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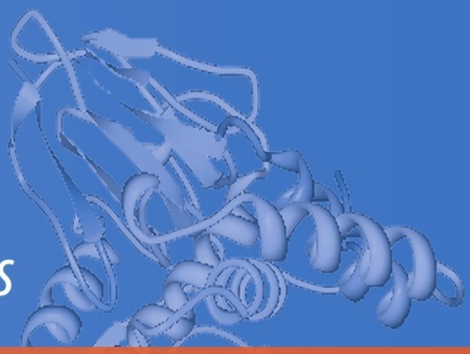

Phosphoinositides I: Enzymes of Synthesis and Degradation

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Preface

When I was approached to shape a book about phosphoinositide signaling, I first felt honored and humbled. On second thought, this appeared to be an impossible task. Phosphoinositides have grown from being just a curious lipid fraction isolated from bovine brain, showing increased radioactive metabolic labeling during intense stimulation protocols, to become the focus of immense interest as key regulatory molecules that penetrate every aspect of eukaryotic biology. The expansion of this field in the last three decades has been enormous: it turned from a basic science exercise of a devoted few to highly translatable science relevant to a large number of human diseases (isn't this the nature of good basic science?). These include cancer, metabolic-, immuno- and neurodegenerative disorders, to name just a few. Reviewing the large number of enzymes that convert phosphoinositides would fill a book—let alone the diverse biological processes in which phosphoinositides play key regulatory roles. Given the interest, a collection of up-to-date reviews compiled in a book is clearly warranted, which was enough to sway me to accept this assignment. As one editor is unable to handle this enormous task, I was delighted when Matthias Wymann and John York were kind enough to join me in this ambitious effort.

When thinking about potential authors, the obvious choice would have been to approach the people whose contributions have been crucial to push and elevate this field to the level it is today. Bob Michell, prophetically placed phosphoinositides in the center of signal transduction in a 1975 Biochem. Biophys. Acta review (Michell 1975), Michael Berridge had a key role in linking phosphoinositides and Ca^{2+} signaling and whose fascinating reviews have inspired many of us (Berridge and Irvine 1984). Robin Irvine, whose group found that InsP_3 was a mixture of two isomers, the active Ins(1,4,5)P_3 and an inactive Ins(1,3,4)P_3, and who described the tetrakisphosphate pathway (Irvine et al. 1986), and who always challenges us with most provocative ideas. Philip Majerus, who has insisted on the importance of inositide phosphatases (Majerus et al. 1999) very early on. The group of Lewis Cantley, with the discovery of PI 3-kinase activities and the mapping of downstream effectors (Whitman et al. 1988; Franke et al. 1997), or the Waterfield lab where the first PI 3-kinase catalytic subunit was isolated and cloned (Otsu et al. 1991; Hiles et al. 1992). Peter Downes, who recognized the translational value of phosphoinositide research. Jeremy Thorner and Scott Emr, whose work in baker's yeast still forms the

foundation of our understanding of the role of inositol lipids in trafficking (Strahl and Thorner 2007) or Pietro De Camilli, whose group documented the central role of inositides in brain and synaptic biology (Cremona et al. 1999). There are many others who made valuable or even greater contributions to phosphoinositide research. The above list reflects my bias, as these researchers had the largest impact on my thinking and the directions of my work. Research is, however, a constantly evolving process and we (now Matthias and John being involved) wanted to involve contributions of scientists who represent a second or third wave of researchers infected with the interest in phosphoinositides. We made an effort to recruit authors who have been trainees of these founding laboratories. With this selection our goal was to sample the view of the current and future generation. By selecting their trainees, we feel that we pay tribute to the "Founding Fathers", and show that the research they put in motion is alive and continues with fresh ideas, new ambitions and a translational and therapeutic value.

Phosphoinositide research in the 1980s went hand in hand with research on Ca^{2+} signaling pursued in "non-excitable" cells and was also marked with the discovery of the family of protein kinase C enzymes, regulated by diacylglycerol, one of the products of phosphoinositide-specific phospholipase C enzymes. These areas of research developed and expanded to form their own fields, and could not be discussed here in detail—even though they are linked historically to the development of phosphoinositide signaling. The enormous work of the groups of Yasutomi Nishizuka on protein kinase C, and Katsuhiko Mikoshiba on cloning and characterizing the Ins(1,4,5)P_3 receptors are prime examples of these achievements. Although we could not cover all these areas, we included a chapter on Ca^{2+} signaling via the Ins(1,4,5)P_3 receptor by Colin Taylor, a trainee of the Michael Berridge's lab, where important links between Ca^{2+} release and Ins(1,4,5)P_3 receptor signaling were discovered. We also decided to allocate some space to inositol phosphates, the soluble counterparts of some of the phosphoinositides. These molecules for long had been viewed only as the metabolic products of the second messenger Ins(1,4,5)P_3 but recently gained significant prominence as regulators of important physiological processes. With the discovery of the highly phosphorylated and pyrophosphorylated inositols and the enzymes that produce them, it became clear that this system represents a whole new regulatory paradigm with exciting new developments.

Finally, it was a difficult dilemma whether to include a Chapter on the early history of phosphoinositides. We decided against it for a number of reasons. First, the really interesting history is traced back to studies that preceded the landmark 1975 Bob Michell review and included the work of the Hokins (1987), Bernard Agranoff (2009) and other pioneers of phosphoinositide research. Nobody could tell these early developments better than Bob Michell in his several recollections (Michell 1995) or Robin Irvine who commemorated the 20 years of Ins(1,4,5)P_3 and the period leading to its discovery (Irvine 2003). We encourage the young readers to go back and read these recollections, as they show several examples of how seemingly uninspiring observations formed the beginning of something that became huge as it unfolded. What came after these landmark discoveries is so overwhelming that each one of us has own views and subjective memories and stories to tell on some aspects of

it. As Editors we felt that our views should not be elevated above others on these historical aspects, and leave it to the authors of the individual Chapters to elucidate the diversity in this respect. The only exception is a Chapter on the history of PI 3-kinases by Alex Toker that we felt deserves special emphasis as it had the most transforming impact on the field since the late 1980s.

One needs to understand that selection of authors is a subjective process and does not always reflect on who contributed the most in a selected field. However, we are confident that proper credit is given in the individual Chapters to each groups and individuals whose work has moved this field forward. It should also be understood that a field that generates over 10,000 entries in PubMed with each keyword that relates to phosphoinositides cannot be covered without missing some aspects that could be important. However, we trust that this collection will be found useful for both the experts and the novices.

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Contents

Abbreviations

AD	Alzheimer's disease
AMPK	5′-AMP-activated protein kinase
ALL	Acute lymphocytic leukemia
ALS	Amyotrophic lateral sclerosis
AML	Acute myeloblastic leukemia
ARNO	Arf nucleotide binding site opener
ASK1	Apoptosis signal-regulating kinase 1
ATM	Ataxia telangiectasia mutated
ATX	Arabidopsis trithorax 1
Bad	Bcl-XL/Bcl-2-associated death promoter
BAFF	B cell activation factor of the TNF family
BCR	B cell receptor
Bcr/Abl	Break point cluster region/Abelson kinase fusion protein
Btk	Bruton's tyrosine kinase
c-Kit	Stem cell growth factor receptor
CAD	Caspase activated DNase
CCR(L)	C-C chemokine receptor (ligand) type
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CERT	Ceramide transfer protein
CIN85	Cbl-interactin protein of 85kD (also Ruk (regulator of ubiquitous kinase), SETA (SH3 domain-containing gene expressed in tumorigenic astrocytes))
CML	Chronic myeloid leukemia
CMT	Charcot-Marie-Tooth
COPI/II	Coatomer protein complex I/II
CXCR(L)	C-X-C chemokine receptor (ligand) type
DAAX	Death domain-associated protein
DAG	Diacylglycerol
DGK	Diacylglycerol kinase
DH	Dbl-homology
DMSO	Dimethyl sulfoxide

DNA-PK_{cs}	DNA-dependent protein kinase, catalytic subunit
DOCK2	Dedicator of cytokinesis 2
Dpm1	Dolichol phosphate mannosyltransferase
EGF(R)	Epidermal growth factor (receptor)
eEF1A	Eukaryotic elongation factor 1A
eIF4E	Elongation initiation factor 4E
EMT	Epithelial-to-mesenchymal transition
EnaC	Epithelial sodium channel
ER	Estrogen receptor, or endoplasmic reticulum
ErbB1	Epidermal growth factor receptor
ERM	Ezrin/radexin/moesin
FAK	Focal adhesion kinase
FAPP1	Phosphoinositol 4-phosphate adaptor protein 1
FAPP2	Phosphoinositol 4-phosphate adaptor protein 2
FcεRI	High affinity receptor for Fc fragment of IgE
FOXO	Forkhead transcription factor, class O
FYVE	Fab1, YOTB, Vac1, EEA-1 homology
G6P	Glucose-6-phosphatase
Gab	Grb2-associated binder
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GIST	Gastrointestinal stromal tumors
GK	Glucokinase
GLUT4	Glucose transporter type 4
GM-CSF	Granulocyte and macrophage colony stimulating factor
GPCR	G protein-coupled receptors
GRK2	G protein-coupled receptor kinase 2 (also βARK1 (adrenergic receptor kinase 1)
Grp1	General receptor for phosphoinositides
GSK-3	Glycogen synthase kinase-3
GST-2xFYVE	Glutathione S-transferase-tagged to tandem FYVE domains
HAUSP	Herpesvirus-associated ubiquitin-specific protease
Hdac2	Histone deacetylase 2
HSCs	Hematopoietic stem cells
IκBK	IκB kinase
ING2	Inhibitor of growth protein 2
Inpp5e/INPP5E	72 kDa inositol polyphosphate 5-phosphatase
Ins	*Myo*-inositol
IGF1(R)	Insulin-like growth factor (receptor)
ILK	Integrin-linked kinase
Ins(1,4)P_2	Inositol 1,4-bisphosphate
Ins(1,4,5)P_3	Inositol 1,4,5-trisphosphate; also used $InsP_3$
IPMK	Inositol polyphosphate multikinase
IRS	Insulin receptor substrate

ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus-activated kinase
JNK	Jun N-terminal Kinase
Kv1.3	Voltage-gated K^+ channel
LAT	Linker for activation of T cells
LOH	Loss of heterozygosity
LSCs	Leukemic stem cells
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
MAPKAP-2	Mitogen-activated protein kinase-activated kinase 2
M-CSF	Macrophage colony-stimulating factor
MDM2	Murine double minute 2
MDS	Myelodysplastic syndrome
MEFs	Mouse embryonic fibroblasts
miRNA	Microrna
MPP(+)	1-methyl-4-phenylpyridinium iodide
MSN	Medium sized spiny projection neurons
MTM	Myotubularin
MTMR	Myotubularin related
mTOR	Mammalian target of rapamycin, see also TOR
MVB	Multivesicular body
MVP	Major vault protein
Nedd4	Neural-precursor-cell-expressed developmentally down-regulated 4
NFκB	Nuclear factor κB
NLS	Nuclear localization signal
NMDA(R)	N-methyl-D-aspartate (receptor)
NOS3/eNOS	NO-synthase 3
NTAL	Non-T cell activation linker, also named LAB (Linker of activation for B cells) or LAT2
OSBP	Oxysterol binding protein
OCRL	Oculocerebrorenal syndrome of Lowe
OGD	Oxygen–glucose deprivation
PAO	Phenyalrsine oxide
PCAF	p300/CBP-associated factor
PDE	Phosphodiesterase
PDGF(R)	Platelet-derived growth factor (receptor)
PDZ	Post synaptic density protein, Drosophila disc large tumor suppressor, zonula occludens-1 protein
PDK1	Phosphoinositide-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboxy kinase
PEST	Proline, glutamic acid, serine, threonine
PH	Plecktrin-homology
PHD	Plant homeodomain
PH-GRAM	Pleckstrin homology glucosyltransferase Rab-like GTPase activator

PHTS	PTEN hamartoma tumor syndrome
PI3K	Phosphoinositide 3-kinase; catalytic subunits of class I PI3K are referred to as p110α, p110β, p110γ and p110δ
PI3Kc	PI3K catalytic domain
PI3Kr	PI3K regulatory subunit
PI4K	Phosphatidylinositol 4-kinase
PI4KII	Type II phosphatidylinositol 4-kinase
PI4KIII	Type III phosphatidylinositol 4-kinase
PICS	Pten-loss–induced cellular senescence
PID	Phosphoinositide interacting domain
PIKE	PI-3-kinase enhancer
PIKK	Phosphoinositide 3-kinase-related kinase
PIP4K	Phosphatidylinositol 5-phosphate 4-kinase (also called type II PIP kinase)
PIP5K	Phosphatidylinositol 4-phosphate 5-kinase (also called type I PIP kinase)
PIPP	Proline-rich inositol polyphosphate 5-phosphatase
PIX	PAK-associated guanine nucleotide exchange factor
PKA	Protein kinase A
PKB/Akt	Protein kinase B, also called Akt after the transforming kinase encoded by the AKT8 retrovirus
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PM	Plasma membrane
PML	Promyelocytic leukemia protein
PPI	Polyphosphoinositide
pRB	Retinoblastoma protein
PRD	Proline-rich domain
P-Rex	PtdIns(3,4,5)P_3-dependent Rac exchanger
PSD95	Post synaptic density protein 95
PtdIns	Phosphatidylinositol
PtdIns4P	Phosphatidylinositol 4-phosphate; short PIP
PtdIns3P	Phosphatidylinositol 3-phosphate; PIP should not be used here
PtdIns5P	Phosphatidylinositol 5-phosphate; PIP should not be used here
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate; short PIP_2
PtdIns(3,4)P_2	Phosphatidylinositol 3,4-bisphosphate; the abbreviation PIP_2 should not be used here.
PtdIns(3,5)P_2	Phosphatidylinositol 3,5-bisphosphate; the abbreviation PIP_2 should not be used here.
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-trisphosphate; short PIP_3
PtdOH	Phosphatidic acid (also used PA)
PTEN	Phosphatase and Tensin homolog deleted on chromosome Ten, [also MMAC (mutated in multiple advanced cancers), TEP1 (TGF-β-regulated and epithelial cell enriched phosphatase 1)]

PX	Phox-homology
RAN	Ras-related nuclear protein
RID	Rac-induced recruitment domain
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
RSK	Ribosomal S6 kinase
R-SMAD	Receptor regulated SMAD
RTK	Receptor tyrosine kinase
Rb2	Retinoblastoma-related gene $p130^{Rb2}$
RYR1	Type 1 ryanodine receptor
Sac	Suppressor of actin
SCIP	Sac domain-containing inositol phosphatases
SCV	*Salmonella*-containing vacuole
SGK	Serum- and glucocorticoid-induced protein kinase
SH2	Src homology 2
SHIP	SH2 domain-containing inositol 5'-phosphatase
SID	Set interacting domain
siRNA	Short-interfering RNA
SKICH	SKIP carboxy homology
SKIP	Skeletal muscle and kidney enriched inositol phosphatase
SNP	Single nucleotide polymorphism
SSC	Squamos cell carcinoma
Star-PAP	Poly(A) polymerase
Syk	Spleen tyrosine kinase, member of the Src tyrosine kinase family
TAC	Transverse aortic constriction
TDLU	Terminal ductal lobuloalveolar units
TGFβ	Transforming growth factor β
Tiam	T-lymphoma invasion and metastasis inducing protein
TNF	Tumour necrosis factor
TopoIIα	Topoisomerase IIα
TOR	Target of rapamycin (also called FRAP or mTOR)
TPIP	TPTE and PTEN homologous inositol lipid phosphatase
TPTE	Trans-membrane phosphatase with tensin homology
TRAPs	Transmembrane adapter proteins, link immune-receptors to downstream signaling cascades. Examples: LAT, NTAL/LAB
TSC	Tuberous sclerosis complex
UTR	Untranslated region
Vps34p	Vacuolar protein sorting mutant 34 protein
WASP	Wiskott Aldrich Syndrome protein
Wm	Wortmannin
WT	Wild type

Chapter 1
The Phosphatidylinositol 4-Kinases: Don't Call it a Comeback

Shane Minogue and Mark G. Waugh

Abstract Phosphatidylinositol 4-phosphate (PtdIns4*P*) is a quantitatively minor membrane phospholipid which is the precursor of PtdIns(4,5)P_2 in the classical agonist-regulated phospholipase C signalling pathway. However, PtdIns4*P* also governs the recruitment and function of numerous trafficking molecules, principally in the Golgi complex. The majority of phosphoinositides (PIs) phosphorylated at the D4 position of the inositol headgroup are derived from PtdIns4*P* and play roles in a diverse array of fundamental cellular processes including secretion, cell migration, apoptosis and mitogenesis; therefore, PtdIns4*P* biosynthesis can be regarded as key point of regulation in many PI-dependent processes.

Two structurally distinct sequence families, the type II and type III PtdIns 4-kinases, are responsible for PtdIns4*P* synthesis in eukaryotic organisms. These important proteins are differentially expressed, localised and regulated by distinct mechanisms, indicating that the enzymes perform non-redundant roles in trafficking and signalling. In recent years, major advances have been made in our understanding of PtdIns4K biology and here we summarise current knowledge of PtdIns4K structure, function and regulation.

Keywords Golgi complex · Membrane traffic · Phosphatidylinositol 4-phosphate · Signalling

1.1 Introduction

The phosphatidylinositol kinases (PtdInsKs) of eukaryotes have a long history. Their existence was originally inferred by the discovery of their enzymatic products, the phosphoinositides (PIs), whose synthesis from phosphatidylinositol (PtdIns) in the exocrine pancreas could be induced with cholinergic agonists (Hokin and

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Table 1.1 Mammalian PI4K encoding genes

Locus	Common name	SwissProt/ Protein (UniProt)	Accession nr. Hs	Interacts with	Reference MIM	Gene Map (Hs/Mm)
PIK4CA	PI4K230, PI4K55	PI4KA_ human	NM_058004 NM_002650		(Wong and Cantley 1994) MIM 600286	22q11.21 16 A3
PIK4CB	PI4K92	PI4KB_ human	NM_002651		(Meyers and Cantley 1997) MIM 602758	1q21 3 F2.1
PI4K2A	PI4KIIα	PI4K2A human	NM_018425	AP-3	Barylko et al. [2001]; Minogue et al. [2001]; Craige et al. [2008]	10q24 19 C3; 19 47.0 cm
PI4K2B	PI4KIIβ	PI4K2B human	NM_018323	Rac1, Hsp90	Minogue et al. [2001]; Balla et al. [2002]; Wei et al. [2002]; Jung et al. [2008]	4p15.2 5 C1;5

Hokin 1953). The enzymes themselves were first identified in membrane fractions and tissue extracts as activities that could transfer the γ-phosphate of radiolabelled ATP to PtdIns (Michell et al. 1967, Harwood and Hawthorne 1969). It soon became apparent that biochemically distinct activities could be detected in these extracts, and these were subsequently named the type I, II and III phosphoinositide kinases (Endemann et al. 1987; Whitman et al. 1987). The type I PI kinase was later shown to phosphorylate the D3 position of the inositol headgroup and these enzymes are now better known as the phosphoinositide 3-kinases (PI3Ks). The type II and III enzymes both phosphorylate at the D4 position but exhibit very different biochemical properties for example, the type II isoforms are inhibited by adenosine but not PI3K inhibitors and have K_{mATP} in the micromolar range; meanwhile, the type III isoforms are larger proteins, insensitive to adenosine and inhibited by PI3K inhibitors, albeit at higher concentrations than PI3Ks (Pike 1992; Balla and Balla 2006; Hunyady et al. 1983). For many years these biochemical characteristics were the only means of distinguishing PtdIns4K activities in experiments and furthermore, results were sometimes confounded by the presence of trace amounts of the highly active type II activity (Yamakawa et al. 1991). Final and unambiguous identification of the isoforms had to wait until the proteins were characterised at the molecular level (Table 1.1) and this was finally made possible during the genomics age of the 1990s. The cloning of the type II (PtdIns4KIIα and PtdIns4KIIβ) and type III isoforms (PtdIns4KIIIα and PtdIns4KIIIβ) revealed that they belong to divergent sequence families with the PtdIns4KIIIs showing a greater degree of similarity to the PI3K/protein kinase superfamily. Thus the biochemical differences, particularly the relative sensitivity to PI3K inhibitors, were finally explained and sequence specific reagents, along with some inhibitors, became available to study PtdIns4K functions. These have contributed to a resurgence of interest in these key activities and the last few years have seen the

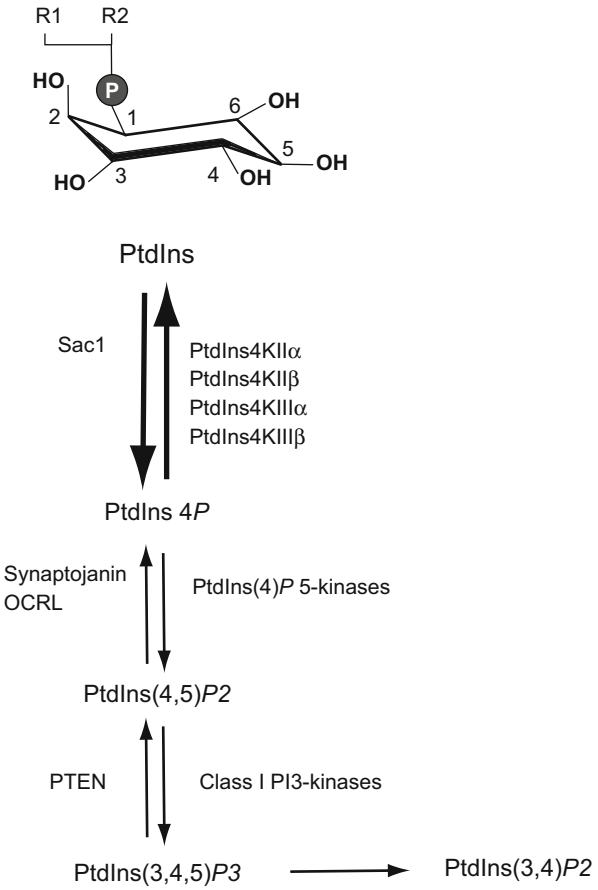

Fig. 1.1 Metabolism of D4 phosphorylated phosphoinositides. The glycerophospholipid phosphatidylinositol (PtdIns) is a component of membranes in all eukaryotic cells. It is made up of a hydrophobic diacylglycerol backbone (R1 and R2, are predominantly stearoyl and arachidonyl in mammalian cells) linked to a polar inositol headgroup. The headgroup can be phosphorylated at the D3, D4 and D5 positions by various kinase activities to generate eight higher phosphoinositides (PIs); PIs phosphorylated by PtdIns 4-kinases (PtdIns4Ks) are shown. Reverse (dephosphorylation) reactions catalysed by specific PI phosphatases are shown

emergence of numerous new and unexpected functions for the PtdIns4Ks, the first PtdInsK activities to be identified all those years ago.

In order to understand PtdIns4K function it is often necessary to study the enzymatic output of individual isoforms, that is, not just PtdIns4*P* but also the D4 metabolic derivatives whose levels may change in response to modulation of PtdIns4K activity. Due regard should also be paid to dephosphorylation reactions by PI phosphatases since together these activities determine the flux of intermediates through PI pathways (Fig. 1.1). Herein lie the central technical problems in PI research: firstly, different PI species are not evenly distributed between organelles and biochemical methods that rely on total PI analysis are unable to distinguish separate pools of PIs which, although chemically identical, may not have equivalent functions. Secondly, the steady-state subcellular localisation of PI kinase protein is not a reliable indicator of its locus of activity. The study of PtdInsK activity is therefore increasingly reliant on protein domains which bind PIs *in vitro* and such reagents have been widely used as overexpressed GFP chimeras to report PtdIns4K activity in cultured cells (Balla et al. 2000b; Szentpetery et al. 2009). However, whilst useful, these tools are sometimes of dubious specificity *in vivo* and their overexpression

may sequester PIs preventing further metabolism or interaction with effector proteins (Balla et al. 2000b). Thus the cell models we rely on may be prone to observer effects where the experimental system can generate complex and uninterpretable phenomena. Fortunately other strategies are available, for instance, using recombinant PI-binding domains to indirectly stain fixed cells to report steady-state levels of PIs (Watt et al. 2002; Hammond et al. 2009). Nevertheless, our inability to accurately image specific pools of PIs and unambiguously attribute results to specific PI kinase activities *in vivo* remains a significant technical limitation. A similar caveat applies to the use of RNAi as a method of studying loss of function. Although convenient, RNAi only reveals phenotypes after 48–72 h of knockdown—a relatively long period of time during which a cell may respond by upregulating other PtdInsK activities or compensating by making previously separate pools of PIs available. This last problem may be circumvented by the use of cell-permeable drugs able to specifically inhibit PI kinases but there are currently few such compounds available. A separate approach, which acutely targets individual PI pools by inducing the heterodimerisation of a specific PI phosphatase with organelle markers has been employed to selectively hydrolyse PIs (Varnai and Balla 2006). No perfect strategy yet exists but used together, approaches such as these have revealed a great deal about PtdIns4K function in cells.

As with many conserved PI kinases, our understanding of PtdIns4K function has been hugely aided by genetic studies in the yeast *S. cerevisiae* and, despite the vast differences between yeast and multicellular eukaryotic biology, these studies have often led the way in elucidating gene function in higher organisms. This fact is perhaps best illustrated by Pik1, the yeast orthologue of mammalian PtdIns4KIIIβ, which was the first PtdIns4K gene identified in any organism and which shares many functional features with its mammalian counterpart. Since several extensive and authoritative reviews have been published recently (Balla and Balla 2006; Strahl and Thorner 2007), this chapter will concentrate mainly on the function of PtdIns4Ks in animal cells and genetic models. However, we will discuss those functions that overlap with or provide particular insight into the relevant mammalian orthologues.

1.2 The Type II PtdIns 4-Kinases

1.2.1 PtdIns4KII Gene Family and Domain Structure

Sequences homologous to PtdIns4KII exist in all metazoan genomes and all vertebrates appear to contain genes encoding the two closely related alpha and beta isoforms. Our analysis indicates that most invertebrate genomes, with the exception of *C. elegans*, contain just a single PtdIns4KII gene. Lower eukaryotic organisms such as the yeasts also contain just one gene and it is difficult to determine whether these are more closely related to the mammalian alpha or beta isoforms.

The mammalian alpha and beta PtdIns4KIIs are both membrane proteins of ~55 kDa and the human proteins share 68% identity in their conserved catalytic

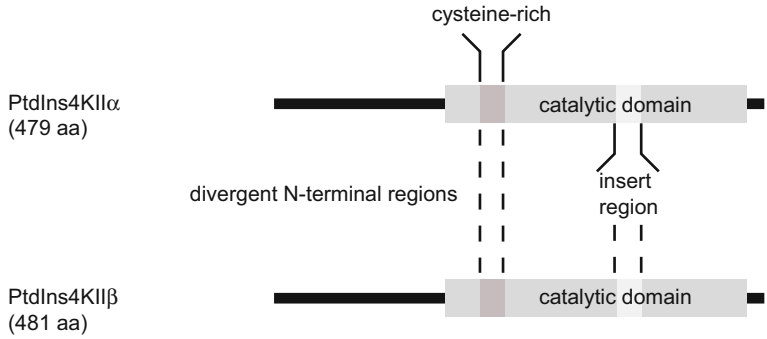

Fig. 1.2 The mammalian type II PtdIns 4-Kinases. A schematic illustration of conserved features in the sequences of mammalian PtdIns4KIIs. The PtdIns4KII isoforms share homology in the conserved catalytic core with the exception of an insert region of varying length in different species. The pre-eminent conserved feature is the cysteine-rich region in the kinase domain which mediates membrane targeting and probably places the enzyme in close proximity to membrane-bound PtdIns substrate. The N-termini are highly dissimilar: whereas PtdIns4KIIα is proline-rich and amphiphillic in character, the N-terminus of PtdIns4KIIβ is highly acidic

domains. No X-ray structures currently exist and the proteins have only weak homology to the PI3K/protein kinase superfamily of phosphotransferase enzymes. Nevertheless, local regions of homology corresponding to recognisable protein kinase subdomains are present (Minogue et al. 2001) and the requirement for invariant residues within these conserved sequences has been tested by site-directed mutagenesis (Barylko et al. 2002). The conserved catalytic core is separated by a non-conserved insert region which varies in length between species (Balla and Balla 2006) (illustrated in Fig. 1.2).

An interesting feature of the otherwise highly related type II PtdIns4K isoforms is that they contain divergent amino termini: PtdIns4KIIα has a proline-rich region (Balla and Balla 2006) followed by a stretch of hydrophobic and charged residues, which are predicted to adopt the structure of an amphipathic helix and may mediate membrane insertion (Barylko et al. 2009); meanwhile PtdIns4KIIβ contains a highly acidic amino terminus. Both PtdIns4KII isoforms contain a conserved CCPCC motif within the predicted kinase domain which undergoes palmitoylation (Barylko et al. 2001). This motif is present across species but acylation may be less important in lower eukaryotes such as the yeasts where the cysteine residues are conservatively substituted by a hydrophobic residue (Barylko et al. 2009).

1.2.2 PtdIns4KII Expression, Localisation and Regulation

The PtdIns4KIIs are widely expressed in human tissues with PtdIns4KIIα somewhat enriched in brain and kidney (Minogue et al. 2001; Balla et al. 2002); meanwhile, PtdIns4KIIβ is relatively enriched in liver and cell lines of haematopoietic origin (Balla et al. 2002). In cultured cells, both isoforms are membrane proteins (Waugh

Table 1.2 The subcellular localisation and functions of PtdIns4K isoforms

PtdIns4K isoform	Subcellular localisation	Function
PtdIns4KIIα	TGN	Recruits adaptors AP-1 and GGA TGN-PM transport
	Late endosome	Interacts with AP-3; regulates degradation of EGFR
	PM	Not known
	ER	Not known
PtdIns4KIIβ	PM	Recruited in response to PDGF and V12Rac
	Endosomes	Not known
PtdIns4KIIIα	ER	Resupply of PM PtdIns4*P*
	Nucleus	Not known
	Golgi	Recruitment of GBF1
PtdIns4KIIIβ	TGN	Golgi-to-PM traffic
	Nucleus	Not known

et al. 2003b) which localise to the *trans*-Golgi network (TGN), endosomes and plasma membrane (PM) (Balla et al. 2002; Wang et al. 2003; Salazar et al. 2005; Minogue et al. 2006) (Table 1.2).

Both PtdIns4KII isoforms can undergo palmitoylation in the conserved CCPCC motif and this appears to be an important, though not exclusive, determinant of membrane localisation and probably therefore, access to PtdIns substrate. Although PtdIns4KIIα lacks identifiable transmembrane sequences it is tightly membrane associated, effectively behaving as an integral membrane protein. Deletion of $_{174}$CCPCC$_{178}$ (human numbering will be used throughout) prevents the incorporation of radiolabelled palmitate and is sufficient to render the enzyme extractable from membranes in sodium carbonate buffers at pH 10–11, but not 1 M NaCl (Barylko et al. 2001; Barylko et al. 2009). This indicates that the deacyl form retains the ability to interact tightly with membranes, albeit in a peripheral manner. Deletion of $_{174}$CCPCC$_{178}$ also dramatically reduces kinase activity. Since the CCPCC motif lies within the conserved catalytic domain, this could be due to gross effects on kinase structure. However, this does not seem to be the case because inhibition of acylation with 2-bromopalmitate or by mutation of all the cysteine residues to serine has identical effects on activity, membrane binding and subcellular localisation (Barylko et al. 2009).

All four cysteine residues in PtdIns4KIIα are capable of accepting [^{3}H]-palmitate in metabolic labelling experiments (Barylko et al. 2009). Site-directed mutagenesis indicates that the first two are preferentially palmitoylated and better able to support integral membrane binding and activity (Barylko et al. 2009). This observation has interesting implications for the regulation of PtdIns4KII enzymes of lower eukaryotes, where the first two cysteines are substituted with hydrophobic residues. The sole PtdIns4KII of *S. cerevisiae* Lsb6 is, however, palmitoylated outside of this conserved motif on a C-terminal cysteine residue (Roth et al. 2006) which may contribute to membrane binding.

Palmitoylation is a potentially reversible modification but as yet, no compelling evidence has been presented to suggest that dynamic palmitoylation regulates

PtdIns4KIIα. Unlike all other PI kinases, PtdIns4KIIα is constitutively membrane-associated, and therefore the mechanism responsible for the observed active pool in membrane fractions (Waugh et al. 2003b) probably occurs within the membrane itself. Indeed, good evidence exists for an intrinsic mechanism of regulation because PtdIns4KIIα activity is extremely sensitive to membrane environment and, in particular, intra-membrane cholesterol concentration. Targeting to cholesterol-containing membranes alters the lateral diffusion kinetics of PtdIns4KIIα and there is strong evidence that a high activity and slowly diffusing pool of PtdIns4KIIα associates with lipid-raft like domains of the TGN (Minogue et al. 2010). Moreover the size of the mobile pool of PtdIns4KIIα is affected by TGN membrane connectivity, indicating that the enzyme's biophysical properties are affected by the overall membrane architecture (Minogue et al. 2010). A further level of cholesterol-dependent control may result from PtdIns4KIIα being highly responsive to oxysterol-binding protein (OSBP)-generated sterol gradients on Golgi and post-Golgi membranes (Banerji et al. 2010). Therefore, PtdIns4P generation by PtdIns4KIIα is a function of sterol-based membrane ordering and topological organisation. In future work, it may be interesting to consider the degree to which PtdIns4*P* and sterol concentrations synergistically determine both the functional and morphological identity of PtdIns4*P* and cholesterol-rich membranes such as those of the TGN.

PtdIns4KIIβ also contains the conserved CCPCC motif but in contrast to PtdIns4KIIα, the enzyme is partially cytosolic. The membrane-bound fraction is palmitoylated and displays ~20-fold higher activity (Jung et al. 2008) suggesting that the enzyme is dynamically acylated resulting in membrane targeting. PtdIns4KIIβ is recruited to the PM in response to PDGF or overexpression of constitutively active Rac; this appears to require the C-terminal 160 amino acids of PtdIns4KIIβ in COS cells and is accompanied by activation of kinase activity (Jung et al. 2008). The enzyme is also phosphorylated *in vivo* on a serine residue in the N-terminus but the significance of this modification is unclear since mutation at this site does not affect kinase activity, palmitate incorporation or membrane localisation (Jung et al. 2008).

Phosphopeptides derived from PtdIns4KIIα have been identified in several proteomic screens and indicate potential for regulation by phosphorylation (Olsen et al. 2006; Villen et al. 2007). In one such study PtdIns4KIIα phosphorylation was agonist-dependent (Olsen et al. 2006), reminiscent of earlier work showing the co-immunoprecipitation of PtdIns4KII activity with activated EGF receptors in a phosphoprotein-containing complex (Cochet et al. 1991; Kauffmann-Zeh et al. 1994). No endogenous protein regulators of PtdIns4KIIα activity have yet been described, however, along with some other interfacial enzymes such as PLD2 (Chahdi et al. 2003), the activity of PtdIns4KIIα can be greatly stimulated by the wasp venom peptide mastoparan and related amphipathic peptides (Waugh et al. 2006). The physiological significance of these biochemical observations have yet to be determined; however, it is noteworthy that the cationic phospholipid phosphatidylcholine has also been shown to be a potent *in vitro* activator of PtdIns4KII activity (Olsson et al. 1993), inferring that alterations to membrane charge may augment PtdIns4*P* synthesis *in vivo*.

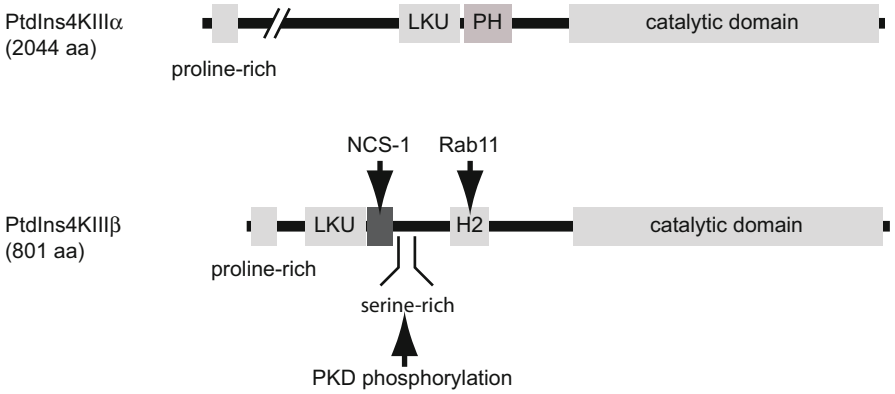

Fig. 1.3 The mammalian type III PtdIns 4-kinases. A schematic illustration of conserved features in the sequences of mammalian PtdIns4KIIIs. The catalytic domains of the PtdIns4KIIIa and PtdIns4KIIIb display minimal homology to the type II PtdIns4Ks and are instead more similar to the PI3K family of enzymes. This similarity includes the presence of the conserved lipid kinase unique (LKU) domain. PtdIns4KIIIa contains an N-terminal proline-rich domain and PH domain. Non catalytic domains in PtdIns4KIIIb include a proline-rich sequence, the LKU, a region of homology (H2) shared with other PtdIns4KIIIb orthologues including the *S. cerevisae* Pik1p, regions implicated in the binding of NCS-1 and Rab11 and a serine-rich region which is phosphorylated by PKD

1.3 The Type III PtdIns 4-Kinases

1.3.1 Gene Family and Domain Structure

PtdIns4KIIIβ and PtdIns4KIIIα were first identified in *S. cerevisae* as the essential gene PIK1 (Flanagan et al. 1993) and STT4, whose mutation confers sensitivity to the protein kinase C (PKC) inhibitor staurosporine (Yoshida et al. 1994). Both PtdIns4KIIIα and -β sequences are present in all metazoan genomes.

The type III PtdIns4Ks are larger enzymes whose catalytic domains display strong sequence similarities to the PI3Ks. Like the PI3Ks and the more closely related serine/threonine kinases TOR, ATM and DNA-PK, they also contain the so-called lipid kinase unique (LKU) domain which is predicted to adopt a helical structure (Balla and Balla 2006) and which, in the yeast PtdIns4KIIIβ orthologue at least, is essential for kinase activity (Strahl and Thorner 2007) (Fig. 1.3). In PtdIns4KIIIα the LKU region is closely juxtaposed with a PH domain (Wong and Cantley 1994) and although this PH domain has poor homology to other such domains, the isolated region from a plant PtdIns4KIIIα binds PtdIns4*P* in lipid overlay assays (Stevenson et al. 1998) and may contribute to product inhibition (Stevenson-Paulik et al. 2003). Binding sites for NCS-1 and Rab11 have been identified in PtdIns4KIIIβ (discussed in Sect. 4.2.4) close to a homology region (Hom2) conserved in other PtdIns4KIIIβ orthologues. This region, situated between the LKU and catalytic domain, also contains a serine-rich domain that is phosphorylated by protein kinase D (Sect. 4.2.4). A splice variant exists which extends this serine-rich region by 15 amino acids (Balla

and Balla 2006). This insert can be phosphorylated by casein kinase II *in vitro* but the modification does not lead to substantial activation of kinase activity (S. Minogue, unpublished data).

The high degree of conservation between the PtdIns4KIII and PI3K proteins confers sensitivity to inhibitors such as wortmannin and LY294002, with *in vitro* IC_{50} values that are approximately 20–100 fold higher than those for class I PI3Ks. Whilst this property has proved useful in distinguishing between type II and type III activities, it is not an effective way to discriminate between PtdIns4KIII activities. However, the recent characterisation of PIK93, a cell-permeable inhibitor relatively selective for the PtdIns4KIIIβ isoform (Knight et al. 2006; Balla et al. 2008) offers a means of selectively inhibiting PtdIns4KIIIβ function.

1.3.2 PtdIns4KIIIα Expression, Localisation and Regulation

PtdIns4KIIIα is widely expressed in tissues but is particularly enriched in brain (Wong et al. 1997; Gehrmann et al. 1999; Zolyomi et al. 2000). PtdIns4KIIIα predominantly localises to the ER in mammalian cells (Wong et al. 1997) but it has also been detected in the pericentriolar Golgi region (Nakagawa et al. 1996). Many studies have been forced to use overexpressed protein to determine the pattern of subcellular distribution because of problems detecting endogenous PtdIns4KIIIα and this may affect the observed localisation of the protein. However, immuno-electron microscopy of endogenous PtdIns4KIIIα in the rat CNS localised the protein to the ER and atypical membranes surrounding mitochondria, and multivesicular bodies (Balla et al. 2000a). It is tempting to speculate that these membranes represent some form of inter-organelle junction (Levine and Rabouille 2005) that permits the communication of PtdIns4 or PtdIns4KIIIα protein between closely juxtaposed compartments. Endogenous PtdIns4KIIIα has also been detected in the nucleolus (Kakuk et al. 2006).

1.3.3 PtdIns4KIIIβ Expression, Localisation and Regulation

PtdIns4KIIIβ is ubiquitously expressed in mammalian tissues (Balla et al. 1997) and localises to *cis*- and *trans*-regions of the Golgi complex (Wong et al. 1997; Godi et al. 1999). Golgi localisation is brefeldin A-sensitive and therefore likely to depend on Arf (discussed in Sect. 4.2.4). PtdIns4KIIIβ binds Rab11 in a region between residues 401–506 (Fig. 1.3) and it appears that this interaction, which is conserved in yeast, is required to recruit Rab11 to Golgi membranes rather than PtdIns4KIIIβ (de Graaf et al. 2004). Like PtdIns4KIIIα, PtdIns4KIIIβ has also been detected in the nucleus (de Graaf et al. 2002). The mechanism of nuclear localisation has not been determined but it is notable that the yeast orthologue Pik1 was identified as a component of the nuclear pore complex (Garcia-Bustos et al. 1994).

1.4 Cellular Functions of PtdIns4Ks

1.4.1 PtdIns4Ks in Signalling Pathways

PtdIns4*P* is the immediate precursor of PtdIns(4,5)P_2, the major *in vivo* substrate for receptor-linked phospholipase C (PLC) and PI3K enzymes. Both PIs have been found at the PM where they can be detected with monoclonal antibodies and PH domains (Hammond et al. 2006, 2009); meanwhile, PtdIns4KIIα, PtdIns4KIIβ and possibly PtdIns4KIIIα all localise to the PM (Table 1.2). Historically, type II PtdIns4K activity was linked to agonist-regulated pathways because of its association with activated EGFR (Cochet et al. 1991; Kauffmann-Zeh et al. 1994). However, these studies were performed prior to the availability of sequence-specific reagents and therefore we cannot rule out the contribution of PtdIns4KIIβ in these results or the possibility that PtdIns4KIIα associates with the receptors in an endosomal trafficking complex (Minogue et al. 2006).

1.4.1.1 PtdIns4KIIs in Signalling Pathways

Although there is evidence for regulated membrane association and kinase activation, relatively little is known about PtdIns4KIIβ function. Partial recruitment in response to PDGF and constitutively active Rac expression is intriguing (Wei et al. 2002) and suggests that the isoform may be acutely regulated by receptor activation and GTP-Rac, although details of the mechanism remain elusive. Rac is a well known signalling molecule regulating cell adhesion and migration (Heasman and Ridley 2008) and one report links PtdIns4KIIβ to hepatocellular cancer cell migration: PtdIns4KIIβ co-immunoprecipitates with overexpressed tetraspannin CD81 from HepG2 lysates and the proteins partially colocalise by immunofluorescence microscopy. The mechanism linking PtdIns4KIIβ and CD81 is as yet unclear but loss of CD81 expression in hepatocellular carcinoma correlates with poorly differentiated and metastatic tumours (Mazzocca et al. 2008).

An interesting development has been the recent finding that PtdIns4KIIα is a component of the canonical Wnt pathway (Pan et al. 2008). The Wnts (from wingless, the *Drosophila* mutation) are a large and conserved family of hydrophobic ligands which control an array of developmental and oncogenic processes (Kikuchi et al. 2009). In the canonical β-catenin-dependent Wnt pathway in the absence of ligand, β-catenin is phosphorylated by GSK3β and CKIα and is subsequently ubiquitylated and targeted for degradation in the proteosome, thereby regulating levels of β-catenin. When Wnt ligands bind to their cognate receptors LRP5 or 6, β-catenin phosphorylation and subsequent proteosomal degradation is suppressed, leading to increased cytosolic levels. β-catenin is then able to translocate to the nucleus where it stimulates expression of a number of genes. PtdIns4KIIα was initially identified in a siRNA library screen aimed at identifying proteins involved in the Wnt3a-dependent phosphorylation of LRP6 and therefore the β-catenin pathway to the nucleus (Pan et al. 2008). PtdIns4*P* 5-kinases were also identified in this screen suggesting that

together the kinases form a PtdIns(4,5)P_2 biosynthetic complex. Supporting this is the finding that exogenous PtdIns(4,5)P_2 stimulates Wnt3a-dependent LRP5/6 phosphorylation *in vitro* and that reduction of endogenous PtdIns(4,5)P_2 levels using a 5-phosphatase has the opposite effect (Pan et al. 2008). Wnt3a also stimulates the production of PtdIns4*P* and PtdIns(4,5)P_2 but this is abolished in HEK293 cells treated with siRNA to knockdown PtdIns4KIIα and PtdIns4*P* 5KIβ (Pan et al. 2008; Qin et al. 2009).

Wnt3a probably regulates PtdIns4KIIα and PtdIns4*P* 5KIβ activities by directly interacting with the scaffold protein dishevelled (Dvl), a critical adaptor protein in Wnt signalling. Dvl co-immunoprecipitates with PtdIns4KIIα and PtdIns4*P* 5KIβ in a tripartite complex when the proteins are overexpressed in HEK293 cells and the activity of both enzymes is activated by Dvl binding *in vitro* (Qin et al. 2009). In the case of bacterially expressed PtdIns4KIIα protein at least, this interaction does not appear to involve the enzyme's divergent N-terminal region (Qin et al. 2009).

1.4.1.2 PtdIns4KIIIs in Signalling Pathways

Despite the association of PtdIns4KII with activated receptors and involvement in Wnt signalling, far stronger evidence supports a role for the type III PtdIns4Ks in agonist-regulated turnover of PIs, indeed the wortmannin sensitivity of angiotensin II (AngII) and PDGF-sensitive pools was identified long ago (Nakanishi et al. 1995). Evidence of a signalling role for PtdIns4KIIIα also exists from *S. cerevisiae* where the orthologue Stt4p localises to the PM and participates in the yeast non-PLC-linked PKC-MAPK cascade (Audhya et al. 2000). More recent work has sought to identify the PtdIns4K isoform responsible for maintaining the PM pool of PtdIns4*P* for PtdIns(4,5)P_2 biosynthesis, using a combination of pharmacological inhibitors and siRNA targeting of specific PtdIns4K isoforms (Balla et al. 2008a). Use of the PH domain from OSH2 fused to GFP and the PLCδ1 PH domain fused to mRFP allowed simultaneous monitoring of PM PtdIns4*P* and PtdIns(4,5)P_2, respectively. The PM localisation of both fusion proteins was sensitive to wortmannin as were the sustained hydrolysis of PM PtdIns(4,5)P_2, changes in levels of total [^{32}P]-labelled PtdIns4*P*, PtdIns(4,5)P_2, [^{3}H]-InsP_3 and Ca^{2+} in AngII-stimulated cells (Balla et al. 2008a). Both PtdIns4KIII isoforms are sensitive to wortmannin but the availability of the new inhibitor PIK93 (Knight et al. 2006; Balla et al. 2008b), relatively selective for PtdIns4KIIIβ, allowed investigators to rule out this isoform in the response to AngII stimulation (Balla et al. 2008b).

This work clearly implicates PtdIns4KIIIα as the isoform responsible for maintenance of the G-protein coupled agonist-responsive pool of PtdIns4*P*. Curiously however, endogenous PtdIns4KIIIα is present on ER and Golgi membranes but cannot be detected at the PM. This means that either only a very small amount of PtdIns4KIIIα is necessary for PtdIns4*P* supply to the PLC signalling pathway, or that PtdIns4*P* is rapidly transported from the ER to replenish that consumed at the PM, possibly via ER-PM junctions. How this might be accomplished is mysterious and, although PtdIns4KIIIα binds to a PtdIns transfer protein (Aikawa et al. 1999), such proteins are not known to transfer PtdIns4*P*. However, there is

also evidence that Golgi PtdIns4*P* can replenish that consumed at the PM during acute signalling, because depletion of the Golgi pool of PtdIns4*P* using targeted Sac1 PtdIns4*P*-phosphatase markedly affects the replenishment of PM PtdIns(4,5)P_2 (Szentpetery et al. 2010). PtdIns4*P* is regarded as the dominant PI species at the Golgi apparatus and the cell's ability to use this pool of precursor during sustained signalling indicates that metabolic compartmentation of PtdIns4*P* may be leaky under certain circumstances. Fascinating though this phenomenon is, the mechanism by which Golgi PtdIns4*P* reaches the signalling pool at the PM remains to be determined and, since no protein is known to transfer PtdIns4*P*, vesicular traffic remains a likely prospect. An earlier study investigating the membrane localisation of PtdIns4*P*-binding PH domains from FAPP1 and OSBP determined that the domains bound PLC-sensitive lipid pools when transfected cells were challenged with Ca^{2+} ionophores. The PM-localised domains re-associated particularly strongly following Ca^{2+} ionophore treatment and this re-association was inhibited by siRNA downregulation of PtdIns4KIIIα but not PtdIns4KIIIβ or PtdIns4KIIα (Balla et al. 2005).

PtdIns4KIIIα has been found in a complex containing the $P2X_7$ ion channel (Kim et al. 2001). The significance of this interaction is not clear but $P2X_7$ activation leads to rapid cytoskeletal rearrangements, suggesting that the receptor is linked, directly or indirectly, to second messenger signalling. PtdIns4KIIIβ is also stimulated by the tumour-associated elongation factor eEF1A2 (Jeganathan and Lee 2007). This occurs through direct interaction of eEF1A2 with PtdIns4KIIIβ and has been mapped to a region between residues 163–320 (Jeganathan et al. 2008). It is not clear whether this interaction takes place in the Golgi complex but it is responsible for a relatively large increase in PM PtdIns(4,5)P_2 accompanied by actin remodelling.

1.4.2 PtdIns4Ks in Intracellular Traffic

Whilst all PtdIns4Ks have been detected at the Golgi complex (Table 1.2), only PtdIns4KIIα and PtdIns4KIIIβ are known to synthesise PtdIns4*P* at this location. The PtdIns4*P* they generate is responsible for the localisation of a number of PtdIns4*P*-binding proteins involved in anterograde traffic, the biosynthesis of sphingomyelin and glycosphingolipids. In many cases the PtdIns4*P*-binding proteins in question have a relatively low affinity and specificity for the lipid *in vitro* and rely on additional protein factors or sequences outside the lipid binding domain to bind membranes specifically and with sufficient affinity (Levine and Munro 2002; Carlton and Cullen 2005).

1.4.2.1 Type II PtdIns4Ks and Intracellular Trafficking Pathways

In cultured cells, endogenous PtdIns4KIIα protein has a broad distribution localising primarily to membranes of the TGN (Wang et al. 2003), endosomes (Balla et al. 2002) and, to a lesser extent, the PM (Minogue et al. 2006) and ER (Waugh et al. 2003a)

(Table 1.2). It is likely that the distribution of the enzyme between these organelles is cell-type specific because PtdIns4KIIα is a component of synaptic vesicles (SVs) (Guo et al. 2003); PtdIns4KIIα is also enriched in Bergmann glia, astrocytes, Purkinje cell bodies and selected populations of neurons in rat brain (Simons et al. 2009). This and the fact that PtdIns4KIIα is dynamically trafficked may partially explain the discrepancies between subcellular localisations in published work.

The first evidence that PtdIns4KIIα is involved in membrane traffic was provided by the finding that overexpression of kinase-inactive mutant PtdIns4KIIα or PtdIns4KIIβ affected the traffic of transferrin and AngII (Balla et al. 2002). Subsequently, knockdown of PtdIns4KIIα was shown to decrease levels of Golgi PtdIns4*P* and block recruitment of the major TGN adaptor AP-1 to Golgi membranes (Wang et al. 2003). AP-1 was shown to be a PtdIns4*P* binding protein *in vitro* and exogenous synthetic PtdIns4*P*, but not other lipids, were sufficient for rescue of AP-1 recruitment to membranes. AP-1 is a clathrin adaptor that mediates TGN-to-endosome traffic but the Golgi is also a key organelle mediating protein processing and late secretory traffic to the PM. Knockdown of PtdIns4KIIα did not affect intra-Golgi traffic of haemaglutinin cargo but did affect the non-AP-1 dependent transport of GFP-VSVG to the PM. Interestingly, this pathway could only be rescued with synthetic PtdIns(4,5)P_2 indicating that PtdIns4KIIα can also contribute PtdIns4*P* for generation of PtdIns(4,5)P_2 in the late secretory pathway (Wang et al. 2003). PtdIns4KIIα knockdown affects the localisation of the Golgi-localised, γ-ear-containing, Arf-binding protein (GGA) family of adaptors which are also involved in TGN-to-endosomal traffic. GGA1, GGA2 and GGA3 binding to the TGN is dependent on a PtdIns4*P* and Arf1 coincidence mechanism (Wang et al. 2007).

1.4.2.2 PtdIns4KIIα and Endosomal Traffic

PtdIns4KIIα localises to endosomal membranes staining positive for the early endosomal autoantigen EEA1, transferrin, AngII (Balla et al. 2002) and particularly well to late endosomal compartments staining for lamp-1, CD63, syntaxin 8 and internalised EGF (Minogue et al. 2006). In live cells, PtdIns4KIIα traffics on highly dynamic endosomal carriers and inhibition of PtdIns4KIIα with an inhibitory antibody or by knockdown with siRNA prevents the correct degradation of the EGFR, presumably by impairing traffic to the lysosome (Minogue et al. 2006). PtdIns4KIIα knockdown also affects the steady-state distribution of lamp-1 and CD63, two other markers of lysosomal traffic (Minogue et al. 2006) and this suggests a general trafficking defect is responsible for the effects on receptor degradation.

PtdIns4KIIα has been identified as a component of AP-3-containing vesicles from PC12 cells and the two proteins show substantial colocalisation together, and with, AP-3 cargoes such as the Zn^{2+} transporter ZnT3 and lamp-1 (Salazar et al. 2005). PtdIns4KIIα also co-precipitates with AP-3 and ZnT3 in *in vivo* crosslinked complexes (Craige et al. 2008). Forms of AP-3 are expressed in both neuronal and non-neuronal cells where they control the selection of lysosomal cargo, often defined by the presence of a dileucine sequence for lysosomal traffic. The normal steady-state localisation of PtdIns4KIIα is AP-3 dependent and conversely, the subcellular

localisation of AP-3 is PtdIns4KIIα dependent, thus the two proteins appear to control their localisations in a reciprocal manner. This relationship is supported by the discovery of a dileucine AP-3 sorting motif in the N-terminus of PtdIns4KIIα required for endosomal localisation; therefore, the enzymatic product of PtdIns4KIIα may regulate AP-3 function through recruitment in a manner analogous to AP-1 at the TGN. However, despite the observation that kinase activity is required for the traffic of lysosomal cargoes (Craige et al. 2008), no evidence for direct interaction of AP-3 with PtdIns4*P* or other D4 PIs exists. Sections of brain tissue from the AP-3-deficient mocha mouse lack PtdIns4KIIα staining in specific areas including nerve termini of mossy fibre neurons of the hilus and hippocampus (Salazar et al. 2005). In this respect it is interesting that PtdIns4KIIα localises to SVs (Guo et al. 2003) because the correct generation of SVs and synaptic-like micro vesicles in neuroendocrine cells is dependent on an AP-3 pathway, suggesting that PtdIns4KIIα functions along with the BLOC-1 and AP-3 trafficking machinery in the biogenesis of SVs (Salazar et al. 2009).

1.4.2.3 Type III PtdIns4Ks and Membrane Traffic

PtdIns4KIIIα

Although PtdIns4KIIIα is the isoform most strongly implicated in agonist-dependent signalling (see Sect. 4.1.2), the protein localises to the ER and Golgi apparatus (Wong et al. 1997). Nevertheless, PtdIns4KIIIα may participate in certain trafficking functions in COS-7 cells because the protein colocalises with the Golgi brefeldin A resistance factor (GBF1) in the Golgi region. siRNA of PtdIns4KIIIα ablates Golgi localisation of GBF1 (and also the adaptor GGA3) (Dumaresq-Doiron et al. 2010), suggesting that the kinase drives recruitment of GBF1. GBF1 is not known to bind PtdIns4*P* but it is an exchange factor for Arf1 and overexpression of constitutively active Arf1 leads to a small increase in Golgi targeting of a FAPP1 PH domain (Dumaresq-Doiron et al. 2010). Another possible role for PtdIns4KIIIα comes from a siRNA screen of membrane trafficking host factors required for hepatitis C virus (HCV) replication. Knockdown of PtdIns4KIIIα resulted in a large reduction in HCV replication and virus production but did not affect virus entry as might be expected for an enzyme responsible for PtdIns(4,5)P_2 production at the PM. GFP-PtdIns4KIIIα localised to intracellular membranes containing viral dsRNA and the HCV protein NS5A in HCV replicon-transfected hepatoma cells. Thus given its known ER localisation, PtdIns4KIIIα may be involved in the formation of an ER-derived membrane domain in which HCV replicates (Berger et al. 2009). Supporting this is the earlier finding that PtdIns4KIIIα was found in a yeast two-hybrid screen for binding partners of the HCV NS5A protein (Ahn et al. 2004).

PtdIns4KIIIβ

PtdIns4KIIIβ is a peripheral enzyme recruited to the Golgi of COS-7 cells by GTP-bound Arf1. This is accompanied by activation of the kinase generating both PtdIns4*P* and PtdIns(4,5)P_2 (Godi et al. 1999). Although PtdIns4KIIIβ localises to other

subcellular compartments (Table 1.2), it functions principally at the Golgi complex where it maintains structural integrity of the organelle and mediates key transport processes such as TGN-to-PM of nascent proteins and sphingolipids. These functions are directly dependent on either PtdIns4*P* or its metabolic derivatives and there is some functional overlap with PtdIns4KIIα, the other major TGN-localised PtdIns4K (Wang et al. 2003). In MDCK cells, PtdIns4KIIIβ regulates multiple trafficking steps (Bruns et al. 2002) including the intra-Golgi traffic of influenza haemagglutinin and the basolateral to apical delivery of vesicular stomatitis virus glycoprotein cargo (VSVG) (Weixel et al. 2005). Further evidence of pleiotropic roles of PtdIns4KIIIβ comes from the observation that it functions in regulated exocytosis from mast cells (Kapp-Barnea et al. 2006) and neuroendocrine cells (de Barry et al. 2006; Gromada et al. 2005) implying that the enzyme can perform PtdIns(4,5)P_2-dependent roles outside of the Golgi in specialised cell types.

The yeast orthologue of PtdIns4KIIIβ, Pik1, accounts for ~45% of total PtdIns4*P* and ~40% of PtdIns(4,5)P_2 production (Strahl and Thorner 2007) and the gene is essential for cell survival. Like PtdIns4KIIIβ, Pik1p is a TGN enzyme which also localises to the nucleus (Strahl et al. 2005). Pik1p is a downstream effector of a small N-myristoylated protein frequenin (Frq1p), which not only determines Pik1p localisation to the TGN but also tightly binds and stimulates PtdIns4K activity (Hendricks et al. 1999). Some aspects of this interaction are evolutionarily conserved because the mammalian orthologue of Frq1p, the neuronal calcium sensor NCS-1, interacts with and stimulates PtdIns4KIIIβ (Weisz et al. 2000; Haynes et al. 2005). NCS-1 and Frq1p belong to a family of myristoylated proteins that bind multiple targets, often in a Ca^{2+}-dependent manner. PtdIns4KIIIβ is actually under the dual control of Arf1 and NCS-1 because the enzyme binds both in a Ca^{2+}-dependent manner and both stimulate kinase activity, however, Arf1 and NCS-1 do not act synergistically to increase activity: Arf1 antagonises NCS-1 stimulation of regulated secretion of recombinant human growth hormone from PC12 cells whilst, conversely, Arf1 overexpression antagonises the constitutive trafficking of VSVG from the TGN (Haynes et al. 2005). The implications of these data are that Arf1 or NCS-1 and Ca^{2+} but not both, can activate PtdIns4KIIIβ. It is interesting that the Golgi complex can act as a Ca^{2+} store and localised Ca^{2+} release may affect numerous trafficking steps (Pinton et al. 1998); PtdIns4KIIIβ function may therefore also be under the control of local Ca^{2+} signals. In this regard it is worth mentioning that the other major Golgi activity, PtdIns4KIIα, is extremely sensitive to Ca^{2+} *in vitro,* suggesting that these two activities may be differentially regulated in order to control PtdIns4*P* synthesis in the Golgi complex.

Many neuronal and secretory functions have been described for NCS-1 and the protein is highly expressed in brain tissues, localising to numerous intracellular membranes including synaptic vesicles in rat axon terminals (Taverna et al. 2002). NCS-1 plays a role in regulated secretion in neuroendocrine cells (McFerran et al. 1998) but is also present in non-neuronal cell types. In different cell systems NCS-1 appears to promote both secretion and endocytic recycling (Kapp-Barnea et al. 2006). It localises to the TGN of rat hippocampal neurons (Martone et al. 1999) and the TGN and PM of COS-7 (Bourne et al. 2001). NCS-1 and PtdIns4KIIIβ increase glucose-induced insulin secretion by increasing the pool of available vesicles in

pancreatic β-cells (Gromada et al. 2005), indicating that the enzyme has roles in regulated exocytosis as well as constitutive secretion.

PtdIns4KIIIβ can be phosphorylated by protein kinase D (PKD) isoforms PKD1 and PKD2, which are known to regulate the fission of TGN-derived transport intermediates (Liljedahl et al. 2001). PKD phosphorylation at PtdIns4KIIIβ S294 stimulates PtdIns4K activity and enhances PM traffic of VSVG compared to a mutant PtdIns4KIIIβ in which the S294 is mutated to an alanine residue. Interestingly, activation of PtdIns4KIIIβ is stabilised by interaction with 14-3-3 proteins, which bind phosphorylated PtdIns4KIIIβ. However, the extensive Golgi tubulation characteristic of PKD mutants is not phenocopied by expression of a PtdIns4KIIIβ(S294A) mutant suggesting that other Golgi-localised PtdIns4Ks are sufficient to supply the PtdIns4*P* required for this trafficking process.

PtdIns4K activities are implicated in the regulation of sphingolipid synthesis via a class of lipid transfer proteins, which contain an N-terminal PtdIns4*P*-binding PH domain along with a C-terminal lipid transfer domain. These unrelated proteins have been dubbed the 'COF' family after the original members, ceramide transfer protein (CERT), oxysterol binding protein (OSBP) and the 'four phosphate adaptor proteins' (FAPPs) (De Matteis et al. 2007). CERT is important in the non-vesicular transport of ceramide from the ER to the Golgi (Hanada et al. 2003) where it is used in the synthesis of sphingomyelin (SM). CERT is targeted to the Golgi via its PH domain but also associates with the ER-bound membrane protein VAP-A. These interactions may define a transport junction between the ER and Golgi membranes or allow CERT to shuttle between the two compartments and PtdIns4KIIIβ appears to be the main activity responsible for generating the pool of PtdIns4*P* required for CERT recruitment (D'Angelo et al. 2007; Toth et al. 2006). Like PtdIns4KIIIβ, CERT is a substrate for PKD phosphorylation, which negatively regulates both its ceramide transfer activity and affinity for PtdIns4*P*. Meanwhile, the synthesis of SM is intimately linked to the generation of diacylglycerol (DAG) at the Golgi that recruits and activates PKD, and together this leads to the inactivation of CERT and the phosphorylation and activation of Golgi-localised PtdIns4KIIIβ (Hausser 2005, p. 69). It is notable that PtdIns4KII, the other Golgi-resident PtdIns4*P*-synthetic activity, interacts with PKD (Nishikawa et al. 1998) therefore the synthesis of SM and PtdIns4*P* appear to be co-regulated in the TGN.

OSBP1 binds and transfers sterols and associates with the Golgi via its PH domain (Levine and Munro 1998), translocating there from cytosolic and vesicular membranes in response to treatment with the cholesterol precursor 25-hydroxysterol (Ridgway et al. 1992). OSBP also interacts with VAP-A and VAP-B in the ER and therefore may act in a similar way to CERT. As well as PtdIns4KIIIβ, evidence exists that PtdIns4KIIα is responsible for the Golgi recruitment of CERT and therefore SM synthesis (Banerji et al. 2010). At steady-state, PtdIns4KIIα localises to cholesterol-poor membranes and the increased sterol content of these, as the result of transfer by OSBP, may stimulate PtdIns4*P* synthesis by PtdIns4KIIα at the TGN where both lipids play important roles.

FAPP1 and FAPP2 are ubiquitously expressed metazoan proteins that are part of a conserved gene family; the proteins play a role in constitutive TGN to PM traffic (Godi et al. 2004) and to the apical surface in MDCK cells (Vieira et al.