate p53 in a stress-dependent manner (18, 35). p53 is regarded as the guardian of the genome, since it controls the DNA damage response. Knowing how p53 is activated is important in understanding how cells execute death pathways in health and disease. The trithorax homologue ATX1 is a SET domain-containing histone methyltransferase and is a master controller of plant development and flowering (36-37). It also regulates gene transcription in response to environmental stress. Drought stress in Arabidopsis induces an increase in PtdIns5P, that is mediated by the plant homologue of myotubularin, a PtdIns(3,5)P\textsubscript{2} 3-phosphatase. The increase in PtdIns5P leads to a decrease in methylation of lysine 4 on histone 3 (H3K4me3) at the promoter of WRKY70, a gene regulated both by ATX1 and drought stress. The decrease in methylation appears to be a consequence of increased PtdIns5P leading to a decrease in the levels of ATX1 associated with promoters and causes a change in the localisation of ATX1 from the nucleus to the cytoplasm, both of which depend on the integrity of the PHD finger (15).

Emerging mechanistic links between phosphoinositides and chromatin modifying enzymes, like ING2 and ATX1 and PtdIns5P may explain how phosphoinositides regulate gene transcription and chromatin structure (38-39).

**PIP4K regulation and signalling**

PIP4Ks phosphorylate PtdIns5P on the 4-position of the inositol ring to generate PtdIns(4,5)P\textsubscript{2}, and in that way they can modulate the abundance of PtdIns5P. There are three isoforms (40-43) of which PIP4K2A and PIP4K2B have been shown to phosphorylate PtdIns5P and PtdIns3P, whereas PIP4K2C appears to be inactive. Deletion of the gene encoding PIP4K2B in mice has no effect on their development or survival, but compared to wild type mice, the knock-out mice are better in controlling their blood glucose levels and do not gain weight when fed diets high in fat (44). PIP4K2B knock-out mice also show increased insulin-induced Protein Kinase B (PKB) activation in muscle and overexpression of PIP4K2B in cells attenuates insulin induced PKB activation (45).

Overexpression of IpgD, a bacterial 4-phosphatase, also increased PKB activation (45). The data point to a role for PtdIns5P and PIP4K in PKB regulation and activation (46). PKB plays an important role in glucose metabolism, cell survival, apoptosis, gene transcription
and migration. PIP4Ks may be implicated in PKB signalling via regulation of PtdIns(3,4,5)P3 (important for localisation and activation of PKB) or direct dephosphorylation of PKB, but the direct mechanism remains unknown. PIP4K activity can be inhibited via activation of the p38 MAPK stress pathway, which leads to increased nuclear PtdIns5P. Inhibition of PIP4K occurs through the direct phosphorylation of PIP4K2B at Ser326 by p38 (18).

An interesting nuclear interaction partner for PIP4K2B was identified by Bunce et al. Using a yeast two hybrid approach, they identified an interaction between PIP4K2B and SPOP, a nuclear speckle associated protein involved in the recruitment of proteins to Cul3-based ubiquitin ligases. They showed that activation of the p38MAPK pathway stimulated the activity of Cul3-SPOP towards substrates and that an inactive version of PIP4K2B stimulates ubiquitination of Cul3-SPOP substrates, suggesting that PtdIns5P stimulates Cul3-SPOP ubiquitin ligase activity. Promoting ubiquitin ligase activity to regulate protein function would be an interesting nuclear function for PtdIns5P, however, additional studies on endogenous targets remain to be identified. Further aspects of PIP4K regulation and signalling are described in chapter 2 and chapter 5 of this thesis.

**Scope of this thesis**

In this thesis, we focused on PtdIns5P and the lipid kinases that can modulate and regulate the cellular abundance of PtdIns5P, the phosphatidylinositol-5-phosphate 4-kinases (PIP4Ks). In Chapter 2, we review oxidative stress signaling, PIP4Ks and modulation of PtdIns5P and PIP4Ks by the prolyl-isomerase Pin1. Mouse embryonic fibroblasts (MEFs) derived from Pin1 -/- mice are resistant to cell death induced by exogenous H2O2 (47). Since phosphoinositides have roles in oxidative stress signalling, we investigated whether Pin1 can regulate phosphoinositide abundance to control adaptive responses and sensitivity to oxidative stress (Chapter 3). We show that upon H2O2 exposure, MEFs derived from Pin1 -/- mice have increased cell viability and an increased abundance of PtdIns5P compared to wild type MEFs. The discovery that Pin1 directly regulates phosphoinositide signalling is novel and may help to understand the complex phenotypes observed in Pin1 knockout mice. In Chapter 4, we continued to investigate Pin1 and phosphoinositides by analyzing whether phosphoinositides can directly associate with Pin1 to alter Pin1 signalling. To evaluate binding of Pin1 to phosphoinositides, we used Surface Plasmon Resonance (SPR). We determined whether manipulation of the abundance of intracellular PtdIns(4,5)P2 affects Pin1 function by measuring its effects on transcriptional output from the CDKN1B and CCND1 promoters. In the review presented in Chapter 5, functions of nuclear phosphoinositides are described and we underline how and why nuclear phosphoinositides differ from their cytosolic counterparts. PIP4Ks have been implicated in a number of cytosolic and nuclear pathways that involve stress-signaling and activation of PKB. However, to date only a few proteins have been identified that can interact with and regulate PIP4Ks. In Chapter 6, we search for proteins that can
regulate PIP4K2B and discover that PIP4K2B interacts with PIP4K2A. We determine that PIP4K2A is 2000-fold more active than PIP4K2B and that PIP4K2B can target PIP4K2A to the nucleus. Intracellular pairing of the two isoforms may be important for their physiological and pathological roles. Mutations in PIP4K2A have been associated with the development of schizophrenia (48) and the gene encoding for PIP4K2B (PIP4K2B) can be amplified in a subset of breast cancers (49). Aside from those studies, little is known about pathological roles for PIP4Ks. Breast cancer etiology is complex, involving the deregulation of many signaling pathways. To stratify groups of breast cancer patients, it is important to identify new molecular markers or drug targets to allow specific and better treatment. In Chapter 7, we focused on PIP4K2B in breast cancer and describe the characterization and utilization of a PIP4K2B specific antibody to interrogate tissue micro-arrays derived from breast cancer patients. We determine PIP4K2B expression in 489 tumours from late-stage disease patients and found a correlation between several clinico-pathological parameters and PIP4K2B expression. Overall, low expression of PIP4K2B correlates with a worse prognosis for breast cancer patients. Next, we sought to determine underlying mechanisms that may in part explain the relationship between low/no expression of PIP4K2B and poor patient prognosis. We therefore tested the effect of PIP4K2B knock-down in the MCF10A normal like mammary cell-line and observed that cells became ‘primed’ for epithelial mesenchymal transition (EMT). EMT is an important process in normal development, enabling cell migration and the consequent differentiation and generation of new organs and tissues. It has also been associated with the development of cancer metastasis and invasion, since many oncogenic pathways may induce EMT. The observed features of PIP4K2B knock down cells towards an EMT phenotype may explain the correlation between low PIP4K2B expression and patient survival.

References

General introduction


