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Daniel G.S. Capelluto *Editor*

Lipid-mediated Protein Signaling

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Preface

The intention of this volume is to provide readers with an update on the role of lipids as signaling molecules and how they direct protein signaling to downstream effectors. To understand the specific mechanisms underlying these processes, we recruited renowned scientists who have contributed relevant work in the various areas of lipid signaling research. The objectives of this volume are to summarize recent developments in our understanding of how lipids provide specificity for signaling and to review the role of compartmentalization in lipid-mediated signaling pathways.

The initial chapters of this volume are dedicated to sphingolipid-mediated signaling pathways. The first chapter by Jiang and colleagues addresses in great detail sphingomyelin-triggered signaling, its role in lipid rafts, regulation of the sphingomyelin synthase 1 and 2, and their relationship with physiological conditions such as insulin-mediated responses. A chapter by Yahi et al. addresses the structural basis for gangliosides and cholesterol recognition by alpha-synuclein and the driving forces for the insertion of this protein into the plasma membrane. The chapter by Capelluto et al. reviews the controversial role of membrane sulfatides in cell signaling with an emphasis on their role in platelet aggregation.

Next, a set of five chapters centers on phosphoinositide-mediated signaling. Zimmermann et al. focuses on the mechanisms by which PDZ domains bind phosphoinositides and the structural basis for specificity, regulation, and significance of lipid recognition. The chapter by Overduin and colleagues deals with phosphatidylinositol 4-phosphate (PtdIns(4)P)-mediated signaling in the Golgi apparatus, with particular emphasis on the functional and structural basis of Golgi-associated PtdIns(4)P-binding proteins. Ross and colleagues review the cellular function of phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2), using the PtdIns(4,5)P_2-binding tumor suppressor Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN) as a model to understand how the lipid controls the membrane binding properties of the protein and introducing neutron reflectivity as a new tool to study the orientation and shape of phospholipid mediated membrane-bound proteins. The chapter by Degterev et al. summarizes the cellular role of phosphatidylinositol 3,4,5-trisphophate (PtdIns(3,4,5)P_3) particularly on what refers to pleckstrin-homology domain functions, implications of this phosphoinositide in health and disease, and a thorough review of current drugs employed for targeting intracellular PtdIns(3,4,5)P_3 levels. Gillaspy highlights the function of

phosphoinositides, the effect of their derivatives, inositol 1,4,5-trisphosphate and inositol hexakisphosphate, and their impact on plant growth and development.

The final three chapters are devoted to the emerging role of non-phosphoinositide phospholipids and their derivatives in signaling. Wang and colleagues give the readers an overview of phosphatidic acid (PA)-mediated signaling, including most recent studies on PA-interacting proteins, effect of PA in membrane structure, and in PA-mediated signaling processes with an emphasis in studies carried out in plants. Grinstein et al. examine phosphatidylserine-mediated cell signaling including current methods of phospholipid visualization in live cells. Changes in the level and relocalization of phosphatidylserine in the cell membrane are discussed as well as its extracellular role under conditions such as hemostasis and apoptosis. The closing chapter by Greenberg et al. describes mitochondrial cardiolipin-mediated signaling, including the relationship of cardiolipin with longevity defects, apoptosis, and cardiolipin-defective remodeling.

The editor thanks and acknowledges the contributors for providing their review chapters in a timely fashion and the Springer SBM staff for their cooperation during the editing process.

Blacksburg, VA, USA Daniel G.S. Capelluto

Contents

Abbreviations

5PTase	Inositol polyphosphate 5-phosphatase
ABA	Abscissic acid
ABI1	Abscisic Acid Insensitive 1
ABC	ATP-binding cassette
ALCAT1	Acyl-CoA:lysoCL acyltransferase 1
AP-1	Adaptor protein 1
aPKC	Atypical protein kinase C
Arf	ADP-ribosylation factor
ASA	Arylsulfatase A
At	*Arabidopsis thaliana*
BAI1	Brain specific angiogenesis inhibitor 1
BAR	Bin/amphiphysin/Rvs
BTHS	Barth syndrome
BTK	Bruton's tyrosine kinase
Cav	Caveolin
CCV	Clathrin-coated vesicle
CD1	Cluster of differentiation 1
CDK	Cyclin-dependent kinase
CDP-DAG	Cytidine diphosphate-diacylglycerol
Cer	Ceramide
CERT	Ceramide transport protein
CGT	UDP-galactose: ceramide galactosyltransferase
CL	Cardiolipin
COI1	Coronatine Insensitive 1
COP II	Coat protein complex II
CP	Capping protein
Crd1	Cardiolipin synthase
CST	3′-Phosphoadenosine-5′-phosphosulfate:cerebroside sulfotransferase
CTR1	Constitutive Triple Response 1
CTX-A3	Taiwanese Cobra cardiotoxin A3
Cyt c	Cytochrome c
Dab2	Disabled-2
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DHR	Disc-large Homology Region
DRM	Detergent resistant membrane

E	Endosome
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF3	Eukaryotic initiation factor 3
ENTH	Epsin amino-terminal homology
EpsinR	Epsin-related protein
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAPP	Four-phosphate adaptor protein
FERM	Four point one/Ezrin/Radixin/Moesin
FoxO	Forkhead-box Class O
FYVE	Fab1/YotB/Vac1/EEA1
G-3-P	Glycerol-3-phosphate
G6P	Glucose 6 phosphate
GAPC	Glyceraldehyde-3-phosphate dehydrogenase
GCS	Glucosylceramide synthase
GEF	Guanine nucleotide-exchange factor
GEP4	PGP phosphatase
GFP	Green fluorescence protein
GGA	Golgi-localized gamma ear-containing, ARF-binding protein
GLTP	Glycolipid transfer protein
GOLPH3	Golgi phosphoprotein 3
GPCR	G protein-coupled receptor
GPI	Glycosyl phosphatidylinositol
GPL	Glycerophospholipid
GRASP	Golgi reassembly and stacking protein
GUK	Guanylate-like kinase
HDL	High-density lipoprotein
HEK	Human embryonic kidney
HOG	High osmolarity glycerol
IGF-I	Insulin-like growth factor I
IL-1	Interleukin-1
IMP	*myo*-inositol monophosphatase
IMPL	IMP-like
$Ins(1,4,5)P_3$	Inositol 1,4,5-trisphosphate
InsP	Inositol phosphate
$InsP_6$	Inositol hexakisphosphate
IPC	Inositol phosphoceramide
IPK	Inositol kinase
IPMK	Inositol phosphate multikinase
IR	Insulin receptor
IRS	Insulin receptor substrate
JA-ILE	Jasmonic acid-isoleucine
JA	Jasmonic acid
JAZ	Jasmonate ZIM-domain
KIND	Kinase non-catalytic C-lobe
L_4-CL	Tetralinoleoyl-CL
LE	Late endosome

Lpa.	Low phytic acid
LPP	Lipid phosphate phosphatase
LPS	Lipopolysaccharide
LTP	Lipid transfer protein
MAPK	Mitogen-activated protein kinase
MD	Molecular dynamics
MDCK	Madin-Darby Canine Kidney
MIK	*myo*-inositol kinase
MIPS	*myo*-inositol phosphate synthase
MLCL	Monolysocardiolipin
MLCKLAT1	MLCL acyltransferase-1
MLD	Metachromatic leukodystrophy
MtCK	Mitochondrial creatine kinase
mTORC	Mammalian target of rapamycin complex
MyD88	Myeloid differentiation primary response gene 88
MYO18A	Myosin-XVIIIA
N	Nitrogen
NBD	7-Nitro-2-1,3-benzoxadiazol-4-yl
NCS-1	Neuronal calcium sensor-1
NeuAc	Neuraminic acid
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear Magnetic Resonance
NO	Nitric oxide
NPC	Non-specific PLC
NR	Neutron reflectometry
OBD	Oxysterol-binding domain
OGT	O-linked β-N-acetylglucosamine transferase
ORP	OSBP-related-protein
OSBP	Oxysterol-binding protein
PA	Phosphatidic acid
Par	Partition-defective
PC	Phosphatidylcholine
PDGF	Platelet-derived growth factor
PDK1	Phosphoinositide-dependent kinase-1
PDZ	PSD-95/Discs large/ZO-1
PDZBM	PDZ binding motif
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGP	Phosphatidylglycerolphosphate
Pgs1	Phosphatidylglycerolphosphate synthase
PH	Pleckstrin homology
PI	Phosphoinositide
PI3K	Phosphoinositide 3 kinase
PI4K	Phosphoinositide 4 kinase
PICK1	Protein interacting with c kinase 1
PIP5K	Phosphatidylinositol 4-phosphate 5 kinase
PK	Protein kinase

PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PLS-3	Phospholipid scramblase-3
PM	Plasma membrane
PP	Diphospho
PP1Cγ	Protein phosphatase 1Cγ
PP2Cε	Protein phosphatase 2 C-ε
PPP	Triphospho
PS	Phosphatidylserine
PSD	PS decarboxylase
PSS1	PS synthase 1
PtdCho	Phosphatidylcholine
PtdEth	Phosphatidylethanolamine
PtdIns	Phosphatidylinositol
PtdIns(3)P	Phosphatidylinositol 3-phosphate
PtdIns(4)P	Phosphatidylinositol 4-phosphate
PtdIns(5)P	Phosphatidylinositol 5-phosphate
PtdIns(3,4)P_2	Phosphatidylinositol 3,4-bisphosphate
PtdIns(3,5)P_2	Phosphatidylinositol 3,5-bisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-trisphosphate
PtdSer	Phosphatidylserine
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PTP-Bas	Protein Tyrosine Phosphatase Basophile
PX	Phox-homology
Pyd	Polychaetoid
r CAS	Ca^{2+}-sensing receptor
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAC-1	Suppressor of actin mutations 1-like protein
SBD	Sphingolipid-binding domain
SBM	Sulfatide-binding motif
SH3	Src Homology 3
SHIP	SH2 domain containing inositol 5-phosphatase
SK	Sphingosine kinase
SLD	Scattering length density
SM	Sphingomyelin
SMase	Sphingomyelinase
SMS	Sphingomyelin synthase
SNCA	Synuclein, alpha, non A4 component of amyloid precursor
SnRK1.1	Sucrose non-fermenting-like kinase 1.1
SnRK2	Sucrose non-fermenting-1-related protein kinase 2
SPHK	Phytosphingosine kinase
SPR	Surface plasmon resonance

SPT	Serine palmitoyltransferase
START	Steroidogenic acute regulatory protein-related lipid transfer
stBLM	Sparsely-tethered bilayer lipid membrane
Taz1	Transacylase tafazzin
tBID	Truncated Bid
TGN	*trans*-Golgi Network
TIM	T cell immunoglobulin mucin
TIR1	Transport Inhibitor Response 1
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TSC	Tuberous sclerosis complex
VHS	Vps27/Hrs/Stam
vWF	von Willebrand factor
ZO	Zonula occludens

Sphingomyelin and Its Role in Cellular Signaling

1

Mahua Chakraborty and Xian-Cheng Jiang

Abstract

Sphingolipid *de novo* biosynthesis is related with metabolic diseases. However, the mechanism is still not quite clear. Sphingolipids are ubiquitous and critical components of biological membranes. Their biosynthesis starts with soluble precursors in the endoplasmic reticulum and culminates in the Golgi complex and plasma membrane. The interaction of sphingomyelin, cholesterol, and glycosphingolipid drives the formation of plasma membrane rafts. Lipid rafts have been shown to be involved in cell signaling, lipid and protein sorting, and membrane trafficking. It is well known that toll-like receptors, class A and B scavenger receptors, and insulin receptor are located in lipid rafts. Sphingomyelin is also a reservoir for other sphingolipids. So, sphingomyelin has important impact in cell signaling through its structural role in lipid rafts or its catabolic intermediators, such as ceramide and glycoceramide. In this chapter, we will discuss both aspects.

Keywords

Sphingomyelin • Ceramide • Diacylglycerol • Sphingolipids • Sphingolipid biosynthesis • Lipid rafts • Cholesterol homeostasis

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1.1 Introduction

Although the discovery of sphingomyelin (SM) was reported more than a century ago, its role as a significant 'bioactive lipid' along with other members of the sphingolipid family have been recognized just couple of decades ago. Technological advances in lipid detection, analysis, and quantitation have played a key role in promoting the

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development of the sphingolipid research field. There have been numerous studies establishing sphingolipids' multifunctional roles in the regulation of various cellular processes such as cell growth, death, senescence, adhesion, migration, inflammation, angiogenesis and intracellular trafficking [1, 2].

However, the concept that SM is involved in cellular signaling is relatively new. We believe that SM mediated cellular signaling can be broadly manifested in two ways:

(i) SM metabolism resulting in the production of various interconvertible bioactive sphingolipids or derivatives such as ceramide, diacylglyceride, and sphingosine-1-phosphate. These bioactive lipids act on their specific targets within the cell and regulate various signal transduction pathways, thereby affecting cellular functions.

(ii) SM-enriched lipid raft mediated cell signaling. The interaction of SM with cholesterol and glycosphingolipid is known to drive the formation of plasma membrane microdomains called lipid rafts [3]. As much as 70 % of all cellular SM is found in these rafts [4, 5] and they have proven to be involved in cell signaling, lipid, and protein sorting, and membrane trafficking [3, 6, 7].

This review specifically aims at deciphering the role of SM as a bioactive lipid in cellular signaling through its metabolism and its contribution to lipid rafts.

1.2 Structure, Sub-cellular Localization, and Measurement of Sphingomyelin Levels

The SM molecule consists of two regions: a phosphorylcholine head group attached to a ceramide molecule (Fig. 1.1). The latter in turn is made up of a sphingosine backbone and a fatty acid (acyl chain). SM usually contains 16:0, 18:0, 22:0, 24:0, and 24:1 acyl chains but the most

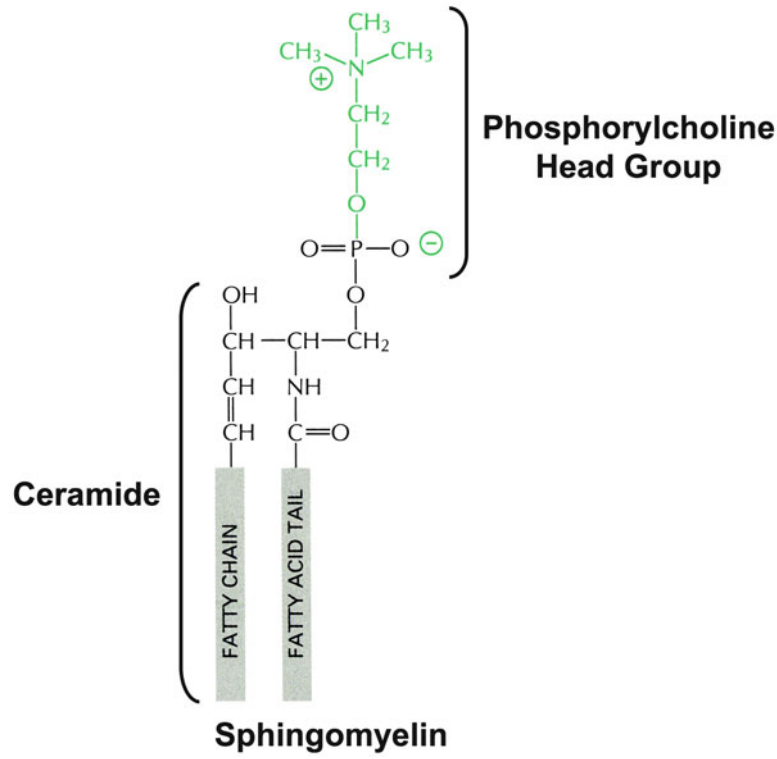

Fig. 1.1 Molecular structure of sphingomyelin

abundant SM species found in mammalian tissues are 16:0 [8]. Whether or not the differing acyl chain lengths in SMs dictate unique functions or important biophysical distinctions has not yet been established.

SM is the most abundant sphingolipid in mammalian cells and the majority of the cellular SM is located in the outer leaflet of plasma membranes [5, 9]. SM is indispensable for mammalian cell viability, as evidenced by the inability of mammalian cells to survive in culture, when they are unable to produce SM [10].

SM levels can be measured by the following methods: (i) enzyme-based assay: tissue homogenates can be incubated with bacterial sphingomyelinase, alkaline phosphatase, choline oxidase, peroxidase, N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, and 4-aminoantipyrine for 45 min. This results in a product with blue color, whose intensity is proportional to the SM present in the tissue sample, and can be measured at an optimal absorption of 595 nm [11]; (ii) liquid chromatography tandem mass spectrometry (LC/MS/MS); and, (iii) lysenin-mediated cell lysis assay. Lysenin is a SM-specific cytotoxin, which recognizes SM only when it forms aggregates or microdomains and eventually leads to cell lysis [12]. Based on the lysenin-mediated cell lysis intensity, plasma membrane SM levels can be indirectly evaluated. More SM on the plasma membrane can cause high cell mortality [12, 13].

1.3 Sphingomyelin Metabolism-Mediated Cell Signaling

1.3.1 De Novo Sphingomyelin Synthesis

SM biosynthesis initiates in the endoplasmic reticulum (ER), utilizing non-sphingolipid hydrophilic precursor molecules, serine, and palmitoyl-CoA (Fig. 1.2). The condensation of L-serine and palmitoyl-CoA to form 3-ketodihydrosphingosine is facilitated by ER membrane associated serine palmitoyltransferase (SPT). The next step in the sphingolipid biosynthesis is the reduction of 3-ketodihydrosphingosine to dihydrosphingosine by a reductase. N-acylation of the dihydrosphingosine gives rise to dihydroceramide, a product that is still relatively hydrophilic. Conversion of dihydroceramide to ceramide is facilitated by ceramide synthases and involves a desaturation step. Ceramides are hydrophobic and therefore become membrane associated. The majority of ceramides are transported from ER to the Golgi by ceramide transport protein (CERT), and the rest are converted to ceramide phosphoethanolamine (CPE). In the Golgi apparatus, ceramides are further converted to sphingomyelin by the sphingomyelin synthase (SMS) [14, 15], to glucosylceramide by the glucosylceramide synthase and, then, to more complex sphingolipids such as glucosylceramide and hematoside (GM3) by their respective synthases (Fig. 1.2). These products are then transported to plasma membrane, the major cellular reservoir for these lipids. SM and other sphingolipids may reach to the blood circulation through lipoprotein secretion or lipid efflux (Fig. 1.2).

1.3.2 Sphingomyelin and Its Related Bioactive Lipids

Sphingomyelin synthase (SMS), utilizing ceramide and phosphatidylcholine as its two substrates to produce SM and diacylglyceride, sits at the crossroads of bioactive lipid synthesis (Fig. 1.3). SM can also be hydrolyzed by sphingomyelinase (SMase) to yield ceramide and choline phosphate. The resulting ceramide can be further converted into sphingosine and sphingosine-1-phosphate (Fig. 1.3). Potentially, manipulating SMS and SMase could influence these bioactive lipid levels, thus influencing cell biological functions.

1.3.2.1 Sphingomyelinase-Mediated Ceramide Production

Twenty years ago, it had been disclosed that SMase-mediated SM hydrolysis (SM cycle) is a novel pathway of transmembrane signal transduction. In response to extracellular agonists, membrane SM can be hydrolyzed by SMase to yield ceramide and choline phosphate [16–20].

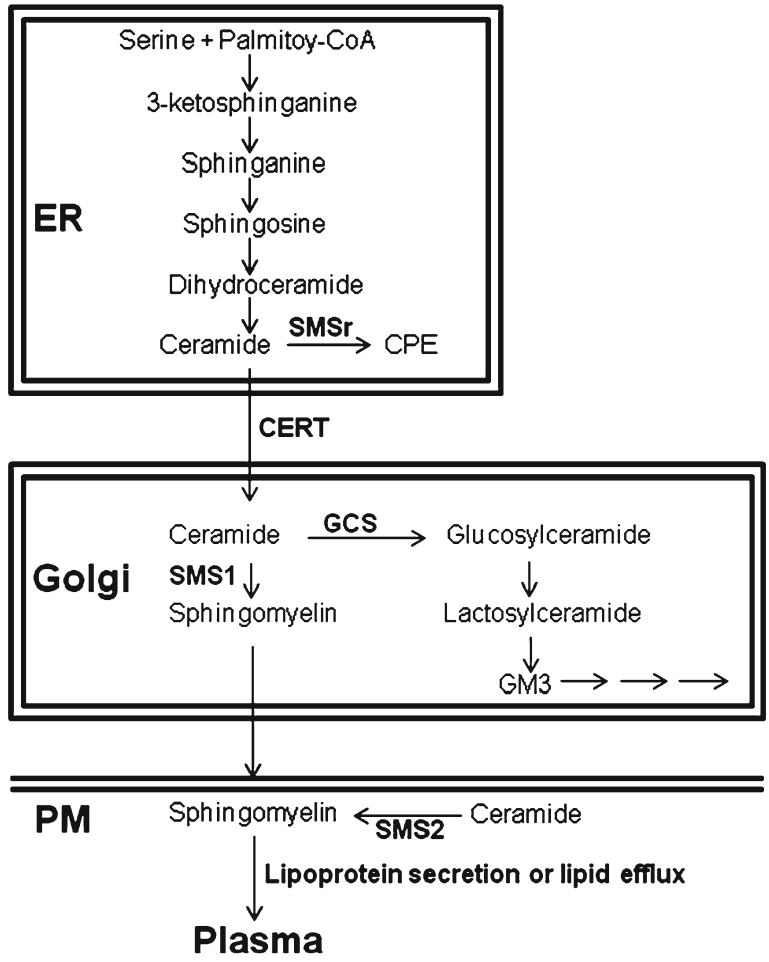

Fig. 1.2 Scheme of sphingomyelin biosynthesis. *SMS* 1 and 2, sphingomyelin synthase 1 and 2; *SMSr*, sphingomyelin synthase related protein; *GCS* glucosylceramide synthase, *CPE* ceramide phosphoethanolamine synthase, *CERT* ceramide trafficking protein, *GM3* hematoside, *ER* endoplasmic reticulum, *PM* plasma membrane

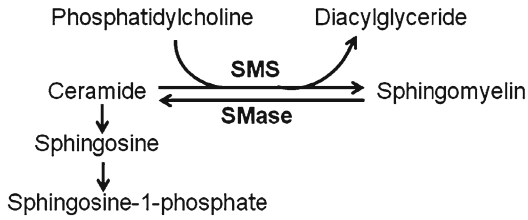

Fig. 1.3 SMS and SMase-related bioactive lipid productions. *SMS* sphingomyelin synthase, *SMase* sphingomyelinase

So far, five type of SMases have been reported and they are classified based on their optimal pH and metal ion dependence activity [21]. They are lysosomal acid SMase, secreted zinc-dependent acid SMase, magnesium-dependent neutral SMase, magnesium-independent neutral SMase, and alkaline SMase. Multiple reviews have summarized the current knowledge about these SMases, from an overview of structure and catalysis to specific properties, roles, and regulation of these enzymes in physiological and pathological contexts [22–24].

Ceramide is a product of SMase reaction (Fig. 1.3) and has been identified as a second messenger, mediating the effects of cell growth, cell differentiation, and apoptosis. Hannun and Obeid [25] have recently summarized a large body of information with regards to metabolism, structure, and function of ceramides.

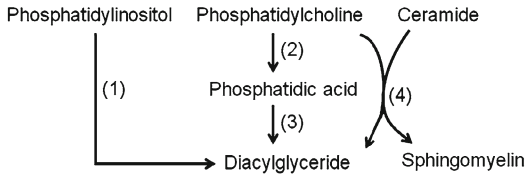

Fig. 1.4 Three pathways for diacylglyceride production. (1) phosphatidylinositol phospholipase C; (2) phosphatidylcholine phospholipase D; (3) phosphatidic acid phosphatase; (4) sphingomyelin synthase

1.3.2.2 Sphingomyelin Synthase-Mediated Diacylglycerol Production

There are three different pathways that can produce diacylglycerol [26] (Fig. 1.4). Many studies have clearly established the significant role of diacylglycerol in the regulation of fundamental cellular functions such as proliferation and apoptosis through the activation of protein kinase C [27–29]. However, we still do not know the importance of the diacylglycerol produced by the reaction catalyzed by SMS. This is because hydrolysis of membrane inositol phospholipids by phospholipase C, or hydrolysis of other membrane phospholipids, particularly choline phospholipids, by phospholipase D and phospholipase A2 can produce diacylglycerol that links extracellular signals to intracellular events through activation of protein kinase C [30]. However, it is conceivable that SMS activity-mediated diacylglycerol can potentially play an important role in maintaining cellular diacylglycerol pools [26].

1.4 Sphingomyelin as a Critical Component of Lipid Rafts in Mediating Signal Transduction

1.4.1 Sphingomyelin-Enriched Cell Membrane Lipid Rafts

Sphingolipids, including SM and glycosphingolipids, together with cholesterol, have been implicated in lateral microdomain or 'lipid raft' formation in biological membranes. These microdomains serve as signaling platforms and are involved in cellular processes, such as signal transduction, membrane trafficking, and protein sorting [31, 32]. Other lipids found in raft structures include phosphatidylethanolamine, glycerophospholipids, phosphatidylserine, arachidonic acid, phosphatidylglucoside, ceramide, and lactosylceramide [33, 34].

The formation of lipid rafts in biological membranes is driven by lipid–lipid interactions, which are largely dependent on the structure and biophysical properties of the lipid components. It is favored by the presence of long-chain saturated sphingolipids and phospholipids as well as by physiological proportions of cholesterol [35, 36]. There is strong evidence suggesting a preferential interaction between SM and cholesterol, stabilized by hydrogen bonding [37–39]. Infrared spectroscopic studies have also confirmed the presence of intermolecular hydrogen bonding between the amide group of SM and the 3-hydroxyl group of cholesterol [40]. The levels of cholesterol and SM in the plasma membrane are also tightly controlled [41, 42]. Greater lateral packing density in SM-containing membranes is known to be responsible for lowering the rate of spontaneous cholesterol transfer from SM-containing membranes [43]. Highly saturated glycosphingolipids are also capable of forming extensive hydrogen-bonding network with cholesterol and are therefore found in lipid rafts. However, in the presence of both SM and glycosphingolipids, cholesterol preferentially interacts with SM [44]. The rafts co-existing with the fluid matrix of the plasma membrane exist in the liquid-ordered phase [45, 46] due to its cholesterol content. Cholesterol promotes phase separation of saturated SMs [47] and SM needs cholesterol to be detergent-insoluble [45].

1.4.2 Lipid Rafts and Cell Signaling

Lipid rafts act as organizing centers for processes such as membrane trafficking and signal transduction [48, 49]. Cytoplasmic proteins that are covalently modified by saturated fatty acids (palmitoyl or myristoyl moieties) and cell surface proteins that are attached via a glycosyl phosphatidylinositol (GPI) anchor are highly concentrated within lipid rafts. Many proteins involved

in signal transduction, such as Src family kinases, G proteins, growth factor receptors, mitogen-activated protein kinase (MAPK), and protein kinase C are predominantly found in lipid rafts [32]. In addition, lipid rafts are dynamic in nature, which tends to scaffold certain signaling molecules, while excluding others. By such spatial segregation, lipid rafts not only provide a favorable environment for intra-molecular cross talk but also aid to expedite the signal relay.

Due to their insolubility in nonionic detergents such as Triton X-100, lipid rafts have been frequently termed as 'detergent resistant membranes' (DRMs). In fact, subpopulations of rafts have been proposed, in part based on their size, constituents, and functional properties [50, 51]. Caveolae are a subset of rafts and are considered to be 50–100 nm flask-like invaginations of the plasma membrane. Rafts and caveolae are dynamic entities, forming and dissipating in response to various external stimuli [52]. Upon stimulation, they internalize and serve a clathrin (coated pit)-independent mechanism of endocytosis of plasma membrane constituents. Raft/caveolae-mediated endocytosis is reported to facilitate transportation of entities to other cellular regions and across the cell (transcytosis) [53–55].

Membrane rafts and caveolae usually express specific proteins like flotillins and caveolins (Cavs) within their structure [56]. Cavs are structural proteins that provide an important, defining feature of caveolae and can be secreted into the extracellular space [57, 58]. Cavs are highly conserved among species and the three different isoforms of Cavs (Cav-1, -2, and -3) are differentially expressed in cells: Cav-3 is restricted to skeletal, cardiac, and smooth muscle, Cav-1 is more ubiquitously expressed, while expression of Cav-2 generally parallels that of Cav-1 [59]. Cavs also undergo covalent modifications like palmitoylation and phosphorylation [57–61]. It is known that insulin receptor (IR) is located in caveolae [62] and insulin receptor can interact with Cav1 [63]. In caveolae, the mobility of IR is increased by dissociation of the IR–Cav1 interaction [63]. It has been reported that SMS2 is able to regulate the dynamic structure of SM-rich lipid microdomains on the plasma membrane [64, 65] and could modify protein function, such as that of CD36 or Cav 1 located in the lipid microdomains [64]. SMS2 gene knockout (KO) mice exhibited disrupted regulation of the lipid microdomains function, leading to a prevention of lipid droplet formations, fatty liver, obesity, and insulin resistance [64, 65].

1.4.3 Role of the Lipid Rafts in Inflammatory Signaling

Toll like receptors (TLRs) are critically involved in inflammatory responses [66, 67]. Lipid rafts appear to provide a platform for the interaction of TLRs with their ligands in cells [68–71].

Each one of TLRs has a unique extracellular domain that allows specific ligand recognition. The intracellular toll/interleukin-1 (IL-1) receptor (TIR) domain of TLRs shares high degree of homology, but there are enough differences to cause diversified functions mediated by different TLRs [66, 67, 72, 73]. Upon ligand-induced stimulation, the TIR domain of TLRs associates with the TIR domain of their respective adaptor molecules to initiate intracellular signaling. Myeloid differentiation primary response gene 88 (MyD88) is a common TLR adaptor used by all TLRs, except for TLR3 [73]. Upon stimulation with a specific ligand, the membrane-associated TLRs (such as TLR2 and TLR4, and other components of the TLR complex) are recruited into the lipid rafts [74, 75]. These rafts aid in the interaction of TLRs with their ligands in macrophages [68–71], initiating nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and MAP kinase activation and proinflammatory cytokine production, thus resulting into inflammatory responses.

Tumor necrosis factor alpha (TNFα) is one of the cytokines involved in systemic inflammation. TNFα can specifically bind to TNF receptors (TNFRs). It is known that lipid rafts play an essential role in TNFR1 clustering [76]. Upon contact with TNFα, TNF receptors form trimers and this binding causes a conformational change to occur in the receptor, leading to the dissociation of the

inhibitory protein silencer of death domain (SODD) from the intracellular death domain. This dissociation enables the adaptor protein TNFR type 1-associated DEATH domain protein (TRADD) to bind to the death domain, serving as a platform for subsequent protein binding. Following TRADD binding, three pathways can be initiated [77, 78]: (1) activation of NFκB, (2) activation of MAPK pathways, and, (3) induction of cell death signaling.

Luberto et al. [79] found that D609, a nonspecific SMS inhibitor, blocks TNFα and phorbol ester-mediated NFκB activation that was concomitant with decreased levels of SM and diacylglyceride. Moreover, this did not affect the generation of ceramide, suggesting SM and diacylglycerol, derived from SM synthesis, are involved in NFκB activation. However, D609 is widely used to inhibit PC-phospholipase C (PLC) (Fig. 1.4), a well-known regulator of NFκB activation via diacylglyceride-mediated signaling [80]. Thus, it remains unclear what pathway is inhibited by D609 in particular that causes a diminished NFκB activation.

1.4.4 Role of Lipid Raft Sphingomyelin in Inflammatory Signaling

Studies from our laboratory [81] indicate that SMS2 knockdown in macrophages results in blockage of ligand-induced internalization as well as recruitment of TNFR1 to lipid rafts, suggesting a mechanism for the modulation of NFκB activity by SMS2. On similar lines, lipopolysaccharide (LPS)-induced plasma membrane recruitment of TLR4-MD-2 (TLR4 co-receptor) complex is also diminished in SMS2-knockout macrophages. As a result, SMS2 deficiency attenuates both NFκB and MAP kinase pathways, both of which are signaled via raft-associated TNFR1 and TLR4 along with their adaptor proteins. These findings strongly suggest the critical role of SMS2-synthesized SM for the normal function of TNFR1 and TLR4 on the plasma membrane following stimulation by their respective ligands (TNFα and LPS) [81].

We also created SMS1 knockout mice and found that SMS1 deficiency significantly decreased SM in plasma, liver, and macrophages but had only a marginal effect on ceramide levels [82]. Surprisingly, we found that SMS1 deficiency dramatically increased glucosylceramide and hematoside (GM3) levels in plasma, liver, and macrophages (4- to 12-fold), while SMS2 deficiency had no such effect. We evaluated total SMS activity in tissues and found that SMS1 deficiency causes 77 % reduction of SMS activity in macrophages [82], while SMS2 deficiency causes 70 % reduction of SMS activity in the liver [13], indicating SMS1 is the major SMS in macrophages, whereas SMS2 is predominant in the liver. We also found that SMS1 deficiency significantly attenuated TLR4-mediated NFκB and MAP kinase activation after LPS treatment.

The content of SM in the plasma membrane can also be modulated by SPT, the first and rate-limiting enzyme of the sphingolipid biosynthetic pathway [83]. SPT deficiency in macrophages also results in lower plasma membrane SM content as evidenced by lysenin-sensitivity assays, making the cells more resistant to lysis when treated with lysenin [81, 84]. LPS treatment of SPT deficient macrophages results in lesser recruitment of TLR4-MD2 complex, thereby attenuating both NFκB and MAP kinase activation. SPT deficient macrophages produce less TNFα and IL-6 *in vitro* when treated with LPS. SM supplementation experiments further prove that exogenous SM can enrich plasma membrane SM levels and can eventually restore the wild-type inflammatory phenotype in SPT deficient macrophages [128]. In general, SMS2 deficiency and SPT partial deficiency yield similar phenotypes, in terms of membrane SM levels, NFκB and MAP kinase activation. Unlike SMS2 deficiency, SPT partial deficiency does not change ceramide at the intracellular level or either in the plasma membrane or its lipid rafts. Thus, ceramide levels may have negligible role in mediating inflammatory signaling [128]. A reduction of plasma membrane SM levels are closely related to inflammation [81, 82].

1.4.5 Role of Lipid Raft Sphingomyelin Content in Cholesterol Homeostasis

Reverse cholesterol transport (RCT) is a multi-step process resulting in the net movement of cholesterol from peripheral tissues back to the liver via the plasma [85] and it plays a major role in cholesterol homeostasis. The first and most crucial step of RCT is cholesterol efflux from peripheral tissues, such as macrophages [85].

Foam cell formation due to excessive accumulation of cholesterol by macrophages is a pathological hallmark of atherosclerosis [86]. Macrophage scavenger receptor class A is implicated in the deposition of cholesterol in arterial walls during atherogenesis, through receptor-mediated endocytosis of modified low-density lipoproteins [87]. A member of scavenger receptor class B, CD36, is also involved in macrophage foam cell formation [88]. However, macrophages cannot limit the uptake of cholesterol, and therefore depend on cholesterol efflux pathways for preventing their transformation into foam cells. Several ATP-binding cassette (ABC) transporters, including ABCA1 [89] and ABCG1 [90], as well as scavenger receptor class B1 (SR-B1) [90], facilitate the efflux of cholesterol from macrophages.

In macrophages, ABCA1 exports cholesterol and phospholipids to lipid-free apolipoproteins, while ABCG1 and SR-BI export cholesterol to phospholipid-containing acceptors [90]. ABCA1-dependent cholesterol efflux requires aid from membrane lipid rafts [91, 92], while ABCG1 is mainly found intracellularly in the basal state, with little cell surface presentation. Under stimulation, for example by liver X receptor agonist treatment, ABCG1 redistributes itself to the plasma membrane, and increases cholesterol mass efflux to HDL [93]. ABCA1 and ABCG1 are known to cooperate in cholesterol efflux [90]. SR-BI also facilitates cholesterol efflux from macrophages [94]. ABCA1, ABCG1, and SR-BI are located in the plasma membrane, and exist either in rafts (SR-BI) [95, 96], or associated with the redistribution of lipids in the plasma membrane (ABCA1 and ABCG1) [90, 97]. It is, therefore, conceivable that fundamental changes in SM and glycosphingolipid levels of the plasma membrane can influence the functions of these proteins and alter cholesterol efflux [98, 99].

SM is also known as a cholesterol-binding molecule and there by plays an important role in cholesterol efflux. There are two possible SM-mediated cholesterol efflux mechanisms. Firstly, SM is involved in the recruitment of efflux-related transporters to the plasma membrane [94]. Indeed, SM-deficient cells enhance apoA-I-dependent cholesterol efflux by ABCA1 [98, 99]. This is further supported by SMS2 deficient and SPT partial deficient macrophage studies, where decrease of SM levels in macrophage plasma membrane increases both ABCA1 and ABCG1 protein levels on macrophage surfaces, thereby increasing cholesterol efflux *in vitro* and *in vivo* [100, 128]. Although ABCA1 is known to be located in a non-raft region, its levels influence lipid raft composition [101]. Overexpression of ABCA1 [97] and treatment of cells with high- density lipoprotein (HDL) or apoA-I [102, 103] disrupts or depletes raft domains, inhibiting raft-dependent signaling. This indicates a possible interaction between ABCA1 and raft-containing lipids.

Secondly, SM is also critical for cholesterol sequestration in the plasma membrane. It is known that lysosomal SMase is involved in cholesterol transport from lysosomes to the plasma membrane [98]. Because SM avidly binds cholesterol [104], SMase deficiency inhibits macrophage cholesterol efflux through promoting cholesterol sequestration by SM. Thus, SPT deficiency, leading to reduced plasma membrane SM levels, produces the inverse effect of SMase deficiency with reference to macrophage cholesterol efflux. SPT deficiency, therefore, aids in cholesterol efflux by inducing less cholesterol sequestration in the macrophage plasma membrane [128]. This is further supported by the finding that exogenously added SM significantly diminishes cholesterol efflux mediated by ABCA1 [99], suggesting that the increase of SM content in the plasma membrane prevents cholesterol efflux.

1.4.6 The Effect of Macrophage Lipid Raft Sphingomyelin Levels on Cholesterol Efflux and Inflammation

It is known that macrophage cholesterol efflux and inflammation are inversely related to each other. Yvan-Charvet et al. reported that macrophage ABCA1 and ABCG1 deficiencies increase free cholesterol accumulation and increase cell signaling via TLRs [105]. Zhu et al. reported that macrophage ABCA1 reduces MyD88-dependent TLR trafficking to lipid rafts by reduction of lipid raft cholesterol [106]. In addition, ABCA1 expression decreases cellular plasma membrane rigidity by reducing formation of tightly packed lipid rafts [97]. Therefore, more cholesterol efflux is related to less inflammation in macrophages. A recent report indicated that IL-6 markedly induced ABCA1 expression and enhanced ABCA1-mediated cholesterol efflux from human macrophages to apoA-I [107]. We found that SPT partial deficient macrophages have significantly lower SM levels in plasma membrane lipid rafts. This reduction not only impaired inflammatory responses triggered by TRL4 and its downstream NFκB and MAPK pathways, but also enhanced reverse cholesterol transport mediated by ABC transporters [128]. Our findings in this study clearly provided the evidence that plasma membrane SM levels are also critical for the inverse relationship between macrophage cholesterol efflux and inflammation.

1.4.7 Significance of Lipid Raft Sphingomyelin in Insulin Sensitivity

1.4.7.1 A Question from SPT Inhibition Studies

A previous study has indicated that treatment with myriocin, a potent SPT inhibitor and an immune suppressor, effectively ameliorates glucocorticoid-, saturated fat-, and obesity-induced insulin resistance [108]. Insulin resistance is a pathological condition where the insulin becomes less effective at lowering blood sugars. The authors attributed that effect to the reduction of ceramide in tissues, but they did not evaluate SM levels, especially in the plasma membrane. We and others have noted that myriocin treatment not only reduces ceramide, but also SM and glycosphingolipid levels [109–111]. This arises a fundamental question from the SPT inhibition studies: which sphingolipid in particular, ceramide, or SM, is responsible for causing the insulin resistance?

1.4.7.2 Ceramide and Insulin Resistance

There are two considerations linking ceramide and insulin resistance. Firstly, ceramide blocks the translocation of Akt/Protein Kinase B (PKB) to the plasma membrane [112]. It has also been reported that ceramide inactivation of Akt/PKB requires the atypical PKC isoform PKCζ [113, 114]. Secondly, ceramide may impair the action of insulin by facilitating signaling pathways initiated by inflammatory cytokines, such as TNFα and IL-6, which are known to impair insulin signaling [115, 116]. However, it has also been reported that various glycosphingolipid synthase inhibitors augment insulin-stimulated phosphorylation of the insulin receptor, as well as Akt/PKB and/or mammalian target of rapamycin phosphorylation, in the skeletal muscle [117] and liver [117, 118] of obese rodents, without altering ceramide levels. While ceramide accumulates in some insulin-resistant models [119, 120], it fails to do so in lipid-infused animals. In fact, the relative increase of ceramide in obese rodents and humans is rather quite small [121, 122]. Moreover, it is not known whether muscle ceramide content is a major factor in muscle insulin sensitivity. Adams et al. demonstrated that ceramide content is increased in skeletal muscle from obese, insulin-resistant humans [121], while Skovbro et al. found that human skeletal muscle ceramide content is not a major factor in muscle insulin sensitivity [123]. In general, the role of cellular ceramide in insulin resistance is controversial. Therefore more studies needs to be done in this field to establish this relationship.

1.4.7.3 Lipid Rafts and Insulin Resistance

Insulin resistance, abdominal obesity, dyslipidemia coupled with high blood pressure, and a

proinflammatory state are common disorders associated with type 2 diabetes and atherosclerosis [124]. An important question is how these interrelated risk factors could be mechanistically coupled in a physiological situation. Considering a simple scenario, lipid raft disruption could affect insulin signaling. It has been suggested that lipid rafts play an important role in the pathogenesis of insulin resistance [125]. Indeed, disruption of caveolae in cultured cells by cholesterol extraction with β-cyclodextrin results in progressive inhibition of tyrosine phosphorylation of insulin receptor substrate 1, as well as reduced activation of glucose transport in response to insulin [126].

Glycosphingolipids are also known to be structurally and functionally important components in the lipid rafts [127]. Pharmacological inhibition of glycosphingolipid synthesis markedly improves insulin sensitivity in rodent models of insulin resistance [118]. Deficiency of GM3 ganglioside (a key glycosphingolipid in the rafts) is also known to enhance insulin receptor tyrosine phosphorylation [62]. Moreover, GM3 could dissociate insulin receptor/Cav-1 complex, thus causing insulin receptor dysfunctionality [63]. Since SM is also one of the major components within lipid rafts, it is conceivable that diminishing SM in the plasma membrane could have a similar impact on insulin signaling.

1.4.7.4 Reducing Plasma Membrane Sphingomyelin Increases Insulin Sensitivity

We utilized two models: SPT partial deficient mice and SMS2 knockout mice for the insulin sensitivity study [65]. We found that: (i) both SPT partial and SMS2 complete deficiency enhances insulin sensitivity; (ii) both deficiencies decrease plasma membrane SM levels, which contribute to the enhancement of insulin sensitivity; (iii) SPT deficiency decreases ceramide, while SMS2 deficiency increases it, therefore, ceramide is probably not the regulator of insulin sensitivity; (iv) there, they were no significant changes of glucosylceramide and GM3 levels in tissues or even cell plasma membrane, so they might not play a significant role in insulin sensitivity in the above models; and finally, (v) this leads us to conclude that SPT or SMS2 inhibition is a promising pharmacological approach for the treatment of insulin resistance and metabolic syndrome.

1.5 Conclusions

The role of SM as a signaling molecule other than its membrane structural properties is recently being recognized in greater depth. And only a few of these important functions have been highlighted in this chapter, however, the scope of SM as a bioactive lipid mediator is enormous. Therefore, it is indeed essential to study and understand how SM is synthesized and degraded via its complex metabolic network. This would further shed light on this enigmatic molecule and its noteworthy roles in physiological processes. On the other hand, accumulating evidence showed that dynamic modification of SM in lipid rafts on cell plasma membrane controls development of obesity, insulin resistance, fatty liver, inflammation, and atherosclerosis. It is important to investigate the relationship between SM and other lipids, and between SM and functional proteins, such as insulin receptor, CD36, TLRs, and so on, in the lipid rafts.

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The Driving Force of Alpha-Synuclein Insertion and Amyloid Channel Formation in the Plasma Membrane of Neural Cells: Key Role of Ganglioside- and Cholesterol-Binding Domains

2

Jacques Fantini and Nouara Yahi

Abstract

Alpha-synuclein is an amyloidogenic protein expressed in brain and involved in Parkinson's disease. It is an intrinsically disordered protein that folds into an alpha-helix rich structure upon binding to membrane lipids. Helical alpha-synuclein can penetrate the membrane and form oligomeric ion channels, thereby eliciting important perturbations of calcium fluxes. The study of alpha-synuclein/lipid interactions had shed some light on the molecular mechanisms controlling the targeting and functional insertion of alpha-synuclein in neural membranes. The protein first interacts with a cell surface glycosphingolipid (ganglioside GM3 in astrocytes or GM1 in neurons). This induces the folding of an alpha-helical domain containing a tilted peptide (67–78) that displays a high affinity for cholesterol. The driving force of the insertion process is the formation of a transient OH-Pi hydrogen bond between the ganglioside and the aromatic ring of the alpha-synuclein residue Tyr-39. The higher polarity of Tyr-39 vs. the lipid bilayer forces the protein to cross the membrane, allowing the tilted peptide to reach cholesterol. The tilted geometry of the cholesterol/alpha-synuclein complex facilitates the formation of an oligomeric channel. Interestingly, this functional cooperation between glycosphingolipids and cholesterol presents a striking analogy with virus fusion mechanisms.

Keywords

Langmuir monolayer • Alpha-synuclein • Parkinson's disease • Glycosphingolipid • Ganglioside • GM1 • GM3 • Cholesterol • Molecular modeling simulations • Weak hydrogen bond • OH-Pi bond • Tilted peptide • Virus fusion • Calcium channel

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2.1 Introduction

Alpha-synuclein belongs to the category of amyloidogenic proteins that are involved in major human neurodegenerative diseases including

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Alzheimer, Creutzfeldt-Jakob, and Parkinson's diseases. These proteins are physiologically expressed in brain tissues but can become pathogenic in case of overexpression or presence of genetic mutations. Three mutations in the alpha-synuclein gene SNCA (synuclein, alpha, non A4 component of amyloid precursor) have been reported in familial Parkinson disease: A30P, E46K and A53T, all associated with increased propensity of these mutant forms of alpha-synuclein to aggregate [1]. Despite intense research efforts these last years, the molecular mechanisms by which wild-type and mutant forms of alpha-synuclein perturb specific brain cells in the substantia nigra are still mostly unknown. Several reports have indicated that alpha-synuclein can selectively interact with membrane lipids such as acidic phospholipids [2–5] and glycosphingolipids [6–9]. On the basis of these data, we and others have proposed that the toxicity of alpha-synuclein is correlated with its capacity to interact with cellular membranes [9, 10]. Several mechanisms can explain the key role of membranes on the behaviour of amyloidogenic proteins such as alpha-synuclein. First, there is a reduction of dimensionality, which occurs when the protein binds to the membrane surface, resulting in a significant increase of concentration [10, 11]. On the other hand, membrane lipids can act as chaperones able to stabilize nonpathological conformations of amyloidogenic proteins [12, 13], or as anti-chaperones promoting disease-associated conformational changes and formation of highly toxic fibrils [14–16].

Despite the widely spread theory of toxic amyloid deposits being responsible for neuron injury in neurodegenerative diseases [17], other mechanisms of toxicity involving oligomers of amyloidogenic proteins have been discovered. In particular, it has been shown that upon binding to membranes, most (if not all) amyloidogenic proteins can self-organize into oligomeric structures forming amyloid pores [18] and/or ion channels [19, 20]. These structures are believed to perturb Ca^{2+} fluxes across brain membranes, leading to severe cellular injury [21]. Upon binding to artificial membranes containing anionic phospholipids, alpha-synuclein has been shown to adopt a helical conformation allowing its insertion in the membrane and the formation of ion channels with well-defined conductance states [20]. Ion channel formation by alpha-synuclein was also observed in membrane bilayers containing gangliosides GM1 and GM3 [7]. Structure-function relationship studies with full-length recombinant alpha-synuclein showed that the protein has a high affinity for gangliosides, especially GM3 [8]. The GM3-binding site was identified as a contiguous fragment of alpha-synuclein located between amino acid residues 34–45 [8]. In the membrane-bound conformation of alpha-synuclein, this fragment forms a loop connecting two alpha-helix domains. This topology of alpha-synuclein is similar to the hydrophobic domain of colicin Ia, a bacterial toxin that inserts into host cell membranes and forms ion channels [22]. Thus it is conceivable that alpha-synuclein channel formation could be initiated by the membrane insertion of the helix-loop-helix motif containing the GM3-binding domain 34–45. Moreover, the membrane-associated topology of alpha-synuclein contains a tilted peptide (67–78) that has been shown to bind cholesterol with high avidity [23]. These data raise the interesting possibility that both a sphingolipid and a cholesterol binding domain could be involved in the translocation of alpha-synuclein to biological membranes.

Deciphering the molecular mechanisms controlling the interaction of alpha-synuclein with brain membranes and the formation of ion channels is of high interest in the context of Parkinson disease research. In a recent study, we have shown that Tyr-39 plays a key role in the interaction between alpha-synuclein and GM3, possibly through the establishment of an OH-Pi hydrogen bond [8]. In this chapter, we will focus our attention on the peculiar geometry parameters of this OH-Pi bond in a GM3/alpha-synuclein complex. We will discuss the role of this weak hydrogen bond in the whole process of insertion of alpha-synuclein in ganglioside and cholesterol enriched plasma membrane domains. This mechanism requires the functional cooperation of glycosphingolipids and cholesterol, which interact sequentially with distinct domains of alpha-synuclein. At the molecular level, it is strikingly similar to the fusion of human retroviruses (HIV-1, HTLV-I), which also display a tilted peptide and penetrate

host cell membranes through a sphingolipid/cholesterol-dependent process [24].

2.2 How Alpha-Synuclein Interacts with Gangliosides

One important aspect of ganglioside recognition by soluble proteins is that these proteins have access only to the part of the lipid that protrudes outside the membrane, i.e., the sugar head group. In this context, protein-ganglioside interactions are similar to protein-sugar interactions [25]. However, the conformation of the sugar head-group of gangliosides is restricted by the part of the lipid embedded in the membrane, i.e., the ceramide moiety [12, 26]. This unique situation considerably simplifies the *in silico* studies of protein-ganglioside interactions. Indeed, molecular dynamics simulations have been of great help in our comprehension of the molecular mechanisms controlling protein binding to gangliosides and related glycosphingolipids [8, 9, 27]. In particular, this has led to the definition of a structural sphingolipid-binding domain (SBD) shared by various cellular and microbial proteins [28]. Briefly, the SBD is a short loop motif containing a central aromatic residue whose role is to stack onto the planar surface of a sugar ring belonging to the glycosphingolipid, generally glucose or galactose [29–33]. This sugar-aromatic interaction is best described as a CH-Pi bond, the aliphatic hydrogen atoms of the sugar being attracted by the Pi cloud of the aromatic ring of the protein [34]. It is particularly operative with phenylalanine and tryptophan residues, which do not bear any chemical group on their aromatic rings. The case is slightly different for tyrosine because its side chain contains a phenol, i.e., a phenyl cycle with a OH group. As discussed below, this hydroxyl further extends the possibilities of molecular interactions between the protein and the glycosphingolipid, as it is the case for ganglioside GM3.

The geometry of a GM3/alpha-synuclein complex is shown in Fig. 2.1. This study is based on the recent characterization of the GM3-binding site of alpha-synuclein as fragment 34-KEGVLYVGSKTK-45 [8]. The structure of the 34–45 fragment was retrieved from the PDB file 1XQ8, which corresponds to an NMR characterization of micelle-bound alpha-synuclein [35]. In this structure, two curved alpha-helices (3–37 and 45–92) are connected by a well ordered, extended linker (fragment 38–44). The linker contains most of the GM3-binding site. The C-terminal part of the protein (93–140) is unstructured. The geometry of the 34–45 fragment retrieved from the pdb file was first optimized with Polak-Ribière algorithm. Then, it was included in a set box and merged with 826 water molecules. After a second round of geometry optimization including the fragment and the water molecules, molecular modeling simulations with the CHARMM force field under the set box conditions were initially conducted for 10 ps. The resulting 34–45 fragment was merged with a dimer of GM3 [8]. The sugar moieties of both GM3 molecules were oriented in two opposite directions so that they did not physically interact. This topology provided a wide space compatible with the insertion of the GM3-binding site of alpha-synuclein between the polar parts of both GM3 molecules (Fig. 2.1a). The ternary complex GM3/alpha-synuclein/GM3 was stabilized by two electrostatic bonds involving the carboxylate groups of the neuraminic acid (NeuAc) residue of each GM3 molecule and the cationic side chain of Lys-45 (Fig. 2.1b) and Lys-34 (Fig. 2.1c). In addition, the aromatic side chain of Tyr-39 acted as an acceptor Pi-group for the hydrogen atom of the OH group linked to the C2 of the glucose residue of GM3 (Fig. 2.1d). This particular type of interaction between an aromatic ring and an OH group has been described as a weak hydrogen bond and referred to as an OH-Pi bond [36].

2.3 Structural Characterization of the OH-Pi Bond Between GM3 and Alpha-Synuclein

The geometrical parameters of this OH-Pi bond have been determined with the Hyperchem program (Fig. 2.2). Since different geometrical descriptors have been used in the past to characterize the X-H…Phenyl hydrogen bond [36], we have performed several measurements. The point of reference is the aromatic centroid M, and

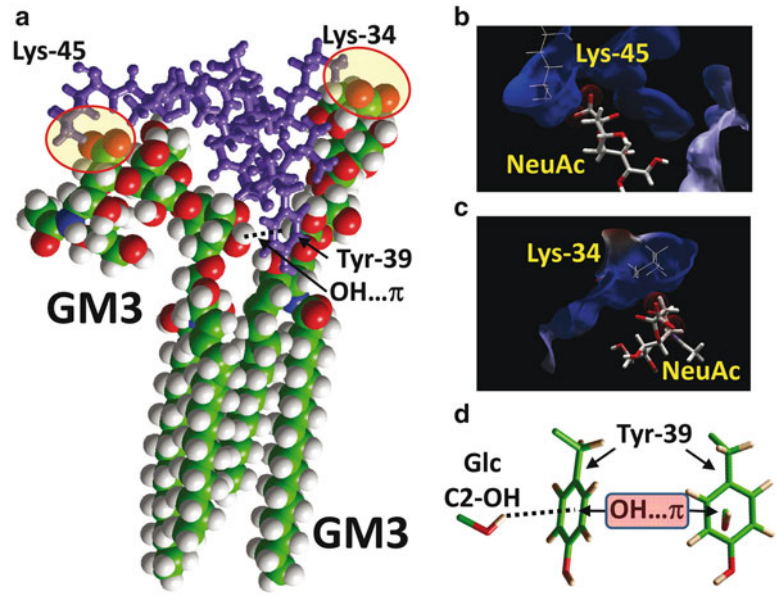

Fig. 2.1 Evidence for an OH-Pi bond between alpha-synuclein and GM3. A general view of a molecular complex between alpha-synuclein (fragment 34–45) and two GM3 molecules is shown in (**a**). The complex is stabilized by two electrostatic interactions involving Lys-45 (**b**) and Lys-34 (**c**) and the carboxylate group of the terminal NeuAc residue of each GM3 molecule. The geometry of the GM3/alpha-synuclein complex is consistent with the establishment of an OH-Pi bond (*dotted line*) between the C2-OH of the glucosyl residue of one GM3 molecule (on the *left* in panel **a**) and the aromatic ring of Tyr-39. Two distinct views of this OH-Pi interaction are shown in (**d**). Note that the phenolic OH of Tyr-39 is not involved in GM3 binding. Molecular dynamics simulations were performed as described in [8]. The molecules were viewed with Hyperchem (**a, d**) or Molegro Molecular Viewer (**b, c**). The *dotted lines* indicate hydrogen bonds

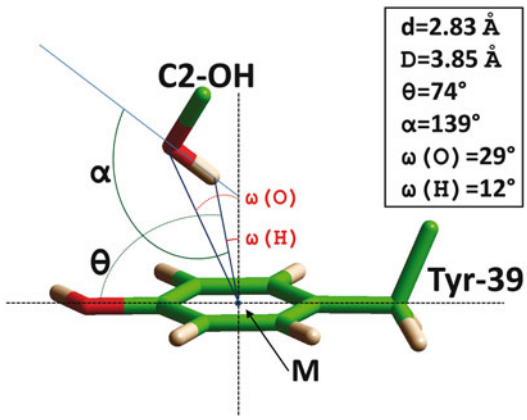

Fig. 2.2 Geometric parameters of the OH-Pi bond between GM3 and alpha-synuclein. M represents the Tyr aromatic *ring midpoint*. C2-OH refers to the OH group linked to carbon C2 of the glucosyl residue of GM3

distances are given in Fig. 2.2 with respect to this point: d = H…M and D = O…M. In the GM3/alpha-synuclein complex, the values for d and D are lower than 3 and 4 Å, respectively. For the sake of comparison, the distance cut-off selected by Steiner and Koellner [37] in a wide survey of XH-Pi bonds in proteins was D < 4 Å. Thus, the OH-Pi bond between GM3 and alpha-synuclein is comparable to those found in the 3D structures of proteins. Omega (H) is the angle between the H…M line and the ring normal. An angle ω(O) is defined analogously with the O…M line. The angle values obtained for the OH-Pi bond described in this study indicate that the hydrogen atom faces the large surface of the aromatic ring, as shown in Fig. 2.1d. Finally, we also used the definition of the geometrical categories of X-H… Phenyl interactions as defined by Malone et al. [38] and determined the values of α and θ (Fig. 2.2). With values of α = 139° and θ = 74°, the geometry of the OH-Pi bond between GM3 and alpha-synuclein falls in the type III category [38]. The frequency of type III OH-Pi bonds have been found to occur is 16 % of the protein

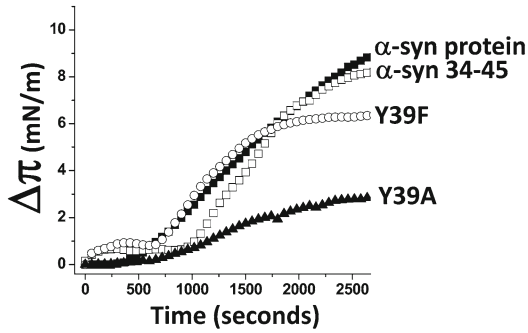

Fig. 2.3 Interaction of recombinant alpha-synuclein and peptides with ganglioside monolayers. The recombinant alpha-synuclein protein or the indicated synthetic peptides were injected underneath a monolayer of GM3 prepared at an initial surface pressure of 20 $mN.m^{-1}$ and the changes in surface pressure were continuously recorded. Each curve is representative of three separate experiments (SD < 10 %). Legend: full-length recombinant alpha-synuclein (*full squares*), fragment 34–45 (*open squares*), Tyr-39/Phe mutant of fragment 34–45 (*open circles*); Tyr-39/Ala mutant of fragment 34–45 (*full triangles*)

studied [38]. Overall the geometrical parameters of the OH-Pi bond described in the present study are typically those of bona fide X-H…phenyl bonds found in proteins.

2.4 Physicochemical Studies of Alpha-Synuclein/GM3 Interactions

Experiments with synthetic peptides have been conducted in order to evaluate the potential role of this OH-Pi bond on alpha-synuclein/GM3 interactions. In these experiments synthetic peptides derived from the 34 to 45 fragment of alpha-synuclein were injected in the aqueous subphase underneath a monolayer of GM3 at the air-water interface. The insertion of the peptide in the GM3 monolayer was followed by surface pressure measurements [8]. This assay is particularly efficient in determining quantitatively the association of a protein with a lipid monolayer [39]. Compared with other assays such as NMR [40], it uses very few amounts of protein (usually in the nM–μM range) in an experimental volume as low as 800 μL [27]. Surface pressure measurements have been successfully used to determine the lipid binding specificity of various amyloidogenic proteins including Alzheimer's Aβ peptides [27], the prion protein PrP [28], amylin [30], and alpha-synuclein [8]. In the later case, we have determined the following order for the affinity of alpha-synuclein/glycosphingolipid interactions: GM3 > Gb3 > GalCer-NFA > GM1 > sulfatide > GalCer-HFA > LacCer > GM4 > GM2 > asialo-GM1 > GD3. The key role of Tyr-39 in these interactions was assessed with mutant synthetic peptides derived from the SBD of alpha-synuclein. A representative experiment with such mutant peptides is shown in Fig. 2.3. In this study, three synthetic peptides were tested: the wild-type 34-KEGVLYVGSKTK-45 and two mutants of Tyr-39: Y39F and Y39A. Before assaying these peptides in the GM3 monolayer assay, a series of molecular dynamics simulations were conducted with these peptides in water. The data indicated that the secondary structure of the peptides was not dramatically affected by the Phe and Ala substitutions [8]. Thus, these peptides could be used to assess the role of the aromatic side chain at position 39 of the GM3-binding site of alpha-synuclein. First, it is important to note that the wild-type 34–45 peptide has retained the ability of full-length alpha-synuclein to rapidly interact with the GM3 monolayers. Interestingly, the Tyr → Phe substitution did not have a major impact on the insertion of the 34–45 peptide in the GM3 monolayer. In contrast, the Tyr → Ala substitution resulted in a dramatic loss of insertion. These data strongly suggested that the insertion of alpha-synuclein between GM3 molecules is dependent upon the presence of an aromatic ring at position 39. This aromatic ring can be the side chain of either Tyr or Phe, so that we can exclude a role for the OH group of the phenol ring. Instead, these data are fully consistent with the establishment of an OH-Pi bond in which the aromatic ring acts as a Pi-acceptor group for a hydrogen atom of an OH group of GM3. This explains why a Phe residue can be a surrogate for Tyr-39, whereas Ala cannot.

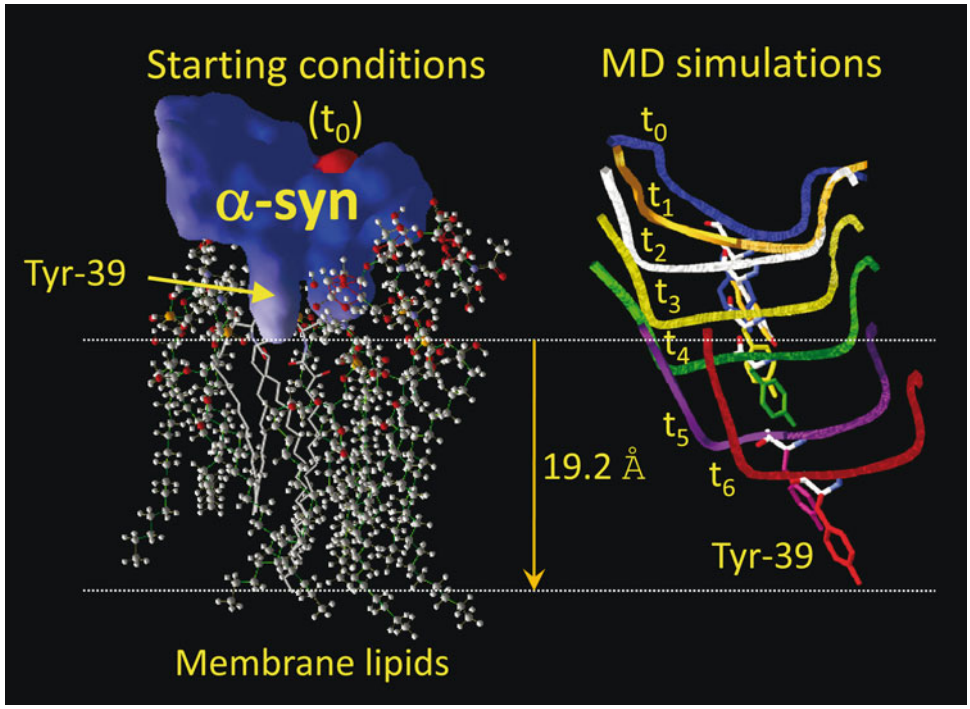

Fig. 2.4 Progressive insertion of alpha-synuclein in a lipid membrane. The SBD of alpha-synuclein protein (fragment 34–45) complexed with two GM3 molecules was merged with a model membrane containing cholesterol and phosphatidylcholine. Energy minimization of the whole system was achieved with the Polak-Ribiere algorithm [8] in order to obtain realistic starting conditions (*left panel*) referred to as t_o time. The OH-Pi bond between GM3 and Tyr-39 of alpha-synuclein was present at this step. Molecular dynamics simulations were then conducted for 10 ns. The OH-Pi bond appeared to be very labile, allowing the peptide to gradually penetrate in the lipid membrane. Snapshots of the insertion process were taken at six different times (from t_1 to t_6) corresponding to 1, 2, 4, 6, 8, and 10 ns, respectively (*right panel*)

2.5 The OH-Pi Bond Between Alpha-Synuclein and GM3 Is Transient

Molecular dynamics simulations were conducted to assess the stability of the OH-Pi bond in the alpha-synuclein/GM3 complex. The SBD of alpha-synuclein bound to the GM3 molecules was introduced in a model membrane containing phosphatidylcholine and cholesterol (Fig. 2.4). After energy minimization of the whole system, molecular dynamics simulations were conducted with the CHARMM force field. After the first nanosecond, the OH-Pi bond did no longer exist and the peptide has slightly penetrated the membrane. Over time, the peptide has been moving steadily towards the apolar region of the membrane, leaving the polar headgroup of GM3 far behind. After 10 ns, the peptide has traveled 19.2 Å and is completely buried into the membrane. This rapid progression can be explained by the polar/nonpolar clash between the OH group and the aliphatic chains of membrane lipids. In the GM3/alpha-synuclein/GM3 complex, the side chain that is closer to the membrane is the phenol group of Tyr-39 (Figs. 2.1 and 2.4). Indeed, the aromatic ring reaches the polar/nonpolar interface of the GM3 membrane, near the ceramide-sugar junction. At this stage, the aromatic ring is transiently stabilized by the OH-Pi bond. Since the OH-Pi bond is moderately strong in energy [36], it will not block the SBD on the sugar head groups of the GM3 dimer. Thus, the loop can penetrate more deeply into the membrane, the polar phenolic

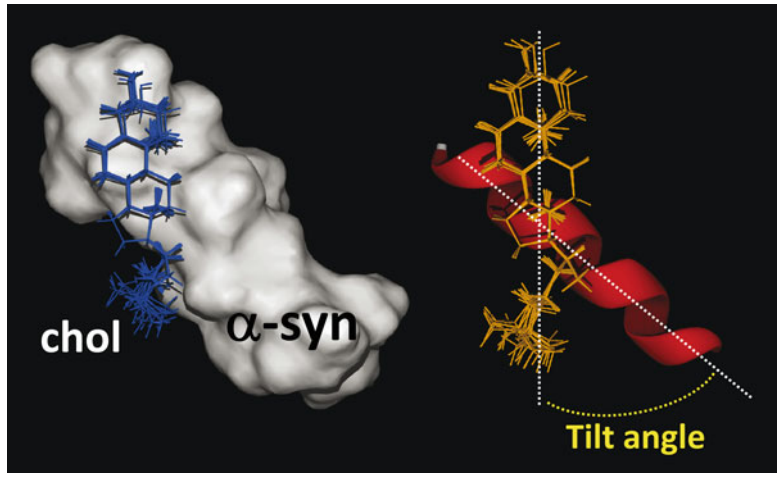

Fig. 2.5 Cholesterol forms a stable complex with the tilted peptide of alpha-synuclein. The surface complementarity between the tilted peptide of alpha-synuclein (segment 67–78) and cholesterol is illustrated by a surface view of the complex (*left panel*). Molecular dynamics simulations of the cholesterol/alpha-synuclein complex conducted for 10 ns showed that the complex was remarkably stable. Iterative snapshots were taken every ns and the successive positions occupied by cholesterol were superimposed on the structure of the tilted peptide. The mean angle between the helix axis of the tilted fragment of alpha-synuclein and the main axis of cholesterol is 46° (*right panel*)

OH group of Tyr-39 repulsing the nonpolar groups of the lipids as the insertion gradually progresses along the aliphatic chains of membrane lipids. Indeed, the OH group of Tyr-39 does not interact at all with GM3 and it faces the nonpolar part of the lipid bilayer (Fig. 2.1).

2.6 The Cholesterol-Binding Domain of Alpha-Synuclein Is a Tilted Peptide

The interaction of alpha-synuclein with negatively charged lipids, such as gangliosides, induces the formation of alpha-helices in the protein [4–6]. One of these helices (45–92) is located upstream the SBD [35]. This alpha-helix domain includes an hydrophobic segment (67-GGAVVTGVT-AVA-78) that has been previously shown to penetrate in the membrane and induces toxicity for neuroblastoma cells and has been structurally characterized as a tilted peptide [41]. Tilted peptides are short helical protein fragments that are able to disturb the organization of the molecular system into which they insert. They are characterized by an asymmetric distribution of their hydrophobic residues, which induces a tilted orientation (around 45°) towards the membrane plane [42]. Because they induce an important distortion of the membrane structure, tilted peptides are involved in the fusion process triggered by viral glycoproteins [43]. By testing a panel of synthetic peptides derived from alpha-synuclein in the lipid monolayer assay, we recently showed that the tilted fragment 67–78 peptide efficiently interacted with cholesterol [23]. Molecular dynamics simulations revealed that this tilted domain displays a high affinity binding site for cholesterol (Fig. 2.5). Moreover, the peptide bound to cholesterol fully retained its tilted orientation. To assess the stability of the alpha-synuclein/cholesterol interaction, molecular dynamics simulations were performed for 10 ns (Fig. 2.5). Snapshots were taken every ns during the 10 ns of simulations to follow the trajectory of cholesterol bound to alpha-synuclein. It can be seen that the geometry of the complex was remarkably stable, cholesterol remaining tightly bound to the tilted peptide. Minor fluctuations of cholesterol position in its binding site were

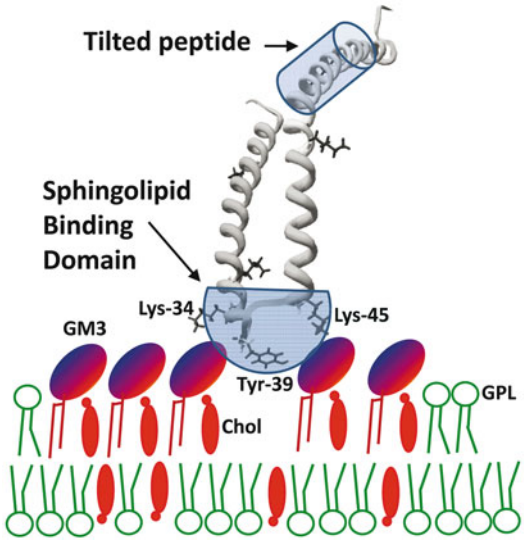

Fig. 2.6 Respective locations of the sphingolipid- and cholesterol-binding domains of alpha-synuclein. Topology of alpha-synuclein at the early steps of its interaction with a lipid raft enriched in GM3, cholesterol, and selected glycerophospholipids (GPL). The SBD of alpha-synuclein (fragment 34–45) interacts with the sugar head groups of GM3, leaving the tilted peptide (67–78) outside the membrane. The SBD has to penetrate into the membrane to allow the tilted peptide to bind cholesterol. The side chain of Tyr-39, with the assistance of lysine residues 34 and 45 of the SBD, plays a central role in this process

noted essentially in the polar head group (OH) and the nonpolar tail (isooctyl chain) of cholesterol. Overall, a mean angle of 46° was measured between the main axis of cholesterol and the peptide. These data, obtained *in silico*, suggested that in marked contrast with the GM3/alpha-synuclein interaction, the association of alpha-synuclein with membrane cholesterol was remarkably stable.

2.7 The Driving Force of Alpha-Synuclein Insertion in the Plasma Membrane

These data shed some light on the mechanisms of insertion of alpha-synuclein in cellular membranes. As shown in Fig. 2.6, when the SBD of alpha-synuclein is bound to cell surface gangliosides, the tilted peptide is far from the membrane. At this stage, two important problems have to be fixed. First, the tilted peptide is globally nonpolar, and it currently faces a polar environment. This was not the case when alpha-synuclein was unstructured, because it is likely that this short fragment could be protected from water molecules by the numerous charged amino acid residues of alpha-synuclein. Although α-synuclein is mostly unfolded in solution, its hydrodynamic dimensions reveal that the protein is more compact than expected from a random coil [44]. Indeed, both circular dichroism and Fourier transformed infrared spectroscopy of alpha-synuclein suggested that the protein has a slightly collapsed conformation, 14 % of its secondary structure corresponding to turns [44]. This could ensure that the nonpolar residues of alpha-synuclein are kept away from water molecules. What precludes the complete folding of the protein is the limited number of nonpolar residues, which renders impossible the formation of a nonpolar core following thermodynamic entropic rules [45]. By inducing the folding of alpha-synuclein in alpha-helical structures containing the tilted segment, membrane gangliosides create *de facto* a clash between the water environment and the nonpolar side chains of the tilted peptide. Consequently, the newly formed tilted peptide has to find a nonpolar milieu that the protein itself cannot provide. Because at this stage alpha-synuclein is bound to the plasma membrane, the closest nonpolar environment that could lower the energy of the tilted peptide is the nonpolar core of the plasma membrane. Thus, the tilted peptide has no "thermodynamic choice" other than inserting into the membrane.

Yet, there is a second obstacle to overcome before the protein can be inserted into the membrane. As a matter of fact, once the SBD is bound to the cell surface it could perfectly remain there if the ganglioside/alpha-synuclein interaction was strong enough. However, this is not the case, and the weak link is the OH-Pi interaction. This weak hydrogen bond could be formed because when the SBD of alpha-synuclein initially interact with GM3, Tyr-39 is the residue closest to the membrane. However, the OH-Pi bond is a weak energy bond and it can be very easily destroyed by the vibrational energy of chemical bonds. This is clearly suggested by molecular dynamics

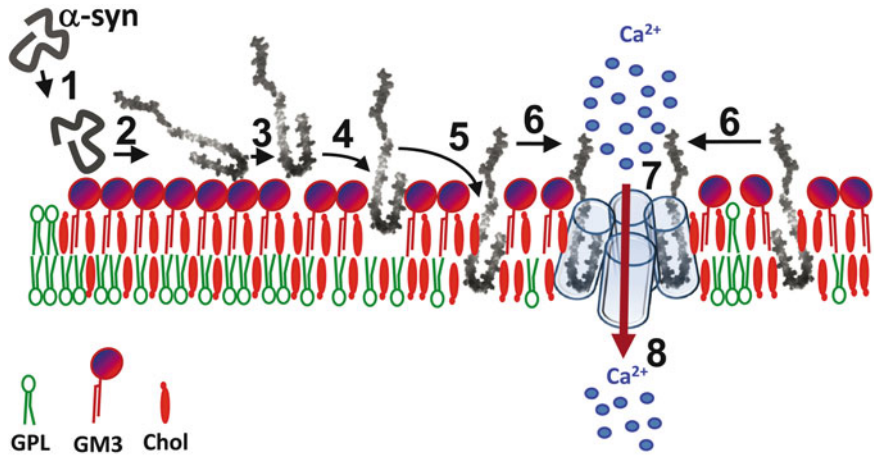

Fig. 2.7 The driving force of alpha-synuclein insertion and channel formation in a lipid raft domain of a neural cell. Alpha-synuclein is an intrinsically disordered protein, which has a marked affinity for negatively charged lipids such as gangliosides (e.g., GM3 in astrocytes) (**1**). Upon binding to gangliosides, the protein adopts an alpha-helical structure (**2**). The insertion process is initiated by the binding of the SBD of alpha-synuclein (segment 34–45) to the sugar head groups of GM3 (**3**). A transient OH-Pi bond is formed and rapidly destroyed, so that the polar OH group of Tyr-39 has to face the nonpolar region of the lipid bilayer. This is probably the main driving force of alpha-synuclein insertion (**4**), which will be complete once the SBD has been translocated through the membrane. The stable interaction between cholesterol and the tilted domain of alpha-synuclein stabilizes the transmembrane topology of alpha-synuclein and forces the protein to remain in a tilted configuration with respect to the membrane (**5**). Under these conditions, the oligomerization of several alpha-synuclein/cholesterol units could occur in the membrane (**6**), eventually leading to the generation of a functional ion channel (**7**). The C-terminal ending of alpha-synuclein contains several acidic residues (Asp and Glu) that can attract calcium ions from the extracellular space and transport them into the cytosol (**8**)

simulations (Fig. 2.4). It is important to note that in the OH-Pi bond, the OH group is provided GM3 and the Pi cloud by Tyr-39. This leaves the OH group of Tyr-39 (the phenolic hydroxyl group) without any stabilizing interaction (Fig. 2.1). Because the phenolic OH is deeply dipped in the membrane, it is directly confronted with an unfavourable nonpolar environment. We propose that this polar/nonpolar clash is the main driving force that allows the rapid insertion of alpha-synuclein in the membrane. In this process, the polar OH group is assisted by the side chains of Lys-34 and Lys-45 that flank each side of the SBD (Fig. 2.6). This triad of polar amino acid residues (Lys-34/Tyr-39/Lys/45) pulls the protein across the membrane until they find a polar environment, the cytosol in this case. When the SBD has totally crossed the membrane, there are two possible options. The protein could pursue its journey and completely cross the membrane, or alternatively, it could remain in the membrane, becoming a transmembrane protein. Our data strongly suggested that the fate of alpha-synuclein in the membrane is controlled by cholesterol. Indeed, the translocation of the SBD across the membrane has allowed the tilted peptide to find membrane cholesterol. If the tilted peptide binds to cholesterol, it is likely that this interaction will efficiently stabilize the protein in the membrane, as suggested by molecular dynamic simulations (Fig. 2.5).

2.8 How and Why Alpha-Synuclein Monomers Self-Organize into Functional Ion Channels

The ultimate step of the complex insertion process of alpha-synuclein is the formation of a functional oligomeric ion channel (Fig. 2.7). This would not be possible if the mode insertion of alpha-synuclein in the membrane was not the same for

all monomers. If cholesterol acts as a wedge, then the tilted peptide will be located at a specific and constant distance from the membrane surface, allowing the recruitment of a pool of homogenous alpha-synuclein monomers. Downstream the tilted peptide, the C-terminal domain of alpha-synuclein contains numerous acidic residues that confer a huge negative charge. This negative charge is naturally repulsive and could preclude the oligomerization process. However, the tilted configuration of the cholesterol-binding domain ensures that the negatively charged domain of alpha-synuclein is rejected sufficiently far from each transmembrane anchoring domain to allow the oligomerization process. In this respect, it can be anticipated that the relative content of cholesterol in a plasma membrane is an important parameter that determines the possibility for amyloidogenic proteins to form amyloid channels. This key role of cholesterol has been previously reported in the literature [9], although the molecular mechanisms controlling this effect remain unclear. Because gangliosides are exclusively located in the extracellular leaflet of the plasma membrane, the extracellular mouth of the channel is negatively charged. Thus, the oligomeric alpha-synuclein channels described are particularly suited to induce intracellular calcium fluxes from the extracellular space to the cytosol (Fig. 2.7). This could elicit important toxic effects in total absence of amyloid fibrils, according to the calcium channel hypothesis proposed by Arispe et al. for explaining amyloid-induced toxicity in the Alzheimer ‘s disease [21].

2.9 Analogy Between Amyloid Channel Formation and Virus Fusion

The insertion mechanism described in this chapter could potentially apply to any protein targeted to lipid rafts and containing a SBD and a tilted peptide. This is the case for various amyloidogenic proteins such as Alzheimer’s Aβ peptide and PrP which, like alpha-synuclein, recognize both sphingolipids and cholesterol and contain both a SBD and a tilted peptide [23]. Interestingly, tilted peptides are also present in the fusion glycoproteins of a several viruses including the human retrovirus HIV-1 and HTLV-I, as well as Ebola, Marburg and Semliki forest virus [43]. Most of these viruses interact with lipid rafts and they require either cholesterol, sphingolipids, or both, to gain entry into host cells [24]. As discussed above, the insertion process is triggered by the sudden unmasking of the nonpolar tilted peptide that is consecutive to glycosphingolipid binding. In the case of alpha-synuclein, the glycosphingolipid can be either GM3 for astrocytes or GM1 for neurons [8]. These gangliosides provide a negatively charged surface onto which the mostly unstructured alpha-synuclein protein attaches through electrostatic interactions. The ganglioside membrane acts as a mould that can induce alpha-helical folding, thereby unmasking the tilted peptide. In the case of HIV-1, the glycosphingolipid can be either GalCer on CD4-negative cells [46], Gb3 on lymphocytes, or GM3 on macrophages [47, 48]. The binding of the surface envelope glycoprotein gp120 on these lipids induces a series of conformational changes that eventually unmask the tilted fusion peptide located at the N-terminal part of the transmembrane glycoprotein gp41. In both case (alpha-synuclein and HIV-1), the tilted peptide is predominantly nonpolar and it inserts into the plasma membrane to escape the polar water environment [49].

2.10 Conclusion and Perspectives

Our work on viruses and amyloidogenic proteins have contributed to a better understanding of the molecular mechanisms controlling the targeting of proteins to lipid raft domains. The discovery of the SBD has been an important step in this comprehension [49]. The study of alpha-synuclein has revealed the importance of the OH-Pi bond in the insertion process leading to ion channel formation. Specifically, the absolute requirement of an OH group in the lipid partner explains why alpha-synuclein present in the extracellular space [50] interacts preferentially with lipid raft domains [51]. Indeed, these

domains are enriched in cholesterol and sphingolipids, both lipids displaying at least one available OH group in their most polar moiety [49]. In contrast, phosphatidylcholine lacks such OH groups, consistent with the lower activity of alpha-synuclein channels in bilayer membranes containing only this lipid compared with ganglioside-containing membranes [7]. This is also consistent with the high affinity of alpha-synuclein for anionic phospholipids such as phosphatidylserine and phosphatidylglycerol [4, 5], since both of these lipids contain free OH groups potentially able to form an OH-Pi bond with Tyr-39. Finally, it is interesting to mention that the non-steroidal anti-inflammatory drug ibuprofen ((RS)-2-(4-(2-methylpropyl) phenyl) propanoic acid) has been associated with a lower risk of Parkinson disease [52]. Although the molecular mechanisms responsible for this prophylactic effect of ibuprofen have not been elucidated, the drug has a central phenyl group that could affect the OH-Pi bond dependent interaction of Tyr-39 of alpha-synuclein with gangliosides. This could prevent the formation of alpha-synuclein ion channels, thereby decreasing alpha-synuclein toxicity. This possibility is currently under investigation in our laboratory.

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The Enigmatic Role of Sulfatides: New Insights into Cellular Functions and Mechanisms of Protein Recognition

3

Shuyan Xiao, Carla V. Finkielstein, and Daniel G.S. Capelluto

Abstract

Sulfatides are sphingolipids commonly found at the surface of most of eukaryotic cells. Sulfatides are not just structural components of the plasma membrane but also participate in a wide range of cellular processes including protein trafficking, cell adhesion and aggregation, axon-myelin interactions, neural plasticity, and immune responses, among others. The intriguing question is how can sulfatides trigger such cellular processes? Their dynamic presence and specific localization at plasma membrane sites may explain their multitasking role. Crystal and NMR structural studies have provided the basis for understanding the mechanism of binding by sulfatide-interacting proteins. These proteins generally exhibit a hydrophobic cavity that is responsible for the interaction with the sulfatide acyl chain, whereas the hydrophilic, negatively charged moiety can be found either buried in the hydrophobic cavity of the protein or exposed for additional intermolecular associations. Since sulfatides vary in their acyl chain composition, which are tissue-dependent, more emphasis on understanding acyl chain specificity by sulfatide-binding proteins is warranted. Importantly, changes in cellular sulfatide levels as well as circulating sulfatides in serum directly impact cardiovascular and cancer disease development and progress. Therefore, sulfatides might prove useful as novel biomarkers. The scope of this review is to overview cell functions and mechanisms of sulfatide recognition to better understand the role of these lipids in health and disease.

Keywords

Sulfatides • Ceramide • Plasma membrane • Sulfatide-binding proteins • Platelet aggregation • Disabled-2 • Cluster of differentiation 1

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3.1 Introduction to Sulfatides

Sulfatides (also known as 3-*O*-sulfogalactosylceramides, sulfated galactocerebrosides, or SM4) are sphingolipids found at the extracellular leaflet of the plasma membrane of most eukaryotic cells. They were first isolated from human brain tissue by Thudichum in 1884 [105]. Sulfatides are not only membrane components but they are also involved in protein trafficking, cell adhesion and aggregation, axon-myelin interactions, modulation of sodium and potassium channels, learning and memory, and neural plasticity [9, 13, 16, 66, 109, 113]. Sulfatides are expressed in a variety of cells, predominantly in the myelin sheath of the nervous system, representing ~4 % of the total myelin lipids [47]. Also, these sphingolipids are largely found at the surface of blood cells such as erythrocytes [57], neutrophils [93], and platelets [85] and they are major component of lipoproteins in blood serum [100]. Sulfatides are esters of sulfuric acid with galactosylceramides at C3 of the galactose moiety, which is connected to the primary hydroxyl group of the *N*-acylated D-*erythro*-sphingosine base via a β-glycosidic bond (Fig. 3.1). The fatty acid chain length of sulfatides varies, with the majority being composed of C16 to C26, including 2-hydroxy fatty acids [47]. Sulfatides containing nervonic acid (C24:1) are the most abundant in myelin, whereas high levels of the lipid with stearic acid (C18:0) are present in the cortical grey matter [46]. Other structural variants of sulfatides (C22:0) are found in kidney tissue [47], with shorter acyl chains (C16:0) being predominant form in pancreas [23]. Sulfatides are also modified by hydroxylation at the α-2 carbon of the fatty acid by the fatty acid 2-hydroxylase [4] and both hydroxylated and nonhydroxylated forms of the lipid are found distinctly distributed in the cerebral cortex [119].

3.2 Sulfatide Synthesis and Degradation

Synthesis of sulfatides occurs in the endoplasmic reticulum and the Golgi apparatus. Initially, a galactose residue is transferred from UDP-

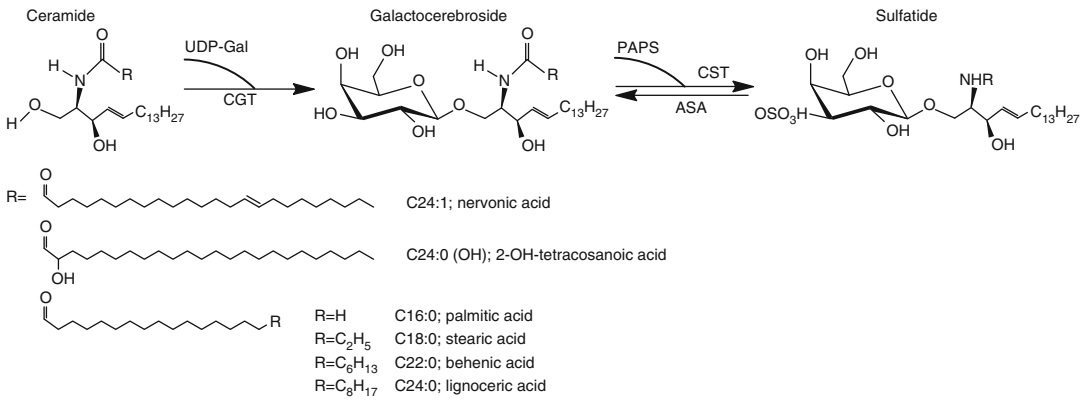

Fig. 3.1 The synthesis and degradation pathway of sulfatides. Ceramide is converted to galactocerebroside by addition of a galactose group from UDP-galactose, a reaction catalyzed by UDP-galactose:ceramide galactosyltransferase (CGT). Galactocerebroside is a substrate of 3′-phosphoadenosine-5′-phosphosulfate:cerebroside sulfotransferase (CST), which adds a sulfate group to the galactose moiety, using 3′phosphoadenosine-5′-phosphosulfate (PAPS), to generate sulfatide. Sulfatide turnover is mediated by arylsulfatase A (ASA), an enzyme that removes the sulfate group and generates galactocerebroside. ASA requires saposin B activity, a cysteine-rich protein that extracts sulfatides from membranes and allows ASA to catalyze the reaction on diffusible protein-lipid complexes. The chemical structure of ceramides is characterized by the presence of a sphingosine group and an additional fatty chain, which usually varies with different lengths and, therefore, depicted with an R group. Commonly found R-groups in ceramides and sulfatides are depicted at the *bottom*

galactose to 2-hydroxylated or nonhydroxylated ceramide at the luminal membrane leaflet of the endoplasmic reticulum, a reaction catalyzed by the UDP-galactose:ceramide galactosyltransferase (CGT; C 2.4.1.45) (Fig. 3.1). The product of this reaction, galactocerebroside, is delivered to the Golgi apparatus where it is modified by sulfation at position 3 of the galactose moiety through the action of a 3′-phosphoadenosine-5′-phosphosulfate:cerebroside sulfotransferase (CST; EC 2.8.2.11) [117]. Tissue-dependent expression of sulfatides correlates with the expression of both CGT and CST genes [35, 39, 123]. Recently, Aoyama and colleagues determined that the CST gene is transcriptionally stimulated by the activated peroxisome proliferator-activated receptor α and this effect directly enhances sulfatide levels in mice [75]. Mice lacking CST or CGT cannot produce sulfatides [38, 89]. Absence of the CST gene leads to disorganized paranodes and a lack of septate-like junctions, defects that promote a reduction of the nerve conduction velocity due to the lack of sulfatides [15, 19, 38]. Degradation of sulfatides is mediated by lysosomal arylsulfatase A (ASA; EC 3.1.6.8), which hydrolyzes the sulfate group from the galactose moiety leading to the formation of galactocerebroside. Sulfatide accumulation by the lack of ASA is associated with demyelination and metachromatic leukodystrophy (MLD), a lethal neurological disease [21, 84]. Overall, accumulated evidences indicate that alteration of sulfatide synthesis has a major impact on the generation of neuronal defects.

The reaction catalyzed by ASA depends upon the presence of saposin B, a sphingolipid activator protein that removes sulfatides from membranes and, thus, allows sulfatides to interact with ASA [56]. The crystal structure of saposin B shows a shell-like dimer of a helical bundle that encloses a hydrophobic cavity [2], a structural organization that is observed in many sulfatide-binding proteins [91]. Saposin B adopts a V-shaped conformation with five amphipathic α-helices, which associates to another saposin B molecule to build a large hydrophobic cavity in the dimer. The structure also reveals a region of elongated electron density that could be a potential lipid-binding site, an association that may require a conformational change of saposin B to expose its inner hydrophobic cavity to membranes [2].

Sulfatides can be intracellularly distributed by action of the glycolipid transfer protein (GLTP), a cytosolic peripheral protein that transfers glycolipids from the cytosolic leaflet of the plasma membrane or the endoplasmic reticulum and acts as a sensor of glycolipid levels [68]. GLTP employs a helical two-layer sandwich motif to transfer glycolipids and is able to recognize the sugar head group using hydrogen bonds and a hydrophobic pocket that associates with most of the nonpolar hydrocarbon chains of the ceramide region of the glycolipid [65]. There are two modes of glycosphingolipid binding by GLTPs [64]: (i) "*Sphingosine in*" mode, in which both the acyl and sphingosine chains are located in the same hydrophobic pocket of GLTP and (ii) "*Sphingosine out*" mode, in which the acyl chain of the sphingolipid remains in the hydrophobic pocket of GLTP, where the sphingosine backbone becomes exposed to the protein surface and allows interaction with another GLTP, forming a dimer. Recently, studies using the crystal structures of the wild-type human GLTP and a mutant ($Asp^{48}Val$; D48V) version of the protein in complex with sulfatides reveal that the D48V mutation favors the transfer selectivity to sulfatides by switching GLTP to the "*sphingosine in*" mode [91]. The D48V GLTP exhibits a cavity that allows the sulfate group to efficiently accommodate the sulfatide molecule in the protein, enhancing sulfatide binding over other neutral glycosphingolipids, such as galactoceramides. Consequently, sulfatides favor dimerization of GLTP, whose dimerization interface resembles the membrane-binding domains of the protein [54, 64].

3.3 Cellular Mechanisms Mediated by Sulfatides

3.3.1 Nervous System

Sulfatides are present in high levels in the myelin sheath, in both the central and peripheral nervous systems [108]. Myelin contains 70–75 % lipid,

4–7 % of which are sulfatides [77]. Sulfatides are also found in other glial cells, astrocytes, and neurons [11, 46, 81] and are myelin-associated inhibitors of central nervous system axon regeneration [114]. Increased cellular concentration of sulfatides is associated with MLD, in which patients exhibit accumulation of the lipid in lysosomes of oligodendrocytes, Schwann cells, macrophages, astrocytes, and neurons [79]; elevated levels of sulfatides are also associated with epileptic and audiogenic seizures [107]. Although unusual, deficiency in saposin B has also been observed in MLD [124]. Nonetheless, MLD leads to a progressive loss of myelin, in which the individual ultimately dies in a decerebrated state. Patients with Multiple Sclerosis or Parkinson's disease exhibit elevated levels of anti-sulfatide antibodies in serum and cerebrospinal fluid compared to healthy individuals [55]. Indeed, sulfatides act as autoantigens in Multiple Sclerosis patients [33]. Overall, these findings indicate that release of sulfatides from myelin is associated with the development of central nervous system diseases. Changes in sulfatide levels have been observed in other neuronal diseases, including epilepsy with mental retardation and Alzheimer's disease (for more details, see [20]).

3.3.2 Platelet Adhesion and Aggregation

Platelets represent an important linkage between thrombus formation and inflammatory processes. First, they prevent post-traumatic blood loss by forming fibrin-containing thrombi at the site of vascular injury, followed by the release of a battery of potent inflammatory and mitogenic molecules within the microenvironment that alters the chemotactic and adhesive properties of endothelial cells. These events facilitate the tethering and rolling of leukocytes over an inflamed vessel wall (activated endothelium [24, 25]), which then either firmly adhere and transmigrate into the arterial intima or simply detach [26, 27]. Among the various glycoproteins involved in these events, selectins are crucial for the initial contact between platelets and the vascular endothelium, and remarkably, mediate rosetting of platelets with monocytes and neutrophils to form platelet-leukocyte aggregates [60, 104]. Despite some contradictory results reviewed by Kyogashima [59], accumulated recent evidence suggests that sulfatides promote platelet adhesion and aggregation [18, 31, 70, 113].

One of the key cell surface receptors mediating leukocyte recruitment and exhibiting pro-aggregatory activity is P-selectin (for a review, see [12]). Most of the P-selectin ligands contain post-translational modifications needed for receptor binding and signal transduction in which sulfate moieties are frequently present [83, 88]. Sulfatides modulate P-selectin activity at the platelet surface [72] leading to further degranulation and increased surface P-selectin expression, which reinforces platelet aggregation [70, 113]. Moreover, P-selectin-sulfatide interaction leads to the formation of stable platelet aggregates and surface sulfatides enhance the formation of platelet-leukocyte aggregates [70]. Platelet P-selectin expression is decreased by fibrinogen deficiency [118].

Frequently, soluble platelet aggregation agonists bind to and induce conformational changes in the extracellular domains of the $\alpha_{IIb}\beta_3$ integrin receptor, triggering the inside-out integrin-signaling pathway [49]. Simultaneously, fibrinogen activates the outside-in signaling pathway by association with the $\alpha_{IIb}\beta_3$ integrin receptor via two Arg-Gly-Asp (RGD) motifs located in its α-chain [82]. In addition to fibrinogen, other integrin receptor agonists include the von Willebrand factor (vWF) and fibronectin and these associations stimulate platelet spreading and aggregation on vascular surfaces [49]. The adaptor protein Disabled-2 (Dab2) negatively regulates fibrinogen-$\alpha_{IIb}\beta_3$ integrin receptor association and, consequently, inhibits cell adhesion and cell signaling [18, 41]. The inhibitory function of the cytosolic pool of Dab2 is mediated by phosphorylation in its Ser24 residue, a post-translational modification that triggers the association of Dab2 to the cytoplasmic tail of the β3 subunit of the integrin receptor [41]. Binding of Dab2 to the integrin receptor is likely to be enhanced by phosphatidylinositol 4,5-bisphosphate-mediated membrane anchoring (Fig. 3.2). Consequently, Dab2 acts as

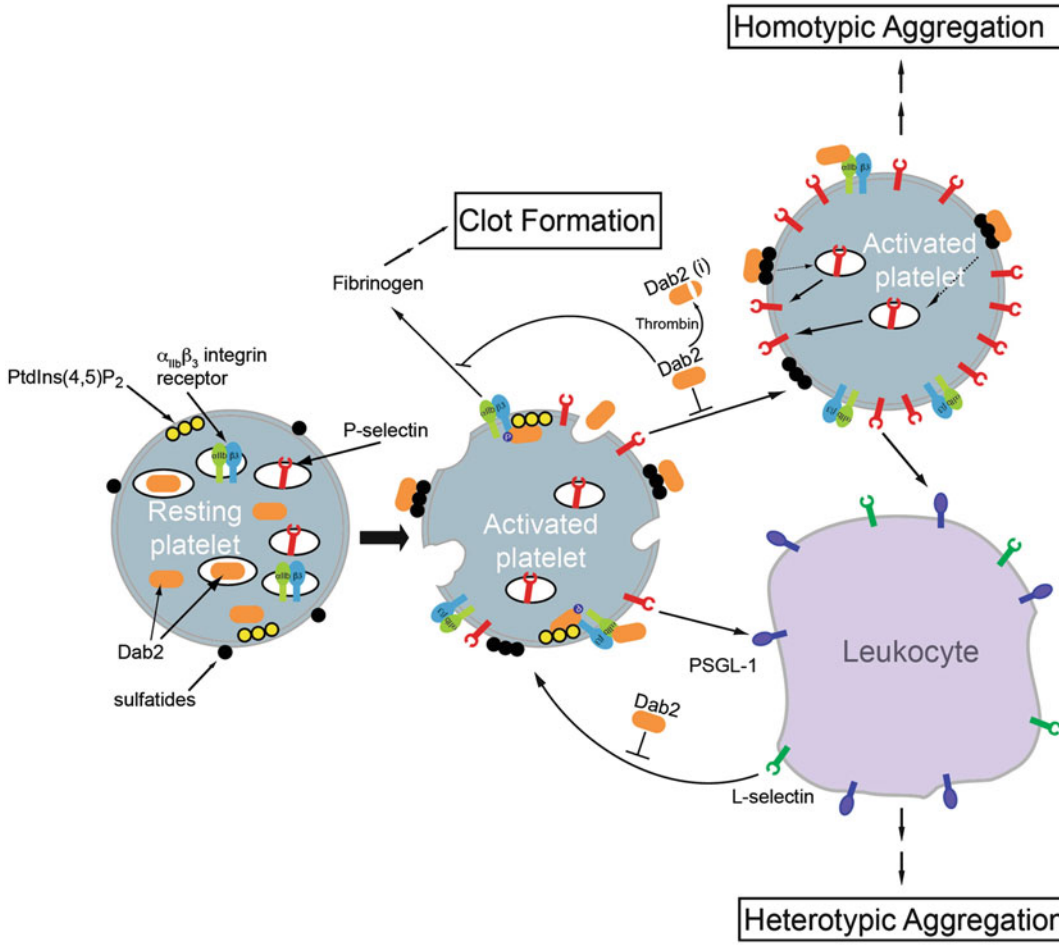

Fig. 3.2 An updated model of sulfatide- and Dab2-mediated modulation of platelet aggregation. Resting platelets are enriched in α-granules, which contain pro-coagulant (*i.e.*, P-selectin, $\alpha_{IIb}\beta_3$ integrin receptor) and anti-coagulant proteins (*i.e.*, Dab2). Another pool of platelet Dab2 is distributed cytosolically. Also, platelets contain signaling lipids including sulfatides (found at the outer leaflet of the plasma membrane) and PtdIns(4,5)P_2 (found at the inner leaflet of the plasma membrane). Upon activation, platelets change shape and release the α-granular content. Released Dab2 is partitioned in two pools: one associates with the α_{IIb} subunit of the integrin receptor through its RGD motif, and therefore, competes with fibrinogen for integrin receptor binding. Consequently, Dab2 negatively controls clot formation by modulating platelet aggregation. The second pool of Dab2 associates with cell surface sulfatides, whose levels are increased upon platelet activation. Upon platelet activation, cytosolic Dab2 is recruited to the plasma membrane in a phosphorylated state where interacts and inhibits the β3 subunit of the integrin receptor. Membrane recruitment of Dab2 is likely enhanced by its association to PtdIns(4,5)P_2. The fate of phosphorylated Dab2 after membrane recruitment is unknown. The function of extracellular Dab2 is modulated by the agonist thrombin, which cleaves Dab2 making it inactive (Dab2(i)). Both P-selectin and L-selectin bind to cell surface sulfatides mediating platelet-platelet platelet-leukocyte interactions, respectively. Furthermore, platelet-leukocyte interactions are enhanced by the association of P-selectin with PSGL-1. Both homotypic and heterotypic interactions are negatively modulated by Dab2. The presence of Dab2 at the cell surface is transient since it has been shown to be internalized back to α-granules (*dotted arrows*)

a negative regulator of integrin receptor inside-out signaling.

Dab2 is also localized in α-granules of both megakaryocytes [41] and resting platelets [18, 42]. Upon activation, Dab2 is secreted to the megakaryocyte and platelet surface via the α-granule secretory pathway where it binds to the $\alpha_{IIb}\beta_3$ integrin receptor, blocking fibrinogen-platelet

interactions [42]. Integrin-binding takes place because of the presence of an RDG motif in Dab2, an association that can be inhibited by the fibrinogen-derived Arg-Gly-Asp-Ser (RGDS) peptide [42]. Dab2 targets platelet surface membranes, as a result of platelet activation, via its N-terminal region containing the phosphotyrosine-binding (N-PTB) domain [18]. N-PTB is necessary and sufficient to inhibit platelet adhesion and aggregation by competing with fibrinogen for binding to the $\alpha_{IIb}\beta_3$ integrin receptor through its RGD motif [18]. In addition, Dab2 binds membrane sulfatides, an association that redistributes the protein at the platelet surface [18]. Dab2 recognizes sulfatides through the residues Lys25, Lys49, Lys51, and Lys53, which are located within the XBBXBX (B, basic residue; X, any residue) and BXBXBX motifs in its N-PTB region [18]. This class of basic clusters also mediates sulfatide binding of other cell adhesive proteins, including thrombospondins, laminins, and selectins [47]. The sulfatide-binding site of Dab2 overlaps with that of the phosphoinositide PtdIns(4,5)P_2 binding site [3], but competition likely does not occur in a physiological context since sulfatides are predominantly found at the plasma membrane surface, presumably in lipid rafts [96], whereas the phosphoinositide is predominantly found at the cytosolic leaflet of the plasma membrane [58]. Whereas sulfatides contribute to Dab2 membrane insertion, which is likely accompanied by a conformational change of the protein, phosphoinositide recognition occurs by electrostatic interactions associated with minor local structural changes in Dab2 [3]. Sulfatide recognition by Dab2 impairs cleavage by thrombin, a strong platelet agonist [18]. Consequently, a pool of Dab2 remains intact at the platelet surface upon activation, and is eventually internalized back to α-granules by an actin cytoskeleton-dependent mechanism [18]. Also, sulfatides modulate the availability of Dab2 for binding to the integrin receptor [18]. Taken together, Dab2 may be distributed in two pools at the platelet surface (Fig. 3.2). One pool of Dab2 competes with fibrinogen for binding to the integrin receptor, whereas a second pool binds sulfatides at the platelet surface. The second pool of Dab2 also exerts an additional layer of modulation of platelet aggregation since sulfatide binding by Dab2 blocks P-selectin-sulfatide interactions (Fig. 3.2) [113], which are required to sustain platelet aggregation [71]. Indeed, sulfatides promote surface expression of P-selectin in activated platelets [70, 113]. The N-PTB region of Dab2 not only blocks platelet-platelet interactions, but also controls the extent of heterotypic cell interactions, such as those with leukocytes *via* its recognition to cell surface sulfatides [113].

We have recently generated a Dab2-derived peptide that contains the two sulfatide-binding motifs (SBMs) of the protein [116]. The Dab2 SBM peptide adopts a helical and amphipathic structure when embedded in dodecylphosphocholine (DPC) micelles. The majority of the sulfatide-interacting residues map to the second sulfatide-binding motif with the basic residues Lys49, Lys51, and Lys53 as well as the nonpolar residues Ala52, Leu54 and Ile55 playing a major role in the interaction with the sphingolipid [116]. Using a combination of paramagnetic probes, we established that the peptide lies in a parallel orientation below the sulfatide-enriched DPC micellar surface but does not cross the hydrophobic core of the micelle. Using microfluidic devices that readily mimic vasculature, we showed that Dab2 SBM displays anti-aggregatory platelet activity, comparable to that described for the fibrinogen-derived peptide, Arg-Gly-Asp-Ser (RGDS) [116]. Thus, by binding to cell surface sulfatides, Dab2 SBM provides the basis for rational design, promising anti-aggregatory low-molecular mass molecules for therapeutic applications.

Sulfatides also interact with homeostatic cell adhesion proteins, such as vWF [86], chemokines [92], laminin [86], and thrombospondin [85]. Sulfatides inhibit vWF's platelet adhesion in flowing blood and under physiological shear stress conditions [9]. The sulfatide-binding site in vWF overlaps with that of the glycoprotein Ib and, consequently, the lipid can inhibit glycoprotein Ib-mediated platelet adhesion [9]. vWF binds sulfatides by a region comprising residues 1,391–1,409 within the A1 domain of the protein [5]. Further site-directed mutagenesis studies

demonstrated that the residues Arg1392, Arg1395, Arg1399, and Lys1423 are critical for sulfatide recognition as shown using ELISA-based plates coated with sulfatides [76]. The residues Arg1392 and Arg1395 within the A1 domain of vWF are also relevant for glycoprotein Ib binding [67], confirming that sulfatides and glycoprotein Ib compete with each other for vWF binding.

Chemokines are cytokines that bind to cell surface sulfated glycosaminoglycans, modulating the activity of chemokine receptors. In addition to glycosaminoglycan binding, chemokines bind sulfatides [92], although the role of sulfatide recognition by these proteins is not clear. Whereas chemokine production is reduced by sulfatides when tested in peripheral leukocytes and fat cells [10, 87], it is stimulated in brain immune cells [52].

Laminins contain a series of G-like modules of about 200 amino acids each that bind to sulfatides, an association that may facilitate the polymerization of the protein into networks [53]. Two XBBXBX and three BXBXBX sequences were initially suggested to be potential sulfatide-binding motifs for the protein [103]. Timpl and colleagues demonstrated that sulfatide binding is increased when laminin G-like modules are in tandem [102], indicating their cooperation in ligand recognition. Structural data indicate that residues K3027 and K3028 within the XBBXBX motif of laminin α2 G-like 4–5 domains are critical for sulfatide binding [37, 102]. Furthermore, residues K3088 and K3091 present in a basic cluster BXXBXXXB of the same protein contribute to sulfatide binding [37]. Likewise, the ^{2831}RAR and ^{2766}KGRTK residues of the related laminin α1 G-like 4–5 domains, which belong to potential BXBXBX motifs, are crucial for sulfatide binding [34]. However, other basic clusters involved in heparin recognition are dispensable for sulfatide binding [34], suggesting that the association of laminin to different ligands may trigger unique biological responses.

Thrombospondins are extracellular calcium-binding proteins that are involved in wound healing, angiogenesis, vessel wall biology, synaptogenesis, and connective tissue organization (for a review, see [1]). Thrombospondins are known to bind many partners [1]. Sulfatides and heparins show strong affinity to thrombospondin-derived peptides containing the WSXW (where X is any residue) sequence with no polybasic motif required for sulfatide binding [32]. Indeed, these peptides strongly inhibit sulfatide and heparin binding to the thrombospondin, blocking binding of this protein to melanoma cells [32].

3.3.3 Innate Immunity and Autoimmunity

T cells recognize antigens, such as foreign and self-lipids and peptides, leading to the production of cytokines and, therefore, contributing to immune responses [7]. T cells also use their cell surface receptor to recognize lipid antigen-bound cluster of differentiation 1 (CD1) molecules at the surface of professional antigen-presenting cells such as macrophages, dendritic cells, and a small group of B cells. There are three groups of CD1 surface proteins: (*i*) CD1a, CD1b, and CD1c (group 1), (*ii*) CD1d (group 2), and (*iii*) CD1e (group 3) [17]. CD1 proteins contain three extracellular domains (α1, α2, and α3), a transmembrane domain, and a cytoplasmic tail. The extracellular domains form a surface groove (named the lipid-binding groove) formed by two α-helices (α1 and α2) on top of a β-sheet [120, 122]. The lipid-binding groove, which is narrow and deep, contains hydrophobic residues that can interact with the acyl chains of the glycolipids [6], whereas the polar head group becomes exposed in the CD1-lipid complex, allowing recognition by T cell receptors [73]. In CD1a proteins, the lipid- binding groove contains two large hydrophobic regions termed A' and F' [120]. In the CD1a-sulfatide complex, the sulfatide adopts an S-shaped conformation in which the A' pocket contributes to the C_{18} sphingosine backbone recognition, and the acyl chain of the lipid emerges from the A' pocket and extends its association into the F' pocket [120]. The galactose moiety forms hydrogen bonds with residues Arg76 and Ser77, whereas the sulfate group forms hydrogen bonds with residues Arg76 and Glu154 and with water that is in complex between residues Arg73 and Glu154 [120]. Consequently,

the sulfated galactose residue becomes exposed at the surface of the complex for T cell receptor recognition. Sulfatides can be presented by all members of the CD1 group 1 and by CD1d [8, 94]. However, the sulfatide-binding affinity varies with each CD1 molecule, with the CD1a-sulfatide being the most stable complex [94].

Sulfatides have also been shown to be self-glycolipid antigens recognized by CD1d, assembling a complex that activates type II natural killer T (NKT) cells [50]. Sulfatides induce proliferation and expansion of memory, but not naïve, T cells [48]. The mechanism by which the T-cell receptor from type II NKT cells (XV19 hybridoma) interacts with the CD1d-sulfatide complex has been recently reported [78]. Whereas the type I NKT T-cell receptor exclusively contacts the F' pocket of CD1d, the type II NKT T-cell receptor binds orthogonally above the A' pocket of CD1d, emphasizing different CD1d points of contact. More importantly, T cells highly reactive to sulfatides are increased in number and CD1d is upregulated in the central nervous system of patients with experimental autoimmune encephalomyelitis [33, 50]. The presence of the sulfate group and the β-anomeric linkage are critical for CD1d activation-dependent T cells [94]. The dominant sulfatide species for CD1d-dependent immune responses is a C24:1 [121], which bears one unsaturation at the 8–9 position (Fig. 3.1). The crystal structure of the CD1d-C24:1 sulfatide complex shows the acyl chain in the A' pocket, whereas the sphingosine chain associates with the F' pocket, leaving the sulfated head group exposed at the protein surface [121].

3.3.4 Host-Pathogen Interactions

The action of protein toxins from pathogenic organisms requires specific sphingolipids at the cell surface to mediate protein endocytosis and to enhance the virulence of the pathogen. Sulfatide recognition by pathogen proteins includes the coli surface antigen 6, the heat-stable toxin b, and the 987P-fimbriae from *Escherichia coli* [14, 30, 51], and heat shock proteins from *Helicobacter pylori* [43, 44]. The only structural data reported for this class of toxins is that for the *Naja atra* Taiwanese Cobra cardiotoxin A3 (CTX -A3) in complex with sulfatides using hexaethylene glycol monodecyl ether detergent as a membrane mimetic [110]. CTX-A3 acts as a toxin by a sulfatide-dependent internalization mechanism that leads to pore formation in the host cell membrane [115]. The crystal structure of CTX-A3 reveals a dimer of two β-sheet proteins, an oligomerization state that is induced upon sulfatide binding. In the CTX-A3-sulfatide complex, the sulfatide head group is buried so that the sulfate group forms a hydrogen bond with the amino group of the residue Lys35, whereas the galactose sugar forms hydrogen bonds with the amino groups of the residues Lys12 and Lys18 and the carbonyl oxygen group on Arg36 and Cys38 [110]. The side chain of Lys44 interacts with the amide region of the ceramide backbone through a single hydrogen bond. The remaining lipid tail becomes exposed to the detergent-enriched solvent that facilitates the dimerization of CTX-A3. Membrane insertion and pore formation by CTX-A3 requires both protein and sulfatide conformational changes [106] and the presence of sulfatide-containing lipid domains [115].

Glycosphingolipids are also employed as receptors for virus infection. Both galactocerebrosides and sulfatides facilitate HIV type 1 virus binding to the $Cd4^{-}$ cell surface *via* the viral envelope gp120 protein [22]. Similarly, sulfatides are thought to be alternative cell surface receptors for the Influenza A virus [99] and the vaccinia virus [80]. In addition, sulfatides have been shown to enhance the formation and release of the progeny of infectious Influenza A viruses as well as translocation of newly synthetized viral nucleoprotein to the cytoplasm [101]. Indeed, sulfatide administration prevents cell viral infection [22, 99, 112] as it has been demonstrated for the bovine immunodeficiency virus, in which its internalization is inhibited by the glycosphingolipid during syncytium formation [112]. More recently, Kumar and colleagues demonstrated that sulfatide administration in mice inhibits HIV type 1 replication more efficiently than treatment with the nucleoside analog reverse transcriptase

inhibitor azidothymidine [98]. This is in agreement with the observation that antibodies that neutralize HIV-1 also recognize sulfatides [69]. Furthermore, the presence of sulfatides enhances mice hematopoiesis, which is usually lost during HIV-1 infection [98]. Overall, this evidence suggests that sulfatides represent novel tools to target viral infections.

3.4 Implications of Sulfatides in Disease Development and Progression

3.4.1 Cardiovascular Diseases

Sulfatides are known to play a critical role in the development of cardiovascular disease. Indeed, the measurement of serum sulfatide levels has been proposed to predict the incidence of cardiovascular disease in patients with end-stage renal disease (ESRD) [40]. The level of sulfatides in ESRD patients undergoing hemodialysis therapy and those with cardiovascular disease is consistently lower than in healthy individuals [40]. Patients with kidney transplantation show a significant increase of serum sulfatides in a time-dependent manner, which is correlated with an increment of platelet levels [111]. The recovery of sulfatide levels may be associated with the attenuation of the systemic oxidative stress triggered by the chronic kidney dysfunction in these patients [111]. Sulfatides are P-selectin ligands and as such mediate platelet-leukocyte interactions via P-selectin and CD11b/CD18 (Mac-1), an integrin receptor localized at the surface of monocytes, neutrophils, and T-cells [28]. Sulfatides increase Mac-1 surface expression in neutrophils, which may contribute to the development of intimal hyperplasia after endothelial injury [95]. Further studies demonstrate that sulfatides contribute to the progress of neointimal thickening after vascular injury, which can eventually trigger atherosclerosis [45]. In the same context, erythrocyte membrane sulfatides significantly increase in sickle erythrocytes and play a relevant role in sickle cell adhesion to endothelial cells [125].

3.4.2 Cancer Diseases

Increased levels of sulfatides have been observed in renal cell carcinoma [90], well-differentiated endometrial adenocarcinoma [97], some types of lung tumors [29], brain tumors [61], and colon [74], hepatocellular [36], and ovarian cancers [62, 63]. Sulfatides have been proposed as early predictors of ovarian cancer [63]. Recently, using a combination of mass spectrometry metabolite analysis and gene expression profiles, it has been established that sulfatide levels are elevated in ovarian cancer compared to normal ovarian tissue [62]. Consistent with this observation, higher levels of mRNA that codifies for the enzymes CGT and CST, required for sulfatide synthesis, are also detected in epithelial ovarian carcinoma cells, whereas the levels of ASA, saposin, and galactosylceramidase remain unchanged [62]. Taken together, measurements of sulfatide levels using mass spectrometry analysis of tumor tissues represent an excellent and sensitive tool to be used as serum biomarkers for early tumors.

3.5 Conclusions and Future Perspectives

As summarized in this review, the role of membrane sulfatides in the nervous system, innate and adaptive immunity, platelet adhesion and aggregation, and bacterial and viral infection is clearly emerging. However, there are several questions about how membrane sulfatides signal that need to be addressed. For example, a precise measurement of membrane sulfatide levels elicited by external cues is required to understand sulfatide-mediated signaling. Also, the levels of the enzymes that participate in the synthesis and degradation of sulfatides should play a key role in the modulation of the membrane levels of the sphingolipid.

The number of identified sulfatide-binding proteins has substantially increased over the past 15 years. The general sulfatide binding mechanism consists of the formation of hydrogen bonds between the acyl chains of the sphingolipid with

residues located in the hydrophobic cavity and accompanied by a few hydrogen bonds and electrostatic interactions between the side chain of basic residues of the protein and the negatively charged sulfate group of the galactose moiety. Perhaps, the key role of sulfatides center on the features of their acyl chains as they interact with protein hydrophobic cavities leaving, in some cases, the head group exposed at the surface of the protein. Thus, development of high-resolution methods for the discrimination of sulfatides with different fatty acid compositions is warranted. This is important as sulfatides with specific acyl chains lengths, unsaturation, or even hydroxylation modifications are tissue-dependent. Furthermore, predicting a sulfatide-binding site from the amino acid sequence of a protein is not an easy task. Whereas sulfatide-binding sites typically exhibit basic clusters of residues that follow the sequence BXBXBX or XBBXBX, some sulfatide-binding proteins exhibit unique sulfatide-binding basic motifs and some others do not employ basic residues at all.

With recent high-resolution structures of sulfatide-binding proteins we may also soon understand the role of sulfatides in protein membrane targeting as well as intra- and extracellular sulfatide-dependent protein dynamics. However, we still lack the information about sulfatide dynamics at membranes, its intracellular distribution of the glycosphingolipid, or its presence and relative concentration in lipid rafts. Moreover, the engagement of sulfatides in cardiovascular and cancer diseases makes this area of research clinically relevant. The identification of additional sulfatide-binding proteins and the appropriate measurement of sulfatide levels in serum and tumor tissues will certainly contribute to early prognosis.

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Phosphoinositides and PDZ Domain Scaffolds

4

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Abstract

The discovery that PSD-95/Discs large/ZO-1 (PDZ) domains can function as lipid-binding modules, in particular interacting with phosphoinositides (PIs), was made more than 10 years ago (Mol Cell 9(6): 1215–1225, 2002). Confirmatory studies and a series of functional follow-ups established PDZ domains as dual specificity modules displaying both peptide and lipid binding, and prompted a rethinking of the mode of action of PDZ domains in the control of cell signaling. In this chapter, after introducing PDZ domains, PIs and methods for studying protein-lipid interactions, we focus on (i) the prevalence and the specificity of PDZ-PIs interactions, (ii) the molecular determinants of PDZ-PIs interactions, (iii) the integration of lipid and peptide binding by PDZ domains, (iv) the common features of PIs interacting PDZ domains and (v) the regulation and functional significance of PDZ-PIs interactions.

Keywords

Scaffold • Cell signaling • Protein-lipid interactions • Dual specificity modules • Nucleus

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4.1 Introduction: PDZ Domains and Phosphoinositides

The regulation of signaling networks is fundamental for accurate and efficient flow of cellular information [1]. Scaffold proteins, composed of modular interacting domains or motifs, provide a simple and elegant solution for determining the specificity of signaling in time and space by acting as hubs coordinating specific physical assemblies of signaling components [2]. PDZ domains containing scaffolds appear crucial for the assembly of multiprotein

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signaling complexes at the plasma membrane and for the establishment and maintenance of cell polarity [3, 4]. PDZ domains were originally identified as repeats of 80–90 amino acids and first referred to as DHR (Disc-large Homology Regions) or GLGF repeats, because of a conserved Gly-Leu-Gly-Phe signature in their sequence [5–7]. The PDZ acronym is derived from the first letters of proteins in which PDZ domains were originally identified, namely (i) the postsynaptic density protein PSD-95, (ii) the *Drosophila* septate junction protein Disc-large and (iii) the epithelial tight junction protein ZO-1 [5, 8, 9]. More than 250 different PDZ domains are present in the human proteome [10, 11] and they are well-known for their property to function as protein-protein interaction modules. They adopt a typical fold of six β strands flanked by two α helices [12] and recognize short peptide stretches generally present at the C-terminus of their targets [13, 14]. PDZ domains can be classified according to their peptide binding specificity. The three main consensus of PDZ binding motifs (PDZBM) are S/T-X-Φ (class I), -Φ/Ψ-X-Φ (class II) and E/D-X-Φ (class III), where X stands for any amino acids, Φ for a hydrophobic residue and Ψ for an aromatic residue [15, 16]. However, more and more PDZ domains appear to display degenerate specificity [17]. Crystallographic studies showed that the peptide binding groove is formed by the second α-helix (α2) and the second β-strand (β2) and that the peptide is bound in an antiparallel manner by the mechanism called β strand addition [18, 19]. Besides the canonical binding mode, other modes of interactions have been documented. Some PDZ domains recognize internal peptide stretches, as has been shown for the PDZ domain of syntrophin interacting with a β-harpin in nNOS [20]. GRASP-1 (Ras guanine exchange factor) interacts with the PDZ7 domain of GRIP1 (glutamate receptor interacting protein) via a hydrophobic region distant from the binding pocket [21]. Moreover, some PDZ domains are also able to homo- and hetero-dimerize [22–24]. PDZ-peptide interactions have low micromolar affinities and tend to be promiscuous as one PDZ domain can interact with various PDZBM and a given PDZBM can be recognized by different PDZ domains [25]. Additionally, PDZ-peptide interactions can be fine-tuned by pH, salt concentration, oxidation [26, 27], phosphorylation [28], and allosteric changes [29]. For more details on PDZ domains see the recent review by Ivarsson [30] and references therein.

Although PIs represent a small fraction of cellular phospholipids, they are involved in nearly all aspects of cell biology, including membrane trafficking, cytoskeleton remodeling, regulation of ion channels and transporters, gene transcription, RNA editing, chromatin remodeling, nuclear export, and cell cycle progression [31–35]. PIs are phosphorylated derivatives of phosphatidylinositol (PtdIns). PtdIns can be reversibly phosphorylated in a combinatorial manner at positions 3, 4, and/or 5 of its inositol ring resulting in generation of seven PIs species, namely PI3P, PI4P, PI5P, $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$, and $PI(3,4,5)P_3$. The spatio-temporal distribution of PIs is tightly regulated by a network of kinases and phosphatases and each of the seven PIs shows specific subcellular compartmentalization. For example, $PI(4,5)P_2$ predominates at the plasma membrane, whereas PI3P and PI4P are, respectively, enriched in early endosomes and in the *trans*-Golgi network [36]. PIs act as second messengers but can also have a direct role in signaling [37–41]. Moreover, they serve as docking sites for proteins containing PI-binding modules such as PH (pleckstrin homology), PX (phox-homology), FYVE (Fab1/YotB/Vac1/EEA1), FERM (Four point one/Ezrin/Radixin/Moesin), ENTH (epsin amino-terminal homology), BAR (Bin/amphiphysin/Rvs), and Tubby domains [42–44]. Studies describing a direct interaction of the PDZ domains of syntenin-1 with plasma membrane $PI(4,5)P_2$ [45] and of the PDZ domains of syntenin-2 with nuclear $PI(4,5)P_2$ [46] originally indicated that PDZ domains might also function as PI-interacting modules.

Several techniques are available for studying protein-PI interactions, each with pro and cons. Experiments where lipids are spotted on nitrocellulose membranes, or on the microtiter plates are simple, fast, and low cost. However, they are prone to false positive and false negative results because the data are governed by k_{off} and lipids are not presented in the complex membrane

environment, which in some cases is a prerequisite for the interaction. To mimic the environment of the membrane, PIs can be incorporated at different concentrations into liposomes resembling the membrane composition and incubated with the protein of interest before ultracentrifugation. Presence in the liposome-fraction in a PI-concentration dependent manner will be indicative of an interaction. A quite reliable method to study protein-lipid interactions is Surface Plasmon Resonance (SPR). In these experiments, PI-containing liposomes and control liposomes are immobilized on a sensorchip and perfused with proteins. Binding to the liposomes results in the change of the surface refractory index and the association and the dissociation phases are visualized 'live' in so-called sensorgrams allowing calculation of apparent K_d values. Yet, this method remains expensive and requires expert knowledge to use the instrument, set-up the experiments, and data interpretation. When applicable, Nuclear Magnetic Resonance (NMR) represents a powerful tool for gaining insight into the molecular determinants of protein-lipid interactions. Analysis of the chemical shifts perturbations enables mapping of PI binding sites and determinations of the affinities and specificities. Applying several *in vitro* techniques to study protein PI interactions should ideally be combined with *in vivo* approaches. For example, live fluorescence microscopy experiments where PIs subcellular pools are acutely manipulated and impact on the subcellular localization of a fluorescently tagged protein of interest can constitute convincing additional evidence for PIs interaction. For further details on this topic the reader is invited to consult excellent reviews [34, 47–49].

4.2 The Prevalence of PDZ-PI Interactions

The first high-throughput study investigating PDZ-lipid interactions analyzed 74 individual human PDZ domains and 14 PDZ tandems [50]. In this study, purified PDZ domains were subjected to co-sedimentation assays with vesicles prepared from bovine brain lipid extracts. Among the 74 domains, 17 were found to interact with lipids, from which five (CASK-PDZ, DLG5-PDZ2, Par3-PDZ2, PICK1-PDZ and X11a-PDZ1) showed strong interaction. PDZ tandems interacted generally stronger with liposomes than their respective domains taken in isolation. This study thus suggests that up to 20 % of PDZ domains may interact with lipids. However, the composition of the bovine brain lipid extracts is poorly defined, so it does not really explore which lipids mediate the interactions. Moreover, it also suffers from a lack of affinity measurements.

In a different approach, combing *in vivo* assays with *in vitro* binding studies, Ivarsson and coworkers addressed the prevalence of the PDZ-PI interaction in the *Drosophila* PDZ proteome. The authors used a cell-based screening assay where they evaluated the ability of 46 PDZ domains and one PDZ tandem to target PI-rich subcellular compartments [51]. As PI-binding domains often act in combination with additional PI-binding domains to confer a specific subcellular localization [52], the authors engineered a screening vector combining a fluorescent protein and the first PDZ domain of syntenin-1 meant to provide low initial affinity for PIs. This approach is reminiscent of methods applied to study the prevalence of PI interactions among PX and PH domains from *Saccharomyces cerevisiae* [53, 54]. Taken in isolation the probe is not sufficient for targeting PI-rich compartments but allows targeting when fused with a second PI-binding module. From this screen, only the second PDZ domain of Polychaetoid was able to target the plasma membrane pool of $PI(4,5)P_2$. This study suggests that high-affinity PI-binding is not a general property of PDZ domains. The same screening strategy was also applied to the human PDZ proteome [55]. From 246 single PDZ domains (almost the entire PDZ proteome [11]), 53 PDZ domains targeted PI-rich compartments including the plasma membrane, cytosolic organelles, and subnuclear compartments. *In vitro* dot blot and SPR experiments were performed for 19 domains and clearly established a new set of PI-interacting PDZ domains.

In a recent systematic study [56], the lipid binding properties of 70 PDZ domains derived

from 35 different mammalian proteins were analyzed by the combination of SPR binding studies and *in vitro* FRET assays. The study indicates that 27 domains have sub-micromolar affinities for vesicles mimicking the composition of the inner leaflet of the plasma membrane. This database was further used to build a high accuracy prediction model for other PDZ domains. The model is based on the structural and sequence properties of the domains including protein features enabling interactions with membranes such as electrostatic interactions between anionic membranes and basic protein residues, association of hydrophobic residues with the membrane hydrocarbon core and hydrogen bonds between protein residues and the lipid head group. The algorithm was applied for prediction of membrane binding of 2,000 PDZ domains from 20 species and up to 30 % of the tested PDZ domains displayed predicted lipid binding.

Thus, although the picture is not totally clear yet, is a common feature of a subset of PDZ domains.

4.3 Molecular Determinants of PDZ-PI Interactions

For a subset of PDZ proteins structural features governing PDZ-PI interactions were characterized and these studies are recapitulated below. The insight into the molecular determinants is emerging but is still far from being complete. The data indicate that PDZ domains can interact with membrane PIs by different and complex modes.

4.3.1 Syntenin-1

Syntenin-1 is a PDZ scaffold supporting the endocytic recycling of transmembrane receptors [57] and the biogenesis of exosomes [58], small extracellular vesicles implicated in trans-cellular communication. It contains two PDZ domains in tandem that are connected by a four-amino acid linker. The PDZ tandem is flanked by an unstructured N-terminus and short C-terminal region [59]. The PDZ tandem of syntenin-1 forms a so-called 'supramodule' displaying distinctive characteristics [60]. For example, high affinity interaction with cognate receptors like syndecans requires both PDZ domains of syntenin-1. As mentioned above, the interaction of PDZ domains with lipids was discovered in studies addressing the biology of syntenin-1 [45]. Live microscopy experiments revealed that syntenin-1 PDZ domains target plasma membrane $PI(4,5)P_2$. Syntenin-1 concentrates at the plasma membrane where it co-localizes with PH domain of PLC, a well-established probe for $PI(4,5)P_2$ [61]. Moreover, the plasma membrane enrichment of syntenin-1 is lost in so-called 'ionomycin experiments' [45], which reduce plasma membrane levels of $PI(4,5)P_2$ and lower the negative charge of the inner leaflet of the plasma membrane by promoting scrambling of phosphatidylserine (PtdSer) [62]. *In vitro* analysis by SPR experiments with reconstituted liposomes demonstrated that the PDZ1 and the PDZ2 domain bind to $PI(4,5)P_2$ with medium and low affinity, respectively. The PDZ tandem has high affinity for $PI(4,5)P_2$-containing membranes. Point mutants of syntenin-1, decreasing $PI(4,5)P_2$ binding were identified [45] (Fig. 4.1a). The mutated residues are located in the peptide binding loop (Lys119Ala) and in the N-terminal region of the α2 helix (Ser171His, Asp172Glu, and Lys173Gln) in the PDZ1 domain. Equivalent mutations in PDZ2 domain (Lys203Ala, Lys250Ser, Asp251His, and Ser252Glu) also decrease $PI(4,5)P_2$ binding, however, to a much lesser extent. In a complementary study, Meerschaert and co-workers identified other mutations in PDZ1 (Lys124Ala, Arg128Ala, and Lys130Ala) that also impaired binding to PIs [63]. Noteworthy, the mutated residues are all located at distinct regions and do not cluster together to form a defined binding site. One explanation could be that some of the residues are involved in electrostatic interactions important for membrane avidity as it has been demonstrated for other PDZ domains like the Polychaetoid PDZ2 domain (see below).

Interestingly, Sugi and co-workers constructed a docking model for syntenin-1 PDZ2-$PI(4,5)P_2$ interaction based on the crystal structure of the

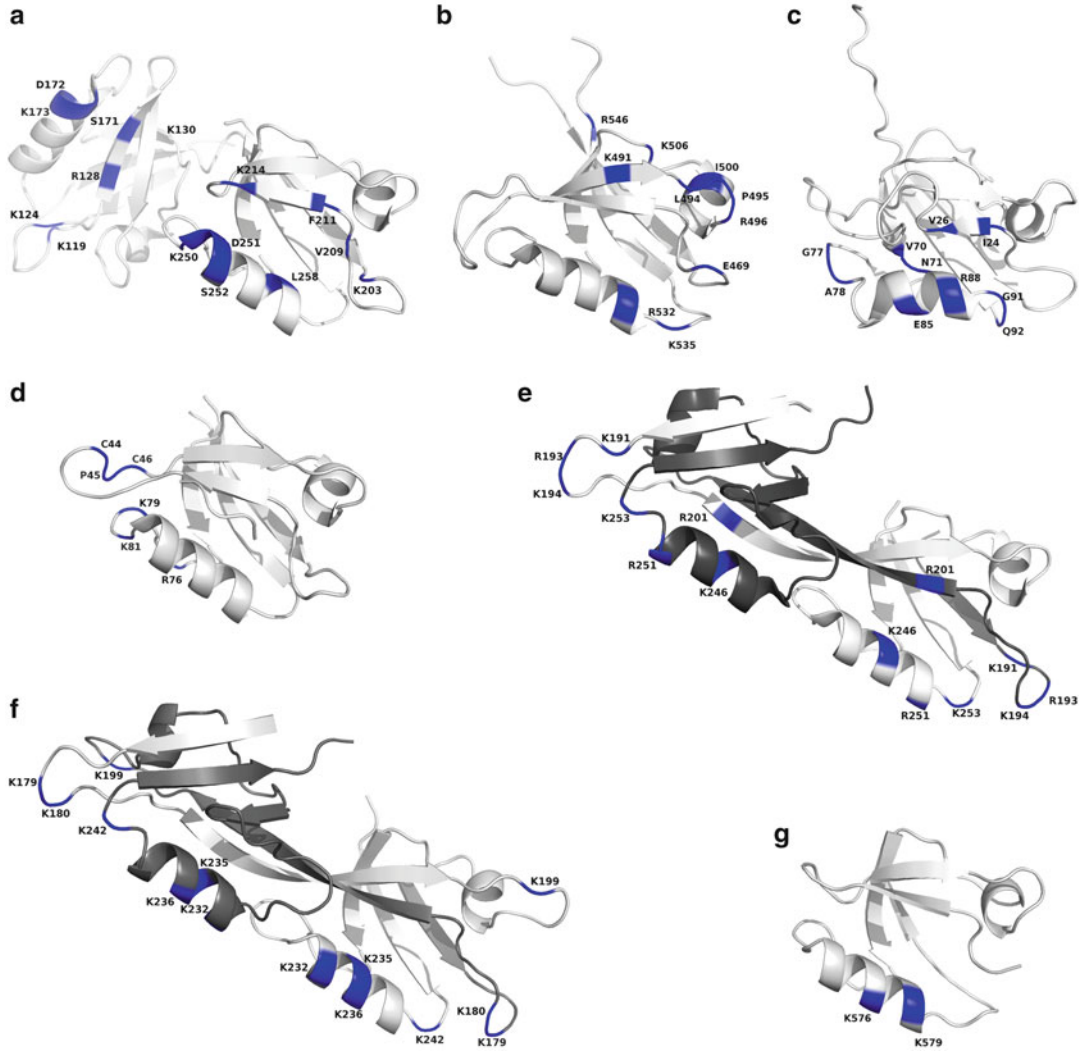

Fig. 4.1 PDZ domain structures and residues implicated in the interaction with membrane-PIs (*blue*) as determined by various experimental approaches; see text for detailed explanation. (**a**) Syntenin-1 PDZ Tandem (PDB ID:1N99). (**b**) PDZ2 domain of Par-3 (PDB ID:2OGP). (**c**) PTP-Bas PDZ2b domain (PDB ID:1Q7X). (**d**) PICK1 PDZ domain (PDB ID:2PKU). (**e**) PDZ2 intertwined dimer of ZO-1 (PDB ID:2RCZ). (**f**) Homology modeling of the PDZ2 domain of Pyd taking ZO1 dimer (PDB ID:2RCZ) as a template. (**g**) Homology modeling of Rhophilin-2 PDZ domain taking Syntenin-1 PDZ2 (PDB ID:1N99) as a template

PDZ domain of tamalin [64]. The fusion protein of tamalin PDZ domain linked to its C-terminal peptide ligand was crystallized in a phosphate buffer. In the crystal structure, two phosphate ions were bound to the PDZ domain in the proximity of the peptide, making contacts with Arg166 in the β5-α2 loop as well as with His167 and Arg168 located in the α helix. According to their docking model, syntenin-1 PDZ2-PI(4,5)P_2 would be explained as such: (i) the 4- and 5-phosphate groups form hydrogen-bonds with Ser252 in the proximity of the α2A position; (ii) the 4-phosphate is engaged in a polar interaction with Lys250 and, (iii) the 3-hydroxyl group of the inositol head group interacts with Lys214 in the β2 strand (Fig. 4.1a). The diacylglycerol backbone was assigned to bind to the hydrophobic cavity in the peptide-binding groove, making van

der Waals contacts with a hydrophobic pocket formed by Val209, Phe211, and Leu258, confirming previous data that PIs and peptide binding sites overlap [45] (Fig. 4.1a). However, the choice of a non-homologous protein for constructing a structural model for syntenin-1 PDZ2-PI(4,5)P_2 interaction might not be an optimal approach considering the structural diversity of such interactions (see below).

Interestingly, the short C-terminal region of syntenin-1 was recently shown to play a supportive role in lipid interaction and in plasma membrane targeting [65]. The apparent affinities, measured by SPR, for 5 % PI(4,5)P_2 embedded in composite liposomes mimicking the composition of the plasma membrane (30 % phosphatidylcholine (PtdCho), 20 % PtdSer, 40 % phosphatidylethanolamine (PtdEth), and 5 % PtdIns) were calculated to be 44 ± 12 μM for the PDZ1-PDZ2 tandem and 11 ± 2 μM when the PDZ1-PDZ2 are flanked by their C-terminal region. The C-terminus contains a highly conserved basic cluster (Lys280, Arg281) which is involved in electrostatic interactions reinforcing the association with lipid membranes as demonstrated by mutational analysis. Therefore, a high affinity interaction with membrane lipids requires both PDZ domains as well as the C-terminal region of syntenin-1.

4.3.2 Syntenin-2

Syntenin-2 is a close homologue of syntenin-1 with an overall sequence identity of 58 % and of 69 % (87 % similarity) for their PDZ domains [66]. Although, syntenin-2 does not interact with the peptide binding partners of syntenin-1, both syntenins share ability to interact with PI(4,5)P_2 [45, 46]. Syntenin-2 binding to PIs was characterized *in vitro* by SPR using composite liposomes mimicking the composition of the plasma membrane and apparent K_d values for PI(4,5)P_2 and PI(3,4,5)P_3 were determined to be 1.5 and 2 μM, respectively. Yet, in *in vivo* experiments solely PI(4,5)P_2, but not PI(3,4,5)P_3, seems to impact on syntenin-2 subcellular distribution [46]. Individually, PDZ1 and PDZ2 have moderate but similar affinities for PI(4,5)P_2. Ala mutation of two Lys located in the carboxylate binding loop of PDZ1 (Lys113 and Lys167) or PDZ2 (Lys197 and Lys244) significantly reduce the binding to PI(4,5)P_2-containing liposomes. When the four mutations are introduced in the PDZ tandem of syntenin-2, the ability to interact with PI(4,5)P_2 is completely lost. The quadruple mutant still interacts with L6A, the sole peptide ligand described for syntenin-2 [67], and is still able to homodimerize [68], indicating that these mutations do not abolish other binding activities of the protein. Thus, as described for syntenin-1, positively charged residues situated in proximity of the peptide binding site are necessary for syntenin-2-PI(4,5)P_2 interaction.

4.3.3 Par-3

Par (partition-defective) proteins are highly conserved regulators of cell polarity controlling landmark developmental processes such as asymmetric cell division and directed cell migration [4]. In mammalian epithelial cells, the Par polarity complex comprises two scaffold proteins Par3 and Par6 together with an atypical protein kinase C (aPKC) and it localizes to the tight junctions where it promotes establishment of the apical-basal boundary [69]. Par 3 contains three PDZ domains and the PDZ2 domain strongly interacts with liposomes prepared from total bovine brain lipid extracts, with lipid strips and with reconstituted liposomes composed of 60 % PtdCho, 30 % PtdSer and 10 % PIs [50]. PDZ2 recognizes all PI species and is not really selective for a given PI. Par3-PDZ2 also slightly interacts with liposomes composed only of PtdCho and PtdSer suggesting nonspecific electrostatic interactions between the positively charged protein surface and the negative charges of PtdCho/PtdSer liposomes. Interestingly, Par3-PDZ2 is highly enriched in basic residues, with the majority of these clustered on one side of the domain. This generates a strong positively charged surface potential which is most probably reinforcing interaction with cellular membranes. Consistently, substitution of the basic residues with either neutral Ala or with negatively charged Glu reduces or abolishes

binding to lipid membranes, respectively. The structure of Par3-PDZ2 determined by NMR revealed that PDZ2 adopts a canonical fold with the peptide binding groove located between the β1 and β2B loops. Using NMR, the PI3P binding site in Par PDZ2 was mapped to the area comprising the α2/β6 and β1/β2 loops (Fig. 4.1b). Residues Arg532 and Lys535 interact with the phosphate groups and the side chain of Glu 469 forms hydrogen bonds with the hydroxyl groups of the inositol ring of the PI. Mutation of any of these residues decreased the affinity by a factor of 10, and the double mutant Arg532Ala/Lys535Ala abolished lipid binding. Interestingly, the PIs head group binding site is situated close to a positively charged patch consisting of Lys491, Lys506, Arg546, and Arg496 and is separated from the polybasic cluster by a stretch of hydrophobic residues (Leu494, Pro495, and Ile500), which directly inserts into the lipid bilayer as demonstrated by fluorescence spectroscopy measurements (Fig. 4.1b). One could then assume that the membrane association of Par3-PDZ2 is driven by (i) specific interaction with the PIs head group, (ii) nonspecific electrostatic interactions, and (iii) membrane penetration. The PI- binding site partially overlaps with the peptide binding site. When the Par3-PDZ2 domain was preincubated with cognate peptide before liposome sedimentation assay, the interactions appear to be mutually exclusive. However, the experimental set-up might not have been optimal as the peptide concentration used in this study was 30 times higher than the concentration of PI3P incorporated in the liposomes and 1,000 times higher than the Par3-PDZ2 domain concentration used in the assay.

4.3.4 PTP-Bas

The non-receptor protein tyrosine phosphatase Basophile (PTP-Bas), also known as PTP1E, PTPL1 and FAP-1, contains N-terminal KIND (kinase non-catalytic C-lobe) domain, followed by a FERM domain, five PDZ domains, and a C-terminal protein tyrosine phosphatase domain [70–72]. The PDZ domains of PTP-Bas mediate a broad range of protein-protein interactions. For example, the PDZ1 binds to the bromodomain containing protein BP75 and IkBα, the PDZ2 binds to Fas and APC, the PDZ3 interacts with PRK2, the PDZ4 interacts with RIL, CRIP2 and ephrin B, and the PDZ5 binds to TRPM2 [73, 74]. The PDZ2, PDZ3, and PDZ5 were proposed to interact with PIs based on gel filtration experiments with $PI(4,5)P_2$-micelles [45]. The second PDZ domain exists as two splice variants, PDZ2a and PDZ2b, differing by the insertion of five amino acids Val-Leu-Phe-Asp-Lys in the loop between β2 and β3 strand [75]. This short amino acid stretch impacts on the recognition of the peptide partners. For example, PDZ2a binds with high affinity to the tumor suppressor protein APC, whereas PDZ2b does not [76]. Although PDZ2b does not interact with any know peptide binding partners of PDZ2a, they both share the ability to interact with PIs. The K_d values of GST-PDZ2a and GST-PDZ2b for $PI(4,5)P_2$ estimated by gel filtration experiments were 55 and 21 μM, respectively [77]. The NMR determination of PDZ2a and PDZ2b structures revealed that, although the global fold of PDZ2a and PDZ2b is similar, the insertion of the five amino acids results in the alteration of the structure [77]. Namely, the five amino acids stretch forms a loop located above β strand 3 that is restricted in its flexibility. Additionally, the length of the β strand 2 is reduced and the orientation of the helix 1 and 2 is altered. Consequently, the groove between β strand 2 and β strand 3 is narrowed changing the properties of the peptide binding pocket. The lipid-binding site was mapped by NMR experiments measuring resonance changes in ^{15}N HSQC spectra upon titration of PDZ2b with soluble forms of $PI(4,5)P_2$ and $PI(3,4,5)P_3$. The K_d value for $PI(3,4,5,)P_3$ was estimated to be 230 ± 30 μM; K_d for $PI(4,5)P_2$ was in the same order of magnitude [77]. Significant chemical shifts perturbations, both for $PI(4,5)P_2$ and $PI(3,4,5)P_3$, were observed for Ile24, Val26, Val70, Asn71, Gly77, Ala78, Glu85, Arg88, Gly91, and Gln92 located in the region of β strand 3, the loop between β strand 4 and 6 and at the both ends of the helix 2 (Fig. 4.1c). As the chemical shifts changes were observed for a broad range of residues it was proposed that

only some of them form the binding site for the PIs head group, whereas others perturbations in resonances would correspond to conformational changes of the protein induced upon PIs binding [78]. Noteworthy, no detectable changes were observed for the loop between β strand 2 and β strand 3 consistent with the fact that the insertion of five amino acids does not influence PI- binding, while having a dramatic effect on peptide binding. However, PIs and peptide binding sites, located on the groove between α helix 2 and β strand 2, overlap.

4.3.5 PICK1

PICK1 (protein interacting with c kinase 1) is a scaffold protein widely expressed in the central nervous system and involved in the trafficking of transmembrane receptors, transporters, and ion channels [79]. For example, synaptic targeting of the AMDA-type glutamate receptors, crucial for synaptic plasticity such as long term potentiation and long term depression, relies on its binding to PICK1 [80, 81]. PICK1 contains a conserved N-terminal PDZ domain, a central BAR domain, and a C-terminal stretch of negatively charged acidic residues. Early studies [82] suggested that the lipid binding of PICK1 solely depends on the BAR domain and that this interaction is positively regulated by the PDZ domain and negatively regulated by the C-terminal acidic region. Positive regulation by the PDZ domain was originally suggested to be the result of a conformational change induced by the PDZ module. However, Pan et al. [83] established that the PDZ domain of PICK1 also displays lipid binding properties. Ligand-free form of PICK1 PDZ domain is unstable and prone to aggregation. To overcome precipitation problems, the C-terminus of GluR2 peptide, a subunit of the AMPA-type glutamate receptors, was linked to PICK1 PDZ domain enabling *in vitro* analysis. The PDZ-GluR2 fusion protein was highly stable and was used to study binding to PIs [83]. The interaction between PICK1 PDZ domain and PIs was demonstrated by co-sedimentation assays with liposomes prepared from bovine brain lipids, lipid strips, and binding assays with reconstituted PtdCho/PtdSer liposomes containing 10 % of selected PIs. According to these approaches, PICK1-PDZ binds to PI3P, $PI(4,5)P_2$, and $PI(3,4,5)P_3$. PIs interaction is reinforced by the negatively charged surface of the membranes. PICK1-PDZ domain contains a positively charged patch on the surface situated at the opposite site of the peptide binding pocket. The positive cluster formed by the charges provided by Arg76, Lys79, and Lys81 was demonstrated to play a critical role in the interactions with the anionic lipids by mutational analysis (Fig. 4.1d). Replacement of Lys79 and Lys 81 with Ala or with Glu almost completely abolished lipid binding [83]. Additionally, the hydrophobic stretch of Cys44-Pro45-Cys46 located in β2-β3 loop (Fig. 4.1d) directly penetrates membrane bilayer reinforcing the interaction. Substitution of both Cys with Gly, as well as their chemical modification, resulted in diminished binding to the lipid membranes but had no impact on the peptide binding properties [83]. Interestingly, H_2O_2-mediated oxidation analysis revealed that the PDZ domain of PICK1 has the propensity to form a disulfide-mediated dimer under mild oxidation conditions [84]. Oxidation induces the broadening of the domain in the region containing Cys-Pro-Cys motif suggesting that oxidation of Cys residues might act as a regulatory switch controlling PDZ-lipid interactions. Consistently, oxidation-mediated dimerization abolishes the lipid membrane-binding capacity of PICK1-PDZ domain. In co-sedimentation assays, the dimeric form, in contrast to the monomeric one, did not exhibit binding to the liposomes. The PDZ-membrane interaction of PICK1 therefore requires a combination of highly conserved Cys-Pro-Cys motif and positively charged patch and can be modulated by the oligomeric state of the protein.

4.3.6 Zonula Occludens

Zonula occludens (ZO) are scaffold proteins involved in the establishment and maintenance of cell polarity, intracellular signaling, and regulation of gene expression [85, 86]. They interact with a myriad of molecules such as occludins,

claudins, alpha-catenin, members of p120 catenin family, connexins, and transcriptional regulators such as Jun, Fos, and C/EBP [87, 88]. To fulfill their biological function, ZO proteins display dual subcellular localization: they are enriched at the plasma membrane, where they are associated with tight, adherent and gap junctions and under certain conditions they are found in the nucleus, where they are enriched in the nuclear speckles [89]. The subcellular localization of ZO-2 depends on cell density; in sparse cultures ZO-2 concentrates in the nucleus, whereas in confluent monolayer is present at the plasma membrane. In addition, heat shock and chemical and mechanical stress promote nuclear accumulation of ZO-2 [89, 90]. ZO proteins contain three PDZ domains; a SH3 (Src Homology 3) domain, a GUK (guanylate-like kinase) domain, and a proline-rich region located either at the C-terminus (ZO-1 and ZO-2) or between the second and the third PDZ domain (ZO-3) [85, 91–93]. The second PDZ domain of ZO-1 and ZO-2 was shown to bind lipids in protein-lipid overlay and ELISA assays and in SPR experiments with PI-containing liposomes [94]. Both domains showed a slight preference for $PI(3,4)P_2$ over $PI(3,4,5)P_3$, $PI(4,5)P_2$, and $PI(3,5)P_2$, indicating that PDZ2 displays only a minor degree of PI specificity. The PDZ2 domains of ZO-1 and ZO-2 form a domain-swapping dimer, stabilized by extensive interaction between antiparallel strands and the peptide binding groove is formed by residues belonging to both monomers [95]. In ZO-1-PDZ2, the peptide binding pocket is surrounded by the cationic residues involved in the interactions with lipids. The lipid binding surface is formed by Lys253, Arg201, Arg251, and Lys246 (Fig. 4.1e) and mutations of these residues to neutral Ala result in decreased PI binding. Mutations of Lys191, Arg193, and Lys194 (Fig. 4.1e) show modest but defined effects on lipid interactions. Binding experiments with PtdSer revealed that Lys253, Arg201, Arg251, and Lys246 are most probably involved in binding to the PI head group, whereas Lys191, Arg193, and Arg194 contribute to non-specific electrostatic interactions [94]. In ZO-1 and ZO-2 PDZ 2, the peptide- and lipid-binding sites overlap, suggesting that peptide and lipid binding are mutually exclusive [94]. Mutations of Arg201 and Lys246 to Ala abolish the peptide binding, whereas mutations of Lys253 and Arg251 to Ala lead to a two-fold decrease of affinity of cognate peptide connexin 43 [96]. Interestingly, mutations of residues involved in the electrostatic interactions (Arg193 and Arg194) had little effect on peptide binding.

4.3.7 Polychaetoid

Drosophila Polychaetoid (Pyd), also known as Tamou [97], is the unique homolog of human ZOs proteins in flies and is required for the regulation of embryogenesis and determination of cell fate decisions [98, 99]. Pyd-PDZ2 domain, as ZOs-PDZ2, forms a stable dimer by a strand exchange mechanism and interacts with PIs [51]. SPR analysis showed that Pyd-PDZ2 binds to all seven PIs with different affinities. Pyd-PDZ2 displays the lowest affinity for the monophosphorylated PIs and the highest for $PI(3,4,5)P_3$. The K_d for $PI(3,4,5)P_3$ was estimated to be seven times higher than that for $PI(4,5)P_2$ (2.2 ± 0.2 and 15 ± 5 μM, respectively). However, when $PI(4,5)P_2$ and $PI(3,4,5)P_3$ were presented to Pyd-PDZ2 in the background of liposomes mimicking the composition of the inner leaflet of the plasma membrane (namely 30 % PtdCho, 20 % PtdSer, 40 % PE and 5 % PIs) the apparent affinities were significantly higher and there was no difference in the affinities for $PI(4,5)P_2$ and $PI(3,4,5)P_3$ (0.7 ± 0.1 and 0.7 ± 0.2 μM, respectively) [51]. Such an increase in apparent affinities indicated that the additional interactions between the positively charged surface of Pyd-PDZ2 and negatively charged membranes can reinforce the interactions. Indeed, Pyd-PDZ2 also interacts with PtdSer (K_d of 20 ± 2 μM) and has a strong positive potential extending along one side of the molecule [51]. In line with this model, mutations of residues from the positive patch (Lys179Ala, Lys180Ala, Lys199Ala, Lys232Ala, Lys235Ala, Lys236Ala and Lys242Ala) (Fig. 4.1f) resulted in a significant loss of binding to $PI(4,5)P_2$ in composite liposomes [51]. The relationship between peptide and lipid binding was investigated using

Crumbs peptide [51]. Crumbs was identified to interact *in vitro* with PydPDZ2 with K_d value of 220 ± 20 μM. Incubation with IP3, the head group of PI(3,4,5)P_3, showed that IP3 does not compete with the peptide binding. Pre-incubation of Pyd-PDZ2 with 500 μM Crumbs peptide before injection over c6-PI(4,5)P_2 resulted in six-fold increase of apparent affinity for the PI suggesting a synergy between peptide and PI(4,5)P_2 interactions. The molecular basis of these effects is unknown and might be the result of induced conformational changes upon peptide binding or additional electrostatic interactions provided by the bound peptide. Noteworthy, this was a first illustration that peptide and lipid can cooperate in binding the same PDZ domain and raised the intriguing possibility that PDZ domain may coordinate protein and phospholipid-mediated signals.

4.3.8 α-Syntrophin

The syntrophin family comprising five members (α, β1, β2, γ1, and γ2) is characterized by a unique domain organization: an N-terminal split PH domain containing an embedded PDZ domain, a central PH domain, and a C-terminal syntrophin unique domain [100]. The two halves of the PH domain fold into a canonical PH domain composed of seven β-strands and one C-terminal α-helix, and the insertion of the PDZ domain is not required for proper folding. However, the PH_N-PDZ-PH_C functions as a supramodule displaying distinct lipid-binding properties [101]. The K_d of α-syntrophin PH_N-PDZ-PH_C for liposomes prepared from bovine brain extracts was estimated to be 5 μM. Interestingly, the binding of the PH_N-PH_C domain is 10 times weaker than the binding of the PH_N-PDZ-PH_C supramodule, and addition of the PDZ domain either at the N-terminus or at the C-terminus of PH_N-PH_C construct does not restore the lipid-binding features. In lipid-strip binding assays, the PH_N-PDZ-PH_C interacts more strongly with PI5P and PI(3,5)P_2 than with PI(3,4)P_2 and PI(4,5)P_2 [101]. In fluorescence perturbation assays, the affinity of the PH_N-PDZ-PH_C for PI(3,5)P_2 (10 %) embedded in 70 % PtdCho/20 % PtdSer liposomes was calculated to be 5.1 μM [101]. Peptide binding of the α-syntrophin-PDZ domain does not affect the lipid binding properties of PH_N-PDZ-PH_C supramodule and *vice versa*, suggesting that the lipid binding and the peptide binding sites do not overlap.

4.4 Common Features and Classification of the PIs Interacting PDZ Domains

The analysis of the properties of the PI-interacting PDZ domains revealed that a high pI value (higher than 9 as compared to an average pI of 7 for the human PDZ domains) and clusters of basic residues are the common features of these [55]. The analysis of the electrostatic potential showed that most lipid-binding PDZ domains contain a surface cationic patch conferring a large net positive charge [50, 56]. The structure based sequence alignment of 22 PI-interacting PDZ domains indicated that there is no general consensus sequence and thus that alternative PI binding modes must exist. Yet, a subgroup of 11 PDZ domains share a basic cluster of three to four Arg or Lys located in the proximity of the peptide carboxylate binding site and mutational analysis confirmed the importance of such basic cluster for PIs binding [55].

As described above, the relationship between peptide and lipid binding varies as competitive (Par3-PDZ2, PTP-Bas-PDZ, ZO-2-PDZ2), independent (PICK1-PDZ), and synergistic (Pyd-PDZ2) binding modes have been described. A recent study proposes a classification of PDZ domains based on the relation between the canonical peptide binding site and the basic cluster involved in the interactions with the anionic surface of the membrane [56]. Class A contains PDZ domains with the cationic patch localized independently from the peptide binding sites, whereas class B includes PDZ domains with a cationic patch localized in the proximity of the peptide binding pocket. SAP103-PDZ3, with a basic cluster on the opposite side of the peptide-binding pocket, represents an example of class A PDZ domains [56]. Mutagenic analysis showed that mutations

of the cationic residues in the lipid binding groove affect $PI(4,5)P_2$ binding, but have no effect on the interaction with the C-terminal peptides [56]. The class B group of PDZ domains is characterized by a cationic patch located around the α2 helix that forms part of the peptide binding pocket, and as a consequence the lipid- and peptide-binding sites can partially overlap or be mutually exclusive. Class B was further subdivided into class B1 with a basic cluster near the C-terminal end of the α2 helix, and class B2 with a cationic patch localized in the N-terminal end of the helix or spread out over the helix [56]. Rhophilin 2 PDZ domain (class B1) can bind lipid and peptide simultaneously, but in contrast to class A where lipid binding has no effect on either the specificity or the affinity of the peptide binding, lipid binding may modulate the peptide binding properties. Indeed, in case of Rhophilin 2 PDZ preincubation with vesicles mimicking the composition of the plasma membrane lead to a 2-fold increase in the affinity for ErbB2. The positive cluster in Tamalin (class B2) is located in the N-terminal end of α2A and mutagenic analysis clearly indicates these residues located in the lipid binding site [56]. Mutation of the cationic residues results in a decrease of lipid binding as well as peptide binding suggesting an overlap between lipid- and the peptide-binding sites [56]. PDZ domains can therefore accommodate different binding modes depending on the structural characteristics of the domain, which can serve as basis for their classification.

4.5 Regulation and Functional Significance of PDZ-PIs Interactions

Interactions with PIs add a new dimension to the biology of PDZ domains as they can rely on PIs for their subcellular targeting, and be regulated by the very dynamic PIs-turnover. Unraveling the functional significance of PIs-PDZ interactions is the ultimate challenge. This has been defined for the syntenin-1 PDZ-$PI(4,5)P_2$ interaction, the first example to be described [45], and was partially addressed in other cases [46, 50, 56, 83, 94].

4.5.1 Syntenin-1

The identification of the syntenin-1-$PI(4,5)P_2$ interaction has been determinant for identifying the role of syntenin-1 in the recycling of syndecans to the plasma membrane, a process with pleiotropic effects on cell behavior [57]. Syntenin-1 was originally identified as a syndecan intracellular ligand [59, 102]. Syndecans are transmembrane receptors carrying heparan sulfate chains on their extracellular domains that function as co-receptors for growth factors and cell adhesion molecules [103]. Syntenin-1 mutants, deficient for $PI(4,5)P_2$ binding, trap syndecans and associated molecules in perinuclear recycling endosomes [57]. These endosomes were shown to be enriched in ADP-ribosylation factor 6 (Arf6) [104, 105]. Further experiments established that Arf6 activation, the recruitment of its downstream effector phosphatidylinositol 4-phosphate 5 kinase (PIP5K), and thereby local $PI(4,5)P_2$ synthesis are essential for the syndecans to be sorted, in a syntenin-1-$PI(4,5)P_2$-dependent manner, to endosomes recycling back to the plasma membrane [57]. When syntenin-1 fails to bind $PI(4,5)P_2$ (or when the Arf6-PIP5K pathway is blocked) syndecan complexes accumulate in perinuclear recycling endosomes, affecting the surface availability of numerous cell adhesion and signaling molecules and resulting in inhibition of cell spreading [57]. The importance of the syntenin-syndecan-$PI(4,5)P_2$-Arf6 complex was later demonstrated *in vivo* in zebrafish. It was shown to be fundamental for the progression of epiboly and to play a critical role in directional cell movements during early stages of development [106]. The syntenin-1 PDZ-$PI(4,5)P_2$ interaction is thus one way for the cell to regulate the output of a plethora of signaling networks by regulating their availability at the plasma membrane.

4.5.2 Syntenin-2

As for syntenin-1, the syntenin-2 PDZ domains interact with the plasma membrane pool of $PI(4,5)P_2$. Additionally, syntenin-2 PDZ domains also concentrate in nucleoli and nuclear speckles,

and mutations abolishing PI(4,5)P_2-binding result in a diffuse nuclear localization. Ionomycin treatment delocalizes fluorescently tagged syntenin-2-PDZ1-PDZ2 from the plasma membrane and PIP5K or Arf6 can increase the plasma membrane recruitment, but these have no effect on the subnuclear enrichment [46]. Yet, nuclear targeting of syntenin-2 relies on PIs as treatment with IGF-I, activating nuclear PLC beta [107, 108], leads to partial delocalization from the nucleus and to an enrichment at the plasma membrane. Additionally, overexpression of yeast PLC1, mutated for its nuclear export signal, promotes a delocalization from the nuclear organelles towards the nucleoplasm and the cytoplasm [46]. Nuclear PIs are crucial regulators of cell signaling in particular gene transcription, chromatin remodeling, mRNA splicing, and editing [35, 109]. Cellular knockdown of syntenin-2 has a drastic effect on nuclear PI(4,5)P_2 organization in nuclear speckles and affects cell viability and the rate of cell division. It has been proposed that syntenin-2 might function as a scaffold protein that shields PI(4,5)P_2, protecting it from a degradation and bringing it in proximity to the speckles components [46, 66]. However, the real function of syntenin-2 in the nucleus, and the importance of its interaction with nuclear PI(4,5)P_2 remains to be discovered.

4.5.3 Zonula Occludens

In sparsely cultured Madin-Darby Canine Kidney (MDCK) cells, endogenous ZO-1 is enriched at the plasma membrane, whereas ZO-2 is found at the plasma membrane, nucleus, and cytosol. Plasma membrane pools of ZO-1 and ZO-2 co-localize with membrane-bound PI(4,5)P_2, as shown by immunostaining with anti-PI(4,5)P_2 antibody and colocalization with the PH domain of PLCδ [94]. Surprisingly, lipid binding is not required for targeting ZO-1 and ZO-2 to the plasma membrane as fluorescently tagged-full length protein carrying mutations abolishing or impairing lipid binding is still enriched at the plasma membrane [94]. The plasma membrane localization is thus most probably driven by protein-protein interactions. Endogenous ZO-2 is enriched in the nuclear speckles and co-localizes partially with anti-PI(4,5)P_2 staining suggesting that ZO-2 might be enriched in the speckles due to its ability to interact with nuclear lipids. Knock-down approach showed that ZO-2 contributes to the organization of nuclear PI(4,5)P_2 as upon ZO-2 depletion the PI(4,5)P_2 staining was dispersed [94]. Therefore, ZO-2 might also act as a scaffold, organizing nuclear PIs.

4.5.4 Polychaetoid

In MCF-7 cells, Pyd PDZ2 is targeted to the plasma membrane in a PI(4,5)P_2-dependent manner, as demonstrated by cellular assays [51]. First, expression of a constitutively active form of Arf6 (Q67L) [110] induces the formation of intracellular macropinosomes surrounded by PI(4,5)P_2-rich membranes. Co-expression of Arf6 Q67L with eYFP-Pyd-PDZ2 results in the concentration of eYFP-Pyd-PDZ2 at the macropinosomal membranes, consistent with PI(4,5)P_2-dependent membrane interaction. Secondly, ionomycin treatment results in the translocation of eYFP-PydPDZ2 from the plasma membrane to the cytoplasm. Finally, translocation of PIs-5-phosphatase to the plasma membrane results in a rapid decrease of PI(4,5)P_2 levels [111], leading to a decrease in the membrane enrichment of eYFP-Pyd-PDZ2. Moreover, mutations impairing interactions with lipids (Lys179Ala, Lys180Ala, Lys199Ala, Lys232Ala, Lys235Ala, Lys236Ala, and Lys242-Ala) confer complete loss of membrane localization. However, *in vivo* both lipid and peptide (Crumbs) appear to contribute to the plasma membrane targeting of Pyd-PDZ2 [51].

4.5.5 Par-3

In MDCK cells, Par-3 is enriched at the plasma membrane and its subcellular localization is driven by Par-3-PDZ2-PI interactions [50]. Deletion of PDZ2, as well as mutations of residues implicated in PI binding, results in a significant decrease of the plasma membrane enrichment of the protein. Noteworthy, these mutations impair lipid interactions but have very little effect on

peptide binding [50]. Moreover, a chimeric protein where Par3-PDZ2 is replaced by the PDZ domain of Mals2/mLin-7b (displaying similar peptide binding properties but showing no PI binding), fails to target Par3 to the plasma membrane. PI-mediated membrane targeting of Par-3 is crucial for regulation of cell polarization. Knock-down of endogenous Par-3 in MDCK cells results in the disruption of tight junction assembly induced by a calcium switch, which can be rescued by RNAi-resistant rat Par3 [112]. Par-3 mutants either with deleted PDZ2 or carrying mutations abolishing PI binding, failed to rescue cell repolarization after calcium switch. Par3-PDZ2/PIs interaction is thus biologically relevant as it mediates plasma membrane localization of Par3 and, consequently, maintains epithelial cell polarization.

4.5.6 Rhophilin 2

Rhophilin 2 inhibits RhoA's activity, leading to the reduction of stress fibers [113]. Overexpression of Rhophilin 2 in HeLa cells results in disassembly of F-actin stress fibers and this activity is mediated by the Rhophilin 2 PDZ domain [114]. In contrast, cells expressing mutant PDZ domains with impaired lipid binding properties (Lys576Ala and Lys579Ala or Lys576Glu and Lys579Glu) (Fig. 4.1g), exhibit normal stress fibers. These results show that ability of the PDZ domain to interact with PIs is crucial for the biological functions of Rhophilin 2 [56]. Thus, PDZ-PIs interactions might be of general importance in the regulation of the cytoskeleton.

4.5.7 PICK1

In Human Embryonic Kidney (HEK) 293 T cells, PICK1 forms small clusters in the cytosol [82]. However, in case of the lipid-binding-deficient mutant (Cys44Gly/Cys46Gly) the clustering is lost and the protein is mainly diffusely localized in the perinuclear region [83]. Fractionation experiments show that the Cys44Gly/Cys46Gly mutant is mainly found in the cytosolic fraction, in contrast to the wild type protein that partitions with membranes. The lipid-binding mediated by the PDZ domain is therefore a crucial determinant of PICK1 clustering and its association with the membrane structures. In neurons, PICK1 is targeted to synapses and this enrichment relies on lipid-binding as the Cys44Gly/Cys46Gly mutant is completely absent from dendritic spines [83]. Moreover, the lipid-binding properties of PICK1-PDZ domain are fundamental for PICK1-mediated clustering and synaptic targeting of AMPA receptors. In HEK 293 cells, PICK1 and GluR2 form co-clusters, which are lost when PICK1-PDZ is impaired for lipid binding [83]. Similarly, wild-type PICK1 targets GluR2 to the neuronal synapses, and Cys44Gly and Cys46Gly mutations lead to a decrease in the number and intensity of synaptic GluR2 clusters. Taken together, the ability to interact with lipids is of high importance for PICK1 to execute its function in protein trafficking.

4.6 Conclusions and Perspectives

PDZ domains are key constituents of scaffold proteins, impacting on a plethora of signaling pathways. Initially discovered and established as protein-protein interaction modules, PDZ domains experienced an unexpected twist when the first report describing a direct interaction with $PI(4,5)P_2$-containing membranes was published, a decade ago. Nowadays, it is becoming more and more evident that membrane/lipid binding is a general property of PDZ domains and that they represent dual specificity binding modules. The nature of the PDZ-lipid interactions is biochemically not fully defined, but PIs are certainly validated partners of some PDZ domains. As revealed by *in vitro* binding studies, the PDZ-PI interactions display a rather low degree of specificity among PIs species and *in vivo* $PI(4,5)P_2$ seems to be the PDZ preferred partner. The interactions between PDZ domains and the plasma membrane, mediated by PDZ-PI interactions, can be/need to be additionally reinforced by electrostatic interactions, in particular with PtdSer, or by membrane insertion sites. Moreover, binding to other membrane lipids cannot be

excluded and should be investigated. Structural studies clearly show that there is no unique PDZ-PI binding motif and PDZ-PIs can impact or not on PDZ-peptide interactions. Crystallographic approaches of PDZ-PI-peptide complexes might help to gain insight into the interdependence between the peptide and PI binding, but are still lacking. A challenge for the future will be to elucidate the signaling consequences of the interplay between peptide and lipid binding by PDZ domains and the broadness of its functional impact for the biology of PDZ proteins, like for example in the nucleus.

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PtdIns(4)P Signalling and Recognition Systems

5

Marc Lenoir and Michael Overduin

Abstract

The Golgi apparatus is a sorting platform that exchanges extensively with the endoplasmic reticulum (ER), endosomes (Es) and plasma membrane (PM) compartments. The last compartment of the Golgi, the *trans*-Golgi Network (TGN) is a large complex of highly deformed membranes from which vesicles depart to their targeted organelles but also are harbored from retrograde pathways. The phosphoinositide (PI) composition of the TGN is marked by an important contingent of phosphatidylinositol-4-phosphate (PtdIns(4)P). Although this PI is present throughout the Golgi, its proportion grows along the successive cisternae and peaks at the TGN. The levels of this phospholipid are controlled by a set of kinases and phosphatases that regulate its concentrations in the Golgi and maintain a dynamic gradient that determines the cellular localization of several interacting proteins. Though not exclusive to the Golgi, the synthesis of PtdIns(4)P in other membranes is relatively marginal and has unclear consequences. The significance of PtdIns(4)P within the TGN has been demonstrated for numerous cellular events such as vesicle formation, lipid metabolism, and membrane trafficking.

Keywords

PtdIns(4)P • Golgi • Phosphoinositide • PH domain • Cellular trafficking • Membrane

5.1 PtdIns(4)P Is the Major Phosphoinositide of the TGN

5.1.1 Metabolic Pathways for PtdIns(4)P

Although PIs represent only a minor fraction of the total lipid content of a cell, they are vitally important for cellular organization. Phosphorylation of the D-3, D-4, and D-5 positions of the

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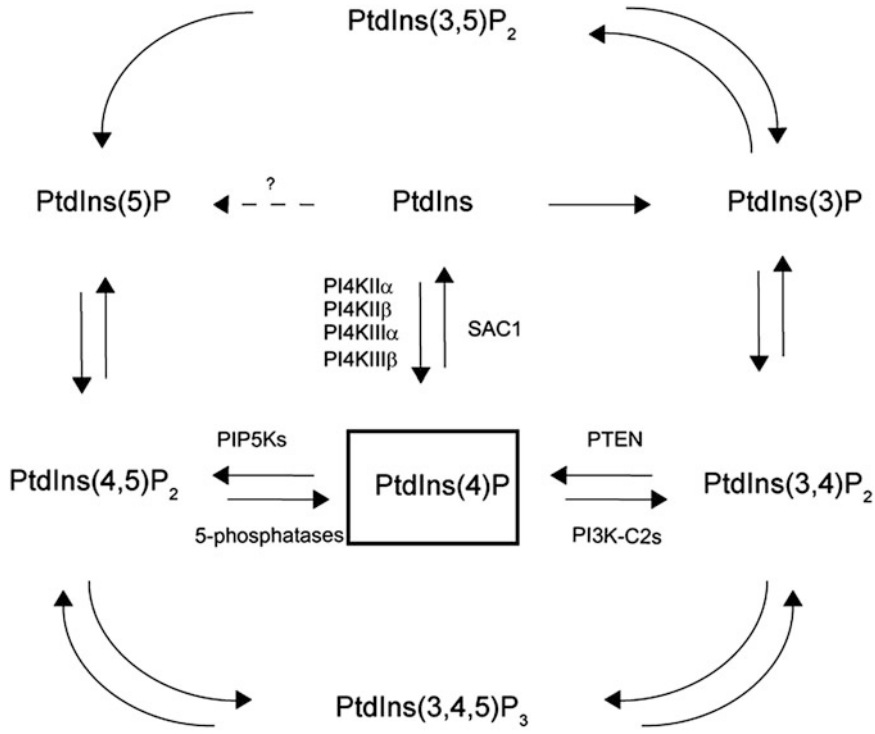

Fig. 5.1 Metabolic pathway of PtdIns(4)P in mammals. The enzymes involved in (de)phosphorylating the 3 and 5 positions of the inositol ring of PtdIns(4)P are indicated. The *arrows* indicate the inter-conversions observed *in vivo*. The conversion of PtdIns to PtdIns(5)P *in vivo* is uncertain and is indicated by a *dotted arrow*

hydroxyl groups of the inositol ring leads to formation of seven distinct PI's (Fig. 5.1). Each exhibits a unique localization amongst the various organelles within mammalian cells, where they are responsible for recruiting different arrays of trafficking and signaling proteins to effect cellular functions (Table 5.1).

The distribution of each PI can be visualized in living cells by expressing a protein that specifically recognizes its structure in a membrane. This reveals a tightly controlled network of PI localizations that mirrors the distribution and regulated activities of the PI kinases and phosphatases responsible for their creation and interconversion. Nonetheless, the limited specificities and affinities of proteins for single lipid molecules and the complexity of assessing dynamic and co-dependent localizations of lipid and protein assemblies in living cells has precluded an easy readout of the lipid code through which a cell's 3D organization is determined.

Progress is being made, with PtdIns(4)P most recently moving to a center stage of PI trafficking. This represents the most abundant of the monophosphorylated phosphoinositides, and is synthesized by phosphorylation of PtdIns or by hydrolysis of the 5- or 3-phosphate of PtdIns(4,5)P_2 or PtdIns(3,4)P_2, respectively. An overview of the mammalian enzymes catalyzing these reactions and their yeast homologues are listed in Table 5.1.

5.1.1.1 Phosphoinositide Conversions

The phosphorylation events mediated by phosphatidylinositol-4 kinases (PI4Ks) have been extensively studied. The reactions are carried out by one of four PI4Ks found in mammals, which have been classified into types II and III according to their sensitivity to the inhibitors adenosine (II) [28] and wortmannin (III) [29]. Together, they constitute the major source of PtdIns(4)P generation in cells, particularly at the Golgi cisternae where most of the cellular PtdIns(4)P is found [30].

Table 5.1 Mammalian PI kinases and phosphatases related to PtdIns(4)P and their yeast homologues

Mammalian enzyme	Yeast homologue	Major cellular localization	References
PI 4-kinases			
PI4KIIα	Lsb6p	TGN, LE, ER, PM	[1, 2]
PI4KIIβ	Lsb6p	PM, E	[3, 4]
PI4KIIIα	Stt4p	ER, N, PM	[5]
PI4KIIIβ	Pik1p	TGN	[6–8]
PI 4-phosphatases			
SAC1	Sac1p	GC, ER	[9]
PI 3-kinases			
PI3K-C2α		GC, E	[10–12]
PI3K-C2β		PM	[13]
PI3K-C2γ		GC	[14]
PI 3-phosphatases			
PTEN1,2	Tep1p	PM, GC, N	[15–17]
PI 5-kinases			
PIP5Kα,β,γ	Mss4p	PM	[18–20]
PI 5-phosphatases			
72-5-phosphatase		GC	[21]
Ocrl		GC, E, Lysosomes	[22, 23]
PIPP		PM	[24]
synaptojanin	Inp52p, Inp53p	Synaptic vesicles	[25, 26]
SKIP	Inp51p, Inp54p	ER, PM	[27]

Abbreviations: *GC* Golgi complex, *E* Endosome, *ER* Endoplasmic reticulum, *PM* Plasma Membrane, *N* Nucleus, *TGN* *Trans*-Golgi Network, *LE* Late Endosome

5.1.1.2 Phosphatidylinositol 4-Kinases Type II

The PI4K type IIα distribution is mainly focused on the TGN but is also present on endosomes and late endosomes (LE) [3, 31, 32]. Both of the PI4KII isoforms are also detected at the plasma membrane (PM): PI4KIIα is present under basal conditions while PI4KIIβ is found after stimulation by a growth factor in a Rac-dependent manner [4]. These enzymes significantly contribute to the pool of PtdIns(4)P found at the PM and are tightly bound to membranes due to their palmitoylation on their cysteine rich region [4, 31], although PI4KIIβ is significantly more cytosolic than its counterpart IIα [33]. PI4KIIα behaves as an integral membrane protein and is unaffected by brefeldin A (BFA), an inhibitor of the ADP-ribosylation factor 1 (Arf1), showing that its Golgi localization is Arf1-independent, which differentiates it from the IIβ form [3, 4]. Consistent with the protein localizations, PI4K type II plays a role in TGN to PM and TGN to endosome transport. The PI4KII proteins have also been found to be phosphorylated by the protein kinase D (PKD), modulating its activity as reported for PI4KIIIβ [34].

The regulatory mechanisms of type II PI4K enzymes are only starting to become clear. Both types are sensitive to calcium [28, 35]. Membrane association in the presence of Rac-GTP increases PI4KIIβ activity and, in its bound state, the enzyme's properties are nearly identical to those of PI4KIIα. Whether the degree of palmitoylation is responsible for the different activities of PI4KII β and α isoforms remains to be determined [4]. At the TGN, the function of PI4KIIα is correlated to the membrane organization and lateral diffusion. The protein's activity increases as it locates in cholesterol-rich membranes under cholesterol-dependent palmitoylation [33, 36]. One model suggests that the activity of PI4KIIα is stimulated by the cholesterol provided by the oxysterol binding protein (OSBP) at the TGN, which results also in the recruitment of

ceramide transport protein (CERT) and an increase of sphingomyelin synthesis [37]. The non-myristoylated PI4KIIα may bind lipids to sample the TGN membrane, and upon palmitoylation by a palmitoyl acyl transferase resides within rafts [33].

5.1.1.3 Phosphatidylinositol 4-Kinases Type III

The type III PI4K's includes two subtypes IIIα and IIIβ. In mammalian cells, PI4KIIIα is mainly found at the ER and the Golgi apparatus [5] and may generate a PtdIns(4)P pool at the PM that is used as the precursor of PtdIns(4,5)P_2. The PtdIns(4)P synthesized by PI4KIIIα is apparently found at the PM despite the ER/Golgi localization of the enzyme [38]. The membrane contact sites (MCS) between a fraction of the peripheral ER and the PM may explain the apparent discrepancies between the enzyme localization and its products, with the enzyme directly phosphorylating the original PI pool in the PM.

PI4KIIIβ is primarily associated with the Golgi complex although it is also found in the endosome. The enzyme, an orthologue of the yeast Pik1p [39], is controlled by a complex system of interactions and regulates Golgi to PM trafficking. PI4KIIIβ is recruited and activated by the GTP form of Arf1. The kinase also interacts with neuronal calcium sensor-1 (NCS-1) and, together with Arf1 and NCS-1, forms a feedback loop that regulates its activity [6, 40]. PI4KIIIβ is phosphorylated on Ser294 by the PKD1 and PKD2 kinases, which are involved in TGN to PM trafficking [41]. The phosphorylation activates the enzyme, which is stabilized by an interaction with 14-3-3 proteins [42]. PI4KIIIβ also recruits Rab11, which contributes to cargo trafficking to the PM [43]. The distribution of PI4KIIIβ is mainly concentrated at the Golgi, and the enzyme is involved in exocytosis. Together with PI4KIIα, it controls the TGN-to-PM transport of cargo, the architecture of the Golgi, and the sphingolipid synthetic pathway.

5.1.1.4 Phosphatidylinositol 4-Phosphatase

Although several phosphatases have been shown to possess a PI phosphatase activity to date, suppressor of actin mutations 1-like protein (SAC1) appears to be the principal protein involved in the specific conversion of PtdIns(4)P to PtdIns. For instance, the total cellular PtdIns(4)P levels increase by up to 10-fold in yeast cells expressing inactivated Sac1p mutants.

SAC proteins contain a Sac phosphatase domain, which is found in several PI phosphatases and can hydrolyze a broad range of PIs. The SAC1 protein localizes to the ER and Golgi compartments, and is anchored to membranes by two transmembrane helices situated at its C terminus [44]. SAC1 localizes to the Golgi of quiescent cells and slows down the rate at which PtdIns(4)P is distributed in the Golgi apparatus. Upon growth factor stimulation, SAC1 then translocates to the ER [45, 46] and in turns attenuates the downregulation of the anterograde PI pathway. The distribution of SAC1 is controlled by its oligomerization, which has been attributed to a putative leucine zipper motif located in its Sac domain structure [47]. Under quiescent condition the oligomer interacts with the coatomer complex of the coat protein complex II (COP-II) and promotes its transport to the cis-Golgi [45]. Upon stimulation by growth factor, the activation of the mitogen-activated protein kinase (MAPK) pathway favors the dissociation of SAC1 complexes, exposing a COP-I binding motif and inducing a retrograde trafficking of SAC1 to the ER [9, 45, 48]. Although the precise functions and regulation of SAC1 remain poorly understood, it appears that SAC1 utilizes PtdIns(4)P as its predominant substrate, and depletion of the protein results in an increased cellular level of PtdIns(4)P [46, 48–51]. Recent studies have also connected the allosteric activity of the enzyme to the lipid content of the membrane [52, 53] and identified a lipid binding groove [47].

5.1.1.5 Other Kinases and Phosphatases

In addition to the PtdIns(4)P kinases and phosphatases, several other enzymes are involved in the metabolism of PtdIns(4)P, although they play comparatively marginal roles. The activities of the PI4P-5K, including its α, β and γ isoforms, catalyze the conversion of PtdIns(4)P to PtdIns(4,5)P_2. The kinase activity of these proteins is stimulated by phosphatidic acid (PA) [18]. The α isoform has been purified with Arf1

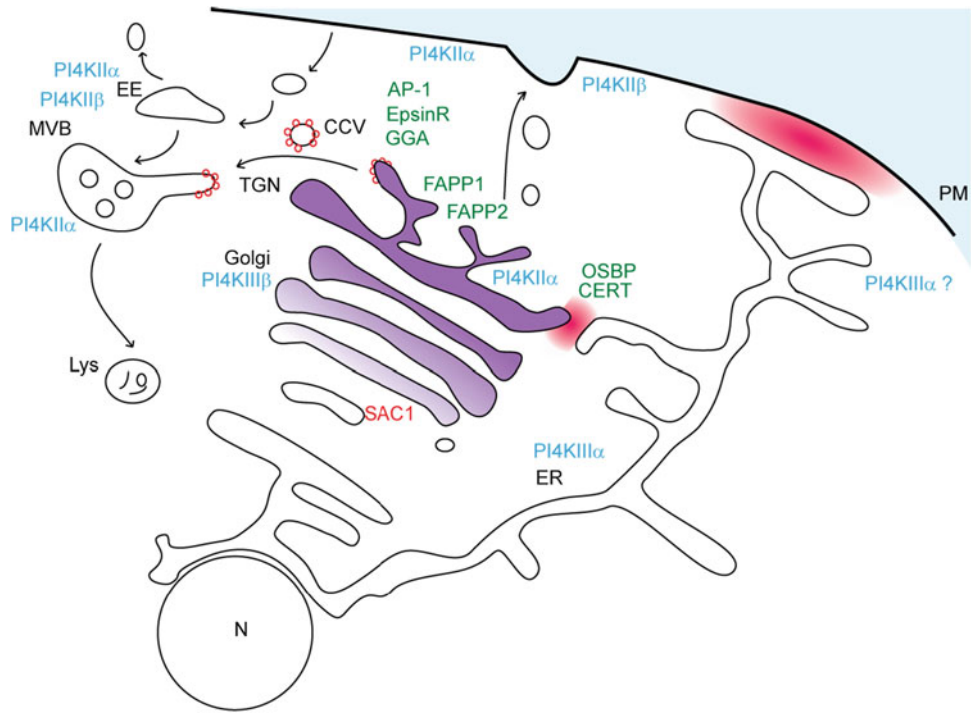

Fig. 5.2 PtdIns(4)P distribution in mammalian cells. Cellular localization of mammalian PtdIns(4)P binding proteins (*green*), kinases (*blue*) and phosphatases (*red*). CCV: clathrin-coated vesicles with the coating protein represented by *red spheres*, EE early endosome, *ER* endoplasmic reticulum, *Lys* lysosome, *MVB* multivesicular body, *N* nucleus, *PM* plasma membrane, *TGN trans*-Golgi network. The membrane contact sites are indicated by *pink areas*. The *purple* color gradient across the Golgi indicates the relative PtdIns(4)P concentrations within the Golgi membranes

which stimulates its activity together with PA [54]. The PI4P-5Ks are localized at the plasma membrane where they use PtdIns(4)P as a substrate to produce PtdIns(4,5)P_2, which is implicated in numerous cellular events such as endocytosis, vesicle formation and [55]. PtdIns(4,5)P_2 can be converted back to PtdIns(4)P by an array of 5-phosphatases [21] which are localized in various cellular membranes.

Several PI3K enzymes convert PtdIns(4)P to PtdIns(3,4)P_2, which is present in very low amounts in quiescent cells. Little is known about these enzymes. The PI3K-C2α is found in clathrin coated pits at the PM and at the TGN, and its overexpression increases the recruitment of AP-2 through PtdIns(3,4)P_2 recognition [10]. The conversion of PtdIns(3,4)P_2 into PtdIns(4)P by the phosphatase and tensin homolog protein (PTEN) has been demonstrated *in vitro* but may be biologically insignificant due to the low levels of PtdIns(3,4)P_2in quiescent cells and its lack of specificity for the substrate [56, 57], hence the *in vivo* relevance of this activity remains unclear.

5.1.2 The PtdIns(4)P Pool at the TGN

Owing to the subcellular distribution of the PI4K, pools of PtdIns(4)P are found in different endomembranes. The major pool of PtdIns(4)P in mammalian cells is found at the Golgi apparatus with a notable heterogeneous spatial distribution. Although PI4K activity has been found across the entire Golgi, PtdIns(4)P is detected in the late apparatus and predominantly at the TGN, suggesting a gradient in the anterograde pathway (Fig. 5.2). Examination of the role of PtdIns(4)P is complicated by the various types of PI4K

present at the Golgi (IIα, IIβ, IIIβ) and the transport between membranes of the lipids, but it appears that the Golgi pool of PtdIns(4)P only marginally contributes to supplying the pool at the PM [58]. PtdIns(4)P is implicated in a variety of functions at the Golgi described hereafter including the recruitment of proteins such as adaptors, coat complexes or lipid-transfer protein but also membrane deformations such as vesicular budding.

The interest in PtdIns(4)P initially focused on its role as a precursor for PtdIns(4,5)P_2 at the PM. However more recently a number of proteins involved in cellular trafficking that bind PtdIns(4)P were discovered [3, 59–64]. This lipid's unique role in Golgi membrane trafficking has recently attracted significant interest as increasing numbers of recognition partners as well as membrane tubulation systems have been identified [65–68].

5.2 Proteins That Recognize PtdIns(4)P

A diverse array of proteins is recruited to the TGN to help mediate trafficking events (Table 5.2). This final subcompartment of the Golgi complex sorts proteins into transport carriers for delivery to distinct destinations within the cell. Some are delivered to the plasma membrane, while others are sorted to endosomes *via* clathrin-coated vesicles (CCVs). The common theme

Table 5.2 Proteins directly interacting with PtdIns(4)P from mammals, yeasts and internalized bacterial pathogens

	Protein	Domain	Function	References
Mammalian	AP-1		Clathrin-mediated trafficking from TGN to endosome	[3]
	epsinR	ENTH	Clathrin-mediated trafficking from TGN to endosome	[69]
	COF	PH	Nonvesicular traffic of lipids, lipid balance in membranes, membrane deformation	[59, 60, 70–72]
	CERT, OSBP1 and ORP9			
	GGAs		Clathrin-mediated trafficking from TGN to endosome	[62]
	GOLPH3		Golgi function and coupling to cell metabolism	[63, 73]
Yeast	Bem1p	PX	Scaffold protein involved in establishment of cell polarity	[74]
	Cla4p	PH-PDB	p21-activated kinase, involved in regulation of cell polarity	[75]
	Drs2p	Split PH	ATPase involved in translocation of phospholipids	[76]
	Gga2p	VHS	Clathrin-mediated trafficking from TGN to endosome	[77]
	Osh1/2/3p	PH	Regulation of PtdIns(4)P levels and MCS, trafficking of sterols	[72, 78, 79]
	Osh4p	Oxysterol binding domain	Regulation of PtdIns(4)P levels and MCS, trafficking of sterols	[80]
	PpAtg26	GRAM	Involved in autophagy and production of ergosterol	[81]
	Sec2p		GEF for exocytic Rab Sec4p	[82]
	Vps74p		Glycosyltransferase retention factor/ retrieval receptor	[63, 73]
Bacteria	SdcA/SidC		Tethering of ER-derived vesicles to *Legionella*-containing vacuole	[83, 61]
	SidM		*Legionella* GEF for Rab1	[84]

Table 5.3 Structures of PtdIns(4)P-binding protein domains

Protein domain	Bound ligands	Methods	PDB	References
FAPP1-PH	C8-PtdIns(4)P, DPC	NMR	2KCJ	[68]
FAPP1-PH	Arf1	NMR	3RCP	[67]
CERT-PH	$Ins(1,4)P_2$, PtdIns(4)P	NMR	2RSG	[71]
FAPP2	(Arf1)	SAXS	–	[65]
AP-1		X-ray crystallography	1W63	[88]
GGA1	Arf1	X-ray crystallography	1NAF	[62]
Epsin-R		X-ray crystallography	1XGW 2QY7 2V8S	[89]
GOLPH3	MYO18A	X-ray crystallography	3KN1	[63]
Bemp1p			2V6V 2CZO	[74]
Osh4p	sterol	X-ray crystallography	3SPW	[80]
Vps74p		X-ray crystallography	2ZIH	[90]
SidM		X-ray crystallography	3N60O 3L0M	[91]
GRASP55 PDZ-PDZ		X-ray crystallography	3RLE	[92]
Sac1p		X-ray crystallography	3LWT	[47]

amongst these systems is the recognition of PtdIns(4)P, although the protein folds involved are diverse and are responsible for distinct biological functions.

5.2.1 Adaptor Protein 1 (AP-1)

Several adaptor proteins found within CCVs serve to link clathrin to membranes by binding PIs as well as cargo proteins. The best characterized of these adaptors are the adaptor protein 1 (AP-1) complex, epsin-related proteins and Golgi-localized gamma ear-containing, ARF-binding proteins (GGAs) [85]. Within yeast cells the AP-1 trafficking hub is complemented by the action of a pair of GGAs proteins (Gga1p and Gga2p) and two Golgi-localized epsin-related proteins (Ent3p and Ent5p) [86]. The populations of AP-1 and Gga2p that accumulate on the TGN are separated in time and space through differential regulation by partners such as the activated form of Arf1 [87].

The AP-1 adaptor is a multi-subunit complex. The interaction of mammalian AP-1 with PtdIns(4)P is mediated by its γ subunit *via* a binding pocket which is conserved in the yeast protein. The TGN localization of the AP-1 complex can be modulated by RNAi silencing of PI4KIIα, and addition of exogenous PtdIns(4)P specifically restores AP-1 targeting in permeabilized PI4KIIα-silenced cells [3], showing that AP-1 membrane targeting is driven by PI recognition.

The structure of the core of the murine AP-1 assembly has been determined at 4Å resolution [88] (Table 5.3), revealing the relationship between fragments of the two large chains, β1 and γ, and the medium and small chains, μ1 and σ1 (Fig. 5.3a). A putative binding site for PtdIns(4)P can be inferred by analogy to the structure of AP-2 co-crystallized with inositol hexaphosphate ($InsP_6$) [93]. The phosphoinositide binding residues are predicted to be Tyr45, Arg48, and Lys52, which form an exposed basic surface in the γ subunit (Fig. 5.3). Mutation of these residues to alanines perturbs TGN targeting, while the Arg48 to Ala mutation abolishes binding to PtdIns(4)P-containing liposomes, suggesting that the basic residues recognize the PI head-group, while the proximal Tyr enhances bilayer affinity. Remaining questions include

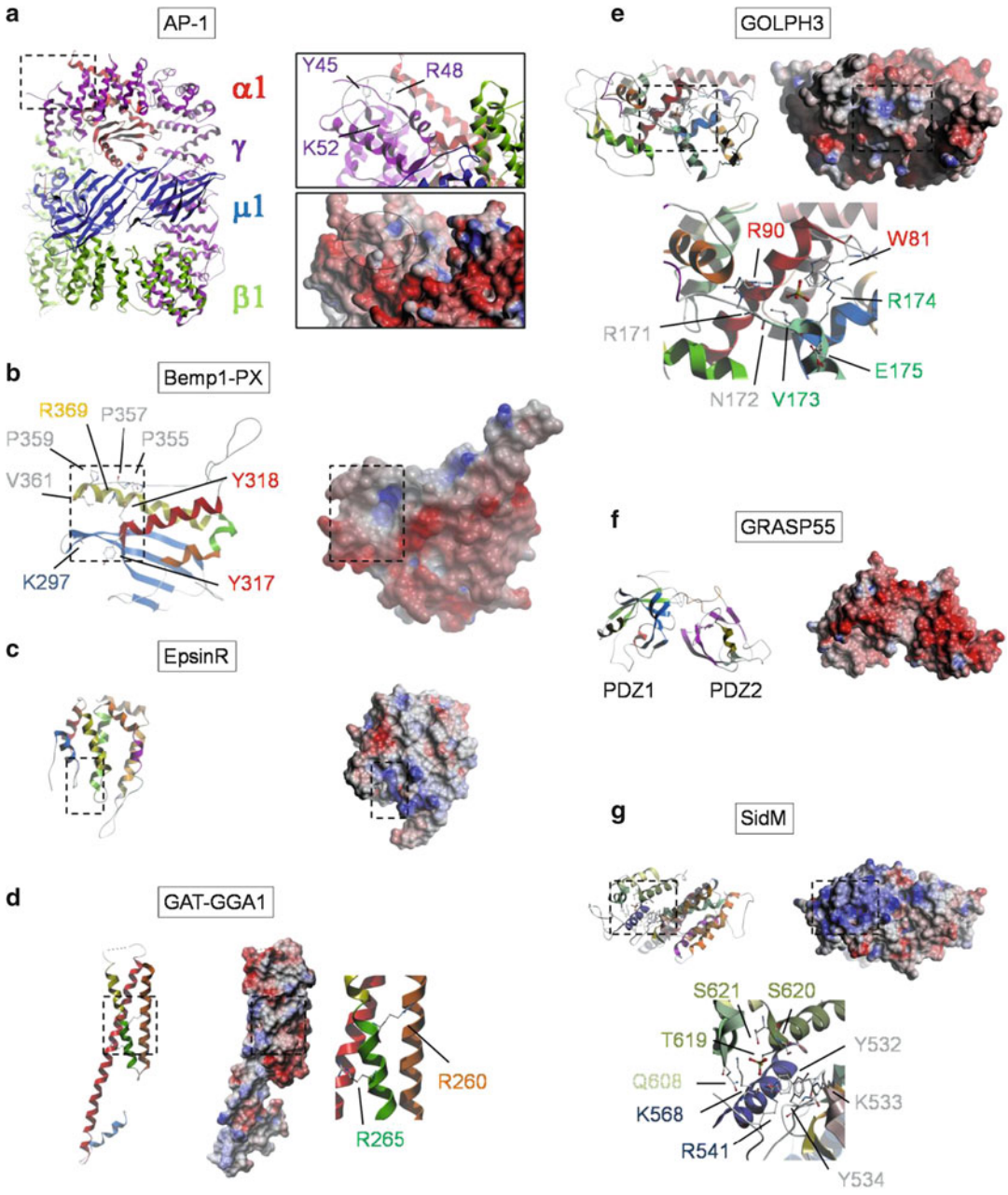

Fig. 5.3 PtdIns(4)P-binding proteins. The proteins AP-1 (**a**), Bemp1-PX (**b**), EpsinR (**c**), GAT-GGA1 (**d**), GOLPH3 (**e**), Grasp55 (**f**) and SidM (**g**) are represented by *ribbons with colored secondary structure segments*. Where known, the locations of the PtdIns(4)P binding sites are indicated by a *dashed box* and the implicated amino acid residues are labeled with their side chains depicted as *sticks*. The corresponding electrostatic surface is represented in the same view for each structure [88]

the mechanistic contribution of ARF · GTP and the membrane anchored YXXØ sorting signal during AP-1:Golgi membrane interaction and deformation [94].

5.2.2 EpsinR (Epsin Related Protein)

Epsins are involved in the physical deformation of membranes in the TGN and generate positively

curved surfaces to contribute to the formation of AP/clathrin-coated vesicles. Immunofluorescence localization and binding assays indicate that EpsinR associates with clathrin, AP-1 and possibly GGA components of the TGN and endosomes both *in vivo* and *in vitro* [95]. EpsinR recruits and promotes clathrin polymerization, but less uniformly than APs. Its Epsin N-terminal homology (ENTH) domain is shared amongst Epsin family members [96], and contains a convex pocket and N-terminal helix that are predicted to bind PI's and engage the membranes respectively [95] (Fig. 5.3c) (Table 5.3). A separate surface of the ENTH domain engages triple helical bundle of SNARE proteins, which are embedded in the membrane through their C-terminal helix [69]. The ENTH domain of EpsinR induces curvature at the outer leaflet of the TGN membrane, exhibiting a preference for the PtdIns(4)P in the Golgi apparatus [69, 95], which is generated by PI4KIIIβ [6]. The knock down of EpsinR dramatically reduces the amount of AP-1 in CCVs and *vice versa*. Hence EpsinR and AP-1 are dependent on each other for maximum incorporation into vesicles formed at the TGN [97], thus promoting membrane curvature and cargo recruitment in a cooperative manner [89].

5.2.3 Golgi Localized, γ-Ear-Containing, ARF-Binding Proteins (GGAs)

The GGAs are monomeric clathrin adaptors proteins that are conserved from yeast to humans. They are involved in vesicular transport between the TGN and endosomal system and have an essential role in lysosomal enzyme sorting [98]. GGAs possess four functional domains: (i) the Vps27/Hrs/Stam (VHS) domain in the N-terminus recruits cargo through interactions with a dileucine acidic cluster DxxLL motif found in the cytoplasmic tail of the integral membrane proteins; (ii) the GAT domain that interacts with PtdIns(4)P, Arf1, and ubiquitin; (iii) a clathrin binding linker, and (iv) a C-terminal appendage domain [93, 99–103]. The cellular localization of GGAs requires a simultaneous recognition of Arf1 and PtdIns(4)P, as the individual contributions are insufficient to drive the TGN localization [62]. The GAT domain recognizes the GTP-bound form of Arf1 but not the cytosolic GDP-loaded form and, when expressed alone, the domain binds to the Golgi apparatus [104]. Accordingly, the knock down of PI4KIIα which has been used to reduce PtdIns(4)P levels in the TGN shows a loss of GGA binding to the Golgi apparatus [62].

The Golgi distribution of GGAs involves distinct TGN-to-endosome pathways from AP-1 [62], ubiquitin-dependent sorting of cargo proteins [105], and TGN-to-endosome retrograde trafficking [106]. The PtdIns(4)P binding site, which was identified by site-directed mutagenesis and tested with liposome sedimentation assays, involves conserved basic residues (Arg276, Arg281, and Tyr310 for GGA2 and Arg260, Arg265, and Tyr294 for GGA1) [62] (Table 5.3). The binding site, which is located on the solvent exposed residues of the three helix bundle of GAT, has no similarities with the other known PtdIns(4)P binding sites (Fig. 5.3d). Mutation of these GGA1 residues to glutamates decreases the protein's association with the TGN, however, the structural basis of the specific lipid interaction remains unknown.

GGAs also occupy a key role in the sorting of protein to the endosome/lysosome system. The GGAs that can bind mannose 6 phosphate receptors (MPRs) cooperate with AP-1 by selecting the cargoes for incorporation into AP-1/CCVs. Furthermore, binding studies reveal a direct interaction between the hinge domains of the GGAs and the γ-ear domain of AP-1 [98, 101].

The mechanism of action of GGAs in cells is unclear. Both AP-1 and GGAs are found in coated buds however, although the GGAs are co-localized with clathrins and partly overlap AP-1 [69, 98, 107], GGAs are not found in isolated CCVs [108] and dissociate readily from membranes when, under the same conditions, AP-1 remains associated [109]. Taken together, several lines of evidence indicate that GGAs play a regulatory role but are not a constant component of the CCV.

5.2.4 Golgi Reassembly and Stacking Protein (GRASP) and Golgin Proteins

The stacked cisternae of the Golgi are maintained by the Golgi Reassembly and Stacking Protein

(GRASP) and Golgin families of proteins. The GRASPs are peripheral membrane proteins which were discovered *in vitro* Golgi assembly assays, and in mammals include GRASP65 and GRASP55 [110, 111]. Their distributions differ, being found in the *cis*- and medial- cisternae within the Golgi stack, respectively, although they have similar sequences and domain organizations. Membrane attachment occurs through an N-terminal myristoyl group, while GRASP65 also binds to the *cis*-localized Golgin GM130 [110, 112] and GRASP55 binds medial-localized golgin-45 [113, 114]. The proteins also self-associate through a conserved N-terminal GRASP domain, which is composed of a pair of PDZ domains with unusual circular permutations and binding pockets, as have been resolved for GRASP55 (Fig. 5.3f) [92]. The Golgins, which interact with GRASPs, are composed of coiled-coils that form extended rod-like structures (Table 5.3). This architecture may allow linking to Golgi membranes [115]. High resolution electron microscopy reveals thin fibers which connect transport vesicles with Golgi cisternae [116], suggesting a tethering role. However, the basis for recognition of specific lipids in Golgi membranes by GRASP or Golgin proteins remains to be defined.

5.2.5 Golgi Phosphoprotein 3 (GOLPH3)

GOLPH3 was identified using a proteomic screening for PtdIns(4)P-binding proteins, and is the human orthologue of the yeast Vps74 protein [117]. Its lipid binding specificity is conserved among yeast, flies, and humans. In mammalian cells, GOLPH3 localizes to the *trans*-Golgi [73, 118, 119]. The Golgi localization is tightly linked to its interaction with PtdIns(4)P. The GOLPH3 proteins represent a conserved family of PI-binding proteins with a unique structure composed of 12 α helices and a pair of β hairpins that mediate oligomerization [90]. GOLPH3 binds to the Golgi membranes through an interaction with PtdIns(4)P, which occurs in the μM range [63]. Binding analysis of mutant GOLPH3 proteins indicates that residues Trp81, Arg90, Arg171, and Arg174 are crucial for PtdIns(4)P recognition, and form a conserved lipid binding site [63] (Fig. 5.3e; Table 5.3).

The complex that mediates the extension of the Golgi includes not only GOLPH3 and PtdIns(4)P, but also MYO18A and F-actin. The interaction with Myosin-XVIIIA (MYO18A) connects the Golgi complex to actin filaments where MYO18A may act as a motor to deform the membrane. Depletion of GOLPH3 results in an unusual Golgi compaction that is also observed for knockdown of MYO18A, or depolymerization of F-actin [73]. The compaction is also a distinct feature of PtdIns(4)P depletion in the Golgi. Together, the results indicate that GOLPH3 links the Golgi to actin cytoskeleton (Fig. 5.6e). Through GOLPH3, PtdIns(4)P may regulate mechanical properties, including the elasticity and elastic limit of the membrane, thus influencing the formation of vesicles and possibly generating the unique morphology of the Golgi apparatus [73]. The role of GOLPH3 in the secretion pathway is illustrated by a study of Hepatitis C virus. These virions are ensembled from ER-derived membranes but their secretion is impaired by the silencing of GOLPH3 or MYOA, which leads to intracellular accumulation of viral particles [120]. The GOLPH3/Vps74 proteins may also be involved in the retrograde pathway via the retention of Golgi material such as mannosyltransferases [63].

5.2.6 SidM

The pathogen *Legionella pneumophila* which is responsible for the Legionnaire's disease, forms a replicative compartment called the *Legionella*-containing vacuole (LCV). This system that produces proteins through a type IV secretion system which are later delivered to the cytosol of the host cell [121]. Amongst these products, SidM (also known as DrrA), is a 647-amino-acid protein that is involved in redirecting ER-derived vesicles to the LCV. The central domain of the protein (amino acids 340–533) possesses a guanine nucleotide exchange factor (GEF) activity for the GTPase Rab1 that is involved in the regulation of vesicular

trafficking [122–124]. In addition, its C-terminal domain, named P4M, specifically recognizes PtdIns(4)P with a nM range affinity and is used to attach SidM to the LCV through a lipid binding [84, 91]. The minimal fragment identified for the PtdIns(4)P binding includes the residues 544–647. The crystal structure of a larger domain spanning residues 340–647 (Table 5.3) reveals six α-helices and one 3_{10}-helix [125]. Within the P4M domain, the PtdIns(4)P-binding site is evidenced from the positioning of the sulfates found within a positively charge grove formed by a perpendicular arrangement of helices. Residues Arg541, His543, and Lys568 provide the positive charges while the opposite side of PtdIns(4)P-P4M binding site which is negatively charged may be responsible for orientating the protein with respect to the membrane; the acidic residues being oriented away from the membrane (Fig. 5.3g). In a cellular context the SidM-PtdIns(4)P interaction is involved in localizing SidM to the PtdIns(4)P enriched LCV membrane compartments that originate from the PM. As a GEF for Rab1, SidM promotes the membrane recruitment of Rab1 which in turn helps to recruit ER-derived vesicles [91, 123].

5.3 COF Family

A family of proteins containing a unique pleckstrin homology (PH) domain have recently been demonstrated to be implicated in the metabolism of sphingolipids and sterols (Table 5.2). These proteins, which include the ceramide transfer protein (CERT), the oxysterol-binding protein 1 (OSBP1), and the four phosphate adaptor proteins (FAPPs), collectively named COF, share a common architecture including a PtdIns(4)P-specific PH domain and typically also a C-terminal lipid carrier domain for cholesterol, ceramide, and glucoceramide (Fig. 5.3). The related FAPP1 protein shares a high identity with FAPP2's N terminal region, localizes to the TGN and interacts with Arf1 [66].

The activities of COF family members as non-vesicular transporters of lipids from the ER to the Golgi or within the Golgi have been extensively studied [60, 66, 126–135]. In contrast, their roles in lipid metabolism and their respective mechanisms of lipid transport are just beginning to emerge. The structural basis of PtdIns(4)P recognition has been most closely studied for FAPP1, providing mechanistic insights into how the Golgi membrane is recognized and manipulated.

5.3.1 Structural Features of COF Proteins

The TGN localization of COF proteins is largely determined by their N-terminal PH domains. The PH domains constitute one of the most common signaling modules, and have a conserved structure composed of a seven β-stranded sandwich capped by a C-terminal α helix (Table 5.3). The roles of most PH domains involves either protein or 3-phosphoinositide recognition, although their diverse ligand specificities remain difficult to predict from structure or sequence alone [136–139].

Assays performed in cells indicate that the PH domains of OSBP1, FAPPs and CERT recognize PtdIns(4)P at the TGN, while *in vitro* experiments give a more complicated picture of PI specificity [70, 137, 140]. Lipid dot blot assays have identified PtdIns(4)P as the unique or at least major ligand [59], while biophysical techniques indicate a more limited intrinsic PI specificity [38, 68, 70], suggesting that extrinsic factors also contribute significantly. In particular, the PH domains of FAPPs and OSBP1 interact preferentially with GTP-bound state of the small GTPase Arf1, which is also present at the TGN [66, 67].

In addition to its PH domain, CERT contains a lipid/sterol-binding steroidogenic acute regulatory protein-related lipid transfer domain (START) in its C-terminal region [130] that transfers ceramide between lipid vesicles *in vitro* [128]. Its START domain can accommodate different acyl chain lengths [130]. The central region contains coiled-coil motifs that mediates oligomerization as well as a FFAT (two phenylalanine in an acidic tract) motif between residues 321 and 327 which is involved in the ER binding through the binding of vesicle-associated membrane protein-associated proteins

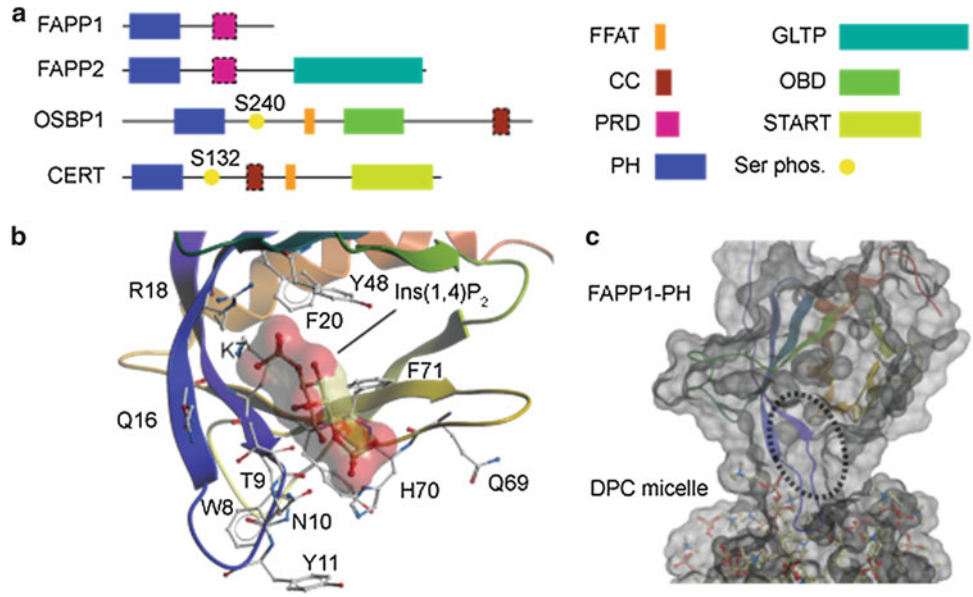

Fig. 5.4 PH-containing proteins which recognize PtdIns(4)P. The architectures of the proteins are compared (**a**) The domains and motifs identified are represented (*FFAT* two phenylalanine in an acidic tract, *C* coiled coil, *PRD* proline rich domain, *PH* pleckstrin homology, *GLTP* glycolipid transfer protein domain, *OBD* oxysterol binding domain, *START*: lipid/sterol-binding steroidogenic acute regulatory protein-related lipid transfer domain, *Ser phos* serine phosphorylation). (**b**) The structure of FAPP1-PH modeled with PtdIns(4)P headgroup ($Ins(1,4)P_2$; *bottom left*) is shown with Ins(1,4) P_2 represented by a *sticks* and a molecular surface, and the backbone of the PH domain by a *ribbon*, with the residues within 5Å of the lipid head group labeled. (**c**) The FAPP1-PH structure bound to a dodecylphosphocholine (DPC) micelle is shown (*bottom right*) with a translucent molecular surface [68]

A and B (VAP-A and VAP-B) [129]. Crystal structures of CERT's START domain complexed with ceramide molecules reveal a long amphiphilic channel that could serve as a hydrocarbon ruler [130]. Recently, the solution structure of its PH domain has been solved and the detailed of the interaction with PtdIns(4)P examined by NMR and surface plasmon resonance [71] (Fig. 5.4).

The OSBP1 protein contains a N-terminal PH domain, FFAT motif and oxysterol-binding domain (OBD) [141]. As with CERT, its FFAT motif links the protein to VAPs. The protein is homodimeric and two coiled-coils are predicted on either side of the OBD. OSBP1 represents the best characterized of the OSBP-related-proteins (ORP) family that localize to the Golgi apparatus [142]. Although the PH domain of ORPs have been solved (ORP8, ORP11) and provides a basis for homology modeling, no structure of the PH domain of OSBP1 has been determined. Only the FFAT motif of OSBP1 in complex with VAP is known and gives a structural insight into the ER localization of the protein [133].

The FAPP1 and FAPP2 proteins share 80% identity in their N-terminal region, and differ primarily in the presence of a glycolipid transfer protein (GLTP) domain at the FAPP2's C-terminus. A proline-rich element in the central region lacks a known partner or function. Recently, a putative FFAT motif has been suggested by data mining and would connect the proteins to the CERT and OSBP1 mechanisms [143]. While the FAPP1 PH domain is monomeric, the full length FAPP2 protein is dimeric and a low resolution structure solved by small angle X-ray scattering implicates the proline-rich region as the dimer interface [65]. The NMR structure of the PH domain of FAPP1 reveals that membrane insertion and PtdIns(4)P recognition are mediated by an elongated hydrophobic

loop and proximal positively charged pocket [67, 68] (Fig. 5.4), features that are conserved across the COF family.

5.3.2 Ceramide Transfer Protein

Ceramides (Cer) are synthesized in the ER and transported to the Golgi where they are converted into sphingomyelin (SM) by sphingomyelin synthetase (SMS) or glucosylceramide by the glucosylceramide synthase (GCS) [144]. In these pathways, the synthesis of SM, but not GlcCer depends on the transport of Cer to the *trans*-Golgi by CERT [128]. The synthesis of SM occurs at the TGN where SMS is localized, and involves transfer of the head group of phosphatidylcholine to Cer which also yields diacylglycerol [128]. Defects in CERT inhibit SM synthesis, and lowering PtdIns(4)P levels also inhibits SM synthesis as a consequence of the impact on the recruitment of the protein to the Golgi apparatus [145].

At least two pathways are responsible for the transport of Cer to the Golgi apparatus; the major pathway is cytosol-dependent whereas the minor route is cytosol-independent [128]. CERT mediates the transfer of Cer *via* the major pathway in a non-vesicular transfer manner [128]. Two models have been proposed for the transfer of ceramide to the TGN: a short distance and a fixed model. In the first hypothesis, CERT alternates the binding to TGN and ER through the PtdIns(4)P and VAP interactions, respectively. In contrast the second model offers a static view at the ER-Golgi contact sites where both the FFAT motif and the PH domains are fixed to the ER and TGN, respectively, and where START domains shuttle the Cer from one compartment to the other [129]. It is generally accepted that GCS is localized in the early cisternae of the Golgi [160] and would transform the totality of the Cer to GlcCer that passes through, while CERT delivers its content to the TGN where SM is produced, bypassing the early cisternae.

A variety of factors directly influence the activity of CERT. The CERT transfer activity depends on the length of the acyl chain of the ceramide substrate and displays a preference for C_{16} or C_{18} over longer chains such as C_{24} [130]. This selectivity mirrors the acyl chain distribution found in many cells and tissues. The Cer is recognized through a polar region at the far end of the hydrophobic cavity that binds Cer through a dense hydrogen bond network.

The association of CERT to the *trans*-Golgi is controlled by binding of its PH domain to PtdIns(4)P [128]. Despite the presence of various isoforms of PI4K at the Golgi apparatus, it is PI4KIIIβ, which is associated with the PtdIns(4)P pool, that is targeted by CERT. Over-expression of the FAPP1-PH or OSBP1-PH, which recognize PtdIns(4)P, leads to inhibition of the Cer transport of these proteins and may also interfere with Arf1 activity [145]. Indeed, it is thought that Arf1 may interact with CERT owing to its similarity with OSBP1 and FAPPs.

The structure of the CERT PH domain has been solved by NMR and a comparative study of the PI affinities reveals a marked specificity for PtdIns(4)P or PtdIns(4,5)P_2 while other monophosphorylated PI such as PtdIns(3)P and PtdIns(5)P are only weakly recognized [71]. The relative affinities for membrane embedded PtdIns(4)P is two order of magnitude stronger than for soluble analogues of PtdIns(4)P [71]. Thus, the affinity for PtdIns(4)P can be enhanced by providing a lipid mixture. An extensive largely basic surface bordering the PI binding site may act as a secondary interacting for PA or phosphatidylserine (PtdSer), which mirrors the observations in FAPP1-PH. A significant difference in the dynamics of the β1–β2 loop between FAPP1 and CERT is apparent and may account for the absence of tubulation induced by CERT [71].

The association of CERT to the Golgi apparatus is regulated by cycles of phosphorylation and dephosphorylation by the TGN-associated PKD and protein phosphatase 2 C-ε (PP2Cε). The phosphorylation of a serine repeat (SR) that follows the PH domain, in particular Ser132, diminishes the binding of CERT to the Golgi apparatus through a concerted interaction between the PH and the START domains [146]. Conversely, the dephosphorylation of CERT by PP2Cε, an enzyme localized in the ER and associated with

VAP-A, promotes the association of CERT to the Golgi apparatus [147] (Fig. 5.6b).

PKD is also known to play a role in the fission of cargo as they are directed from the TGN to the PM. This kinase is recruited to the TGN which binds DAG, a product of SMS reaction between PC and Cer via its cysteine-rich C1 domains. Over-expression of CERT promotes the activation of PKD, while PKD negatively regulates CERT [126]. PKD also activates PI4KIIIβ enhancing the production of PtdIns(4)P at the TGN and the recruitment of PH domains of CERT, OSBP and FAPPs. A model of regulation of CERT has emerged based on the subcellular localization of both PKD and PP2Cε at the MCS where both enzymes successively activate and inhibit the TGN interaction of CERT. This suggests that this protein could be dynamically localized when these two enzymes are present [147, 148].

5.3.3 Oxysterol Binding Protein

Vesicular- and non-vesicular routes are used to maintain the cholesterol homeostasis in cells through mechanisms that are yet to be determined. OSBP1 was first identified as a cytosolic protein capable of binding oxysterols, and later it was found to bind cholesterol in the micro- to nano- molar range [149]. The protein is localized to the TGN and possesses a FFAT domain that allows VAPs to interact with the protein, as with CERT [150]. Though its PH domains can simultaneously recognize PtdIns(4)P, its intrinsic specificity may be limited based on binding experiments showing similar affinities for liposome embedded PtdIns(4,5)P_2. Consequently its TGN targeting may be explained by the coincident binding of the PtdIns(4)P and Arf1-GTP [38, 67, 70], as well as secondary lipid interaction.

OSBP1 has been described as a sterol sensor that transports sterols in a non-vesicular manner between membranes between the ER and the Golgi apparatus [141, 151]. The over-expression of OSBP1 showed an effect on cholesterol homeostasis [152] although the precise role of OSBP1 in cholesterol and oxysterol homeostasis are still not resolved. The localization of OSBP1 is dictated by oxysterols and by the functional integrity of its OBD. Depletion of its C-terminal lipid transfer domain results in constitutive binding of the protein to the Golgi apparatus, as mediated by its PH domain. The OSBP protein also translocates to the Golgi upon binding to 25-hydroxycholesterol [141, 153] and interacts with Arf1 [66, 70].

The OSBP1 protein undergoes cycles of phosphorylation and dephosphorylation within a serine-rich motif that regulates its subcellular localization. The phosphorylation of Ser242 by PKD results in a loss of Golgi apparatus binding, and depletion of OSBP1 induces fragmentation of the Golgi apparatus [131]. The modulation of this activity also has implications for CERT activity, and suggests a regulatory function of sterol and SM synthesis at the Golgi apparatus. Indeed, it was shown that OSBP mediates the activation of SM synthesis in response to a change sterol levels, with OSBP1 appearing to connect the SM and the cholesterol pathways [154] which in turn could affect or be dependent on lipid raft assembly in the late Golgi. It has been suggested that the protein acts a cholesterol sensor through the oxysterol binding and results in an increased CERT-dependent ceramide transfer from the ER to the Golgi apparatus [155] (Fig. 5.6b).

A lipid dependent signaling pathway involving OSBP1 has recently been identified. The cellular levels of cholesterol regulate its association with two phosphatases HePP and PP2A, thus modulating the phosphorylation state of the extracellular signal-regulated kinase (ERK) [151]. The cytosolic OSBP1 assembles into a 440 kDa oligomer that contains the phosphatases and cholesterol. Conversely, cholesterol depletion promotes the disassembly of the oligomer and enhances the activity of phosphorylated ERK.

The overexpression of OSBP enhances the synthesis of SM in response to oxysterol in a mechanism that implies a sterol binding to OSBP and an increased of CERT translocation from the ER to Golgi apparatus [132]. The role of OSBP in SM synthesis is also evidenced from mutation of Trp174 to Ala within the PH domain, which results in a loss of PtdIns(4)P binding and

significantly reduces SM synthesis [156]. Recently, OSBP was found to activate PI4KIIα at the TGN, resulting in an enhance PtdIns(4)P level and subsequently an increase of CERT recruitment and SM synthesis [37].

Finally, several ORPs are similar to OSBP, having FFAT and PtdIns(4)P-specific PH domains, and as such OPR9 and ORP11 could overlap OSBP1 functions. ORP9 also localizes at the Golgi and promotes the Golgi localization of ORP11 but does not appear to be involved in the SM synthesis or translocate to ER under sterol simulation [60, 64, 156]. However, the phosphorylation of ORP9 by PKD2 does not affect its Golgi localization [157]. Owing to the overlapping functional role of the ORPs and the size of this protein family, the distinct roles of the individual proteins and their precise functions in cells remain unclear.

5.3.4 Four Phosphate Adaptor Proteins

Both FAPP1 and FAPP2 associate dynamically with the TGN where they localize at the exit sites from which cargoes leave for the PM [66]. This localization is dictated by the coincident recognition of PtdIns(4)P and Arf1-GTP through their PH domain [65, 66, 135] (Fig. 5.6c). The depletion of the GLTP domain of FAPP2 and the mutations within the PtdIns(4)P recognition site demonstrate that the PH domain is directly responsible for the localization to the Golgi apparatus [66, 67]. The overexpression of FAPP1-PH domain also shows a deformation in cells that involves generation of long tubules [66]. Subsequent experiments *in vitro* also show that both FAPPs can achieve a membrane deformation and elongation of flat membrane sheets in a PtdIns(4)P-dependent manner [65, 68].

The mechanism of coincident recognition of Arf1 and PtdIns(4)P by FAPP1-PH has been investigated. The Arf1 protein binding interface spans the β5-β6 loop of the FAPP1 PH domain [67]. Structural studies also show that the protein's affinity for a unimolecular the PtdIns(4)P headgroup is weaker than for the PI embedded in bilayer environments [68]. The extremity of the β1-β2 loop inserts into the hydrophobic interior to provide additional stability of the membrane complex and helps to deform the bilayer and induce tubules to form. According to the NMR titrations and site-directed mutagenesis analyses [67, 68], the specific interaction between the FAPP1-PH domain and the PtdIns(4)P involves the Lys7 residue. The molecular model of the interaction suggest that the inositol ring is positioned differently compared to the PIP3-recognising PH domain, with an orientation of the ring parallel rather than perpendicular to the β1 and β2 strands since in the crystal structures of PI:PH domain complexes.

The FAPP1-PH domain is used for mapping PtdIns(4)P in cells due to its biological specificity [38, 73, 158, 159]. The intrinsic ligand specificity for PtdIns(4)P is evident using protein-lipid overlay assays, however, although PtdIns(4,5)P_2 can also interact *in vitro* based on other techniques including NMR spectroscopy or liposome binding assays [68, 72], suggesting coincident recognition of Golgi membranes, with PtdSer and Arf1 enhancing the biological interaction [68].

FAPP1 and FAPP2 have been identified as key players in the post-Golgi trafficking. However knock-down experiments show also that the depletion of these proteins does not significantly alter the morphology of the Golgi apparatus [66, 135]. In polarized MCDK cells, the overexpression of either FAPP protein results in the loss of cargo to the apical and basolateral membranes, while under low expression levels cells are unaffected [135]. On the other hand, the knock-down of either FAPP1 or FAPP2 shows that only the latter inhibits the transport to apical membrane and this effect could not be rescued by FAPP1 [135]. This suggests that FAPP2, by specifically targeting the apical membrane, participates in the formation of lipid rafts at the TGN through its lipid transfer function. Indeed, in these polarized cells, the rafts formed at the TGN join the apical membrane [135].

FAPP2 function is associated with the metabolism of GlcCer, the precursor of complex glycosphingolipids (GSL). The synthesis begins in the ER from Cer that is subsequently glycosylated at the cytosolic leaflet of the *cis*-Golgi to GlcCer. The GlcCer is then transferred to the *trans*-Golgi where it is translocated to the lumi-

nal leaflet and undergoes further glycosylations. FAPP2 can specifically transfer GlcCer from a donor to an acceptor membrane *in vitro*. The transfer rate increases significantly when the vesicles contain Arf1 and PtdIns(4)P, with inactivation of PI4K or Arf1 in cells, thus confirming the *in vitro* experiments [134]. Furthermore, the distribution of GlcCer remains mainly at the cis-Golgi when FAPP2 is knocked down. In addition, depletion of FAPP2 shows a significant decrease of the complex GSLs that are derived from GlcCer. Based on the cellular mapping of the GlcCer synthase mainly found in the median-trans Golgi it is likely that natural GlcCer does not flip at the *trans*-Golgi but undergoes a shuttling process between the PM and ER where it is then passed to the Golgi apparatus [160]. FAPP2 protein shuttles GlcCer from the various cellular membranes back to the ER where it is flipped inside the lumen of the ER and is further glycosylated while it travels through the Golgi.

To date, the details of the recognition of PtdIns(4)P by the members of the COF family has been described for FAPP1 and CERT PH domains using NMR spectroscopy. The residues identified by NMR titrations match remarkably well and supports a common mechanism of recognition for the entire family with however a possible difference in the flexibility of the hydrophobic membrane insertion loop [71]. The studies also confirm that the mechanism of membrane recognition is far more complex than a simple PtdIns(4)P binding event as the affinity of the head-group or short chain PtdIns(4)P is in the mM range while the embedded PtdIns(4)P is bound in the μM range.

5.4 Integration of the Role of PtdIns(4)P in Vesicular Trafficking at the Golgi

The pivotal role of PtdIns(4)P appears through numerous examples of proteins targeting the Golgi apparatus. A lipid-based machinery has emerged that involves PtdIns(4)P as a regulator for membrane trafficking or vesicle formation. PtdIns(4)P is known to be involved in the recruitment of protein that participates to TGN-to-PM [3, 66] events and TGN-to-endosome, such as EpsinR and AP-1 [3, 95] and the transport of lipids to the TGN (Cer, GlcCer, and sterol).

5.4.1 PtdIns(4)P in Sphingolipid Metabolism

The production of PtdIns(4)P in the Golgi is mainly controlled by two enzymes (PI4KIIα and PI4KIIIβ) that phosphorylate PtdIns to PtdIns(4)P and is at the heart of a complex system of regulation that still remains poorly defined and incomplete (Fig. 5.6a). Three proteins – CERT, PKD and OSBP – form a network that controls the production of sphingolipids and influences the levels of PtdIns(4)P (Fig. 5.5).

PtdIns(4)P is a hallmark of the Golgi apparatus and is recognized by CERT through its PH domain. CERT shuttles Cer from the ER to the TGN in a PtdIns(4)P-dependent manner (Fig. 5.5). The transported Cer is then metabolized to SM and DAG. DAG, in addition to Arf1 binding, recruits PKD to the TGN membrane by binding to its C1 domain and DAG association controls PKD activity [161, 162]. Furthermore, PKCη, which also recruited to the TGN in a DAG-dependent manner [163], activates PKD by phosphorylating Ser744 and Ser748 within an activation loop that releases the auto-inhibition mediated by the PH domain of PKD [138, 164–166]. PKD has a central role in the regulation of PtdIns(4)P-dependent mechanisms at the TGN. Its kinase activity targets PtdIns(4)P adaptors as well as the production of PtdIns(4)P: the phosphorylation of the lipid carriers OSBP and CERT impairs their TGN localization while PI4KIIIβ is activated by PKD phosphorylation of Ser294. PKD plays two antagonistic roles at the TGN towards CERT and OSBP functions [126, 131, 148]. It promotes PtdIns(4)P synthesis through the activation of PI4KIIIβ and, therefore, contributes to CERT and OSBP recruitment. On the other hand, when PKD phosphorylates these proteins, it negatively regulates their Golgi localization and consequently

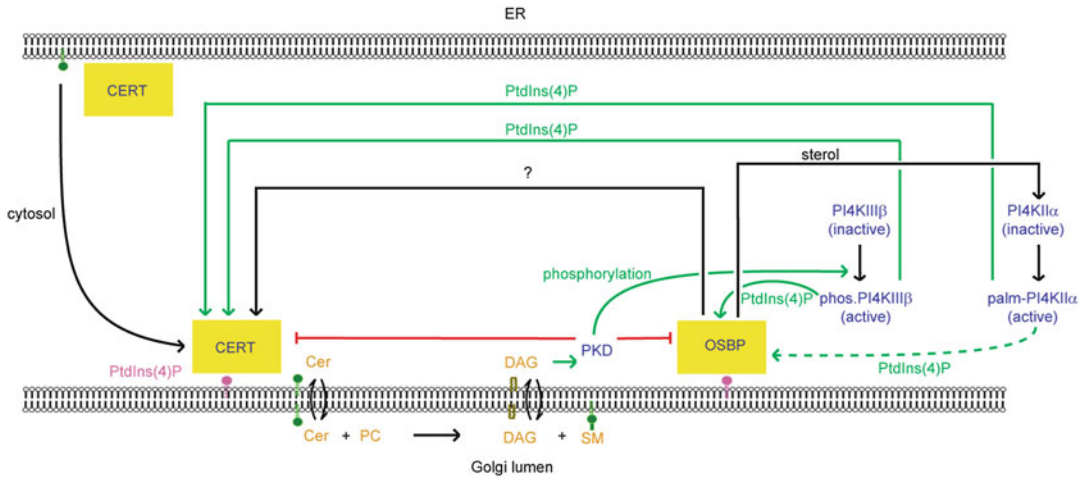

Fig. 5.5 The CERT-PKD-OSBP network at the Golgi apparatus. Proteins are indicated in *blue* and lipids in *orange*. The positive feedback loops are shown in *green* whereas the negative loops are indicated in *red*. Proteins that bind PtdIns(4)P are indicated by *yellow squares*. Ceramides are produced at the ER and transported to the TGN where they are translocated to serve as precursors for sphingomyelins. The DAG produced by the reaction translocates from the luminal to the cytosolic leaflet of the TGN to activate PKD that inhibits CERT and OSBP activities

Cer delivery. The phosphorylation of CERT at Ser132 induces a conformation change that leads to an interaction between the PH and the START domain. It also compromises the recognition of PtdIns(4)P and dissociates CERT from the TGN. The ER resident phosphatase PP2Cε dephosphorylates CERT which activates again the transport of Cer and the association to the TGN. A similar pattern applies for OSBP with the phosphorylation of Ser240 [131]. OSBP localizes at the TGN upon sterol binding and stimulates the CERT functions at the Golgi apparatus. OSBP also stimulates the activity of PI4KIIα in a sterol-dependent mechanism which results in the recruitment of CERT, and probably OSBP, at the TGN. The activation of PI4KIIα is achieved within cholesterol-rich phases by palmitoylation and is stimulated by the activity of OSBP. PtdIns(4)P is also active in the sphingolipid metabolism through FAPP2 that it recruits at the TGN. Although the transport mechanisms are less clear than for CERT studies, it is established that the depletion of PtdIns(4)P prevents FAPP2 recruitment to the Golgi and causes the protein to instead remain largely cytosolic (Fig. 5.6b).

The importance of the co-regulation of the sterol and sphingolipid metabolisms is their propensity to associate in lipid rafts that orient the sorting of proteins at the Golgi apparatus, for example, by directing them to the apical membrane (raft-rich) or the basolateral membrane (non-raft) in polarized MCDK cells. PtdIns(4)P has a determinant role in the recruitment of the proteins that transfer locally both sphingolipids and cholesterol [37, 128, 134, 142]. To date, a role FAPPs in the network described above has not been established but owing the similarities between the scaffolds of the COF proteins, it seems likely that FAPPs activities may be regulated by the activities of OSBP, CERT, and PKD.

5.4.2 Role of PtdIns(4)P in the Formation of Coated Vesicles

PtdIns(4)P is crucial for vesicular biogenesis for sorting cargoes into vesicles and for membrane budding. Adaptor and coat complexes (AP, GGA, EpsinR) facilitate budding of clathrin-coated

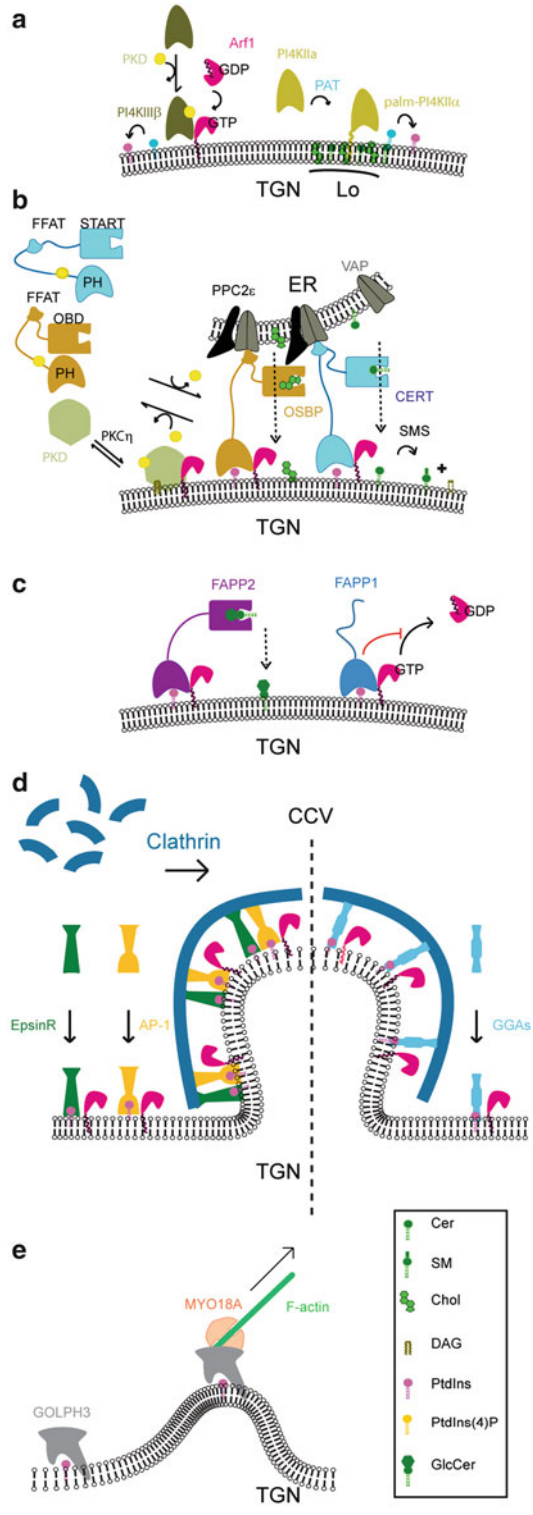

Fig. 5.6 Overview of the interacting proteins at the TGN. (**a**) The major source of PtdIns(4)P in the Golgi apparatus, PI4KIIα is activated after palmitoylation in cholesterol/SM-rich regions (Lo) by palmitoyl acyl-transferase (PAT).

vesicles via direct binding to PtdIns(4)P [3, 62, 69] (Fig. 5.6d). The recruitment of clathrin *via* the adaptor proteins and GGAs relies on the coincident detection of PtdIns(4)P and Arf1 and these functions together in generating adaptor/clathrin-coated vesicles [167–169]. These adaptors constitute a layer between the clathrin and the membrane and tether the two entities together. As for lipid transfer proteins (LTPs), the regulation of the PI4Ks plays an essential role in the recruitment of the adaptors and coat proteins, and can be arranged in Arf1-dependent and -independent routes for PI4KIIIβ and PI4KIIα, respectively. These two routes are complementary since none of the routes are individually sufficient for the recruitment of AP-1. For the Arf1-dependent mechanism, Arf1 activates PI4KIIIβ. AP-1 then binds both Arf1 and PtdIns(4)P of the TGN membrane together with sorting signals on the selected cargo [170]. Proteins are then recruited on the TGN and eventually deform the membrane. For the Arf1-independent signal, the knock down of PI4KIIα prevents the recruitment of AP-1 at Golgi by a decrease of PtdIns(4)P levels in this compartment [3]. One mechanism attributes the PI4KIIα route a lipid docked mechanism that performs the initial recognition. In this case, AP-1 directly interacts with PtdIns(4)P produced by PI4KIIα. On the other hand, the PI4KIIIβ route would promote a protein docked mechanism with the recruitment of multiple low affinity proteins [86], such as EpsinR, that together with AP-1, enhances the affinity of the complex for PtdIns(4)P rich-regions.

5.5 Conclusion – Perspectives

The differential partitioning of PtdIns(4)P across the Golgi stack is used by several peripheral proteins to target the TGN where PtdIns(4)P is most abundant. Although the three-dimensional structures of these proteins are diverse they all recognize PtdIns(4)P through a basic pocket decorated by an exposed patch of basic and aromatic residues. However, a consensus binding site is not evident from the heterogenous folds, although the analysis is further complicated by the low intrinsic PI specificity and the paucity of structural data on many of the lipid complexed states.

The binding to the TGN via the single recognition of PtdIns(4)P is unlikely and the functions of the proteins, the regulation of their activities may be modulated by effectors, such as PKD or Arf1, but also by the lipid composition. The latter remains the most challenging question to address due to the technical difficulties, for example to observe the dynamic lipid assemblies and rafts which are present in cells. However, increasing evidences suggest that the composition of the membrane can be cumulative determinant for the dynamic sorting of proteins between compartments, as illustrated by the activities of PI4KIIα and PI4KIIIβ. Future challenges include understanding how these Golgi-specific proteins can specifically target and manipulate the complex lipid surfaces and curvatures as they find residence and mediate trafficking between the plasma, endosomal, and Golgi membrane compartments.

Fig. 5.6 (continued) PI4KIIIβ is regulated by Arf1 and the phosphorylation (*yellow disk*) by PKD1. The production of PtdIns(4)P influences directly the binding of the COF family proteins. (**b**) The OSBP-CERT-PKD network and transfer of Cer/sterol between the ER and the TGN are depicted. A cycle of phosphorylation/dephosphorylation by PKD1 and PPC2ε, respectively, inactivates and activates CERT and OSBP. Both CERT and OSBP are linked to the ER through the interaction of their FFAT motif with the VAP protein. Activated CERT and OSBP transport Cer and cholesterol to the TGN in an Arf1-dependent manner. Cer is then a substrate for the SMS to produce DAG and SM. DAG promotes TGN binding of PKD1 in an Arf1-dependent manner. (**c**) FAPP2 transports GlcCer at the TGN, while FAPP1 inhibits the nucleotide exchange by ARF-GTPase activating protein (GAP); both TGN recognition events are Arf1-dependent. (**d**) The formation of CCVs for EpsinR, AP-1, and GGAs in two distinct pathways. After recruitment of the protein by PtdIns(4)P and Arf1, the clathrin coat the vesicles deform the TGN membrane. (**e**) The role of GOLPH3 in bridging F-actin and TGN membrane through the interaction with MYO18A and PtdIns(4)P is represented. MYO18A acts as a motor protein to deform the membrane of the Golgi apparatus (**e**)

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PtdIns(4,5)P_2-Mediated Cell Signaling: Emerging Principles and PTEN as a Paradigm for Regulatory Mechanism

6

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Abstract

PtdIns(4,5)P_2 (phosphatidylinositol 4,5-bisphosphate) is a relatively common anionic lipid that regulates cellular functions by multiple mechanisms. Hydrolysis of PtdIns(4,5)P_2 by phospholipase C yields inositol trisphosphate and diacylglycerol. Phosphorylation by phosphoinositide 3-kinase yields PtdIns(3,4,5)P_3, which is a potent signal for survival and proliferation. Also, PtdIns(4,5)P_2 can bind directly to integral and peripheral membrane proteins. As an example of regulation by PtdIns(4,5)P_2, we discuss phosphatase and tensin homologue deleted on chromosome 10 (PTEN) in detail. PTEN is an important tumor suppressor and hydrolyzes PtdIns(3,4,5)P_3. PtdIns(4,5)P_2 enhances PTEN association with the plasma membrane and activates its phosphatase activity. This is a critical regulatory mechanism, but a detailed description of this process from a structural point of view is lacking. The disordered lipid bilayer environment hinders structural determinations of membrane-bound PTEN. A new method to analyze membrane-bound protein measures neutron reflectivity for proteins bound to tethered phospholipid membranes. These methods allow determination of the orientation and shape of membrane-bound proteins. In combination

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with molecular dynamics simulations, these studies will provide crucial structural information that can serve as a foundation for our understanding of PTEN regulation in normal and pathological processes.

Keywords
Phosphoinositide • Phosphatidylinositol 4,5-bisphosphate • Lipid membrane • PTEN • Phosphatase

6.1 Introduction

The functional diversity of cellular membrane compartments in eukaryotic cells requires that distinct membranes have distinct recognizable surface compositions, comprised of lipid headgroups that are presented to targets in the cytoplasm. Phosphoinositides are phosphorylated derivatives of phosphatidylinositol, which vary in the number and arrangement of additional phosphate residues around the six carbon inositol ring headgroup [1]. This group of lipids is a minor constituent of most cell membranes, yet has important signaling roles and is required for many cellular processes, including endocytosis, autophagy, remodeling of the actin cytoskeleton, cell growth, and proliferation [2]. Seven phosphoinositides have been described in many divergent eukaryotes, with all combinations of one, two or three phosphates in the 3, 4 and 5 positions of the inositol ring. These seven species have distinct functions and cellular distributions [1], some of which are reviewed in other chapters of this volume. This chapter addresses the functions of PtdIns(4,5)P_2, which in most cells is the most abundant phosphoinositide with about one mole percent of the plasma membrane (PM) lipid [3]. PtdIns(4,5)P_2 interacts with diverse proteins, in many cases acting to regulate membrane remodeling and trafficking events and in other cases binding or directly regulating specific proteins such as small GTPases and ion channels. This phosphoinositide also acts as a metabolic hub, in that it has multiple routes of synthesis and metabolism and in particular is the substrate for two important signaling enzyme families, Phospholipase C (PLC) and Phosphoinositide 3-Kinase (PI3K).

6.1.1 Routes of PtdIns(4,5)P_2 Synthesis and Cellular Localization

Several enzymes produce PtdIns(4,5)P_2, with the major route of synthesis seemingly mediated by the PIP5K group of phosphoinositide kinases (Fig. 6.1) that phosphorylate PtdIns(4)P [4]. An alternative route of synthesis is from PtdIns(5)P by the PIP4K enzymes. However, cellular levels of PtdIns(5)P are usually far lower than those of PtdIns(4)P, and current data imply that the function of the PIP4K enzymes is more dedicated to the removal of PtdIns(5)P than the generation of PtdIns(4,5)P_2 [5, 6]. PtdIns(4,5)P_2 is also produced by dephosphorylation of PtdIns(3,4,5)P_3 by PTEN. However, since most, if not all, PtdIns(3,4,5)P_3 is produced by class I PI3K from the far larger cellular PtdIns(4,5)P_2 pool and turns over rapidly, it seems unlikely that the action of PTEN generates functionally significant pools of PtdIns(4,5)P_2. The three isoforms of PIP5K that generate PtdIns(4,5)P_2 have overlapping but distinct cellular distributions. All are enriched on the PM, but additionally, PIP5Kα is enriched at sites of membrane remodeling such as lamellipodia and nascent phagosomes, PIP5Kβ, at perinuclear vesicles and at least one spliced form of PIP5Kγ, at focal adhesions and epithelial cell-cell junctions [4, 7–9].

Several experimental approaches show that most cellular PtdIns(4,5)P_2 is located in the PM [10–12], and several functions of PtdIns(4,5)P_2 imply that the lipid acts as a defining marker for the cell plasma membrane environment. In accordance with this concept, evidence shows that PtdIns(4,5)P_2 is rapidly metabolized during

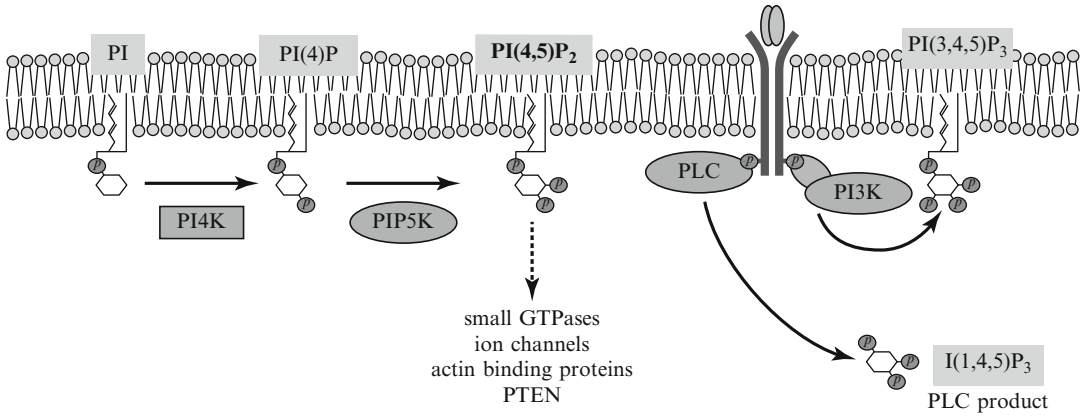

Fig. 6.1 Synthesis and functions of PtdIns(4,5)P_2 (shown as PI(4,5)P_2). The route of synthesis of most cellular PI(4,5)P_2 from phosphatidylinositol (PI) via the sequential phosphorylation of PI and PI(4)P is shown. Two routes of receptor-stimulated metabolism of PI(4,5)P_2 are also shown, being further phosphorylated to PI(3,4,5)P_3 by class I PI 3-kinase and being cleaved to form I(1,4,5)P_3 and diacylglycerol (not shown) by the action of Phospholipase C. For clarity, these reactions are shown within the same membrane compartment, but although all are present on the plasma membrane, PI, PI(4)P and both PI4K and PIP5K activities are abundant on intracellular membranes and the metabolic relationship between these cellular pools is unclear. Some of the recognized functions of PI(4,5)P_2 are indicated by a dashed line, reflecting the reversible binding of the lipid to proteins that include many small GTPases of the Ras superfamily, ion channels, actin binding proteins, and the tumor suppressor phosphatase PTEN

endocytosis as membrane is internalized [13, 14]. There has also been accumulating support for models in which PtdIns(4,5)P_2 (and other lipids) are clustered into small PM microdomains [15–17]. However, the most enigmatic data regarding PtdIns(4,5)P_2 localization imply the existence of a small lipid pool in the nuclear matrix [12, 18–21]. The functions of this and other nuclear phosphoinositide pools and even their physical environment are unclear [18].

6.1.2 Direct PtdIns(4,5)P_2-Mediated Regulation of Protein Function and Membrane Recruitment

Many proteins have been identified that bind to PtdIns(4,5)P_2 with various degrees of selectivity. In some cases, binding is mediated by dedicated lipid-binding domains, such as the Pleckstrin Homology (PH) domain of PLCδ1 or the eponymous Tubby domain from the human Tubby protein [22]. In the case of PLCδ1, it is likely that the PH domain allows competitive binding between PtdIns(4,5)P_2 and the PLCδ1 product, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) [11, 23]. However, in many other cases, selective binding to the relatively abundant and highly charged PtdIns(4,5)P_2 lipid is provided simply by short stretches of amino acids rich in basic residues [24, 25]. This relatively selective interaction of PtdIns(4,5)P_2 with polybasic stretches has been identified in many ion channels, the C-termini of a number of small GTPases, many actin-binding proteins and the PTEN tumor suppressor phosphatase [24, 26, 27]. These PtdIns(4,5)P_2-polybasic interactions depend not only upon electrostatics but also a precise spatial coordination of the lipid phosphate groups [28, 29]. The simplest initial interpretation of protein binding to PtdIns(4,5)P_2 suggests a role in PM recruitment, in particular for the small GTPases, in which a polybasic motif is often combined with a membrane-targeting lipid modification such as prenylation [24]. However, for some proteins, a more complex interaction affects both localization and activity, for example the actin binding protein, ezrin, and PTEN [26, 30, 31].

6.1.3 PtdIns(4,5)P_2 as a Platform for Plasma Membrane Based Lipid Signaling

The PM of eukaryotic cells is the key location that mediates cellular responses to the extracellular environment. PtdIns(4,5)P_2 serves as a substrate for two families of enzymes that are acutely activated in response to extracellular stimuli via cell surface receptors, namely phosphoinositide-specific PLC and phosphoinositide 3-kinase (PI3K).

6.1.3.1 Phosphoinositide-Specific Phospholipase C

PtdIns(4,5)P_2 is the only phosphoinositide that is efficiently cleaved by PLC to form diacylglycerol (DAG) and Ins(1,4,5)P_3, and both cleavage products have significant, independent signaling roles (Fig. 6.1) [32]. The best recognized roles for these PLC products are the ability of Ins(1,4,5)P_3 to promote the release of calcium ions from intracellular stores and the recognition of membrane localized DAG by modular C1 domains. Notably, the presence of both tandem C1 domains and a calcium-responsive C2 domain in some members of the Protein Kinase C family underpins the roles of these enzymes as downstream mediators of signaling initiated by PLC cleavage of PtdIns(4,5)P_2 [33]. Humans have 13 different *PLC* genes, which, through alternative splicing, encode a very large number of enzyme isoforms that share a conserved catalytic region [34]. The structural diversity of these multi-domain proteins allows the activation of PLC by several different classes of cell surface receptors, including many G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs). Indeed, it seems likely that more cell surface receptors couple to PLC than to any other intracellular receptor-activated signal transducer [32, 34]. The biological responses to PLC activation are almost always mediated by large changes (>10-fold) in the concentrations of DAG and Ins(1,4,5)P_3, which are present at much lower concentrations in cells than PtdIns(4,5)P_2. In most cases, PLC activation only modestly reduces cellular PtdIns(4,5)P_2 levels. However, maximal activation of receptors that very strongly activate PLC, such as the PAR-1/Thrombin receptor, can transiently deplete PtdIns(4,5)P_2 levels by as much as 75 %. Even in such cases, PtdIns(4,5)P_2 pools recover within tens of seconds or at most a few minutes [35]. However, there is evidence, albeit controversial, that spatial segregation of PtdIns(4,5)P_2 pools can result in the local depletion of PtdIns(4,5)P_2 by PLC and the selective regulation of targets such as ion channels [17, 36].

6.1.3.2 Class I Phosphoinositide 3-Kinase

The phosphorylation of PtdIns(4,5)P_2 by the class I PI3K generates the signaling lipid, PtdIns(3,4,5)P_3. Similar to PLCs, PI3Ks have low basal activity, but can be acutely activated by several classes of cell surface receptors. PI3K is most strongly activated by RTKs, in particular the insulin receptor and many growth factor receptors as well as some cytokine and chemokine receptors. Humans have four catalytic PI3K isoforms, each of which is approximately 110 kDa in size. They exist in cells as heterodimers, bound to one of several regulatory subunits. These catalytic isoforms differ in the receptor classes by which they are activated, with some activated by tyrosine kinase linked receptors (p110α and p110δ), p110γ activated by GPCRs and the p110β isoform activated by phosphotyrosine-based recruitment or GPCRs [35, 37]. The PI3K signaling system has been intensively studied because it is a strong driver of cell proliferation, growth and survival, along with associated changes in metabolism. The PI3K pathway is also activated in many, perhaps most, cancers [38]. One mechanism of oncogenic activation is via mutations that activate PI3K activity either directly or by the activation of upstream proteins that themselves stimulate PI3K, such as RTKs or Ras. Another, similarly significant mechanism by which PI3K pathway activation occurs in tumors is loss of function of the PtdIns(3,4,5)P_3 phosphatase, PTEN. This tumor suppressor protein will be the focus of the rest of this chapter, as it is an excellent example of PtdIns(4,5)P_2 regulation, *i.e.*, promoting PTEN activity on the plasma membrane by localizing and directly activating the phosphatase.

6.2 PTEN: A Lipid Phosphatase and PtdIns(4,5)P_2-Binding Protein

PTEN is a phosphatidylinositol phosphate (PIP) phosphatase that is specific for the 3-position of the inositol ring [39]. Even though PTEN can dephosphorylate PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 *in vitro* [40], it is likely that PtdIns(3,4,5)P_3 is the principal substrate *in vivo*. PtdIns(3,4,5)P_3 affects many cellular processes by inducing phosphorylation and activation of the Akt kinase [41–43]. In addition, PTEN may dephosphorylate itself [44].

PTEN has two major structural domains, the phosphatase and C2 domains (Fig. 6.2) [45]. The C2 domain binds anionic lipids, such as phosphatidylserine (PtdSer), independent of Ca^{2+}. In addition, there is a PtdIns(4,5)P_2-binding domain at the N-terminus and the phosphatase domain binds the substrate, PtdIns(3,4,5)P_3 [26, 40, 46]. There is a C-terminal tail that when phosphorylated, negatively affects phosphatase activity by binding to the C2 and phosphatase domains [47, 48]. Finally, the C-terminus includes a PDZ ligand sequence that binds to linker proteins with PDZ domains [49].

In functional terms, PTEN is a unique protein with many critical functions [50]. PTEN is the second most commonly mutated protein in human cancers [51]. PTEN mutations are associated with autism and macrocephaly [52–58]. In the plant *Arabidopsis*, PTEN is required for pollen maturation [59]. In the slime mold *Dictyostelium discoideum*, PTEN accumulates at the trailing edge of migrating cells and is required for chemotaxis [60]. Honeybee PTEN plays a role in nutrient sensing and queen-worker differentiation [61]. Finally, a PTEN homolog in *Caenorhabditis elegans* worms regulates aging [62].

Given that PTEN plays a role in so many processes, a natural question is whether the PTEN signaling pathways can compensate when something goes wrong with the phosphatase. Mice with one, but not two, PTEN gene knocked out are viable [63]. However, these mice develop a lymphoid hyperplasia and autoimmune disease and display enhanced tumor formation as they age [64]. In fact, substitution of one PTEN gene with a mutant gene with partial activity is sufficient to enhance tumor formation. This result mirrors Cowden's disease in humans born with one intact PTEN gene develop multiple hamartomas [65].

Can we compensate for a deficit in PTEN activity? Because PTEN mostly exists as a phosphorylated proenzyme-like protein with a closed conformation in the cytoplasm [47, 66],

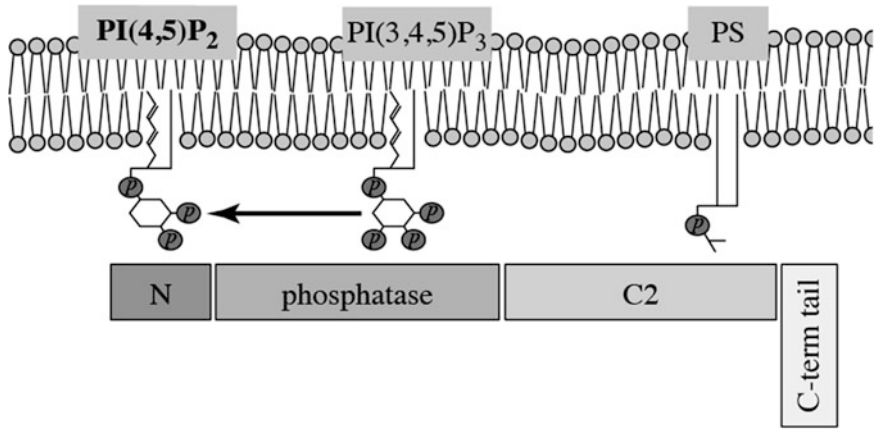

Fig. 6.2 PTEN binds to multiple anionic lipids in biological membranes. The N-terminus domain binds PI(4,5)P_2, whereas the phosphatase domain binds its substrate PI(3,4,5)P_3. The C2 domain binds anionic lipids such as PtdSer (shown as PS). The C-terminal tail is presumed to have regulatory functions

it may be possible to activate this reserve pool of soluble PTEN. We are just beginning to understand the equilibrium between soluble and membrane-bound PTEN [67, 68]. A better understanding of the binding of PTEN to PtdIns(4,5)P_2 in membranes may allow us to activate PTEN and, thereby, reduce the risk of tumor recurrence.

6.2.1 Anionic Lipids and PTEN Phosphatase Activity

Interactions with cell membranes, notably the PM, likely regulate PTEN phosphatase activity by several mechanisms. Binding of PTEN to biological membranes is dominated by anionic lipids, including PtdSer and PtdIns(4,5)P_2 [45, 69, 70]. First, anionic lipids recruit PTEN to the surface of the membrane, where it can laterally diffuse until it encounters the substrate, PtdIns(3,4,5)P_3, and hydrolyzes several molecules before returning to the cytoplasm [67]. PtdIns(3,4,5)P_3 is present in very low quantities in the membrane and, hence, in the absence of PtdSer and PtdIns(4,5)P_2, would be unlikely to mediate the initial membrane binding. Furthermore, anionic lipids influence the dissociation rate from the membrane. Compared to individual anionic lipids, the combination of PtdSer and PtdIns(4,5)P_2 leads to much longer membrane residency [71]. Second, binding of PtdIns(4,5)P_2 to the PTEN N-terminal domain increases phosphatase activity [26, 48, 72]. These studies were carried out with short chain PI phosphates used below their critical micelle concentrations. PtdIns(4,5)P_2, and to a lesser degree PtdIns(5)P, activated the phosphatase activity [26]. Another interfacial enzyme, phospholipase A_2, also undergoes conformational changes upon membrane binding [73–75]. Myotubularin is a PIP phosphatase that dephosphorylates PtdIns(3,5)P_2 [76]. It is intriguing that similar to PTEN, the product, PtdIns(5)P, activates myotubularin. Anionic lipids may also create asymmetric distributions of PTEN. Devreotes and coworkers showed that PTEN is localized in the trailing edge of migrating cells [77]. Also, the distribution of anionic lipids is dynamic and can be perturbed by physiologic processes, such as phagocytosis in macrophages [78].

6.3 PTEN Binding to Anionic Lipids and Its Function in Creating Phosphoinositide Gradients in Cells

PTEN binds to the membrane through at least two major lipid binding sites: PTEN's N-terminal end binds to PtdIns(4,5)P_2, while a stretch of basic amino acids in the CBR3 loop of the C2 domain interacts with PtdSer in a Ca^{2+}-independent manner. Binding of anionic lipids, particularly PtdIns(4,5)P_2, can principally contribute to PTEN membrane association, however, binding of PtdIns(4,5)P_2 to the phosphatase active site must be weak enough so that PtdIns(4,5)P_2 can be easily replaced by PtdIns(3,4,5)P_3 and the produced (or previously bound) PtdIns(4,5)P_2 can be cleared out of the active site.

PTEN's N-terminal end preferentially interacts with PtdIns(4,5)P_2, *i.e.*, binding to other phosphoinositide derivatives, including those that carry a similarly high negative charge like PtdIns(4,5)P_2, (PtdIns(3,5)P_2, PtdIns(3,4)P_2, or PtdIns(3,4,5)P_3) [79], is significantly weaker [29]. This observation suggests that the interaction between PtdIns(4,5)P_2 and PTEN's N-terminal end involves both electrostatics and hydrogen bond formation, apparently, requiring a distinct phosphoinositide headgroup geometry. It has been found that the interaction of PTEN's N-terminus and PtdIns(4,5)P_2 triggers a conformational change towards slightly increased α-helical secondary structure elements. At first glance one might expect that this structural change is localized at PTEN's N-terminal end, however, infrared spectroscopy measurements utilizing a peptide derived from PTEN's N-terminal end that exhibits the same PtdIns(4,5)P_2 binding preference as the full length protein showed that this part of the PTEN protein in all likelihood remains unstructured upon interaction with PtdIns(4,5)P_2 [29].

A truncated $PTEN_{16-403}$ protein did not show activity towards PtdIns(3,4,5)P_3 embedded in a lipid bilayer [26], consistent with *in vivo* experiments that showed reduced or no membrane association [46] and with *in vitro* measurements that showed strongly reduced binding to PtdCho/PtdIns(4,5)P_2 mixed vesicles. $PTEN_{12-16}$ (NKRRY) is highly conserved across different species and among various phosphoinositide phosphatases, such as Ci-VSP, TPTE and TPIP (*Ciona intestinalis* Voltage Sensing Phosphatase, Transmembrane phosphatase with tensin homology, and TPTE and PTEN homologous inositol lipid phosphatase). In addition, the cancer relevant K13E PTEN mutant does not bind to PtdIns(4,5)P_2-containing model membranes [29]. It appears that not only the proper lipid headgroup geometry is an important factor for the interaction between PTEN and phosphoinositides, but that this distinct headgroup geometry requires an equally distinct amino acid sequence (*i.e.,* reversing the sequence of adjacent amino acids affects binding; Redfern, Ross, Gericke, unpublished results). It is likely that R11 and K6 participate in the binding, however, it is unresolved whether these amino acids merely provide a suitable electrostatic environment or participate more directly in the binding event.

The C2 domain has been shown to interact with PtdSer through a lysine-rich string of amino acids (PTEN residues 259–269) in the CBR3 loop. Alanine mutations of lysine residues in this region led to a significantly reduced binding of PTEN to PtdSer-containing model membranes [69]. It is important to note that the interaction of PTEN with PtdSer (C2 domain) and PtdIns(4,5)P_2 (N-terminal end) is synergistic rather than competitive [29, 71].

PTEN association with the membrane is dynamic. Single molecule TIRF microscopy measurements [67] showed the enzyme binds to the membrane for a few hundred milliseconds, which is a time frame that allows the protein to dephosphorylate several PtdIns(3,4,5)P_3 molecules. The mechanisms (or reasons) that cause the protein to dissociate from the membrane are largely unknown at this point.

While mutations in these primary lipid-binding modules lead to a reduced membrane association, other mutations have been reported to increase lipid binding and membrane association. The autism-related H93R PTEN mutation exhibited in *in vitro* experiments enhanced binding to PtdSer containing (but not PtdIns(4,5)P_2) model membranes [71, 80] and *in vivo* experiments revealed a significantly increased PM localization in comparison to wild-type PTEN [80]. Surprisingly, this enhanced membrane association did not translate into increased overall activity, but rather the opposite was observed. Similarly, the PTEN E307K mutant was present in higher concentrations in the membrane fraction, however, also in this case, this enhanced membrane association did not translate into an overall higher activity (the activity was largely unchanged in comparison to wild type PTEN) [72].

PtdIns(3,4,5)P_3 has been associated with cytoskeletal rearrangements and the PI3K/PTEN enzyme system has been found to play an important role in the regulation of phosphoinositide gradients in migrating cells and during cytokinesis [81, 82]. The Devreotes group investigated the role of PtdIns(3,4,5)P_3 gradients in chemotaxis [77, 83, 84]. For migrating *D. discoideum* cells, they found elevated PtdIns(3,4,5)P_3 levels at the leading edge of the migrating cell, while at the trailing end PtdIns(3,4,5)P_3 levels were depleted. Correspondingly, PI3K and PTEN accumulated at the leading edge and trailing end of the cells, respectively. This functional role of PTEN was mirrored in HL60 cells, a cell line that can be differentiated in neutrophil-like cells *in vitro* [85]. However, $pten^{-/-}$ neutrophils did not show elevated PtdIns(3,4,5)P_3 levels. In this case, the 5-phosphatase SHIP1 (SH2 domain containing inositol 5-phosphatase 1) was found to be essential for maintaining the PtdIns(3,4,5)P_3 gradient [86].

Phosphorylation/dephosphorylation at the C-terminal end (S380, T382, T383, and S385) has been identified as an important regulator for PTEN's competency to bind to membranes. Vazquez et al. [47, 66] proposed a phosphorylation-dependent open/closed model for PTEN. In this model, which was recently verified by

Radhar et al. [68], the phosphorylated C-terminal end folds back onto the phosphatase domain, presumably with one of the phosphorylated amino acids acting as a pseudo-substrate. Independent of tissue, it appears that almost all cellular PTEN is phosphorylated on some or all of these residues [87, 88] and Maccario and Leslie (unpublished).

6.3.1 Voltage-Sensitive Phosphatases

Voltage-sensitive phosphatases (VSP) and PTEN may be regulated by related mechanisms [89]. The VSP from the sea squirt *C. intestinalis* (Ci-VSP) (mentioned above) was the first member of this family to be characterized. This protein has a voltage-sensing domain and PTEN-like phosphatase domain with 5'-phosphatase voltage-dependent activity [90]. Remarkably, a hybrid protein with the Ci-VSP voltage-sensing domain and human PTEN-like phosphatase domain show voltage-dependent phosphatase activity [91]. Furthermore, the linker region appears to play a critical role in this regulation [92, 93], as it may bind PtdIns(4,5)P_2, followed by a voltage-dependent conformational change in the Ci-VSP phosphatase domain [94, 95]. A detailed comparison of the regulation of PTEN and Ci-VSP may allow us to better understand both enzymes.

6.4 Structure of Membrane-Bound PTEN

Attempts to enhance PTEN activity are hindered by the paucity of data on the structure of membrane-bound PTEN. The structure of membrane-bound peripheral proteins are difficult to obtain on physiologically relevant, thermally disordered membranes, because x-ray crystallography is not compatible with the disordered state of the membrane. NMR techniques only provide a window into local contacts, making it difficult to investigate large protein-membrane complexes. However, neutron reflectometry (NR) [96] of PTEN bound to substrate-supported bilayers recently provided a first glimpse into the interaction of this phosphatase with bilayer surfaces and has led to a low-resolution structure [71]. This structure has subsequently been refined and characterized in more detail with all-atom MD simulations [97].

An interesting new twist on the membrane binding of PTEN comes from a recent report by Huang and coworkers [98] who presented evidence that SUMOylation of the protein is essential to recruit the phosphatase to the membrane. There are two SUMOylation sites, K254 and K266, but the K266 site is more influential. They carry out MD simulations of a PTEN-SUMO hybrid in solution from which they suggest that SUMO acts as an electrostatic adhesion promoter. Despite an impressive amount of data, there remain several important questions. First, bacterial recombinant PTEN lacking SUMOylation binds to membranes and is active [29]. Moreover, two K266 mutants showed normal activity in a U87MG cell model [99]. Second, even though they conclude that the SUMOylation is essential for tumor suppressor activity, there are no cancer-associated mutations of SUMOylation site K266 in the Sanger Center Cosmic Database (http://www.sanger.ac.uk/genetics/CGP/cosmic/).Third, we have carried out extensive MD studies of PTEN and anionic membranes [97] and find PTEN-membrane interactions consistent with our binding studies and NR analysis, *i.e.*, these simulations suggest PTEN readily associates with the membrane surface without a SUMO modification. Resolution of this controversy requires additional biophysical characterization of PTEN-membrane interactions.

6.4.1 Experimental Approaches

Recent developments in membrane-mimetic model systems [100, 101] facilitate structural investigations of membrane-associated proteins in sample environments that capture the relevant features of biological membranes in highly simplified, well-controlled formats. The original designs of substrate-supported, planar bilayer membranes [102, 103] have been considerably refined in the past decade [101, 104–107], as it

was realized that the chemical 'tethering' of membranes to an underlying substrate increases the robustness of the resulting model systems [108]. Systems have been reported in which bilayers are decoupled from solid supports by ultrathin, hydrated polymer cushions [109–112] or short linear tethers of hydrophilic oligomers [113–115]. Both tethering approaches lead to the stabilization of well-defined hydrated cushion layers between membrane and carrier substrate [114], important to maintain in-plane fluidity of the bilayers, to protect the protein from denaturation or aggregation following unspecific adsorption to the otherwise bare substrate surface and to permit the incorporation of transmembrane proteins [116–118]. A system developed in the Lösche group [113], the sparsely-tethered bilayer lipid membrane (stBLM), is widely applicable for the biophysical characterization of lipid-protein interactions and is particularly useful for high-resolution neutron scattering investigations [116–119]. Thiolated tether lipids [114, 115], laterally diluted by coadsorption with β-mercaptoethanol to atomically flat gold films supported by Si or glass substrates, are used to anchor bimolecular phospholipid films. The lipid bilayers in stBLMs are essentially defect-free and hence highly insulating [114, 120], as shown with electrochemical impedance spectroscopy [121]. Any bilayer platform that is used to study lipid-protein interactions by NR must be stable for the duration of the experiment and at the same, must mirror the in-plane fluidity of biological membranes. To combine both of these aspects in one model system has been a challenge for many years and arguably, stBLMs meet this crucial requirement like no other lipid model system:

1. Phospholipid bilayers in stBLMs are thermally disordered, exhibit in-plane fluidity and hence, resemble biological membranes in these aspects. Adsorbed peripheral proteins or incorporated membrane-spanning proteins [116] therefore reside in a near-natural environment. In fact, fluidity of the stBLMs leads to biological activities of associated proteins similar to those observed in biological membranes, as shown by measuring enzymatic turnover as a function of chain composition [122].
2. The resilience of stBLMs permits *in situ* manipulation of the sample, such as the exchange of the fluid buffer bathing the bilayer for isotopic contrast variation [114–116]. This capability permits multiple subsequent NR measurements on the same physical sample. stBLMs, therefore, can be measured as neat bilayers, prior to protein incubation, with the protein adsorbed, and after a final rinse, with all measurements performed at a standardized series of $^1H_2O/^2D_2O$ buffer compositions. Thereby, even though the number of adjustable parameters in the NR experiments is large, this model system is still overdetermined because of multiple NR spectra independently measured with varying conditions. For example, it has been shown that the position of a protein reconstituted in the bilayer membrane can be determined with ≈1 Å precision if the internal structure of the protein is known [116].
3. The same lipid model system can be used for a variety of characterization techniques, such as surface plasmon resonance (SPR) [123] and electrochemical impedance spectroscopy [121] if the gold film that covers the carrier substrate is sufficiently thick (typically 100 nm). Fluorescence microscopy and fluorescence correlation spectroscopy, as well as NR, are compatible with thin gold films (typically 10 nm).

Neutron (or x-ray) scattering – in distinction to diffraction, which requires ordered molecular arrays as in a protein crystal – has the capability to characterize intrinsically disordered samples, including disordered membranes. NR and x-ray reflectometry are surface-sensitive variants of the generic scattering process that take advantage of the refraction and reflection of a beam directed toward an interface. These techniques are therefore particularly sensitive to the molecular structure near surfaces and interfaces. Their physical principles have been exhaustively covered. Significantly, because of the lack of phase information, the molecular structures that give rise to the scattering are most conveniently described in models whose parameters are adjusted to fit the experimentally observed results [96, 119, 124, 125].

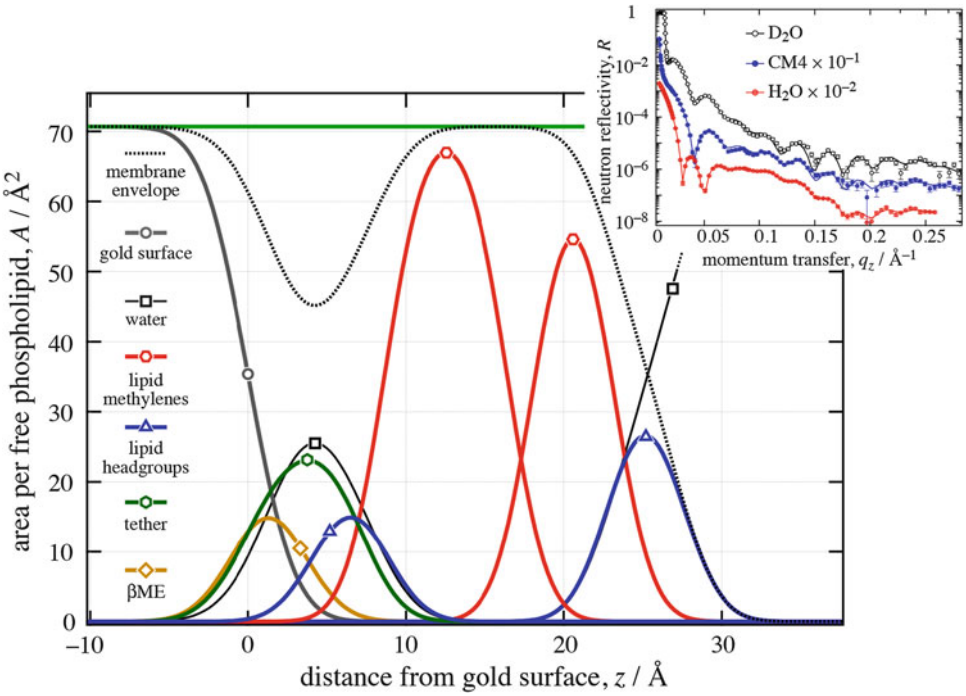

Fig. 6.3 Exemplary NR data set (proportion of reflected neutrons *vs.* momentum transfer, $\vec{q}_z = \vec{k}_{out} - \vec{k}_{in}$) and its interpretation in terms of the thermally broadened distribution of molecular fragments across the interface between a gold film on Si and aqueous buffer. Shown is an stBLM with its bilayer composed of (chain-deuterated) DMPC (dimyristoylphosphatidylcholine) [124]. The NR of the stBLM (inset) has been measured in D_2O, H_2O, and a mixture of the two with a neutron SLD (nSLD) of 4×10^{-6} Å^{-2}, termed 'CM4'. A model was then refined by simultaneously fitting three data sets. The resulting decomposed nSLD profile is shown in the main figure. For clarity, distributions of the lipid methyl groups in the bilayer center and the glycerol backbone of the tether lipid, which were also quantified in the model, have been omitted in the plot. By accounting for the chemical connectivities and volumes of the molecular subfragments shown in the distributions, the parameter space could be greatly constrained, so that the decomposed nSLD profiles can be determined with high confidence

In *specular* reflection, where incoming and outgoing wave vectors, $\vec{k}_{in}$ and $\vec{k}_{out}$, have the same overall length and in-plane component, the generic output of such models are scattering length density (SLD) profiles, *i.e.*, projections on the surface normal z of the three-dimensional scattering length distributions.

For samples such as the stBLMs used to investigate protein interactions with phospholipid bilayers, the interfacial structure of the membrane system and its carrier is already fairly complex in the absence on any protein. A typical sample consists of a Si wafer that bears a sputtered chromium bonding layer and a terminal Au film. Chemisorbed to the gold surface is a mixed monolayer of β-mercaptoethanol and thiolated oligo(ethyleneoxide) lipid. The lipid's hydrophobic chains intercalate the phospholipid leaflet proximal to the gold surface, thus tethering the membrane to the substrate, and the bilayer is terminated with a distal phospholipid monolayer (Fig. 6.3).

Molecular dynamics (MD) simulations of wild-type PTEN were performed with the protein deeply embedded in buffer (≈74,000 TIP3P water molecules) and on a large patch of PC/PtdSer bilayer membrane (≈104,000 TIP3P water and 648 lipid molecules). The CHARMM22/CMAP and CHARMM36 parameterizations were used for the protein and lipid, respectively. Protein conformations started in the crystal structure of the truncated protein [45] with the clipped protein regions added back in energy-minimized

conformations. The long (52 residues) C-terminal stretch was initially artificially extended into the buffer from the protein body, and the compact protein domains were located >10 Å away from the membrane surface in roughly the expected orientation for membrane binding. Simulated annealing procedures were applied to equilibrate these initial structures followed by production runs in excess of 300 ns duration [97].

6.4.2 PTEN Affinity for Anionic Membranes

Used as biomimetic surfaces in SPR to quantify protein affinity to membranes, stBLMs have low unspecific protein adsorption because stBLMs exhibit low defect densities [71]. In a recent study of PTEN interactions with anionic stBLMs, we reported that the wild-type protein binds to PtdSer-containing membranes with a dissociation constant, $K_d = 11.9 \pm 0.4$ μM, with binding curves evaluated as Langmuir isotherms. For membranes that contained PtdIns(4,5)P_2 but no PtdSer, $K_d = 0.4 \pm 0.1$ μM. stBLMs that contain both PtdSer and PtdIns(4,5)P_2 show pronounced synergy in protein binding and increased protein affinity by another order of magnitude. In three-component systems (PtdCho:PtdSer:PtdIns(4,5)P_2 with the PtdIns(4,5)P_2 as a minority component in the 1–3 mol% range), binding curves were reported to be bimodal with one $K_d = 40 \pm 10$ nM and another $K_d > 5$ μM, with the latter value similar to that observed for stBLMs with PtdSer as the sole anionic component. In all cases, protein loading depends on the amount of anionic lipid in the membrane, whereas K_d does not. Specifically, bilayers that included 30 mol% DOPS in DOPC, with or without PtdIns(4,5)P_2, were observed to bind wild-type PTEN in amounts ($B_{max} > 150$ ng/cm^2) consistent with the formation of a fairly densely packed monolayer of the protein that may be suitable for investigations with NR. Protein binding depended pronouncedly on bilayer fluidity. While the K_d value reported above ($K_d = 0.4 \pm 0.1$ μM) refers to stBLMs composed of lipids with unsaturated acyl chains (dioleoyl for PtdCho and PtdSer and purified natural PtdIns(4,5)P_2), the affinity was at least a factor of 5 lower ($K_d \approx 2$ μM) to stBLMs composed of DPPC and dipalmitoyl-PtdIns(4,5)P_2.

6.4.3 PTEN Density Distribution on stBLMs by Neutron Reflectometry

NR results for PTEN bound to bilayers composed of PtdCho and PtdSer, as well as PtdCho and PtdIns(4,5)P_2, were recently reported [97] for the wild-type protein and the H93R mutant, which is associated with autism. Subsequently, all-atom MD simulations of membrane-bound wild-type PTEN were reported and complemented with simulations of the protein in buffer [71]. Because the simulations have so far not included PtdIns(4,5)P_2, we concentrate in this review on the structure of wild-type PTEN on PtdCho/PtdSer-containing bilayers and show how the combination of NR with MD results yields a reference structure for this important phosphatase on a fluid, thermally disordered membrane.

NR spectra were measured first for the pristine stBLM and then for the same lipid bilayer after protein binding. Since all data were collected on one physical sample, the spectra could be modeled with one consistent framework in which many parameters, for example those that describe the multilayered carrier structure, were shared between spectra. Because the structure of the membrane-bound PTEN protein is *a priori* unknown, its contribution to the overall neutron SLD (nSLD) profile was fitted with a set of spline functions. While these protein contributions were allowed to penetrate the bilayer structure, it was observed that they are only peripherally associated with the membrane surface in models that are consistent with the experimental data (Fig. 6.4). The nSLD profile can be further interpreted to yield a tentative structure of the protein–membrane complex. The crystal structure of the truncated PTEN protein (from which residues 1–6, 286–309, and 354–403 are missing and a few more, residues 7–13, 282–285,

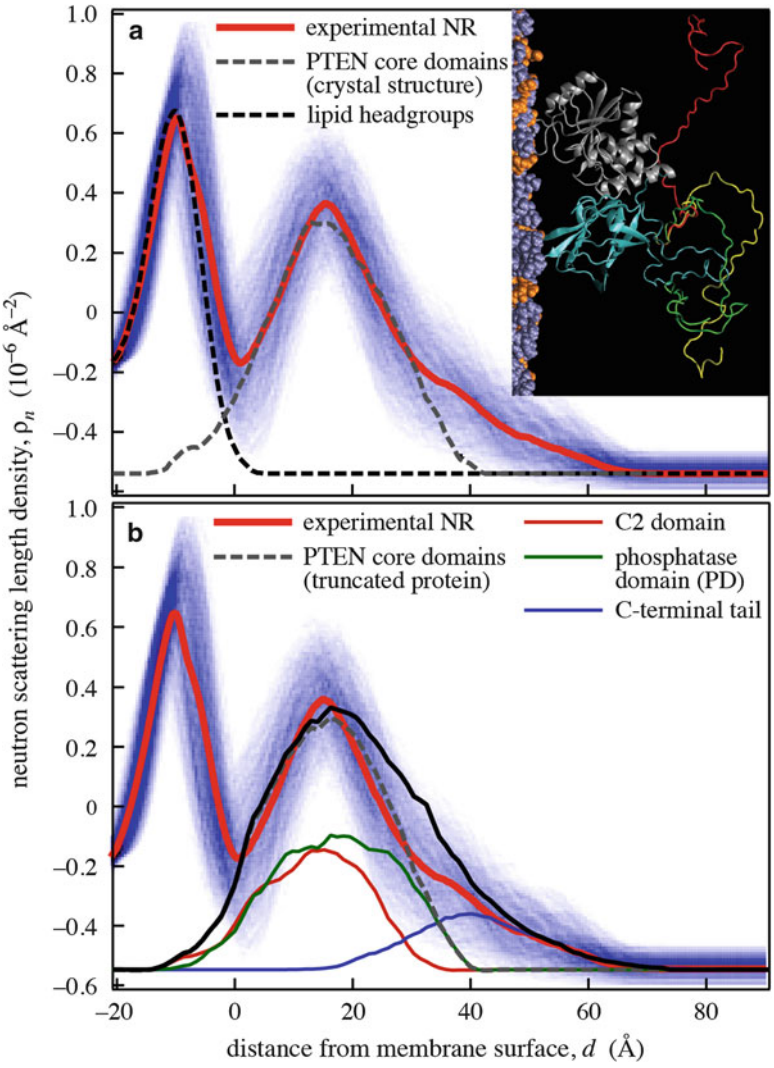

Fig. 6.4 Neutron scattering length density distribution of a PtdCho/PtdSer stBLM with bound wild-type PTEN from experimental results (*red*) and its interpretation (**a**) in terms of placing the crystal structure of a truncated PTEN variant [71] and (**b**) in terms of all-atom MD simulations [97]. The *shaded blue band* indicates the confidence intervals around the best-fit model (*red line*) determined by Monte-Carlo data resampling [114]. The inset in *panel A* shows a PTEN model based on the crystal structure placed on the bilayer with a few alternate conformations of the C-terminal tail (*colored red, yellow, green, and blue*) that are consistent with the experimental nSLD profile

and 352–353, are not resolved) could be placed on the membrane model in the orientation proposed by Lee and coworkers [45] and its projection scaled and overlaid on the experimental nSLD profile (dashed line in Fig. 6.4). This procedure provides an astounding agreement between the two projections in the region close to the bilayer surface (z=0–35 Å in Fig. 6.4, where the z axis originates at the membrane surface), while the region further away (z=35–60 Å) shows significantly higher nSLD, attributed to the PTEN protein, in the experimental profile. It was suggested [71] that the discrepancy between these densities is due to the truncations of the PTEN protein used for the crystal structure – most notably the C-terminal tail that constitutes the major

part of the clipped protein sections, ≈20 % of the total weight, and is predominantly anionic with ten excess negative charges. It is then tempting to speculate that electrostatic repulsion keeps the unstructured, presumably flexible anionic C-terminal tail away from the acidic membrane surface. An inset in Fig. 6.4 shows a schematic visualization of the structure of wild-type PTEN on the bilayer that is consistent with the data and displays the C-terminal tail in the hypothetical conformations generated with a Monte-Carlo procedure [125]. In conclusion, the NR data on wild-type PTEN on a PtdCho/PtdSer bilayer in connection with the crystal structure [45] of the truncated protein suggest that (1) PTEN scoots on the membrane, (2) the crystal structure is indeed a good starting point for an atomic-scale interpretation and (3) that the C-terminal tail is tugged away from the bilayer surface in the membrane-bound protein [71].

6.4.4 MD Simulations and Their Correspondence with NR Results

Subsequent to the NR investigations reviewed above, all-atom MD simulations of wild-type PTEN were performed on PtdCho/PtdSer bilayers and in solution [97]. Simulations of the membrane-bound PTEN (PtdCho:PtdSer = 2:1, as compared to a 7:3 mixture in the NR experiments) were initiated with the protein >10 Å distant from the bilayer surface, and it settled quickly into a more stable position with the protein close to the lipid/buffer interface. At the same time the C-terminal tail, which pointed away from the protein core and the membrane initially, contracted and settled in a compact, yet highly dynamic conformation located near the face of the protein body that is distal to the membrane surface. The organization of the two core domains, C2 and phosphatase domain, and their mutual arrangement was conserved in the MD protein structure on the membrane but deviated slightly from the crystal structure. Overall, these deviations were too small to be resolved in NR measurements. Figure 6.4 compares the nSLD profiles derived from the averaged projections of the full-length protein on the local z axis as seen in the MD simulations with those derived from NR. Various partial protein components, *i.e.* the C-terminal tail, the phosphatase domain, and C2, as well as the sum of the two core domains as a visualization of a dynamic projection of the protein's truncated crystal structure are also shown in the panel. It is evident that the overall protein nSLDs derived from the simulations and from NR experiments corresponded to each other extremely well. The only adjustable quantity in this comparison was the amount of protein per unit area, which was different in both situations. On the one hand, this suggested that the parameterizations of both the protein and the lipid have matured to a performance where they can reproduce experimental situations without any "steering", even for highly charged bilayers. On the other hand, the observed correspondence corroborated the tentative interpretations of the NR results discussed in the previous section. In other words, the experimental and simulation results cross-validate each other and provide confidence in a reference structure of a partially disordered phosphatase protein associated with a thermally disordered lipid membrane at Ångstrom resolution.

Indeed, the MD simulations provided atomic-scale insight into structure and interactions of the protein with the membrane, and complementing simulations of full-length PTEN in buffer revealed important structural differences of the protein in these distinct environments [97].

1. While the C2 domain led the docking of the core protein to the charged membrane by penetrating the lipid headgroups locally with its CBR3 loop and anchoring the protein firmly, there were also transient electrostatic interactions observed between the phosphatase domain and charged lipid headgroups. Electrostatic interactions provided the driving force for protein anchoring between a patch of cationic residues on CBR3 and PtdSer, but hydrophobic contacts between M264 and K265 and PtdSer glycerol backbones reinforced these interactions. As a result, specific PtdSer–C2 contacts prevailed for the entire duration of the MD trajectory (>300 ns). In

distinction, electrostatic contacts between PtdSer and the phosphatase domain were more dynamic, lasting only tens of nanoseconds in the simulation.

2. Lipid diffusion was severely restricted by PTEN binding. While the diffusion coefficient derived from the simulation was $D = 7.5\ \mu m^2/s$ for phospholipids in the absence of protein, it was reduced to 3 and 1.5 $\mu m^2/s$ for DOPC and DOPS, respectively, for lipids in the footprint of the protein.
3. The simulation also suggested that both the solution and the membrane-bound structures of the PTEN core differ slightly from the published crystal structure. While the solution structure was on average slightly more closed, the protein flattened upon binding to the membrane surface, thus locating the substrate-binding pocket closer to the lipid headgroups. An analysis of the flexibility of the protein core suggested that the phosphatase domain's pα1 helix is the hot spot of displacements with average deviations of the Cα positions of >3 Å from those in the crystal structure [97]. Generally, the observed deviations from the crystal structure are too small to be resolved in NR measurements.
4. The most significant difference between the solution and membrane-bound structures of wild-type PTEN concerned the C-terminal tail and provided a functional scenario that might implement a control mechanism of the membrane binding of the phosphatase, and hence of its activity [97]. While the tail was displaced from the membrane in the MD simulations, presumably by repulsive electrostatic interactions between the anionic membrane surface and the anionic excess charges on the peptide, it wrapped tightly around the C2 domain in solution, leading to a more compact, globular structure of the protein, as illustrated in representative simulation snapshots shown in Fig. 6.5. The tight "hugging" of the C2 domains leaves the dangling tip of the C-terminus in a position where it obstructs the membrane-binding interface of the core domains. While the conformation of the flexible tail is apparently reversible upon membrane binding of the protein (Fig. 6.5a), it might be locked into place through phosphorylation of the tail. The right hand side of Fig. 6.5b shows various known Thr and Ser phosphorylation sites. In particular S380 and S385 are located on a prominent kink in the tail as it wraps around the C2 domain, suggesting that it could be very well amenable to phosphorylation.

6.4.5 Reference Structure for a Membrane-Bound Phosphatase

Progress in the structural characterization of membrane-bound proteins on thermally disordered, fluid lipid bilayers has been made by combining neutron scattering experiments on dedicated sample formats with all-atom MD simulations. The studies reviewed here provide a reference structure for a phosphoinositide-specific phosphatase and provide a first atomic-scale glimpse into the mechanics of the membrane binding of the PTEN tumor suppressor. Moreover they suggest a mechanism that could provide a control of the membrane association, and therefore the activity, of this important regulatory enzyme in the PI3K/Akt signaling pathway.

6.5 Summary and Future

PtdIns(4,5)P_2 is a major component for signaling by several mechanisms. It is the source of PtdIns(3,4,5)P_3 and Ins(1,4,5)P_3. Furthermore, PtdIns(4,5)P_2 is localized in the PM where a number of integral and peripheral membrane proteins can bind it. We have chosen PTEN as an example for this mechanism of regulation. The lack of structural data has been a major impediment in understanding how PtdIns(4,5)P_2 activates PTEN. Although just a first step, we summarized how NR can provide structures for membrane-bound proteins in their natural, disordered membrane environment. In combination with NMR and x-ray crystallography, this technique perhaps offers

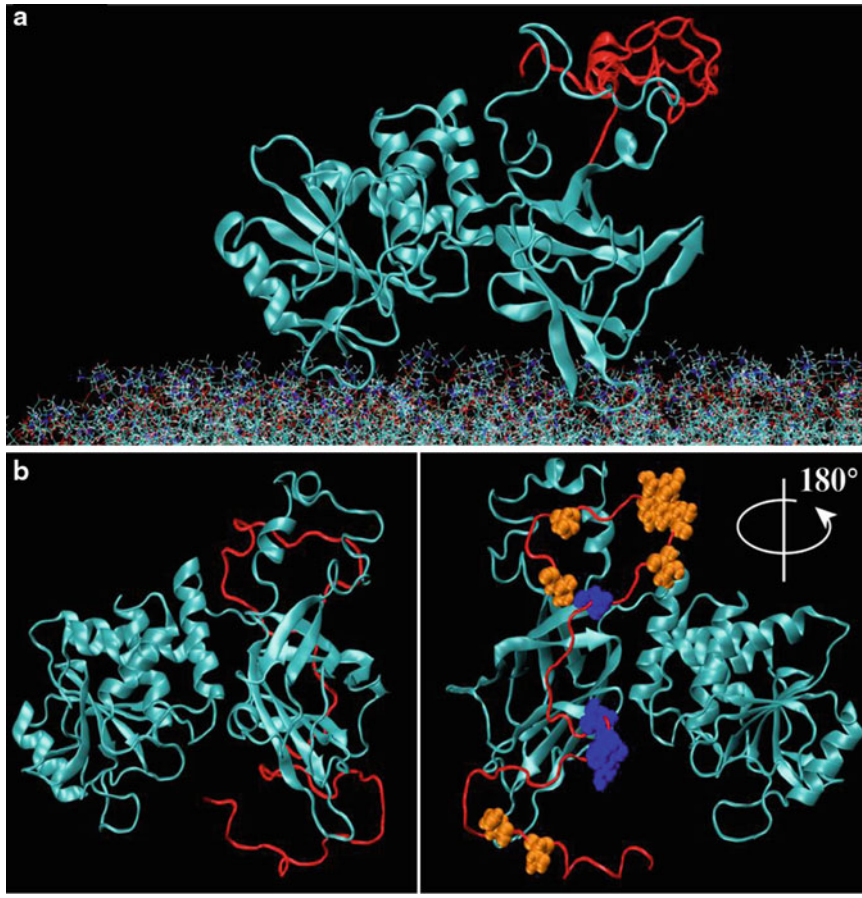

Fig. 6.5 All-atom MD simulation snapshots of wild-type PTEN on a PtdCho/PtdSer (2:1) membrane (**a**) and in solution (**b**). For clarity, water molecules and dissolved ions (100 mM NaCl) are not shown. The general organization of the two core domains, the C2 and the phosphatase domain, is very similar, but in the membrane-bound structure, the protein is slightly more flattened against the membrane surface. Both core protein structures differ slightly from the crystal structure [45]. The flexible C-terminal tail (*red*), which is absent in the crystal structure, shows grossly different organization on the membrane-bound and the dissolved protein. On the membrane-bound protein, the net negatively charged tail is electrostatically repelled from the anionic bilayer surface (**a**). In distinction, it wraps around the C2 domain in solution and obstructs the membrane-binding interface partially (*left in panel B*). In the rotated view (*right in panel B*), Ser and Thr residues are shown in *blue and maroon*, respectively. Known protein phosphorylation sites such as S370, S380 and S385 are located in exposed positions on the tail and well amenable to phosphorylation. In particular S380 and S385 define a prominent kink in the wrapped tail, which suggests that phosphorylation could lock this conformation in place

great promises to understand how PtdIns(4,5)P_2 controls PTEN activity and, consequently, many downstream physiological processes.

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Role of Phosphatidylinositol 3,4,5-Trisphosphate in Cell Signaling

7

Robert D. Riehle, Sinziana Cornea, and Alexei Degterev

Abstract

Many lipids present in cellular membranes are phosphorylated as part of signaling cascades and participate in the recruitment, localization, and activation of downstream protein effectors. Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) is one of the most important second messengers and is capable of interacting with a variety of proteins through specific PtdIns(3,4,5)P_3 binding domains. Localization and activation of these effector proteins controls a myriad of cellular functions including cell survival, proliferation, cytoskeletal rearrangement, and gene expression. Aberrations in the production and metabolism of PtdIns(3,4,5)P_3 have been implicated in many human diseases including cancer, diabetes, inflammation, and heart disease. This chapter provides an overview of the role of PtdIns(3,4,5)P_3 in cellular regulation and the implications of PtdIns(3,4,5)P_3 dysregulation in human diseases. Additionally, recent attempts at targeting PtdIns(3,4,5)P_3 signaling via small molecule inhibitors are summarized.

Keywords

Phosphatidylinositol (3,4,5)-trisphosphate • PI3-kinase • Akt • PTEN • Tumorigenesis

7.1 Introduction

Phosphoinositides (PIs), phosphorylated derivatives of phosphatidylinositol (PtdIns), have been investigated as agonist-dependent second messengers since their discovery in the mid 1950s by Hokin and Hokin [1]. Initial focus was made on PtdIns(4)P and PtdIns(4,5)P_2 and the enzymes responsible for their synthesis and degradation. The discovery of a family of PI3-kinases and its lipid products PtdIns(3)P, PtdIns(3,4)P_2 and most

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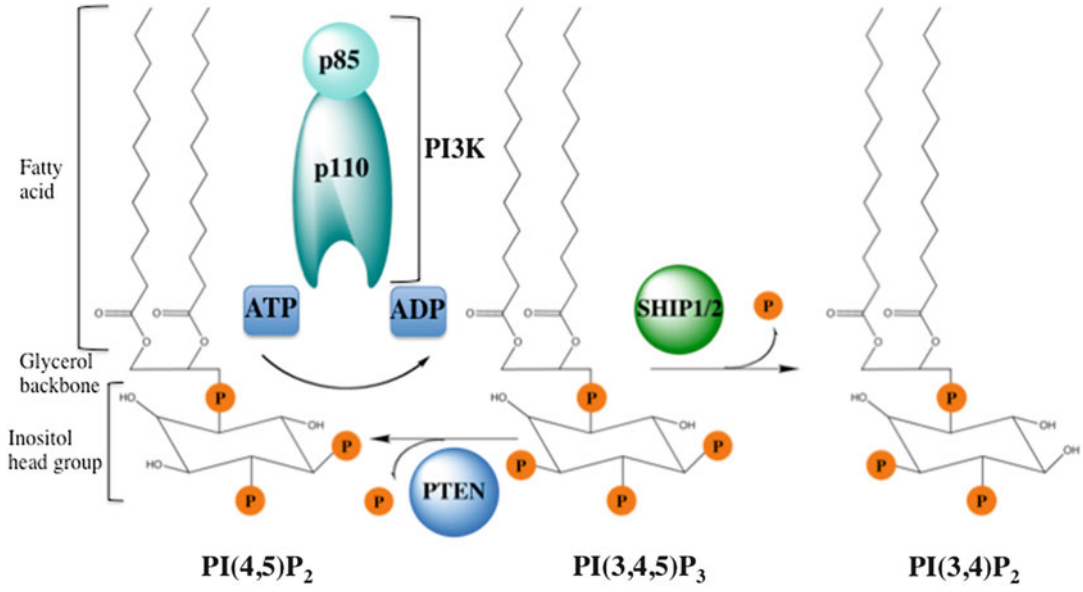

Fig. 7.1 Phosphatidylinositols are composed of long chain fatty acid groups attached to a phosphorylated inositol head group via a glycerol moiety. Fatty acids anchor the molecule in the membrane, while the inositol is exposed to the cytoplasm. Various kinases catalyze the addition or removal of phosphates to the head group at specific locations. Above, PI3K phosphorylates PtdIns(4,5)P_2 (shown as PI(4,5)P_2) at the 3′ position to form the second messenger PtdIns(3,4,5)P_3 (shown as PI(3,4,5)P_3). The phosphatases PTEN and SHIP breakdown PtdIns(3,4,5)P_3 by removing phosphates from the 3′ and 5′ positions respectively

importantly phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) uncovered additional complexity to PI-mediated signal transduction [2–4]. Further work lead to the elucidation of the mechanisms of PI3-kinase signaling and the critical importance of this class of lipid kinases in a variety of cellular functions including directional motility, metabolism, growth, and cell survival. Additionally, many diseases can be traced back to aberrations in the PI3-kinase pathways. For this reason, efforts have been made to understand the complex regulation of PIs and PtdIns(3,4,5)P_3, in particular. PI3Ks are subdivided into three classes: I, II and III, differing in their structure, products, and regulation. PtdIns(3,4,5)P_3 is the product of Class I of PI3-kinases, which will be referred to as PI3K throughout the text.

PtdIns(3,4,5)P_3 is a member of the PI class of membrane lipids. Typically, PtdIns found in cell membranes account for approximately 5–10 % of the total lipid with only approximately 1 % being phosphorylated, indicating that their primary function is regulatory in nature [5]. PtdIns can be phosphorylated at either the 3′, 4′ or 5′ hydroxyl sites (Fig. 7.1). These hydroxyl groups are phosphorylated alone or in combination to create seven possible PIs with individual stereospecificity and charge. PIs are localized on the endosomal, Golgi, nuclear, and plasma membranes, depending on their structure. Of the 7 PIs only PtdIns(4)P and PtdIns(4,5)P_2 are found in relatively high abundance [6]. Unique inositol head groups coupled with extremely low PI concentrations in the cell offer deliberate, specific, sensitive, and localized signaling.

Various kinases and phosphatases regulate the formation and turnover of PIs depending on cellular stimuli [7]. Of the PtdIns present in the cell only 0.25 % are phosphorylated at the 3′ position indicating a highly specific regulatory function [5]. The PI3-kinase family of proteins is responsible for the formation of these PIs and four separate lipid products can be formed: PtdIns-(3)P, PtdIns(3,4)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 is predominately synthesized by the phosphorylation of PtdIns(4,5)P_2 by class

I PI3K in response to extracellular stimuli [8]. The precursor of PtdIns(3,4,5)P_3, PtdIns(4,5)P_2, has been shown to have some affinity for various effector proteins and to control the activity of several integral membrane proteins. However, it primarily serves as a pool for formation of second messengers: inositol trisphosphate (Ins(1,4,5)P_3), diacylglycerol (DAG), and PtdIns(3,4,5)P_3 [9–11]. Though only present at less than 1 % of all plasma membrane phospholipids, PtdIns(4,5)P_2 is found at high localized concentrations due to its accumulation into sphingolipid/cholesterol-based rafts [12].

PtdIns(3,4,5)P_3 functions both as a protein activator as, for example, in the case with Akt (also known as Protein Kinase B) and as a membrane anchor, recruiting proteins to the plasma membrane where they localize and perform their actions.

7.2 PtdIns(3,4,5)P_3 Signaling Pathway

PtdIns(3,4,5)P_3 lipid second messengers serve to amplify and propagate signals from receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs), and the small GTPase Ras, as well as to confer signaling specificity by its temporal and spatial distribution in cellular membranes [13]. Tight regulation of PtdIns(3,4,5)P_3 levels in the plasma membrane is critical for the prevention of hyper- or hypo-responsiveness to many extracellular stimuli. Since the original discovery of a novel PI3K activity associated with cellular transformation by polyoma middle T antigen in 1985 [2], major insights have been gained into the mechanism of PtdIns(3,4,5)P_3 signaling, including initiation and termination of the signal, its effector preferences and its role in cellular regulation.

7.2.1 Signal Initiation and Termination

PtdIns(3,4,5)P_3 signaling is initiated by extracellular stimulation of a variety of plasma membrane receptors, including RTKs and GPCRs, leading to the activation of Type I PI3K (Fig. 7.2). Cellular homeostasis is dependent in part on tight regulation of PtdIns(3,4,5)P_3 levels in the plasma membrane and is controlled by growth hormones, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin, and insulin-like growth factor I (IGF-I). PI3Ks specifically phosphorylate the D-3 inositol ring position of PIs in the plasma membrane, generating PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3. Synthesis of PtdIns(3,4,5)P_3 is the primary cellular function of Type I PI3K, while Type II and III PI3Ks are the key regulators of the cellular PtdIns(3)P pool. Dephosphorylation of PtdIns(3,4,5)P_3 by SHIP phosphatases is an important mechanism of PtdIns(3,4)P_2 generation [14].

Type I PI3Ks are heterodimers consisting of the p110 catalytic subunit and the p85 regulatory subunit. The two SH2 domains in the p85 subunit recruit the p110 subunit to the cytoplasmic domains of activated receptors at the plasma membrane through a variety of adaptor molecules. These SH2 domains are connected by a coiled coil domain, which constitutively interacts with and stabilizes the p110 subunit. There exist three isoforms of Type I_A PI3K (p110α, β and δ) and one Type I_B PI3K isoform (p110γ), which is controlled by an alternative regulatory subunit (p101) and was previously believed to be activated exclusively by GPCRs, but more recently was found to respond to RTKs as well [15]. Type I_A PI3Ks are mainly activated by growth factor receptors and Ras proteins. p110α and β isoforms are expressed ubiquitously, while p110γ and p110δ are expressed primarily in leukocytes [16].

Under basal conditions, the levels of PtdIns(3,4,5)P_3 in the plasma membrane are extremely low and almost undetectable, constituting about 0.0001 % of the plasma membrane's lipid content. Phosphorylation of PtdIns(4,5)P_2 by PI3K results in a rapid, approximately 40-fold increase of PtdIns(3,4,5)P_3, to about 10 % of the basal level of PtdIns(4,5)P_2, which is relatively abundant in the plasma membrane with an approximately 500-fold greater concentration than basal PtdIns(3,4,5)P_3 levels [17]. The low level of PtdIns(3,4,5)P_3 reflects its potent effect on key cellular regulatory pathways, requiring a tight

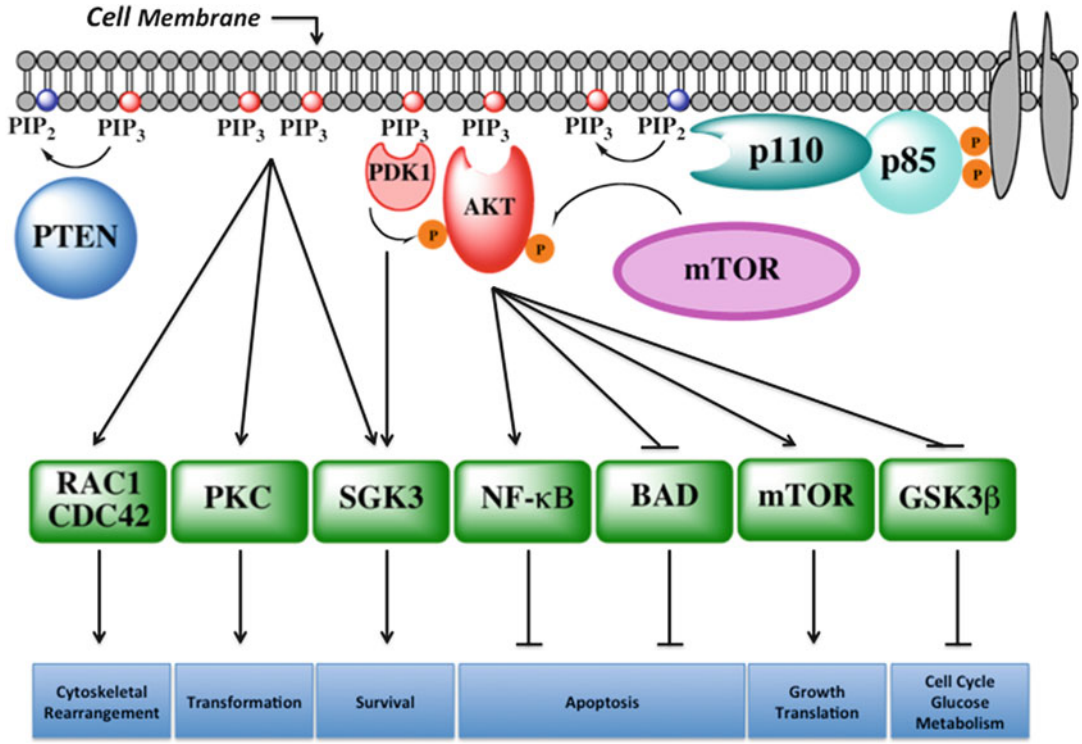

Fig. 7.2 The general model of PtdIns(3,4,5)P_3 (shown as PIP_3) activation and downstream signaling. Upon growth hormone stimulation of receptors in the plasma membrane, PI3K phosphorylates PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3, leading to the recruitment of PH-domain containing effector proteins, such as Akt/PKB and PDK1. Activation of these effector proteins leads to signaling cascades controlling a multitude of cellular pathways, including cell survival, metabolism, and cell migration. PTEN and other phosphatases quench PtdIns(3,4,5)P_3 signaling

control to prevent hyperactivation by extra- and intracellular cues.

Dephosphorylation of PtdIns(3,4,5)P_3 is required to prevent its accumulation and constitutive signaling. This is under the control of the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and SH2-containing inositol 5-phosphatases (SHIPs), which produce PtdIns(4,5)P_2 and PtdIns(3,4)P_2, respectively [17]. The structural basis of PtdIns(4,5)P_2 recognition and membrane binding of PTEN is discussed in detail in Chap. 6. The main function of PTEN is as a lipid phosphatase, dephosphorylating the D-3 position of PtdIns, and negatively regulating the PtdIns(3,4,5)P_3 pathway [18]. It is well established to serve as one of the most important tumor suppressors, which is inactivated as frequently as p53 in some forms of cancer. The phosphatase consists of a central phosphatase domain, two C-terminal PEST sequences that serve to stabilize the protein, and an N-terminal tensin- and auxilin-homology region. The gene coding for PTEN is located on human chromosome 10q23, and is often found affected in a multitude of disorders [19]. While SHIP1 is present primarily in hematopoietic cells, SHIP2 is ubiquitously expressed and utilizes PtdIns(3,4,5)P_3 as a main substrate [20]. There is some evidence that PTEN plays a more important role in controlling basal levels of PtdIns(3,4,5)P_3, while SHIPs counteract stimulus-induced increases [21]. It is also worth noting that another phosphatase, Inositol Polyphosphate 4-phosphatase type II, has emerged more recently as an important tumor suppressor [22]. This enzyme dephosphorylates PtdIns(3,4)P_2, which can bind both Akt and PDK1. This lipid is either synthesized *de novo*

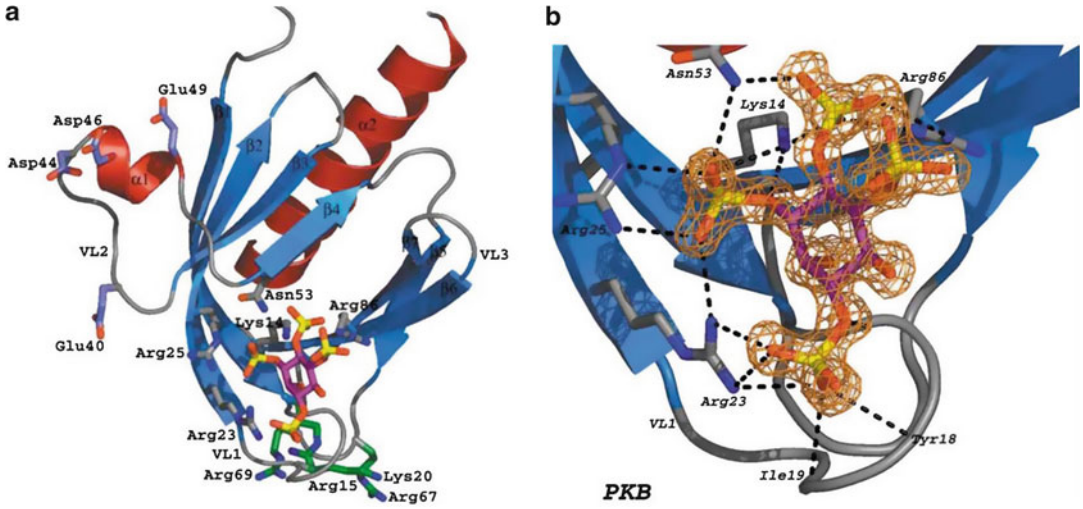

Fig. 7.3 Ribbon drawings of PtdIns(3,4,5)P_3 bound to the PH domain of Akt. (**a**) Seven-stranded beta-barrel structure formed from the β2-β3, β4-β5, and β6-β7 loops (*blue*), capped at one end by an amphipathic α-helix (*red*), and bound to the inositol (1,3,4,5)-phosphate head group of PtdIns(3,4,5)P_3 (*purple*). The basic residues proposed to bind PtdIns(3,4,5)P_3 are drawn in *green*. (**b**) Close up of the PtdIns(3,4,5)P_3 head group interactions, with *dotted lines* indicating hydrogen bonds (Reproduced with permission from Ref. [1])

from PtdIns(3)P and PtdIns(4)P, or is a result of SHIP dephosphorylation of PtdIns(3,4,5)P_3. Therefore, concerted actions of PI3Ks and several phosphatases control signaling by multiple PI species in a highly dynamic fashion in response to growth factor signals.

7.2.2 PH Domains: Primary Sequence vs. Protein Folding and Recognition Preferences

PtdIns(3,4,5)P_3 serves to anchor its effector proteins to the membrane surface, as well as to activate, stabilize, and co-localize proteins. The best-known mechanism of action involves direct binding of the PtdIns(3,4,5)P_3 inositol head-group to the pleckstrin homology (PH) domain of proteins resulting in their recruitment to the plasma membrane [17]. The effector network of PtdIns(3,4,5)P_3 includes only about 40 of the approximately 250 known PH domain-containing proteins, and the members bind PtdIns(3,4,5)P_3 with varying degrees of specificity and affinity. The most commonly studied of these include Akt/Protein kinase B, phosphoinositide-dependent kinase-1 (PDK1), guanine nucleotide-exchange factors (GEFs) GRP-1 and ARNO, and Bruton's tyrosine kinase (Btk), all of which are considered relatively specific for PtdIns(3,4,5)P_3.

The PH domain consists of approximately 120 amino acid residues arranged into a bowl-like binding pocket that closely fits the inositol head group of PtdIns(3,4,5)P_3. While the tertiary structure of this pocket remains highly conserved between different classes of PtdIns(3,4,5)P_3 effector proteins, the primary structure is known to vary significantly. It is believed that this variation contributes to the specificity and affinity for PtdIns(3,4,5)P_3 versus other membrane PIs [23]. The binding pocket is a seven-stranded anti-parallel beta-barrel formed by the β2-β3, β4-β5, and β6-β7 loops and capped at one end by an amphipathic α-helix [24] (Fig. 7.3). Basic residues creating a highly positive electrostatic environment capable of attracting negatively charged PtdIns(3,4,5)P_3 line the interior of the PH domain. The pattern of phosphate groups on the inositol head group is also very important in binding [17].

7.2.3 Mechanisms of Effector Activation – Localization and Conformational Change

PtdIns(3,4,5)P_3 in cellular membranes serves to recruit effector proteins from the cytoplasm and anchor them by binding to their PH domains. The action of binding to PtdIns(3,4,5)P_3 often induces a conformational change, allowing protein activation and propagation of downstream signaling. The induced conformational change, along with membrane localization, both contribute to activation of PtdIns(3,4,5)P_3 effectors.

Activation of Akt by PtdIns(3,4,5)P_3 has been studied extensively and is best understood. Binding of PtdIns(3,4,5)P_3 to Akt produces a conformational change within the protein that allows for its phosphorylation, and full activation, by PDK1 and mammalian target of rapamycin complex 2 (mTORC2) at residues Thr308 and Ser473, respectively. PDK1 is also bound and recruited to the membrane to allow full Akt activation following growth factor signal. Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance in mice [25], by preventing its co-localization with Akt and subsequent activation downstream pathways. Membrane localization is not the only mechanism of Akt activation. Rather, PtdIns(3,4,5)P_3 binding also promotes dissociation of the autoinhibitory binding of PH domain to the catalytic domain, thus exposing the catalytic loop residue Thr308 to PDK1 phosphorylation [26, 27].

Regulation of other PH domain-containing proteins is less well characterized. However, complex regulation involving changes in protein conformation and localization, acting in concert, is emerging as a common theme. In the case of PDK1 kinase, initial work has suggested that PtdIns(3,4,5)P_3 binding to its PH domain does not affect catalytic activity of the kinase [28]. Hence, inability of the PH domain mutant of PDK1 to promote activation of Akt in response to growth factor signaling primarily reflected defects in membrane localization. The normal cytosolic phosphorylation of S6K by PDK1 in PDK1 PH domain mutants indicates that they retained its kinase activity [29]. However, further detailed analysis suggested a complex role of the PH domain in the regulation of PDK1 activity in the cells. The PH domain has been proposed to both promote Ser241 autophosphorylation, which is required for activity, and to autoinhibit the catalytic activity of phosphorylated kinase under basal conditions, creating a pool of primed kinase [30]. Binding of PtdIns(3,4,5)P_3 releases PH domain-dependent inhibition, allowing PDK1 to affect its substrates.

In case of another PH-domain containing factor, Grp1, elements proximal to PH domain have been shown to potently autoinhibit the Sec7 exchange domain of this Arf6 GEF. Membrane localization through PtdIns(3,4,5)P_3 binding in concert with complex formation with membrane-targeted Arf6-GTP was shown to result in full catalytic activation of Grp1 towards Arf6-GDP molecules [31].

7.3 Cellular Processes Controlled by PtdIns(3,4,5)P_3

PtdIns(3,4,5)P_3 is directly involved in a variety of processes within mammalian cells, including cell cycle progression, regulation of cell death, cytoskeleton rearrangement, chemotaxis, and metabolic control. The role of PtdIns(3,4,5)P_3 in both plasma and nuclear membranes has been identified. As new cutting edge cell imaging techniques have become widely available, increasingly intricate roles and mechanisms of action for PtdIns(3,4,5)P_3 are being elucidated. It is clear that tight regulation of PtdIns(3,4,5)P_3 is fundamental to cell function, and deregulation along any of its effector pathways leads to pathogenesis, including cancer, autoimmune disease, cardiovascular problems, and diabetes.

7.3.1 Cellular Growth, Proliferation, and Apoptosis

Regulation of cell growth is one of the key functions of PtdIns(3,4,5)P_3 pathway. The growth of cells requires coordinated regulation of proliferation,

cell death, and metabolism, all processes involving signaling from PI3K.

7.3.1.1 Progression Through the Cell Cycle

During interphase of the cell cycle, cells increase in size, synthesize lipids and proteins, and replicate DNA, before the cell can enter the division phase, also known as mitosis (or meiosis in the case of reproductive cells). PtdIns(3,4,5)P_3 is involved in the regulation of the critical factors (cyclins and cyclin-dependent kinase (CDK) inhibitors) involved in the regulation of the progression through interphase. The necessary conditions for a cell to transition from the G_1 checkpoint to the S phase of interphase are dependent in part on the activity of Forkhead-box Class O (FoxO) transcription factors in the nucleus, which are responsible for the repression of cyclins D1 and D2 as well as the transcription of CDK inhibitors, including $p21^{Cip1}$ and $p27^{Kip1}$ [32]. Cyclins make up of a family of proteins that oscillate throughout the cell cycle, controlling its progression via interactions with CDKs. Cyclin D is G_1/S-specific molecule and is a regulatory subunit of CDK4 and CDK6 [33]. FoxOs also promote the synthesis of cyclin G2, an atypical cyclin highly expressed in quiescent cells.

The binding of growth factors activates the PI3K/PtdIns(3,4,5)P_3/Akt pathway, followed by a translocation of active Akt to the nucleus, where it phosphorylates FoxO and promotes its exclusion from the nuclei [34]. Phosphorylation of different FoxOs by Akt occurs at the conserved T1, S1, and S2 residues and promoting the coordinated action of nuclear export machinery (Crm1, Ran GTPase) and 14-3-3 regulatory proteins resulting in the export FoxO into the cytosol and preventing the phosphorylated protein from re-entering the nucleus. Absence of FoxO proteins from the nuclei releases inhibition of cyclin D synthesis, while also decreasing levels of CDK inhibitors (p21, p27, etc.), thus, triggering a G_1-S phase transition [35].

Another mechanism involved in the promotion of proliferation by Akt is its direct phosphorylation of $p21^{Cip1}$ at Thr145 [36]. Interestingly, Akt signaling has been recently shown to also increase expression of cell cycle inhibitor $p57^{Kip2}$, limiting proliferative responses of breast epithelial cells to insulin and IGF-1, thus, highlighting a system of checks and balances established by Akt in controlling proliferative responses by growth factors [37].

7.3.1.2 Cell Growth

The majority of cell types spend most of their cell cycle growing in size and preparing to proliferate. Nutrients and growth factors in the extracellular matrix initiate the PI3K/PtdIns(3,4,5)P_3 pathway, leading to increased biosynthesis of lipids and proteins, which are required for rapid cell growth.

PtdIns(3,4,5)P_3-activated Akt phosphorylates and inhibits the action of tuberin, which binds to hamartin, generating a protein complex known as the tuberous sclerosis complex (TSC). The TSC complex suppresses activity of Complex 1 of mTOR (mTORC1) [38]. Two of the best studied downstream effectors of mTORC1 are p70S6Ks, AGC kinases required for cell growth and progression through G_1, and 4EBP1, which are inactivated in the absence of mTORC1 activity through the binding to eukaryotic initiation factor 3 (eIF3) [39]. Activated mTORC1 binds to eIF3 and phosphorylates p70S6K and 4EBP1, relieving their inhibition. p70S6K, in turn, phosphorylates multiple effectors required for cell growth and protein biosynthesis including ribosomal S6, promoting translation of a subset of mRNAs containing an oligopyrimidine tract at the 5′ end [40]. Another important target of mTORC1 is 4EBP1, which dissociates from its target eIF4E upon phosphorylation, leading to an increase in cap-dependent translation.

Though regulation of translation is an important function of mTORC1 on cell growth, it is not the only contribution. It has also been linked to the control of the availability of endogenously produced amino acids needed for biosynthesis, mitochondrial biogenesis, and *de novo* lipogenesis [41, 42].

Cellular metabolism is also altered in the transition of cells from quiescence to rapid proliferation. Activation of PI3K/PtdIns(3,4,5)P_3/Akt

pathway plays a major role in these changes as well, activating glucose uptake, promoting aerobic glycolysis, and suppressing fatty acid oxidation, thus preserving a source of lipids for rapid membrane expansion [41, 42].

7.3.1.3 Cell Survival and Apoptosis

PI3K, acting through a PtdIns(3,4,5)P_3-dependent activation of Akt kinases, provides one of the most important growth and survival signaling circuits in a wide range of cell types. Overactivation of PI3K/PtdIns(3,4,5)P_3/Akt signaling plays a central role in the enhanced proliferation and reduced cell death of tumor cells (see below). PI3K signaling also plays a key role in mediating growth factor-dependent "tonic" survival signals in many lineages of normal cells. For example, important early insights into the growth and survival signaling by PI3K and Akt were obtained in the models of interleukin-dependent survival of hematopoietic cells [43–46].

Akt has many well-characterized connections to the basic apoptotic machinery. Early findings suggested that Akt phosphorylates the pro-apoptotic BH3-only Bcl-2 family member Bad, leading to its retention in the cytosol through the binding to 14-3-3 and blocking translocation to mitochondria. Thus, interaction with other Bcl-2 family members is prevented resulting in the inhibition of the mitochondrial step in apoptosis activation [44, 47]. Notably, Akt phosphorylation of Ser-136 residues of Bad, acts in concert with other pro-survival kinases, such as A-kinase anchor proteins, p90 ribosomal S6 kinase and Protein kinase A, to control pro-apoptotic activity of Bad [47–52]. Another critical role of Akt in apoptosis is mediated by the phosphorylation of FoxO transcription factors, including ubiquitously expressed FoxO1, FoxO3, and FoxO4 [53–55]. FoxOs promote transcription and synthesis of a variety of proteins involved in apoptosis, including TNFα family members TRAIL and Fas-ligand, and pro-apoptotic BH3-only family members BIM and PUMA [32].

Glycogen synthase kinase 3 (GSK3) is another class of direct Akt target, playing important and complex roles in the regulation of cell death as well as many other cellular functions. Energy metabolism, transcription, microtubule dynamics and Wnt and Hedgehog pathways are all influenced by GSK3 activity [56]. Akt phosphorylates GSK3α/β on Ser-21/9 residues respectively, targeting them for ubiquitination and degradation. Akt has also been shown to directly promote nuclear factor (NF)-κB activation in response to growth factors and TNFα activation through association with IκB kinase (IKK) complex and subsequent phosphorylation of the Thr23 residue of IKKα [57, 58].

Akt is not the only PtdIns(3,4,5)P_3 target involved in the control of cell growth and survival. Bruton's tyrosine kinase (Btk), expressed in B lymphocytes, is recruited into activated B-cell receptor "signalosome" in a PtdIns(3,4,5)P_3-dependent manner where it undergoes activating phosphorylation by the Src family Lyn and Syk kinases [59]. Btk, in turn, activates phospholipase C γ (PLCγ), which regulates Ca^{2+} mobilization and activation of mitogen-activated protein kinase (MAPK) and NF-κB pathways [60]. Activation of NF-κB in a protein kinase C β (PKCβ)-dependent manner is one of the more important mechanisms of cell survival regulation by Btk [61]. Activity of Btk is required for cell survival during development and provides "tonic" pro-survival signaling in mature resting B cells [62, 63]. Btk has been also reported to promote survival macrophages, but only following stimulation with lipopolysaccharide (LPS) or interferon-γ (IFNγ)[64].

PDK1 is another PtdIns(3,4,5)P_3 binding kinase critically involved in the regulation of cell survival [65]. PDK1 plays a key role in the survival of cancer cells with perturbed PI3K signaling, which is mediated by Serum/glucocorticoid regulated kinase 3 (SGK3), rather than Akt activation [66]. In general, PDK1 regulates a large number of downstream kinases linked to the regulation of growth and survival, including Akt, several PKC isoforms, p70S6Ks and SGKs. However, only Akt and SGK3 have been linked to date to PDK1 signaling in plasma membrane, indicating the involvement of PtdIns(3,4,5)P_3.

7.3.2 Cytoskeleton Rearrangement and Chemotaxis

Human cell migration is an important process beginning with conception and continuing throughout adulthood. Cell migration is a critical process in a variety of biological responses including cell renewal and tissue repair, immune and inflammatory responses, and angiogenesis. Aberrant changes in cell migration directly contribute to a variety of pathologies. Most importantly, tumor vascularization, aberrant immune responses, osteoporosis, chronic inflammatory diseases, and loss of correct neuronal network formation are all characterized by dysregulation of cellular migration. In most cells, cell migration is highly dependent on the actin cytoskeleton [67]. PtdIns(3,4,5)P_3 along with other regulators such as cAMP and cGMP, is the key signaling molecule contributing to the regulation of polymerization and rearrangement of the actin cytoskeleton that occurs during chemotaxis, with a marked enrichment of PtdIns(3,4,5)P_3 at the leading edge of migrating cells [68].

The appropriate direction of migration is established by receptor molecules at the cell surface, which sense the locations and intensities of extracellular signals, subsequent activation of the receptors and signal propagation leads to a rearrangement of the actin cytoskeleton. This rearrangement is regulated by Rho GTPases, a subfamily of small signaling G-proteins in the Ras superfamily, including Rho, Rac, and Cdc42 [69]. Activation of PI3K by extracellular cues initiates binding of PtdIns(3,4,5)P_3 to GEFs, which, in turn, activate Rho GTPases. Formation of protrusions at the front of the migrating cell, specifically lamellipodia and filopodia, are stimulated by Rac and Cdc42 respectively, while Rho is responsible for the cells posterior retraction [67]. Activation of Cdc42 and Rac has been shown to decrease the activity of Rho at the leading edge, as well as enhance PI3K activity. Cdc42 is also implicated in microtubule growth and recruitment of vesicles to the leading edge, a process dependent on the localization of the microtubule-organizing center and the Golgi apparatus between the nucleus and the front of the cell [70].

The final downstream effectors of Rho GTPase signaling are the Wiscott-Aldrich syndrome protein (WASP) and the WASP verprolin homologous proteins (WAVE), which form links between the GTPases and the actin cytoskeleton. PtdIns(4,5)P_2-activated WASP binds via its newly exposed VCA (verprolin homology, cofilin homology and acidic) region to the actin-related protein (Arp)2/3 complex, leading to rapid actin polymerization [67]. Of the three WAVE isoforms present in human tissues, WAVE2 is ubiquitously expressed, localizes to the plasma membrane, possibly by specific binding to PtdIns(3,4,5)P_3 to its basic region (rather than through a canonical PH domain), and has been shown to be critical for lamellipodia formation at the leading edge of the cell [69]. More recent evidence indicated that PtdIns(3,4,5)P_3 acts in concert with the microtubule binding complex of EB1 and stathmin in promoting localization of WAVE2 to the leading edge of the cell [71]. Furthermore, another PtdIns(3,4,5)P_3 target IRSp53, rather than WAVE2 itself, may directly interact with PtdIns(3,4,5)P_3 and is responsible for membrane localization of WAVE2, its constitutive binding partner [71].

Regulation of actin polymerization is not the only contribution of PtdIns(3,4,5)P_3 to directional migration. The actin motor myosin has also been found to interact with PtdIns(3,4,5)P_3 and contributes to lamellipodia formation. Non-muscle myosin IIA [72], several Class I myosins in *Dictyostelium* and Class IF myosin in mammalian cells are all able to bind PtdIns(3,4,5)P_3 through their tail homology domain 1 [73].

An impressive aspect of cell migration is the ability of cells to respond to very shallow chemical gradients, at times experiencing less than 10 % variation in concentration between the leading and trailing edge of the cell. It is thought that this is possible due to positive feedback loops that amplify the signal at the leading edge of the cell, as well as sequestration of PTEN to the sides and rear of the cell, resulting in an anterior focusing the Rho GTPase signaling [70].

It is also worth noting that Akt, PDK1, and PTEN are all also implicated in the regulation of cell migration and motility. For example, Akt1

has been shown to inhibit cell migration through phosphorylation of actin-binding factor, palladin, as well as through the regulation of nuclear factors of activated T-cells, Erk, and TSC2. Alternatively, Akt2-specific regulation of β1 integrin can promote cell migration [74]. *Pdk1*$^{-/-}$ cells display defects in cell migration [75]. The mechanisms of this regulation have been proposed to involve direct binding of PDK1 to ROCK1 and PKN kinases, the latter being a direct substrate of PDK1 [76, 77]. However, the role of PtdIns(3,4,5)P_3 in the regulation of this function of PDK1 has not been investigated. Curiously, while PTEN certainly contributes to the regulation of migration through the control of PtdIns(3,4,5)P_3, its lipid-independent protein phosphatase activity towards Focal adhesion kinase (FAK) and SHC-transforming protein 1 (Shc) has also been proposed to play an important role in the regulation of cell migration and metastasis [78]. These data establish very profound, but complex roles of PtdIns(3,4,5)P_3 and its effectors in the regulation of cell migration, motility, chemotaxis, and metastasis.

7.3.3 Nuclear Function

The discovery of the existence of PtdIns(3,4,5)P_3 in cellular membranes other than the plasma membrane has led to an investigation of the role of this lipid in other cellular compartments, especially in the nucleus. Components of the PI3K signaling cascade, including PI3K itself, Akt, and PDK1, have been detected in nuclear membranes. In one reported mechanism, nerve growth factor (NGF) stimulation was found to activate PI3K through the activity of nuclear GTPase, PIKE, acting as an equivalent to the cytoplasmic PI3K activator, Ras. PtdIns(3,4,5)P_3 in the nuclear membrane plays a critical role in several processes, including cell survival, cell cycle regulation, and DNA repair [79]. Similar to the plasma membrane pool, dephosphorylation of PtdIns(3,4,5)P_3 is coordinated by PTEN and SHIP phosphatases, although the nuclear pool of PtdIns(3,4,5)P_3 may be less sensitive to PTEN compared to that observed at the plasma membrane [80]. It is interesting to note that while PTEN is found localized to the nucleus in many primary cells, its nuclear pool is dramatically reduced in a number of tumor cells, including exocrine pancreatic tumors and melanomas [81].

It has been established that following growth factor stimulation at the plasma membrane, activated Akt detaches from the plasma membrane and translocates to the nucleus. Nuclear Akt appears to retain activity independently of PtdIns(3,4,5)P_3, and was shown to promote cell survival, in part by direct interaction with the nuclear PtdIns(3,4,5)P_3 target, nucleophosmin (NPM)/B23. NGF-stimulation promotes PtdIns(3,4,5)P_3 binding to B23, recruiting the protein from the nucleoli to the nucleosomes, where it can interact with and stabilize nuclear Akt [82]. Nuclear PtdIns(3,4,5)P_3, Akt, and B23 have been shown to protect PC12 cells from apoptosis via the formation of a complex capable of inhibiting DNA fragmentation activity of caspase-activating DNAse (CAD). It is also clear that the spatial and temporal dynamics of PtdIns(3,4,5)P_3 following NGF stimulation are critical to the interaction between B23 and Akt, and deregulation of these dynamics leads to impaired biological function [83].

Class I PI3Kβ is also involved in the DNA damage response (DDR) that eukaryotic cells utilize to repair double-stranded breaks (DSB), which are among the most disastrous lesions that affect the human genome. Experiments carried out on irradiated NIH 3 T3 cells revealed that PI3Kβ and PtdIns(3,4,5)P_3 localize to damaged DNA sites, and are necessary for the early detection of DSB. PI3Kβ functions to promote recruitment of the Mre11-Rad50-NbsI (MRN) complex, of which Nbs1 is considered the earliest sensor of DNA damage, and is critical for the proper association of the complex. It was also shown that PI3Kβ is necessary for the activation of G2/M cell cycle arrest, during which DNA damage is assessed and repaired, or else apoptosis is induced. Loss of PI3Kβ function results in an impairment of MRN recruitment and inefficient G2/M arrest, resulting in an accumulation of DSB in irradiated cells. While the exact role of local PtdIns(3,4,5)P_3 accumulation to DSB sites is not

yet completely understood, it is thought that besides its normal role in protein recruitment, it may serve to stabilize DNA in an open conformation in order to facilitate the DDR, either by repelling the negatively charged DNA strands, or by recruiting positively charged histones to chromatin [84].

Finally, a role of the nuclear PtdIns(3,4,5)P_3 pool in the differentiation of myeloid cells through a mechanism involving association of the p85 subunit of PI3K with tyrosine phosphorylated Vav1 GEF has also been proposed [85]. Notably, Vav1 is a PH domain-containing protein, which can be activated by PtdIns(3,4,5)P_3 *in vitro* [86, 87]. Novel nuclear PtdIns(3,4,5)P_3 targets continue to emerge, making it clear that it has important functions in the control of nuclear processes.

7.3.4 Neuronal Development and Function

The typical characteristic morphology of a neuronal cell consists of one long axonal process, through which the signal is transmitted, and several shorter tapered dendrites, through which neighboring cells activate the neuron. Once a neuronal progenitor cell attaches to the substratum, a five-stage process of neuronal differentiation is initiated [88]. The first stage involves the formation of several lamellipodia along the surface of the cell, which in stage 2 develops into immature neurites of approximately equal length. During the third stage, the neuron becomes polarized with one of the immature neurites lengthening rapidly into a long neurite possessing axonal characteristics (*i.e.*, growth cone). Following development of the axon, the neuron enters the fourth stage, in which the remaining neurites transform into dendrites and all processes continue to mature. In the fifth and final stage, the neuron forms synaptic connections with surrounding neurons and a neuronal network is established [89].

While the exact trigger for the development of a single neurite into a mature axon remains largely unknown, it has been shown that PI3K/PtdIns(3,4,5)P_3/Akt signaling is involved in this process, as well as in the maintenance of neuronal polarity [88]. Akt regulates neuronal polarity by inhibiting GSK3β. Inhibition of GSK3β occurs equally among all the neurites during the first two stages of development. During the third stage, Akt localizes to the cell body (the soma) and to tip of the neurite newly designated as the axon. This results in the inhibition of GSK3β primarily at the axonal tip. It has been experimentally shown that the expression of constitutively active Akt throughout the neuron leads to the formation of multiple axons [90] and inhibiting GSK3β in existing dendrites causes their conversion to axons [88]. Thus, spatially-specific accumulation of PtdIns(3,4,5)P_3 and activation of Akt is critical for neuronal polarization and development of neuronal networks.

The activation of Akt at the distal tip of neurites is dependent on the local accumulation of PtdIns(3,4,5)P_3. Transport of PtdIns(3,4,5)P_3 to the neurite tip occurs via guanylate kinase-associated kinesin (GAKIN), a member of the kinesin-3 family of microtubule-based motor proteins, which binds the PtdIns(3,4,5)P_3 binding protein (PtdIns(3,4,5)P_3-BP), also called centaurin-α, via its forkhead-associated (FHA) domain. PtdIns(3,4,5)P_3-BP exhibits ADP-ribosylating factor GTPase activating protein (Arf GAP) activity, and contains two PH domains through which they specifically interact with PtdIns(3,4,5)P_3. Using *in vitro* motility assays, it has been shown that the accumulation of PtdIns(3,4,5)P_3 at the distal tip of developing neurons occurs at least in part via transport of PtdIns(3,4,5)P_3-containing vesicles bound to PtdIns(3,4,5)P_3-BP complexed to GAKIN [91].

7.4 Implications in Disease

As discussed in detail above, PtdIns(3,4,5)P_3 mediated signaling is a highly ordered and conserved pathway in a variety of tissues and cells. The relatively low abundance and precise control over the levels of PtdIns(3,4,5)P_3 ensure ordered and deliberate activation of downstream targets, which contribute to many core functions in the

cells. It is therefore not surprising that a significant number of human diseases can be linked to perturbations in PtdIns(3,4,5)P_3 signaling. In fact, approximately 15–30 % of human cancers have been shown to have disruptions in this pathway [92]. Additionally, diabetes, cardiovascular diseases and various inflammatory disorders have also been linked to PtdIns(3,4,5)P_3 signaling abnormalities.

In normal cells, proper signaling is reliant on low basal levels of PtdIns(3,4,5)P_3 with rapid and controlled production by PI3K in response to extracellular signals. Additionally, a return to basal levels requires the activity of either PTEN or SHIP phosphatases. Activating mutations in PI3K (or its downstream activators) or the loss of PTEN result in elevated cellular levels of PtdIns(3,4,5)P_3. Promiscuous and constitutively present PtdIns(3,4,5)P_3 is a trademark of tumorigenesis in cells of many tissue types [92]. Alternatively, a loss of PI3K function has been implicated in insulin resistance and the resulting diabetic phenotype [93]. A discussion of specific alterations in PtdIns(3,4,5)P_3 signaling and their implications in diseases such as cancer, inflammation, cardiac diseases, and diabetes are presented below.

7.4.1 Cancer

Cancer is a complex disease requiring genetic alterations in the cell resulting in diverse changes including enhanced proliferation, uncontrolled growth, resistance to apoptotic signals, and invasion of surrounding tissues [94]. PtdIns(3,4,5)P_3 signaling is implicated in these oncogenic traits and is demonstrably overactivated in a wide range of tumor types [95]. Many excellent in-depth reviews, detailing the role of PI3K signaling pathway in cancer, are available [93, 95, 96], therefore just the major points will be outlined.

As previously discussed, PtdIns(3,4,5)P_3 production results in the activation of various downstream proteins, most notably Akt, and is responsible for enhanced cell growth and proliferation, survival, and motility. Upon PtdIns(3,4,5)P_3 production and Akt activation, multiple downstream processes promote cell survival and resistance to apoptosis [97]. This occurs through both activation of cellular signaling and transcriptional changes. Akt phosphorylates BAD, thus inhibiting its association with downstream apoptosis factors and decreasing apoptotic tone [98]. Additionally, Akt has been shown to activate NF-κB resulting in transcription of pro-survival and proliferation genes [58, 99]. The FoxO family of transcription factors is phosphorylated by Akt, leading to their sequestration in the cytosol and attenuation of transcription of pro-apoptotic proteins including BIM and Fas-ligand [100], thus, any increase in Akt activity has major implications on the ability of cells to undergo apoptosis. Tumorigenic effects of Akt are not limited to the regulation of cell survival. Activation of Akt pathway contributes to major remodeling of cellular metabolism promoting aerobic glycolysis, reducing β-oxidation of fatty acids and maximizing lipid biosynthesis, all critical for rapid proliferation of cancer cells [101, 102]. Akt is also a major mediator of mTORC1 regulation downstream from growth factor and oncogenic signals, providing a critical link to the robust requirement for protein biosynthesis during proliferation. Inhibition of the $p21^{Cip1}$ and $p27^{Kip1}$ CDK inhibitors, through direct phosphorylation by Akt, as well as transcriptional regulation of p27 and Cyclin D1 contribute to increased cellular proliferation [103]. More recent evidence indicates a critical role of Akt2 in epithelial-mesenchymal transition through the regulation of specific miRNAs [104]. These are some of the key examples of the multitude of pro-oncogenic mechanisms controlled by overactivated Akt pathway in cancer cells [95].

Multiple factors contribute to the overactivation of PtdIns(3,4,5)P_3 synthesis in cancer cells. Genetic mutations resulting in increased expression and/or activity of RTKs, PI3K, or loss of PTEN activity are some of the most common alterations of the PtdIns(3,4,5)P_3 pathway noted to drive tumorigenesis. These mutations can often occur redundantly [92]. RTKs have been repeatedly shown to be dysregulated in a wide variety of cancers [105]. The epidermal growth factor receptor (EGFR) in particular is commonly

overexpressed or mutated in gliomas and non-small cell lung cancer [106, 107]. HER2 is overexpressed in approximately 25 % of breast cancer patients and is commonly associated with high recurrence rates and increased mortality [108]. Gain-of-function mutations in the tyrosine-protein kinase c-Kit are evident in gastrointestinal-stromal tumors, acute myeloid leukemia, mast cell leukemia, and melanoma [109, 110]. These are examples of some of the frequent RTK mutations leading to an increased PI3K activation and subsequent PtdIns(3,4,5)P_3 production.

Since the discovery of cancer-specific mutations in the *PI3KCA* gene encoding the p110α subunit of PI3K, oncogenic mechanisms of mutated PI3K has been intensely investigated [111]. Mutations in *PI3KCA* gene have been implicated in head and neck, squamous cell lung carcinoma, gastric, and cervical cancers [93]. Three hot spot, non-synonymous, missense mutations of amino acids in the helical (E542K and E545K) or kinase (H1047R) domains make up approximately 80 % of the mutations in the *PI3KCA* gene [112–114]. PtdIns(3,4,5)P_3 generation is amplified several fold as a result of increased PI3K kinase activity [115–120]. Increase in PtdIns(3,4,5)P_3 concentration is the primary driver of oncogenic signaling [121]. Additionally, constitutively active PI3K uncouples PtdIns(3,4,5)P_3 production from growth factor-induced RTK activation. Several mutations in *PI3KR1*, the gene encoding p85, have also been shown to induce oncogenic transformation in the cell [122]. These mutations typically occur in regions of the protein responsible for p110 binding, thus preventing inhibitory effects on the catalytic activity of p110 [123].

Since the discovery of its role as a potent tumor suppressor in 1997, decreased PTEN function has been correlated with a number of human cancers [124, 125]. Subsequent studies of *Pten* knockout mice confirmed PTEN tumor suppressor functions in a variety of tissues [126–128]. Additional studies of *Pten*$^{+/-}$ mice revealed that PTEN functions as a haploinsufficient tumor suppressor gene [129, 130]. Thus, any inhibitory effect on the expression or activity of PTEN has implications in cancer with PTEN levels inversely correlating with disease severity [131, 132]. Additionally, many molecular mechanisms have been associated with decreased expression and/or activity of PTEN, making any correlations of simple step-wise changes in the expression of *Pten* on cancer progression incomplete [133]. For example, allelic loss, epigenetic silencing, functional mutations, miRNA silencing, and post-translational modifications have all been demonstrated to decrease PTEN expression and/or function. These discoveries have led to the proposal of a "continuum model of tumor suppression" relating overall level and function of PTEN with tumor suppression [131, 134]. The obvious role of PTEN as an inhibitor of PtdIns(3,4,5)P_3 signaling is well established, though novel functions of PTEN are continuously being elucidated [133]. For example, nuclear localization of PTEN is associated with tumor suppression independent of its phosphatase activity [80, 135, 136]. Additionally, PTEN has been implicated in fibroblast-mediated shaping of the tumor microenvironment [137, 138].

While Akt plays a critical role in PtdIns(3,4,5)P_3 contribution to tumorigenesis, other mechanisms contribute as well. The contribution of PtdIns(3,4,5)P_3 to the increased cell motility through regulation of the activity of small GTPases and actin cytoskeleton remodeling should not be discounted. Recent siRNA screens revealed, unexpectedly, that PDK1 may be an even more critical driver than Akt in *PI3KCA* tumors. This has been linked to the Akt-independent regulation SGK3 as mentioned above [66].

The obvious role of PtdIns(3,4,5)P_3 signaling in cancer has lead to a dramatic increase in research into the mechanisms of increased PtdIns(3,4,5)P_3 production and expression, while therapies targeting this pathway are concomitantly being developed. Several drugs are currently on the market targeting RTK activity upstream of PtdIns(3,4,5)P_3 production and there is a race to develop isoform specific inhibitors of PI3K. Additionally, increased PtdIns(3,4,5)P_3 signaling has been associated with drug resistance in tumors, therefore, drugging this pathway is extremely important

clinically [139, 140]. Further discussion of these efforts will be presented below.

7.4.2 Inflammation

There are four different isoforms of Class I PI3K, depending on the identity of the catalytic subunit. Class I_A PI3Kα and PI3Kβ isoforms are ubiquitously expressed, while class I_A PI3Kδ and class I_B PI3Kγ are predominately expressed in the hematopoietic cells [141]. As a result of their restricted expression, it has been proposed that these isoforms are important regulators in different populations of immune cells including neutrophils, macrophages, mast cells, eosinophils, T-cells, and B-cells. Current research indicates that PI3Kδ and PI3Kγ-mediated production of PtdIns(3,4,5)P_3 is an important druggable target in inflammatory diseases [142]. In the immune system, PtdIns(3,4,5)P_3 signaling is initiated in response to antigen, cytokine, and chemokine receptors [19]. Increased PtdIns(3,4,5)P_3 signaling results in differing phenotypes depending on the cell type.

Neutrophils and macrophages respond to microbial invasion and are crucial players in inflammatory reactions. In response to inflammatory signals, these cells migrate from the circulation to the site on injury through a process known as chemotaxis [143]. Extracellular chemokine signaling results in the local activation of cellular receptors translating into dramatic localized increases in PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 then recruits and activates GEFs for Rac and Arf GTPases, promoting actin cytoskeletal rearrangement and directional cell movement [144]. Several studies have indicated that this process is dependent on PI3Kγ [145–147]. The precise role of PI3Kδ is still unclear. An isoform specific inhibitor of PI3Kδ was shown to inhibit chemotaxis in neutrophils [148]. On the other hand, subsequent studies have supported the function of PI3Kγ as the primary isoform responsible for cellular migration in response to chemoattractants [149]. PTEN stability and activity is essential for proper inflammatory cell migration, further illustrating the importance of PtdIns(3,4,5)P_3 signaling [150]. In addition to chemotaxis, the production of reactive oxygen species (ROS) at the site of inflammation also depends on PtdIns(3,4,5)P_3 signaling [145–147, 151]. PDK1 and Akt are both implicated in the phosphorylation of various NADPH oxidase proteins leading to their assembly, subsequent production, and release of ROS [152]. Increased neutrophil and macrophage activation has been associated with atherosclerosis, lupus, and rheumatoid arthritis indicating PtdIns(3,4,5)P_3 signaling as a potential druggable target in these inflammatory diseases [153–155].

Mast cells are an additional player in mediating inflammatory activities in response to infection or parasites and are important in amplifying adaptive immunity [156]. These cells are perhaps best known for their detrimental effects in allergic diseases [146]. In response to allergens, the high-affinity IgE receptor is crosslinked and immunoreceptor tyrosine-based activation motifs are phosphorylated. Class I PI3Ks are then activated upon binding to the phosphorylated immunoreceptor tyrosine-based activation motifs. PtdIns(3,4,5)P_3 production results in the activation of Btk and, subsequently, PLCγ, ultimately initiating the opening of plasma membrane Ca^{2+} channels and granule release [157]. Initial degranulation and release of cytotoxic effector molecules is dependent on PI3Kδ activity while subsequent waves of degranulation are dependent on PI3Kγ [158, 159]. In addition, shRNA knockdown or genetic deletion of PTEN increased PtdIns(3,4,5)P_3 signaling in mast cells leading to mastocytosis and heightened allergic responses in mice [160, 161]. Shenker et al. have demonstrated that a chimeric toxin containing a PtdIns(3,4,5)P_3 phosphatase was able to inhibit mast cell degranulation, further illustrating the importance of this pathway in mast cell pro-inflammatory responses [162]. Increased PtdIns(3,4,5)P_3 signaling in mast cells has implications in a variety of immune disorders including allergic disease, asthma, anaphylaxis, autoimmunity, and mastocytosis [163].

T-cells and B-cells play a central role in the adaptive immune responses. T-cells are responsible for cell-mediated immunity while B-cells

produce antibodies and comprise humoral immunity. Class I PI3K have been shown to be activated in response to T-cell receptors, cytokines such as IL-2, IL-4, IL-7 and IFNγ, and CD28 signaling [16]. PI3Ks are also essential for proper lymphocyte development [164, 165]. Okkenhaug and Fruman review the importance of PI3K in the development of lymphocytes, in particular, illustrating the importance of p110δ [165]. Thymocyte development is dependent on both class I_A and class I_B PI3Ks. Loss of class I_A PI3Kδ results in aberrant pre-T-cell receptor signaling, though it has no effect on overall thymocyte numbers [166]. PI3Kγ knockout mice display an increase in thymocyte apoptosis and double knockout (PI3Kδ/γ negative) mice display a dramatic reduction in thymocyte numbers [167, 168]. Additionally, PI3Kδ regulates the differentiation and expansion of helper T-cells [169]. The role of PtdIns(3,4,5)P_3 in directional lymphocyte migration is still a matter of debate, but it has been suggested that PI3Kγ mediates chemotaxis in T-cells while PI3Kδ is responsible for B-cell migration [170, 171]. Notably, PI3Kγ has not been implicated in B-cell development or survival and all PtdIns(3,4,5)P_3 signaling has been shown to be dependent on PI3Kδ [172].

Counterintuitively, both increases and decreases in PtdIns(3,4,5)P_3 signaling have been implicated in the development of autoimmunity [173–175]. This is likely due to the alterations in the attenuation of peripheral immune functions mediated by regulatory T-cells. Several studies have linked the contribution of PtdIns(3,4,5)P_3 signaling in autoimmunity to its regulation of functions of regulatory T-cells (reviewed in [19]).

The evidence outlined above gives plenty of weight to the importance of PtdIns(3,4,5)P_3 signaling in immune function and disease. Current research in this area is focused on elucidating the role and importance of PI3K isoforms and effects of PTEN/SHIP activity on the immune system, in particular, defining the areas where drug intervention could provide the most benefit, given the complex role of PI3K signaling in immune regulation as well as in many other tissues [157, 176]. Targeting the correct PI3K isoform is critical and several isoform-specific inhibitors are currently under clinical investigation for various inflammatory disorders [142].

7.4.3 Cardiovascular Disease

PtdIns(3,4,5)P_3 plays an important role in cardiac physiology and pathophysiology of heart disease. PI3K and PTEN are expressed throughout the heart including cardiomyocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells [177]. Cardiac PtdIns(3,4,5)P_3 signaling has been shown to have an important role in cell survival, hypertrophy, contractility, metabolism, and mechanotransduction [178]. If normal PtdIns(3,4,5)P_3 signaling is enhanced through either overactivation of PI3K or a loss of PTEN, myocardial hypertrophy and decreased contractility can result, thus impairing normal cardiac function. In contrast, a decrease in PtdIns(3,4,5)P_3 signaling results in increased areas myocardial infraction and prevents myocardial preconditioning.

Class I_A and class I_B PI3Ks have both been demonstrated to have functions in cardiac PtdIns(3,4,5)P_3 signaling. Class I_A PI3K catalytic isoforms p110α and p110β are expressed in the heart and vasculature [179–181] while the class I_B isoform p110γ is found in cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells, and endothelial cells [177, 182, 183]. Each isoform has a distinct role in cardiac regulation and function. Class I_A isoforms are thought to regulate physiologic growth mediated by activation of RTKs [177, 179, 184]. Class I_B γ isoform is required for proper contractility of the myocardium [179, 185]. Termination of PtdIns(3,4,5)P_3 signaling in the heart is dependent on the function of PTEN. Loss of PTEN leads to increased PtdIns(3,4,5)P_3 signaling ultimately resulting in various pathologies including hypertrophy and decreased contractility [177, 179, 186, 187]. The downstream targets of PtdIns(3,4,5)P_3 signaling described above are similarly involved in cardiac regulation. In particular, Akt/GSK3 pathway plays an important role in heart disease [188, 189].

All three Akt isoforms are expressed in the heart, though Akt1 and Akt2 are the most prevalent [179, 190, 191]. Normal Akt activation in the myocardium is required for cell proliferation, metabolism and inhibition of apoptosis [192]. A transgenic mouse model with cardiac specific knockdown of *Akt1* resulted in a reduced number of cardiomyocytes and smaller overall heart sizes, though there was no effect seen on contractility [193]. At the same time, overexpression or increased Akt activation can result in cardiac hypertrophy and abnormal contractility [193–195].

Both GSK3 isoforms, GSK3α and GSK3β are expressed in the heart though more emphasis has been placed on the impact of GSK3β [189]. GSKβ is a constitutively active enzyme, phosphorylating and inactivating glycogen synthase and, thus, inhibiting glycogen synthesis [196]. Upon PtdIns(3,4,5)P_3 production and Akt activation, GSK3β is phosphorylated and rendered inactive, thus promoting glycogen synthesis. GSK3β is also shown to be a regulator of important transcription factors, in particular, nuclear factors of activated T-cells, β-catenin, CREB, and the Jun family of proteins [189]. Overall, constituently active GSK3β has been shown to act as a hypertrophic restraint in the heart [197], while induction of hypertrophy by PtdIns(3,4,5)P_3 has been linked to the inhibition of GSK3β [189].

As discussed above, PtdIns(3,4,5)P_3 signal alteration has variable effects on the physiology of the myocardium. PI3Kα signaling in response to exercise has been shown to provide protective effects in patients with heart failure [198]. Notably, myocardial ischemic preconditioning, a process whereby a brief period of ischemia is able to protect the heart from further ischemic events, has been linked to the changes in PI3K pathway [199–201]. This occurs through the reduction in apoptosis and increased proliferation of preconditioned cells. Activation of PtdIns(3,4,5)P_3 signaling pathways, or loss of PTEN, has been shown to mimic the cardio-protective nature of ischemic preconditioning [200, 202–204]. Additional heart pathologies such as diabetic cardiomyopathy [205], adriamycin-induced cardiomyopathy [206], chronic β-adrenergic receptor stimulation [184], or pressure overload induced hypertrophy [207] have all been shown to involve altered PI3K/Akt signaling.

Overall, similar to immune regulation, PtdIns(3,4,5)P_3 signaling pathway has multiple effects on the regulation and pathophysiology of the cardiac system. There is still much work to be done to unravel the exact role of each PI3K isoform in the pathophysiology of different heart diseases. Particular care should be taken to avoid cardiovascular toxicity following prolonged exposure to various isoform specific PI3K inhibitors [208].

7.4.4 Diabetes

A number of tissues are involved in the complex process of metabolic regulation. Liver, skeletal muscle, adipose tissue, pancreatic beta cells, and several CNS neuronal populations are all involved in glucose sensing and regulating insulin signaling. Insulin receptors (IR) are coupled to downstream effects in part by inducing PI3K production of PtdIns(3,4,5)P_3 [93]. Recent research has implicated the generation and breakdown of PtdIns(3,4,5)P_3 as a prime mediator of insulin receptor signaling and alterations in this pathway have been shown to result in metabolic syndromes and the diabetic phenotype [209]. Potential inhibition of glucose metabolism is one of the most important considerations in developing PI3K inhibitors.

Insulin receptors belong to the RTK class of receptors. Upon activation by their ligands and subsequent dimerization, IR and IGF receptors phosphorylate insulin receptor substrate (IRS) proteins [210]. Several IRS proteins displaying differences in tissue expression have been identified [211]. Tyrosine phosphorylated IRS proteins bind to the SH2 domains of the regulatory p85 subunit of Class IA PI3Ks. The p110α catalytic subunit is thought to be the primary subunit involved in IR/PI3K metabolic signaling as inactivation of this subunit results in embryonic death. Heterozygote mice display small body size, insulin resistance, and glucose intolerance [212]. Similarly, isoform-selective p110α inhibitors

blocked IR/PI3K signaling, while p110β inhibitors showed little effect [213]. On the other hand, emerging evidence indicates p110β may still play a role by changing the kinetics of PtdIns(3,4,5)P_3 production, prolonging insulin induced signaling, resulting in the prolonged Akt activation [214].

Peripheral insulin resistance is thought to be the product of disrupted PI3K signaling in the effector cells [210, 215]. Any mechanism resulting in decreased PtdIns(3,4,5)P_3 production or expression can result in diabetic phenotypes. Mice lacking either p110α or p110β die in early embryogenesis while mice heterozygous for p110α and p110β display glucose insensitivity [212, 216]. In contrast, mice lacking PTEN expression in either muscle, adipose, or liver tissue leads to an increase in insulin sensitivity and glucose tolerance [217, 218]. Additionally, studies of SHIP2 heterozygous deletion mice have been shown to result in increased insulin sensitivity indicating SHIP2 is a key regulator of glucose homeostasis [219].

Several reports suggested that decreased expression of p85 regulatory subunit of PI3K resulted in enhanced insulin sensitivity in mice [220]. Subsequent investigation uncovered an IR/PI3K inhibitory function of p85 [221]. p85 is present in excess to p110 in many cell types and free p85 has been shown to sequester activated IRS1 in the cytoplasm, preventing IRS1 from interacting with PI3K at the membrane [222]. Notably, elevated levels of p85 have been found in pregnancy-induced diabetes in women and in skeletal muscles of type-2 diabetic patients [223, 224]. Interestingly, the levels of p110 were not altered indicating the importance of the p85-p110 ratio. At the same time, a complete loss of p85 in either the muscle or liver resulted in compromised insulin signaling in those tissues due to its critical role in linking PI3K to IR [225, 226]. Additionally, chronic inflammation present in adipose tissue of obese patients leads to release of TNFα, which can activate JNK and block IRS thus decreasing PtdIns(3,4,5)P_3 signaling [227, 228]. p70S6K activation downstream of mTOR is also able to inhibit PtdIns(3,4,5)P_3 signaling by phosphorylation of IRS-1 thus providing a negative feedback and contributing to insulin resistance [229, 230].

Following activation of IR/PI3K and production of PtdIns(3,4,5)P_3, a variety of downstream events are regulated by Akt, including GLUT4-mediated glucose uptake [231], glycogen synthase activation through GSK3 inhibition [196], and inhibition of FoxO-mediated gene transcription [232]. In particular, Akt2 has been shown to play a key role in glucose homeostasis. Disruption of *Akt2* in mice results in the diabetic phenotype [233]. In addition, full activation of Akt in response to insulin signaling is reliant on PtdIns(3,4,5)P_3-dependent phosphorylation by PDK1. Genetic mutation of the PH domain of PDK1 in mice resulted in glucose and insulin intolerance, indicating the critical role of PtdIns(3,4,5)P_3 in the connection between PDK1 and Akt [25].

PtdIns(3,4,5)P_3 presence in the membrane is able to both propagate IR/PI3K signaling and initiate events leading to the attenuation of the signal. O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT) has been recently shown to bind PtdIns(3,4,5)P_3 and to translocate to the membrane upon insulin induced PtdIns(3,4,5)P_3 production [234]. Once at the membrane, OGT is able to catalyze the addition of GlcNAc to the key insulin signaling intermediates downstream of PtdIns(3,4,5)P_3 such as ribosomal proteins. It also competes with other PH-containing proteins for PtdIns(3,4,5)P_3 binding at the membrane, resulting in attenuation of their signal [235]. O-GlcNAc levels were also shown to be elevated under hyperglycemic conditions. Another protein with affinity for PtdIns(3,4,5)P_3 is prohibitin, a protein shown to bind PtdIns(3,4,5)P_3 and modulate insulin signaling *in vitro* [236].

Although there is ample evidence to implicate perturbed PtdIns(3,4,5)P_3 signaling in diabetes, it remains a difficult pathway to directly target therapeutically. The complex pathophysiology of diabetes covers several tissue types, each with individual expression of PI3K isoforms and unique pathway elements. Furthermore, any attempts to increase PtdIns(3,4,5)P_3 signaling could have oncogenic implications, as this pathway is highly associated with tumorigenesis.

Conversely, any PI3K pathway inhibitors should be analyzed for their potential effects on metabolic signaling.

7.5 Approaches to Target PtdIns(3,4,5)P_3 Signaling

The importance of PtdIns(3,4,5)P_3 regulation has been described in detail above. Dysregulation of the pathway is implicated in a wide variety of disorders and, therefore, targeting the pathway pharmacologically represents a great promise. Thus, it is not surprising that the development of small-molecule inhibitors of various components in the PtdIns(3,4,5)P_3 signaling cascade is a hot topic, especially in the fields of cancer and inflammation. At present there are over 100 clinical trials underway of PI3K inhibitors alone (http://www.clinicaltrials.gov). This does not include clinical trials on drugs targeting other proteins in the PtdIns(3,4,5)P_3 pathway, such as mTOR, Akt, or PDK1, or drugs inhibiting upstream targets, such as activity of RTKs. Discussion of upstream inhibition of PtdIns(3,4,5)P_3 pathway by targeting RTKs such as EGFR or IGFR has been extensively reviewed elsewhere and will not be covered here [237, 238].

7.5.1 PI3K Inhibitors

The most straightforward method to inhibit PtdIns(3,4,5)P_3 signaling is to prevent the PI3K mediated conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. Many small molecule PI3K inhibitors have been developed with several advancing to clinical trials (Fig. 7.4) [96, 239]. Wortmannin, a sterol like fungal metabolite, was the first PI3K inhibitor and one of the most extensively studied. It was initially discovered to have an inhibitory effect on respiratory burst in neutrophils [240]. Subsequent studies illustrated wortmannin functions as an irreversible inhibitor of PI3K [241]. Nonspecific inhibition of other protein kinases, poor stability and high toxicity were all limiting factors preventing its use in the clinic [242, 243]. The first synthetic PI3K inhibitor was developed by Eli Lily in 1994 [244]. LY294002 has been most widely utilized as a research tool delineating PI3K signaling in cells. The clinical development of LY294002 has been limited by low potency and poor aqueous solubility [35, 245].

Second generation PI3K inhibitors based on the structures of wortmannin and LY294002 have been developed and are currently undergoing clinical trials. PX-866, in development by Oncothyreon, is an irreversible PI3K inhibitor based on the structure of wortmannin [243]. It has shown to have good oral bioavailability and an improved toxicity profile in a phase I trial. SF-1126 developed by Semaphore Pharmaceuticals is a LY294002 pro-drug with improved pharmacological properties and tumor targeting [245]. This compound is currently in clinical trials as well.

An emerging strategy in PI3K inhibition is to design isoform specific inhibitors. Previous discussion has demonstrated the importance of individual isoforms in various disorders. By selectively inhibiting the isoform implicated in disease, an improved therapeutic index is possible [142]. Initial efforts focused on specific inhibition of p110α and p110β due to their role in tumorigenesis. However, these isoforms are ubiquitously expressed and play critical roles in normal homeostasis, such as glucose metabolism. Therefore, development of p110δ and p110γ inhibitors has attracted increasing attention [246]. p110δ is an important isoform present in immune cells and is commonly overexpressed in inflammatory disorders and lymphomas. IC-87114 (ICOS Corporation), CAL-101 and CAL-263 (Calistoga Pharmaceuticals) are all specific p110δ inhibitors with 40–300 fold increased potency versus other isoforms [148, 247, 248]. CAL-101 in particular has shown promising clinical results in patients with various hematologic malignancies [249]. CAL-101 and CAL-263 have both entered clinical trials for the treatment of allergic rhinitis, potentially indicating utility in inflammatory diseases. p110γ specific inhibitors were initially thought to be effective against several inflammatory disorders [250]. AS-605240 (Merck Serono) was tested for isoform specific

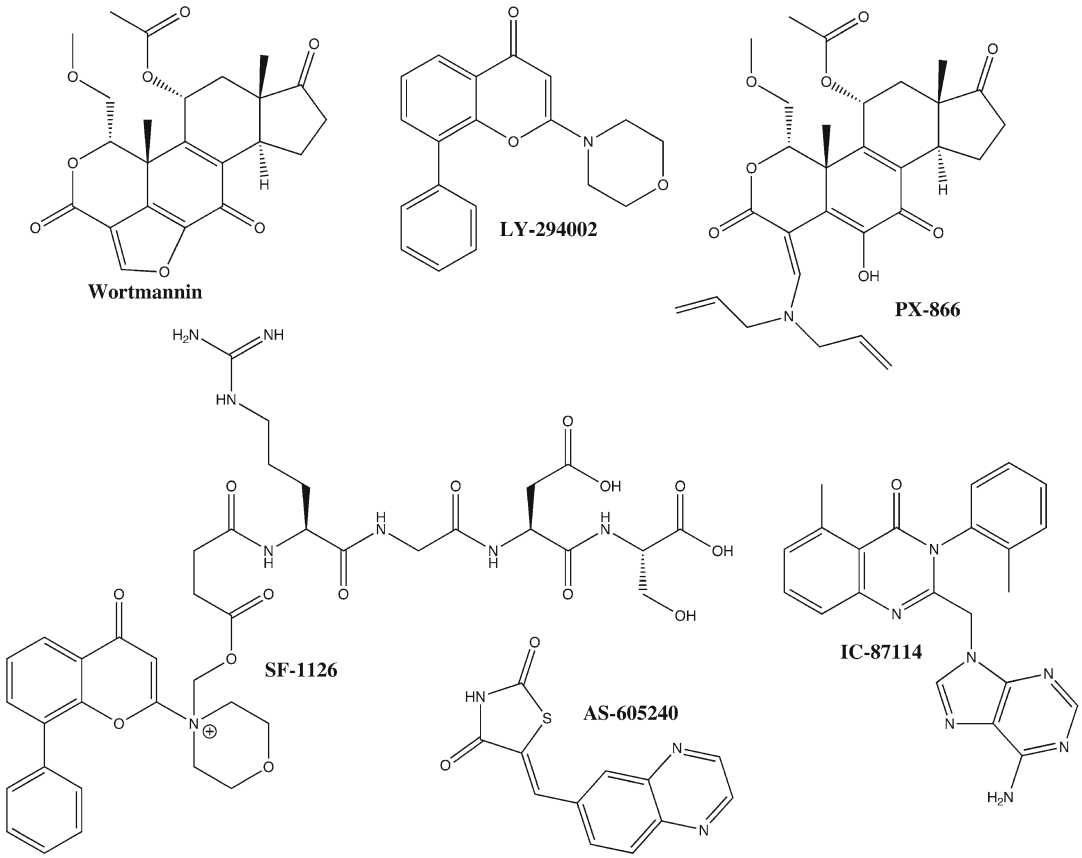

Fig. 7.4 Structures of several PI3K inhibitors

inhibition of p110γ with some success [251]. However, subsequent studies have been disappointing as selectivity to p110γ is insufficient to overcome off-target effects [142].

7.5.2 Inhibitors of Downstream Targets of PtdIns(3,4,5)P_3

Targeting specific isoforms of PI3K is only one way to improve therapeutic index and tolerability. Several downstream activators of PtdIns(3,4,5)P_3 signaling have also been targeted for inhibition. It is thought that targeting the critical components specifically contributing to particular pathologies could lead to improved efficacy and reduced toxicity compared broad PI3K inhibition. Akt and mTOR are the two most commonly targeted proteins downstream of PtdIns(3,4,5)P_3 [252], although other proteins, such as PDK1, have been targeted as well.

7.5.2.1 Akt Inhibitors

Akt is one of the primary downstream effectors of PtdIns(3,4,5)P_3 signaling contributing towards increased cell survival, proliferation and metabolic alterations in cancer cells. Several inhibitors have been developed that target all three isoforms of Akt (Fig. 7.5). Both Perifosine and triciribine inhibit Akt activation by binding to its PH domain, thus preventing the membrane localization and activation by PDK1 [253, 254]. Both of these agents have seen limited success in the clinic due to poor response rates and adverse events, though there remains potential for use as part of combination therapies [252]. The allosteric inhibitor MK-2206 (Merck) showed promising results in combination therapy in xenograft

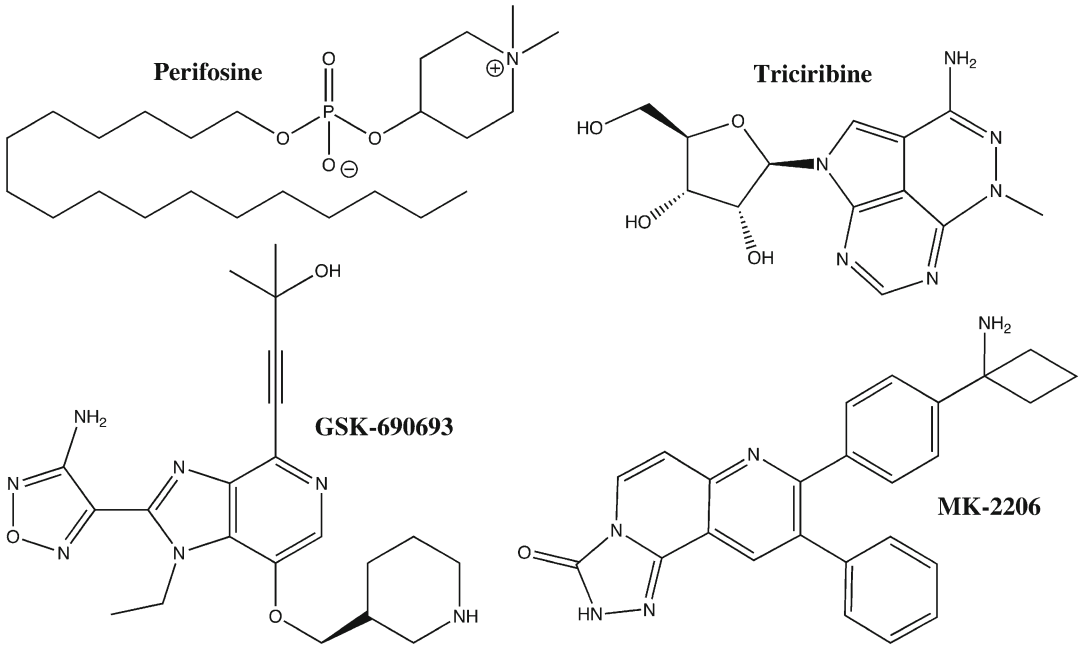

Fig. 7.5 Structures of several Akt inhibitors

models and is currently in Phase I trials [239, 255]. GlaxoSmithKlein has also developed two ATP competitive inhibitors of Akt, GSK-690693 and GSK-2141795 [256–258]. GSK-2141795 in particular has shown promising results in several phase I trials.

7.5.2.2 mTOR Inhibitors

Rapamycin (Wyeth) is a relatively selective mTORC1 inhibitor originally investigated as an antifungal agent. It has now been clinically approved as an immunosuppressant used to prevent rejection in organ transplantation. Analogs of rapamycin (known as rapalogs) such as temsirolimus (Wyeth) and everolimus (Novartis) have been approved for the treatment of renal cell carcinoma (Fig. 7.6) [259, 260]. Other cancers have lower response rates following treatment of rapalogs, thus indicating the heterogeneity of mTOR signaling in human cancers [252, 260]. mTORC1 is responsible for several but not all of mTOR-dependent functions. It has therefore been proposed that targeting the kinase activity of mTOR is a more effective means of PtdIns(3,4,5)P_3 signal inhibition [261, 262]. Very potent class ATP-competitive mTOR inhibitors (Torin-1 and Torin-2) have shown promise in pre-clinical development [263, 264]. Other ATP-competitive inhibitors, AZD-8055 (AstraZeneca), INK-128 (Intellikine), and OSI-027 (OSI Pharmaceuticals) are also under development and currently undergoing clinical trials [252, 265].

7.5.2.3 Dual PI3K/mTOR Inhibitors

Many PI3K inhibitors under development are also active against structurally similar proteins, including mTOR [265]. mTOR is activated by both nutrient signaling and PI3K dependent growth factor signaling. Therefore, inhibition of PI3K does not completely abolish mTOR activity. Additionally, mTOR inhibition can increase PI3K activity through suppression of p70S6K-dependent negative feedback loop [96]. Therefore, strategies targeting both PI3K and mTOR have been pursued and several dual PI3K/mTOR inhibitors have been described (Fig. 7.7) [266]. PI-103 in particular was developed as a dual inhibitor with much preclinical success [267, 268]. Despite its potency, PI-103 has poor solubility and rapid clearance. Therefore several

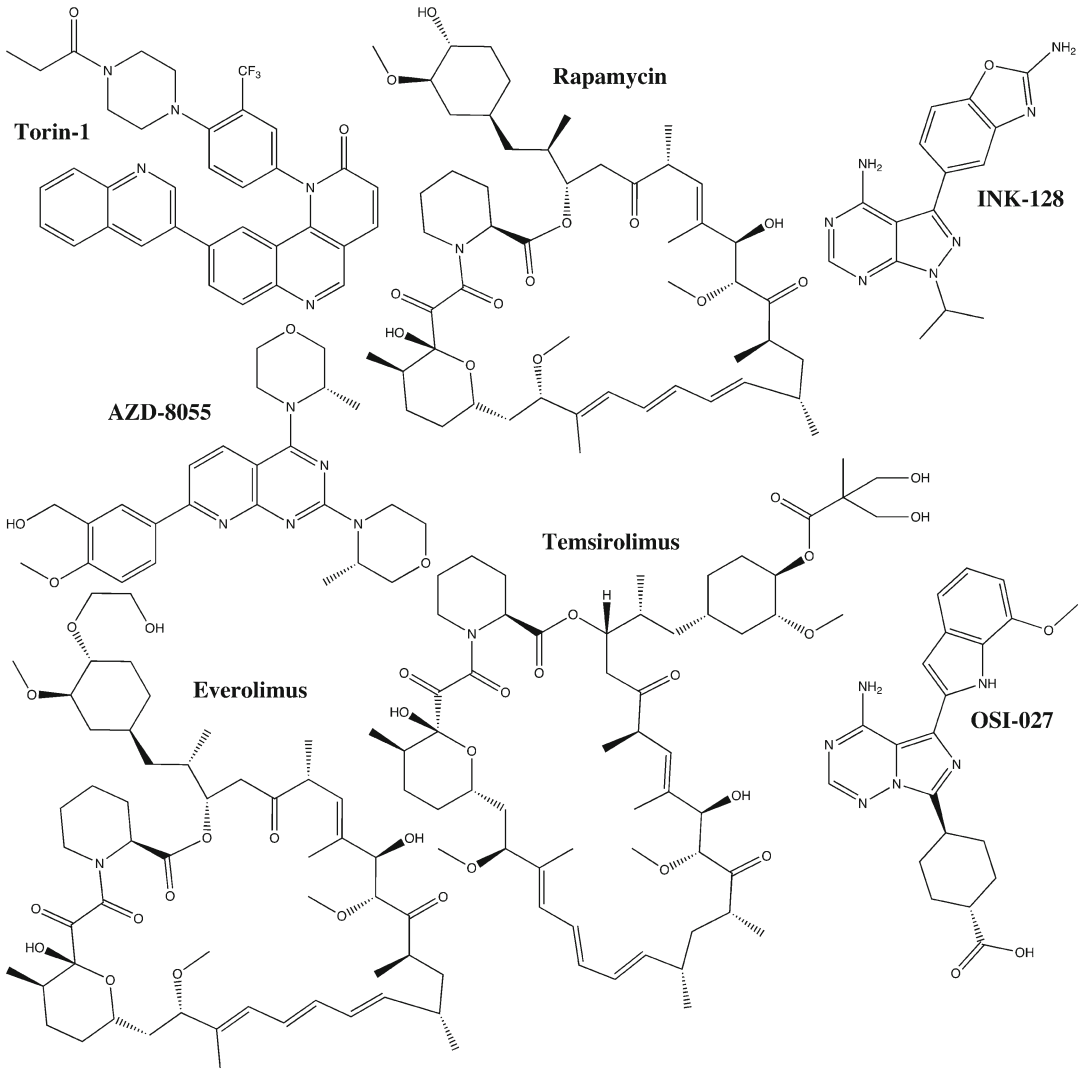

Fig. 7.6 Structures of several mTOR inhibitors

analogs, GDC-0980 and GDC-0941, have been developed by Genentech and are currently in clinical trials [252, 265]. Two other PI3K/mTOR inhibitors are being developed by Novartis, BEZ-235 and BGT-226. BEZ-235 is an orally available ATP-competitive inhibitor with low nanomolar IC_{50} values against both PI3K and mTOR [269, 270]. Phase I trials have indicated this compound is well tolerated with significant activity against breast cancer cells with high levels of PI3K signaling [271]. BGT-226 has also showed promising results both *in vitro* and *in vivo* and the clinical development of this compound is also underway [272, 273].

7.5.2.4 Btk Inhibitors

Another important target in the treatment of B-cell malignancies and autoimmune arthritis is the PtdIns(3,4,5)P_3-dependent Btk. As discussed above, Btk is a member of the Tec family of cytosolic tyrosine kinases and is activated upon recruitment to the inner leaflet of the membrane through interaction with PtdIns(3,4,5)P_3 and its PH domain. Two inhibitors of note

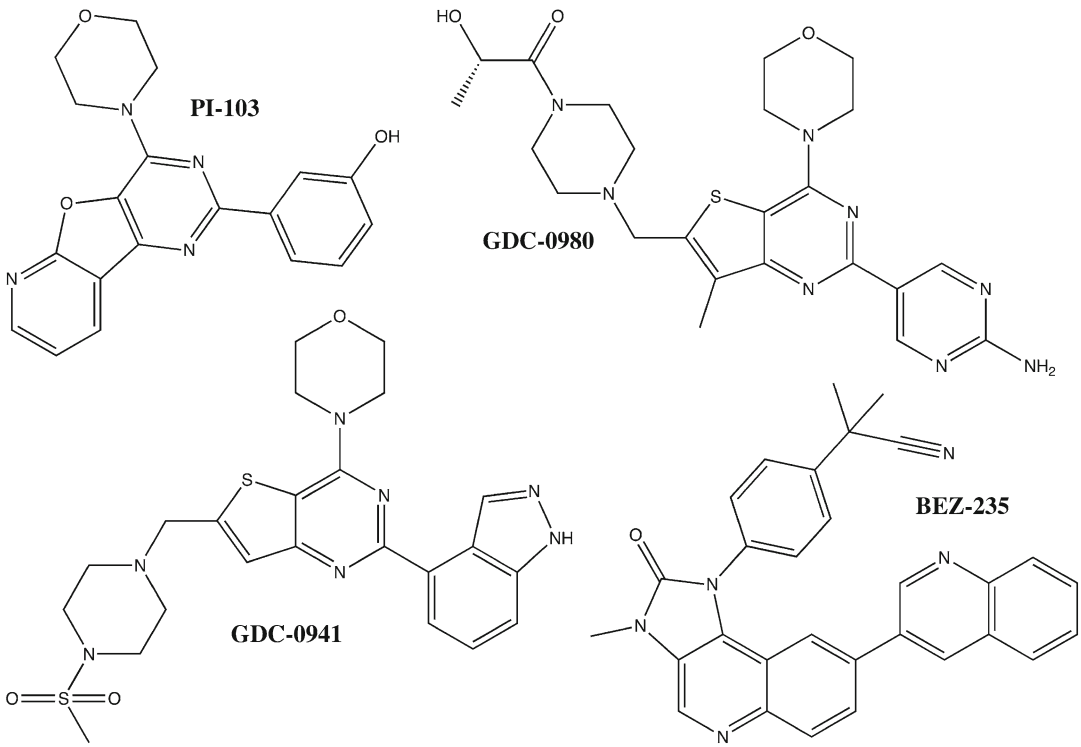

Fig. 7.7 Structures of several dual PI3K/mTOR inhibitors

have been developed targeting Btk, primarily for the treatment of B-cell malignancies, Pharmacyclics' PCI-32765 and Avila's AVL-292 (recently purchased by Celgene) [274]. PCI-32765 in particular has advanced through the clinic with positive Phase II results and is also being investigated as a treatment in autoimmune arthritis [275].

7.5.3 PH Domain Inhibitors

Another approach to inhibit PtdIns(3,4,5)P_3 signaling involves preventing the interaction of various proteins with membrane associated PtdIns(3,4,5)P_3. Blocking this interaction prevents the proper localization and activation of downstream effectors. Research into the structure and binding properties of the PH domain of Akt has provided insights into PH-PtdIns(3,4,5)P_3 interaction and development of inhibitors [17, 24, 276]. Inhibiting PtdIns(3,4,5)P_3 signaling at this step should universally block downstream activation of PI3K mediators.

PtdIns(3,4,5)P_3-PH inhibition can be achieved either by lipid-based antagonists or by non-lipid small molecule antagonists (Fig. 7.8) [265]. Initially, modified water-soluble head groups of PIs, inositol polyphosphates, were investigated for their PtdIns(3,4,5)P_3-PH inhibition [277, 278]. Later optimization revealed a compound capable of increased specificity for PDK1 and inhibition of mTOR [279]. The previously discussed perifosine is another lipid based PtdIns(3,4,5)P_3-PH inhibitor with specific activity against Akt, preventing recruitment to the membrane. Non-lipid antagonists under investigation include metabolite of triciribine (triciribine-phosphate), NSC-348900, PHT-427 and API-1 [265]. Additionally, a novel class of compounds known as PITENINs has been developed [280, 281]. Chemical modification of the lead compound DM-PIT-1 has resulted in more potent and selective inhibition of Akt-PtdIns(3,4,5)P_3 interaction [281, 282].

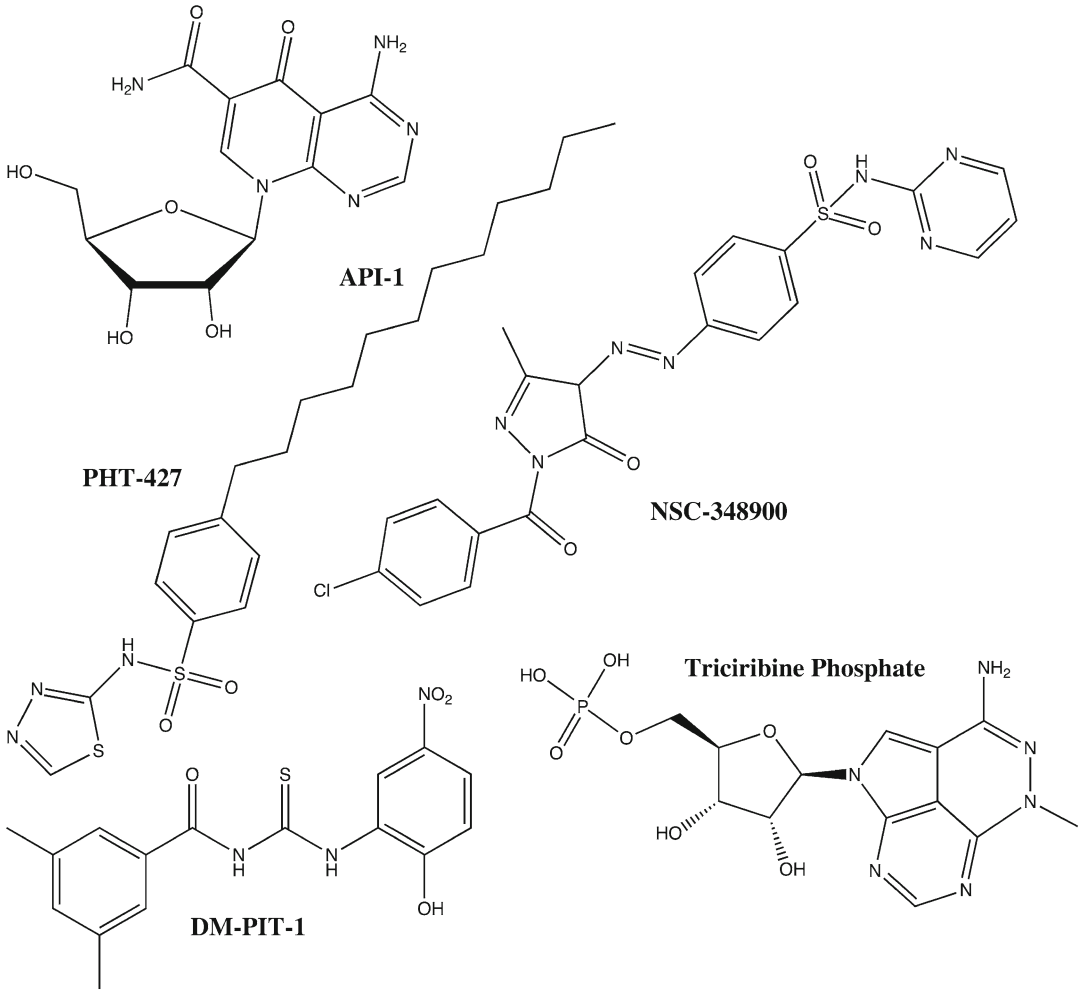

Fig. 7.8 Structures of several inhibitors of PtdIns(3,4,5)P_3 /PH interactions

7.5.4 Drug Resistance

Many *in vitro* studies have demonstrated that PtdIns(3,4,5)P_3 signaling is associated with decreased response to therapy and drug resistance. Constitutively active Akt signaling is implicated in both paclitaxel and TNFα-related apoptosis inducing ligand resistance [140, 283]. Additionally, breast cancer cell lines demonstrating activated HER2/PI3K/Akt signaling were less susceptible to chemotherapeutic apoptosis and inhibiting either PI3K or Akt restored drug sensitivity [284]. Furthermore, clinical evidence implicates PI3K and PTEN mutations with Herceptin resistance in breast cancer [285, 286]. Increased PtdIns(3,4,5)P_3 signaling has also been linked to colon cancer resistance to EGFR inhibition [287]. As a result of the increasing evidence implicating PtdIns(3,4,5)P_3 signaling in drug resistance, combination therapy strategies involving the targeted inhibition of the PtdIns(3,4,5)P_3 pathway and the Raf/MEK/ERK and BRAF pathways are being pursued [288, 289]. Several clinical trials are underway investigating the utility of PtdIns(3,4,5)P_3 pathway inhibition in avoiding drug resistance.

7.6 Conclusion

PtdIns(3,4,5)P_3 has emerged as an important second messenger in many cellular processes with implications for a wide range of diseases. Genetic studies in mice have illustrated the complexity of PtdIns(3,4,5)P_3 formation with various PI3K isoforms uniquely contributing to cellular signal propagation. PTEN expression and regulation has also emerged as an important mediator of PtdIns(3,4,5)P_3 activity. Following the formation of PtdIns(3,4,5)P_3, effector proteins such as Akt, PDK1, Btk, and others are able to bind to PtdIns(3,4,5)P_3 via PH domains and assemble at the inner plasma membrane where they are able to exert their effects resulting in cell survival, proliferation, cytoskeletal rearrangement, and gene expression. To better understand the effects of PtdIns(3,4,5)P_3 signaling in human diseases, additional elucidation of PI3K isoform activities needs to be performed. The ubiquitous expression of PI3K in healthy tissue necessitates the use of therapies targeting specific isoforms to reduce off target effects. Additionally, while a lot of progress has been made in understanding the regulation of key PtdIns(3,4,5)P_3 effectors, the contribution of many of the putative targets remains to be fully characterized.

As we begin to understand the complex relationships between upstream receptors, PI3K and downstream effectors, new small molecule inhibitors targeted at specific components are being developed with several successfully advancing through the clinic. Development of key biomarkers indicating which elements of the pathway to target will be a key step in the advancement of PtdIns(3,4,5)P_3 pathway inhibitors.

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The Role of Phosphoinositides and Inositol Phosphates in Plant Cell Signaling

8

Glenda E. Gillaspy

Abstract

Work over the recent years has greatly expanded our understanding of the specific molecules involved in plant phosphoinositide signaling. Physiological approaches, combined with analytical techniques and genetic mutants have provided tools to understand how individual genes function in this pathway. Several key differences between plants and animals have become apparent. This chapter will highlight the key areas where major differences between plants and animals occur. In particular, phospholipase C and levels of phosphatidylinositol phosphates differ between plants and animals, and may influence how inositol second messengers form and function in plants. Whether inositol 1,4,5-trisphosphate and/or inositol hexakisphosphate (InsP6) function as second messengers in plants is discussed. Recent data on potential, novel roles of InsP6 in plants is considered, along with the existence of a unique InsP6 synthesis pathway. Lastly, the complexity of myo-inositol synthesis in plants is discussed in reference to synthesis of phosphoinositides and impact on plant growth and development.

Keywords

Auxin • Jasmonic acid • *myo*-inositol • Inositol kinase • Inositol phosphate • Inositol hexakisphosphate • Inositol 1,4,5-trisphosphate • Phospholipase C • ABC transporter • Phosphatidylinositol phosphate • Ca^{2+} • Inositol polyphosphate phosphatase

8.1 Introduction

In Chinese philosophy Yin-Yang refers to combination of polar opposites that are interconnected, interdependent, and form a dynamic system. The phosphoinositide signaling pathway exemplifies such a system with its production of two types of signaling molecules that in some ways are opposite

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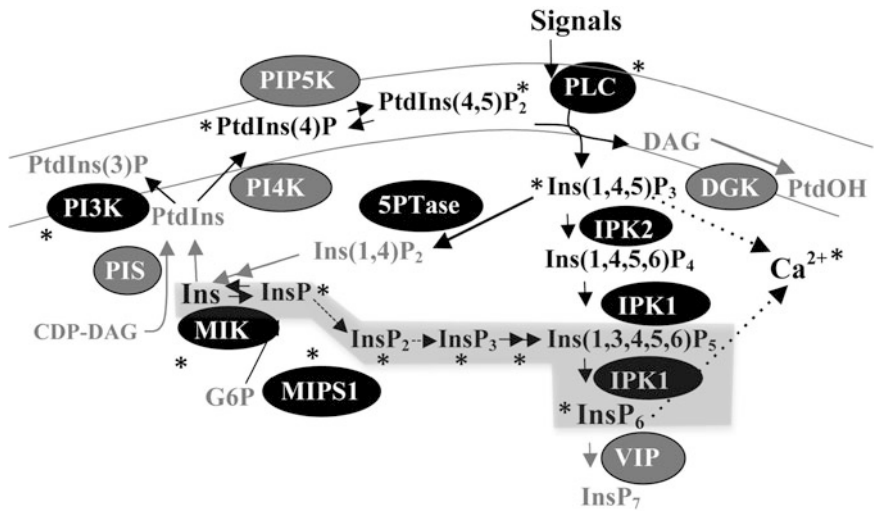

Fig. 8.1 Overview of phosphoinositide signaling in plants. Enzymes and molecules with differences from the animal pathway are in *black* and are denoted with an *asterisk*. *Dashed arrows* indicate uncharacterized or tentatively assigned steps. The *gray box* indicates the novel, lipid-independent pathway for $InsP_6$ synthesis. Abbreviations are defined in the text. MIPS1 is one of three genes encoding the *myo*-inositol phosphate synthase enzyme

in nature, including the water-soluble inositol phosphates (InsPs) and the lipid-soluble phosphatidylinositol phosphates (PtdInsPs). In addition, these two types of molecules are interdependent in that the PtdInsPs are used as substrates by phospholipases to produce the InsPs, and InsP breakdown produces *myo*-inositol that is used as substrate to produce the PtdInsPs.

In plants, the phosphoinositide signaling pathway has been studied by a diligent group of plant physiologists, biochemists and geneticists for years, and many recent, excellent reviews exist in the literature [1–4]. As the model system *Arabidopsis thaliana* offers the possibility of understanding gene function through analysis of genetic knock-out mutants, more researchers have joined the effort to understand how this signaling pathway enables plant growth, development and certain stress responses. There are key differences in how plants utilize and regulate the phosphoinositide pathway, and the focus of this chapter will be on molecules and enzymes involved and will highlight the differences in the plant pathway as compared to the pathways in animals and yeast. Figure 8.1 contains an overview of the phosphoinositide pathway in plants, and will be used as a reference throughout this chapter. Portions of the pathway that have unique aspects in plants are in black with asterisks, while conserved portions with a mostly similar function to that in animals or yeast are in gray.

8.2 The Biological Context

Phosphoinositide signaling in plants begins as it does in animals, with an external signal. Although the field of inositol signaling in plants has, in many ways, lagged behind that of animals, many studies over the years have lead to identification of signals that perturb either PtdInsPs or InsPs in plants. Two signals that are known to stimulate these events in plants is addition of the plant drought hormone, abscissic acid (ABA), and salt [5–8]. After ABA is given to *Arabidopsis* seedlings, one can measure a transient increase in inositol (1,4,5) trisphosphate ($Ins(1,4,5)P_3$) [5, 7]. In addition to elevating $Ins(1,4,5)P_3$, salt appears to also stimulate an increase in $PtdIns(4,5)P_2$ which later declines [6]. There is also ample evidence drought, which acts via ABA, stimulates phosphoinositide signaling in plants [9–11].

Gravitropism is probably the best understood signal that utilizes inositol signaling in terms of impact on InsP changes in plants with sustained increases in Ins(1,4,5)P_3 preceding growth in graviresponsive maize and oat pulvini [12]. Other signals for which data exist include light [13, 14], gibberellic acid [15], anoxia [16], cold [17], heat [18, 19], drought [10], and various plant pathogens and elicitors [20–22]. In some of these cases, Ins(1,4,5)P_3 levels have been shown to increase transiently in response to the signal. This list of signals is by no means complete, and may only be limited by what investigators have been willing to test. Developmental transitions most likely also require various inositol signaling molecules, as seen by the fact that many mutants in genes that encode enzymes required for inositol signaling have developmental alterations (for review see [23] and Sect. 9.5.1). This need for sustained basal levels of Ins(1,4,5)P_3 has recently been referred to as "basal signaling", which denotes the fact that the constant flux of on-going signaling is important for homeostasis [2].

8.3 Plant Phospholipase C Differences

One major difference in plant phosphoinositide signaling, as compared to that in other organisms, is the type, function, and regulation of phospholipase C (PLC) (EC 3.1.4.11) [24]. Plants contain multiple genes that encode a single epsilon-type of PLC, which is sensitive to Ca^{2+} *in vitro* [25]. In addition, plants contain non-specific PLCs, which function in hydrolysis of phosphatidylcholine [26]. One key difference in the epsilon-type plant PLCs is that they might not exclusively hydrolyze PtdIns(4,5)P_2. When animal PLCs hydrolyze PtdIns(4,5)P_2 in the plasma membrane, a diacylglycerol (DAG) product is generated that remains associated with the membrane, and a water-soluble Ins(1,4,5)P_3 molecule is generated that can interact with other proteins or cellular components to propagate the transfer of information within the cell [27, 28]. This is a key event in PtdInsP signaling, and it provides movement of information from the membrane to the cytosol. In fact, some plant PLC enzymes may hydrolyze PtdIns(4)P or PtdIns [29], which differs from the animal PLC enzymes that mostly hydrolyze PtdIns(4,5)P_2. This key difference results in the possibility of a combination of second messengers being produced by the different plant PLC enzymes. For example, if PtdIns(4)P is used as a substrate by PLC, then production of DAG and Ins(1,4)P_2 results. In contrast, If PtdIns is used as a substrate by a PLC enzyme, then DAG and Ins(1)P result.

The *Arabidopsis* genome contains 10 *AtPLC* genes [21, 30], while mammals have 11, and yeast has only 1 gene [31]. In *Arabidopsis*, the PLC genes are differentially regulated during development and in response to various environmental stimuli, including cold, salt, nutrients, dehydration, and ABA [24]. In tomato, expression of two PLC genes, *SlPLC4* and *SlPLC6*, are increased in *Cladosporium fulvum*-resistant tomato lines [32], suggesting a role for these genes in pathogen signaling. Mutants lacking AtPLC9 are deficient in production of Ins(1,4,5)P_3 in response to a heat stress treatment and have compromised responses to heat stress as well [19]. In addition, overexpression of AtPLC9 leads to increased Ins(1,4,5)P_3 and increased thermotolerance [19]. Together, these studies strongly suggest that PLC genes are important for adapting plants to both abiotic and biotic stress.

Post-translational control of PLC enzymes may also be important for signaling. Application of the plant pathogen elicitor, flg22, has been shown by proteomics experiments to result in phosphorylation of PLC [33]. This suggests that some signals can modify PLC directly, although whether this modification results in different PLC activity is not known.

8.3.1 Is PLC Signaling-Gated in Plants?

Perhaps one of the most interesting differences in the plant phosphoinositide signaling pathway is the potential lack of signaling-gated regulation of PLC. The fact that PLC may be regulated by transcriptional processes supports the idea that merely changing the amount of PLC in plant cells may be important. In addition, critical studies using

transgenic expression of the human PtdIns4P 5-kinase (PIPKIα) in tobacco cells resulted in an 100-fold increase in plasma membrane PtdIns(4,5)P_2 and a >40-fold increase in mass Ins(1,4,5)P_3 [30]. This indicates that conversion of PtdIns4P to PtdIns(4,5)P_2 is rate-limiting in the pathway. Further, the large basal increase in Ins(1,4,5)P_3 suggests that activation of PLC is not rate-limiting for Ins(1,4,5)P_3 production. A separate study involving characterization of a loss-of-function in the *Arabidopsis* SAC9 phosphoinositide phosphatase revealed mutant plants also contained elevated PtdIns(4,5)P_2 and Ins(1,4,5)P_3 [34]. Thus, in both of these studies, changes in PtdIns(4,5)P_2 yielded large changes in Ins(1,4,5)P_3, indicating the possibility that increasing substrate for PLC is enough to trigger activation of phospholipase activity.

8.4 Differences in PtdInsP Levels in Plants

An additional factor that impacts PLC function in plants is the difference in presence and amount of specific phosphoinositides. By radiolabelling with ^{3}H-inositol or ^{32}P-orthophosphate, plants have been characterized as having ~20-fold more PtdIns4P as compared to PtdIns(4,5)P_2 [1, 35]. A similar finding is apparent when GFP biosensors are used to visualize PtdIns4P as compared to PtdIns(4,5)P_2 within the plant cell [36]. This ratio is closer to 1:2 in signaling animal cells [35]. This difference has prompted some to speculate that both PtdIns4P and PtdIns(4,5)P_2 may have different functions in plant cells [37]. One intriguing speculation is that PtdIns4P, as the more abundant molecule, serves as the predominant substrate for PLC hydrolysis [37]. Interesting, a recent report investigating the role of both molecules in animal cells shows that the majority of plasma membrane PtdIns4P is not required for PtdIns(4,5)P_2 synthesis. Instead, the authors suggest that most of the PtdIns4P present serves to fulfill requirements for polyvalent anionic lipids, such as binding to plasma membrane proteins, leaving PtdIns(4,5)P_2 free to undergo rapid turnover and regulate specific effector proteins [38]. Thus the ratio of signaling PtdIns4P to PtdIns(4,5)P_2 in any organism might be different than expected from the absolute mass values.

There is an incredible expansion in the number of genes plants use to synthesize PtdIns4P and PtdIns(4,5)P, and as well, in the number of genes that encode phosphoinositide phosphatases [37, 39]. There are 12 predicted PtdIns4-kinase genes and 8 predicted PtdIns4P-5-kinases, although not all have been yet examined biochemically. Some of these genes are regulated at the level of transcription within specific tissues or organs, thus giving tissue-specific ability to synthesize these molecules.

As is true in animal cells, PtdInsPs may function as second messengers independent of their role in InsP production. Many studies in plants have indicated roles for PtdInsPs [22, 36, 40–52]. Specifically, phosphate addition and removal on PtdIns and PtdIns(4)P likely drives some types of membrane and vesicular trafficking in plant cells as seen by the impact of mutations in various PtdIns kinases and PtdInsP phosphatases on root hair development, a tip growth process driven by membrane trafficking [53]. In addition, proteins that bind to PtdInsPs have been identified and found to play a role in diverse signaling and developmental processes [4]. One interesting and controversial role for PtdIns(3)P is in binding of certain Oomycete pathogen effector proteins, which suggests that PtdInsP-binding is required for the intracellular virulence-enhancing activity of the effector [54–56]. Another intriguing difference between plants and animals with respect to PtdInsP function is the absence of PtdIns(3,4,5)P_3, which has never been found in plant cells, even though a recombinant plant PtdInsP kinase can produce this molecule *in vitro* [57].

8.5 The Roles of DAG and Ins(1,4,5)P3 May Be Different in Plants

When PLC hydrolyzes PtdIns(4,5)P_2 in response to signals, the result is production of diacylglycerol (DAG) and Ins(1,4,5)P_3 [27]. DAG activates protein kinase C near the membrane, and protein kinase C can phosphorylate many target substrate

proteins in response [58]. These classic second messengers in animal cells are also present in plant cells, although key differences exist. Even though several labs have identified plant PKC-like activities or genes [59, 60], no obvious, highly homologous protein kinase C protein or gene that would encode such a protein has ever been identified or purified from plants. Even more important, DAG has not been shown to activate any purified plant protein kinase. Thus, the canonical role of DAG as a protein kinase activator probably does not exist in plants. DAG has been demonstrated to induce both ion pumping in patch-clamped guard cell protoplasts and to promote opening of intact stomata [61], so it can perturb plant cell biology. Evidence exists that DAG produced from PLC hydrolysis of PtdIns(4,5)P_2 can be phosphorylated by a DAG kinase resulting in phosphatidic acid (PtdOH) production [62]. PtdOH is an abundant second messenger and has been linked to many diverse cellular processes, including the response to drought, cold, and pathogens [63].

The role of Ins(1,4,5)P_3 in plant signaling is also controversial. There is no evidence of a canonical Ins(1,4,5)P_3 intracellular receptor in plants, although Ins(1,4,5)P_3 binding to the plant vacuole was nicely delineated several years ago, and Ca^{2+} increases in response to external stimuli have been well-studied in plants [64, 65]. This may be similar to the situation in the related parasites, Apicomplexa, in which Ins(1,4,5)P_3 can be measured, and is seen to effect intracellular Ca^{2+} release, yet no Ins(1,4,5)P_3 receptor has been identified [66]. Even with many different sequences of plant genomes available, no genes have been identified which are predicted to encode a Ins(1,4,5)P_3 receptor, even though a putative Ins(1,4,5)P_3 receptor has been identified in the flagellar proteome of *Chlamydomonas* [67]. Therefore, if such a gene product exists in plants, it must be very different from the animal counterpart in nucleic acid sequence [68].

Guard cells are a set of specialized cells that form a pore and allow for gas exchange in plants, and important function especially during drought. Guard cells have been well-studied with respect to their phosphoinositide signaling pathway, and offer evidence for Ins(1,4,5)P_3 function. Guard cells undergo an initial increase in cytosolic Ca^{2+} concentration that occurs within minutes of ABA exposure [69–71]. This rapid Ca^{2+} increase is preceded by an increase in Ins(1,4,5)P_3 [72], and is dependent on increased PLC activity in guard cells [73] and in suspension cells [74]. Microinjection of caged Ins(1,4,5)P_3 into guard cells has been shown to be sufficient for closure [75]. Other studies in plant and algae cells also support a role for Ins(1,4,5)P_3 in triggering physiological events [76–79]. A Ca^{2+}-sensing receptor (CAS) has been identified that may regulate the concentration of Ins(1,4,5)P_3 via indirect means [80]. Since CAS has been localized to the chloroplast it has been suggested that CAS facilitates calcium release from this organelle [81].

8.5.1 Differences in Enzymes That Metabolize Ins(1,4,5)P_3

Supporting an independent signaling role for Ins(1,4,5)P_3 in plants, is the presence of a large family of inositol polyphosphate 5-phosphatases (5PTases; EC 3.1.3.56). These 5PTases exist in yeast and animals, where they function to metabolize Ins(1,4,5)P_3 and PtdInsPs containing a 5-phosphate [82, 83]. However, one key difference is that all plant genomes queried contain two types of predicted 5PTases [23]. Both types of plant 5PTases contain the highly conserved catalytic domain shared in animal and yeast 5PTases that allow for hydrolysis of 5-phosphates from inositol-containing substrates including Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, and PtdIns(5)P, PtdIns(4,5)P_2, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3 [84, 85]. Plants also encode a second set of 5PTases, that contain an additional large, N-terminus containing five to six WD40 repeats [86]. WD40 repeats are found in a number of eukaryotic regulatory proteins, where they form a stable β-propeller-like platform to which other proteins can bind either stably or reversibly. My group has hypothesized that the presence of WD40 domains in plant 5PTases reflects the ability of these 5PTases to participate in signaling protein complexes. Evidence supporting this idea

comes from studies on the WD40 domain of the At5PTase13 protein that binds to the sucrose non-fermenting like kinase 1.1 (SnRK1.1) [87]. This interaction appears to protect SnRK1.1 from proteasomal degradation when sugars are in limited supply [87]. Since SnRK1.1 is an important energy/stress sensor that has been linked to lifespan determination in plants [88–90], the regulation of SnRK1.1 by At5PTase13 may indicate a role for inositol signaling in these processes. It is not known if other At5PTases (Group B; At5PTase12, At5PTase14, and FRA3) have protein binding partners, however, the WD40 domains are different enough such that it is possible that binding and protection of different proteasomal target proteins is a common function of these 5PTases [91].

The substrate preferences of many of the 15 *Arabidopsis* 5PTases have been characterized, and the biochemistry of these enzymes is similar to what has been reported for the yeast and animal enzymes [5, 51, 86, 92–96]. By examining plants containing either a gain or loss of function in specific 5PTases, it has been established that 5PTase hydrolysis of Ins(1,4,5)P_3 or PtdIns(4,5)P_2 are critical for plant development and signaling. Several studies link 5PTase alterations to cotyledon vascular pattern [51, 97], fiber cell development within the stem [96], root hair initiation [98], blue light responses and phototropin 1 signaling [99], nutrient/energy signaling [87], gravitropism, vesicular trafficking required for PIN1 and PIN2 movements and subsequent polar auxin transport [100], oxidative stress signaling [95], ABA signaling [5, 7, 101], and drought tolerance [9, 11]. Together, the mutants and transgenic plants containing altered inositol signaling and the others described in this section illustrate that proper regulation of steady-state Ins(1,4,5)P_3 and PtdIns(4,5)P_2 levels is required for normal growth and development in plants.

8.6 Inositol Hexakisphosphate Is a Signaling Molecule in Plants

It may seem odd that Ins(1,4,5)P_3 is being debated as to whether or not it serves as a second messenger in plants. It is interesting to note that that yeast most likely does not use Ins(1,4,5)P_3 to stimulate intracellular Ca^{2+} release. In fact, yeast appears to use the Ins(1,4,5)P_3 resulting from PLC-driven hydrolysis of PtdIns(4,5)P_2 solely for the purpose of producing Ins(1,2,3,4,5,6)P_6, or inositol hexakisphosphate ($InsP_6$) [102]. $InsP_6$ is found in many organisms; in plants $InsP_6$ is the primary storage form of phosphorus in seeds [103], although all plant cells likely contain much lower amounts of $InsP_6$. In yeast and perhaps other organisms, $InsP_6$ serves to regulate several nuclear processes, including mRNA export from the nucleus, chromatin remodeling, and telomere length [104]. Given the evolutionary relationship between yeast and plants, it would not be surprising if plants used Ins(1,4,5)P_3 to build $InsP_6$ second messengers, which has been suggested by others [37].

The evidence for $InsP_6$ as the key second messenger arising from production of Ins(1,4,5)P_3, is contained in elegant work from two publications investigating $InsP_6$ signaling. From this work, it was shown that ABA results in an increase in $InsP_6$ in guard cells, and microinjected $InsP_6$ results in intracellular Ca^{2+}release from the vacuole in these same cells [105, 106]. Given that $InsP_6$ was more effective at Ca^{2+} release in this work, the authors speculated that Ins(1,4,5)P_3 may be converted to $InsP_6$ in this system [106]. One unresolved issue is the mechanism of how $InsP_6$ triggers intracellular Ca^{2+} release. Indeed, no $InsP_6$ receptor has been found in plants, or in any other organism. The lack of an InsP-regulated calcium channel in plants, thus, is a major difference between plants and animals. Clarification of this issue awaits a biochemical and/or genetic strategy to delineate the molecules required for InsP-gated Ca^{2+} release in plant cells.

8.6.1 Plants Have a Unique $InsP_6$ Synthesis Pathway

Besides a role as a second messenger effecting intracellular Ca^{2+} release, $InsP_6$ is also a major storage form of phosphate in the plant cell [103]. A major difference between plants and animals and yeast is the existence of a unique plant pathway for $InsP_6$ synthesis. $InsP_6$ is gaining

prominence in animals as an anti-cancer compound on one hand [107, 108], and an anti-nutrient in the GI tract of some animals [109]. $InsP_6$ is a highly charged molecule, and as such, can chelate cations such as Fe^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+}. In plants, large amounts of $InsP_6$ are synthesized in seeds, and much of this probably serves as a phosphate storage reserve [103, 110]. Other plant tissues also synthesize $InsP_6$, although seeds can accumulate 1,000-fold $InsP_6$ more than vegetative tissues [111, 112]. An intense area of investigation currently is to aimed at understanding the role of $InsP_6$, such as where and when is it synthesized, how does it get transported throughout the cell, and how does it affect signaling processes? For each of these questions tissue-specificity may be important, as different mechanisms may exist to regulate the signaling versus storage pool of $InsP_6$ in plants.

The route of synthesis of $InsP_6$ in plants is not completely characterized, but it is known that two pathways exist. The first is shared with animals and yeast and is called the lipid-dependent pathway, and has already been partially described (Fig. 8.1). This pathway utilizes PtdInsP precursors acted on by PLC to produce $Ins(1,4,5)P_3$. Subsequent phosphorylation of $Ins(1,4,5)P_3$ by a group of specific inositol kinases (IPKs) takes place, resulting in $InsP_6$ [113]. This process can be reconstituted *in vitro* by adding two purified IPKs to substrate $Ins(1,4,5)P_3$ [113], although the details of the *in vivo* pathway and IPK action are still not clear in various tissues, even though the IPKs have been well-studied in plants [114]. Thus, Fig. 8.1 offers only one possible route for $InsP_6$ synthesis in the lipid-dependent pathway. *Arabidopsis* contains two different IPK2 genes (*IPK2*α and *IPK2*β) [115]. Recently the X-ray crystal structure for the *Arabidopsis* IPK2α protein was solved, and it is important to note that this enzyme shares amino acid similarity with the animal inositol phosphate multikinases (IPMKs) although the plant enzymes have important differences in substrate specificity [116]. Conversion of $Ins(1,4,5)P_3$ to $Ins(1,4,5,6)P_4$ can be catalyzed by the *IPK2* gene products utilizing IPK2's 6-kinase activity [115, 117]. Function of IPK2β was delineated in the whole plant by knocking out the *IPK2*β gene. Seeds from *ipk2b* mutants contain a 35 % reduction in seed $InsP_6$ [113], indicating a role for this enzyme in $InsP_6$ synthesis.

The next IPK in the pathway is called IPK1 and is encoded by a single gene in *Arabidopsis* [113]. Disruption of *IPK1* gene function results in an 83 % reduction in seed $InsP_6$ levels, and knocking out both IPK2β and IPK1 together reduce seed $InsP_6$ by >95 % [113]. Thus, these two IPK enzymes are necessary for synthesis of seed $InsP_6$. The IPK1 enzyme is also required for seedling $InsP_6$ synthesis, with *ipk1-1* mutants containing a 93 % reduction in seedling $InsP_6$. In contrast the *ipk2*β mutant had no alterations in seedling $InsP_6$, which suggests that the *IPK2*α gene functions to maintain seedling $InsP_6$ in *ipk2*β mutants [113].

The other possible route from second messenger $Ins(1,4,5)P_3$ to $InsP_6$ would require phosphorylation at the C3-position on the inositol ring, however no purified protein or gene can be found to support this. The final evidence considered for linking $Ins(1,4,5)P_3$ and $InsP_6$ comes from transgenic plants studies. Transgenic plants and plant cells engineered to constitutively break down $InsP_3$ or synthesize $PtdInsP_2$ contain changes in $InsP_6$. In both of the studies performed, changes in $Ins(1,4,5)P_3$ are mirrored by changes in $InsP_6$, thus there is a correlation between these two molecules [11, 30, 44].

The second pathway for $InsP_6$ production is unique to plants and related organisms (gray box, Fig. 8.1). In the lipid-independent pathway, $InsP_6$ is synthesized *de novo* through the sequential phosphorylation of *myo*-inositol [118], the penultimate product being $InsP_6$ [119] (Fig. 8.1). There are several unique aspects of this pathway that bear mentioning. While the latter part of the pathway makes use of the previously mentioned IPK1 and IPK2 enzymes, the beginning steps in the pathway utilize novel gene products. Foremost is the *myo*-inositol kinase (MIK) enzyme, which was discovered as part of efforts to identify mutant plants with low phytic acid (*lpa*) [111, 112]. Given the chelating and anti-nutritional properties of $InsP_6$, several excellent programs have been focused on identifying such plants over the years [112]. $InsP_6$ also presents a problem in non-ruminating animal production (*i.e.*, chicken

and pigs), as these animals consume large amounts of seed meal, and then have no ability to digest $InsP_6$. The result is phosphate pollution of soils and nearby waterways [120], which in the Chesapeake Bay watershed and other areas of the U.S., has resulted in water quality issues. Maize (corn) plants deficient in the *MIK* gene accumulate 50 % less seed $InsP_6$, and do not have a corresponding build-up of other InsPs [118]. The MIK gene product was shown to phosphorylate free *myo*-inositol *in vitro* [118]. The impact of an MIK mutation on vegetative $InsP_6$ levels has not been yet reported.

An alternative route for initiating the lipid-independent pathway of $InsP_6$ synthesis could take place with the action of the *myo*-inositol phosphate synthase enzyme (MIPS) (Fig. 8.1). In plants, the first MIPS mutants were identified as *lpa* mutants [121]. As crop species typically have an even greater number of *MIPS* genes as compared to the three *MIPS* genes in *Arabidopsis*, mutation of seed-specific *MIPS* genes can reduce seed *myo*-inositol and $InsP_6$ levels without greatly impacting levels of *myo*-inositol elsewhere in the plant. Characterized maize and barley MIPS mutations give rise to an approximate 30–50 % decrease in seed $InsP_6$ levels, although uncharacterized alleles in barley have an even greater reduction in seed $InsP_6$ [122]. Since these mutations in crop species disable a seed-specific MIPS, this 30–50 % decrease in seed $InsP_6$ could represent the total amount of $InsP_6$ synthesized from the lipid-independent pathway. Mutant plants with a loss of function in PLC could, in theory, address the $InsP_6$ produced by the lipid-dependent $InsP_6$ pathway. Genetic redundancy in PLC genes along with the lethality of key *Arabidopsis PLC* genes are significant issues in such a strategy, although groups are most likely working on this important point.

Two critical steps in the lipid-independent pathway are still uncharacterized. The genes and enzymes functioning to phosphorylate InsP and $InsP_2$ have yet to be identified (Fig. 8.1). It is possible that on-going low *lpa* genetic screens in crop species will shed light on one or both of these steps. In addition, studies on *Arabidopsis* mutants impaired in various inositol phosphatases might also present an avenue for understanding regulation of the so-called lower inositol phosphates [101, 123]. Of interest here is the fact that a potato IPK2 enzyme has been shown to phosphorylate an $InsP_2$ substrate *in vitro* [124]. It has also been suggested that unique InsP or $InsP_2$, resulting from PLC activity, might contribute to substrates used for $InsP_6$ synthesis [37].

8.6.2 Potential Unique Signaling Roles of $InsP_6$ in Plants

One $InsP_6$ has been synthesized in the plant cell, there are some unique functions that can impact signaling. The first is the issue of transport. An ABC transporter has been identified that specifically transports $InsP_6$ across a plant membrane, most likely the vacuole. The MRP5 $InsP_6$ transporter was elegantly shown to be capable of this transport in a heterologous system, and a genetic loss of function in *Arabidopsis* and maize results in an $InsP_6$ reduction in seeds [125]. Work in *Arabidopsis* indicates the likelihood that during seed development, lack of MRP5 transport of $InsP_6$ results in less $InsP_6$ accumulation in the vacuole, the precursor to the location for stored $InsP_6$ in the seed [125]. *Atmrp5* mutants also have insensitivity to ABA, perhaps because, in addition to high expression in developing seed, the *MRP5* gene is highly expressed in guard cells (a major site of ABA action) [125]. How this altered transport impacts InsP signaling throughout the *Arabidopsis* or maize (ZmMRP4) mutants is not yet known.

A second intriguing role for $InsP_6$ is its conversion to the high energy diphospho (PP)- or triphospho (PPP)-InsP molecules, including $InsP_7$ and $InsP_8$ [126–128]. These molecules have recently been linked to energy-sensing in yeast and various functions in animal cells [129–132]. In plants, molecules with the hallmarks of $InsP_7$ and $InsP_8$ have been reported, but not yet carefully examined [122, 133]. Given that plant seeds contain tremendous amounts of $InsP_6$, it seems plausible that conversion to $InsP_7$ and

$InsP_8$ could occur, if these signaling molecules are used in plants. It is noteworthy that plant genomes contain genes predicted to encode kinases related to the VIP kinases in yeast that phosphorylate $InsP_6$.

A final unique role for $InsP_6$ in plants concerns the potential for regulation of plant hormone receptors [134, 135]. The discovery that higher InsPs, including Ins(1,2,4,5,6)P_5 and $InsP_6$, bind to a similar type of F-box protein involved in jasmonic acid (JA) and auxin reception, suggests a role for these InsPs in JA and auxin signaling. Specifically, the Transport Inhibitor Response 1 (TIR1) protein functions as the auxin receptor, by binding auxin and mediating protein degradation of transcription repressors [136]. The crystal structure of the *Arabidopsis* TIR1 complex revealed that the leucine-rich repeat domain of TIR1 is complexed to $InsP_6$ [135]. The crystal structure of the JA receptor revealed a similar binding pocket comprised of Coronatine Insensitive 1 (COI1), bound to transcriptional repressors known as jasmonate ZIM-domain (JAZ) proteins. This pocket also contains $InsP_5$ [134]. Remarkably, the $InsP_6$ binding site on TIR1 and the $InsP_5$ binding site on COI1 are strikingly similar [134], perhaps indicating the status of $InsP_5$ and $InsP_6$ as co-factors. The recent JA receptor study explored this concept using basic biochemical approaches to remove $InsP_5$ from the receptor, followed by addition experiments to detect InsPs that were capable of restoring ligand (*i.e.*, jasmonic acid-isoleucine; JA-ILE) binding. The results indicate that Ins(1,2,4,5,6)P_5 and Ins(1,4,5,6)P_4 support JA-ILE ligand binding, but $InsP_6$ and Ins(1,4,5)P_3 do not [134]. A recent study utilizing HPLC separation of ^{3}H-InsPs verified that potato leaf discs are capable of generating a potential pool of the Ins(1,4,5,6)P_4 ligand by breaking down ^{3}H-Ins(1,3,4,5,6)P_5 [137].

The functionality of $InsP_5$ within the JA receptor complex has also been investigated using the yeast two-hybrid system, which showed that COI1 binding to JAZ9, is influenced by $InsP_5$ [138]. Mutated COI1 variants altered in the putative $InsP_5$ binding site displayed a reduced capability to rescue JA-mediated root growth inhibition or silique development in *Arabidopsis coi1* mutants. As well *ipk1* mutants [113] were shown to have increased JA responses and defensive capabilities via COI1-mediated processes, as seen by elevated wound-induced gene expression, defense against caterpillars or root growth inhibition by JA [138]. Together these data indicate that $InsP_5$ contributes to COI1 function in plants.

8.7 Synthesis of *myo*-Inositol Is More Complex in Plants

The MIPS enzyme has already been described as an entry point into understanding lipid-independent $InsP_6$ synthesis. Figure 8.1 indicates the central role MIPS has in other aspects of phosphoinositide signaling as in animals and yeast, both phosphoinositides and inositol phosphates are derived via the synthesis of *myo*-inositol. In plants, *myo*-inositol has historically been added to plant tissue culture media to stimulate growth and *myo*-inositol has been documented to stimulate cell division in certain plant cells grown in culture [139]. Additionally, plants use *myo*-inositol as a scaffold to synthesize many other unique metabolites. Some of the unique roles of *myo*-inositol and methylated derivatives are to serve as osmoprotectants in certain species [140]. There is evidence that plants utilize other isomers of *myo*-inositol, such as D-*chiro* inositol found in abundance in soy milk, however, the chief isoform in most plant tissues is the *myo* isoform.

One chief difference in inositol synthesis in plants is that the rate-limiting enzyme, MIPS, is encoded by a multigene family [141–149]. This gives plants a way to differentially regulate *myo*-inositol synthesis, which is useful considering that inositol has different functions apart from phosphoinositide signaling. In the model plant, *Arabidopsis thaliana*, there are three MIPS genes that encode enzymes with very similar substrate binding and rate constants [143]. Most of the regulation of MIPS has been shown to occur at the level of transcription, with the MIPS1 gene

having a broad pattern of expression throughout different cell types and tissues, and the MIPS2 and MIPS3 genes being spatially restricted to vascular cells at specific stages of development [143]. This pattern indicates the possibility that MIPS2 and MIPS3 specifically contribute to *myo*-inositol synthesis within or near vascular tissues, which may facilitate transport of *myo*-inositol throughout the plant. All three MIPS enzymes are located in the cytoplasm of the plant cell [143], which suggests that they act on glucose 6 phosphate (G6P) substrate and produce InsP product within the cytoplasm. Other studies have revealed that individual MIPS genes in other species are also regulated at the level of transcription, with spatially restricted patterns for individual genes in various species [142, 143, 145, 146, 150, 151].

There is also expansion of the genes encoding the second enzyme required for *myo*-inositol synthesis, the *myo*-inositol monophosphatase (IMP; EC 3.1.3.25) [152–157]. IMP catalyzes the dephosphorylation of InsP to *myo*-inositol. Both plants and animals have multiple IMP genes, although, in plants besides the canonical, well-conserved IMP there are two other IMP-like (*IMPL*) genes [147, 158]. Both *Arabidopsis IMPL* genes encode chloroplast-localized enzymes. It has been shown that one of the *Arabidopsis IMPL* genes most likely acts to dephosphorylate histidinol phosphate, one of the final steps required for histidine biosynthesis in plants [159]. The substrate for the IMPL1 enzyme is currently unknown, although it has been reported to have activity with InsP substrates [158]. The chloroplast location of IMPL1 suggests that *myo*-inositol synthesis may occur within this organelle. A previous report of a smaller, chloroplastic MIPS isoform in bean [142] may indicate accumulation of MIPS in this organelle under certain conditions not present in the *Arabidopsis* localization experiments. Thus, it is possible that chloroplasts contain both enzymes needed to synthesize *myo*-inositol from G6P. Given the prokaryotic nature of the chloroplast, and the lack of other critical enzymes in the phosphoinositide signaling pathway, it is unlikely that chloroplasts utilize InsPs for signaling purposes.

8.7.1 Genetic Mutants Lacking Inositol

Lack of *myo*-inositol synthesis in genetic mutants has yielded much information on the importance of *myo*-inositol. As in the yeast *ino1* mutants deficient in the single yeast MIPS gene [160], *Arabidopsis mips1* mutants have defects in accumulation of PtdIns [143]. In plants, this decrease in membrane PtdIns has two important developmental consequences. The first is related to the fact that plants synthesize inositol phosphoceramide (IPC) via a condensation reaction involving PtdIns and ceramide [161]. When PtdIns levels become limiting, as they do in *Atmips1* mutants, ceramide levels increase, resulting in unrestricted cell death [143]. This spontaneous cell death is similar what occurs in lesion-mimic mutants, which can lead to enhanced basal resistance to certain plant pathogens. Cell death is *Atmips1* mutants is dependent on the amount of light mutants receive [143], which is often the case with other lesion-mimic mutants [162]. Interestingly, these same *Atmips1* mutants have decreased *myo*-inositol [143], raffinose and galactinol levels, indicating the importance of MIPS1 overall, to driving cellular *myo*-inositol, raffinose and galactinol synthesis. A loss-of-function in *MIPS2* or *MIPS3* genes does not induce cell death [143], underscoring the minor role these enzymes have on overall inositol synthesis due to restricted patterns of expression.

Interestingly, *Atmips1* and *Atmips2* mutants, as well as potato antisense MIPS plants, contain reduced $InsP_6$ levels in vegetative tissues [163, 164]. *Atmips2* have increased susceptibility to pathogens, while *Atmips1* mutants containing lower leaf $InsP_6$ levels were not altered in their pathogen susceptibility [163]. Together, these data suggest that changes in overall $InsP_6$ levels alone cannot account for changes in pathogen susceptibility, and suggest that the MIPS2 enzyme may channel *myo*-inositol into synthesis of a specific pool of higher InsPs (*i.e.* $InsP_6$, $InsP_7$ or $InsP_8$) that regulate key defense pathways.

Depletion of *myo*-inositol in *Arabidopsis* mutants also impacts the transport of the major plant growth regulatory hormone, auxin. Auxin is synthesized and then transported to specific areas

of the plant, which results in normal growth and development [165]. Transport is dependent on efflux of auxin out of the plant cell, and is controlled by efflux proteins called PINs. PINs are integral membrane proteins that utilize vesicular trafficking to restrict their location to one side of the cells [166]. The lack of PtdIns in *Atmips1* mutants results in alterations in vesicular trafficking of specific PIN proteins [167, 168]. Interestingly, the embryo defective phenotype of *mips1/mips3* double mutants, which are reminiscent of auxin mutants, can be rescued by overexpression of the Phosphatidylinositol synthase 2 (*AtPIS2*) gene, which shows the dependence of trafficking defects on PtdIns and/or related molecules [167]. Interestingly, AtPIS1 and AtPIS2 have been shown to have different substrate preferences for cytidinediphospho-DAG species differing in fatty acid composition, with PIS2 preferring unsaturated substrates *in vitro* [169]. In addition, overexpression of either gene yields different PtdIns species with AtPIS1 overexpression resulting in lipids enriched in saturated or monounsaturated fatty acids [169]. This indicates that different AtPIS enzymes may preferentially produce different PtdIns species that could function in different signaling pathways in plants.

8.8 Summary

Plants, as mostly sessile organisms, have evolved different types of signal transduction pathways. Genome sequencing, along with biochemical and physiological approaches, have revealed conservation in many of the genes required to synthesize and metabolize InsP and PtdInsP molecules in plants. In many cases, expansion of gene families has resulted in much more genetic redundancy for genes such as the MIPS, PtdIns 4-Kinases and PtdIns4P 5-kinases in plants. However, in some cases, readily apparent homologues are not present (*i.e.,* an InsP-gated receptor), and unique genes are present that encode new synthetic pathways (*i.e.*, $InsP_6$ synthesis). This genomic information is supported by many excellent studies that have delineated key differences in the plant phosphoinositide pathway. Together, this information forms a picture in which plants share many of the yeast elements, and perhaps build on these to enable unique, plant-specific mechanisms. These unique plant-specific mechanisms include transport, storage, and hormone receptor binding of $InsP_6$, and formation of protein signaling complexes with InsP and PtdInsP-metabolizing enzymes.

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Phosphatidic Acid-Mediated Signaling

9

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Abstract

Phosphatidic acid (PA) is recognized as an important class of lipid messengers. The cellular PA levels are dynamic; PA is produced and metabolized by several enzymatic reactions, including different phospholipases, lipid kinases, and phosphatases. PA interacts with various proteins and the interactions may modulate enzyme catalytic activities and/or tether proteins to membranes. The PA-protein interactions are impacted by changes in cellular pH and other effectors, such as cations. PA is involved in a wide range of cellular processes, including vesicular trafficking, cytoskeletal organization, secretion, cell proliferation, and survival. Manipulations of different PA production reactions alter cellular and organismal response to a wide range of abiotic and biotic stresses. Further investigations of PA's function and mechanisms of action will advance not only the understanding of cell signaling networks but also may lead to biotechnological and pharmacological applications.

Keywords

Phosphatidic acid • Phospholipase D • Phospholipase C • Diacylglycerol kinase • Lipid signaling • Stress response

9.1 Introduction

Phosphatidic acid (PA) is a minor class of membrane lipids with phosphoryl glycerol esterified with two fatty acid chains (Fig. 9.1). PA has been known as a key intermediate in glycerolipid biosynthesis, in which PA is dephosphorylated to diacylglycerol (DAG) for the synthesis of phospholipids, galactolipids, and triacylglycerol. Only in recent years PA has been recognized as a class of lipid messengers. PA has been found to interact with various effector proteins to modulate their catalytic activities and/or membrane associations. The cellular levels of PA can be controlled by various phospholipases, lipid kinases, and phosphatases (Fig. 9.1). Genetic and pharmacological manipulations of PA levels in the cell have revealed a broad spectrum of cellular and physiological roles in cell signaling. PA and enzymes associated with

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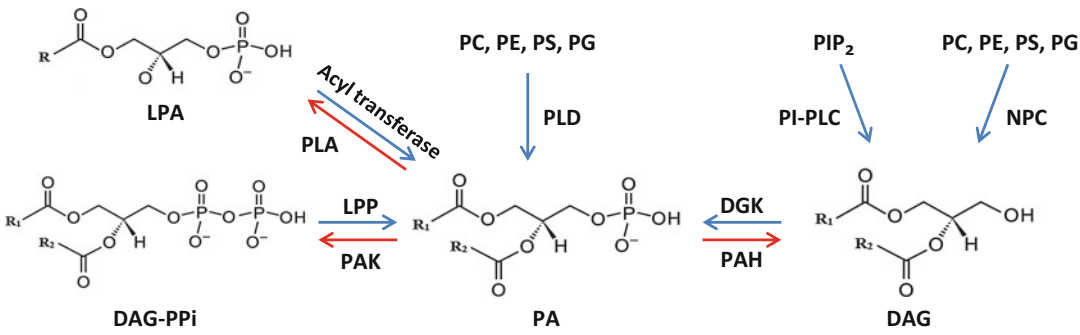

Fig. 9.1 Production and removal of phosphatidic acid (PA). Metabolic reactions and enzymes that produce and metabolize PA in the cell. The *blue arrows* indicate PA-producing pathways whereas the *red arrows* indicate PA-removing pathways. Enzymes are abbreviated as follows: *PLA* phospholipase A, *PLD* phospholipase D, *PI-PLC* phosphoinositide-phospholipase C, *NPC* non-specific PLC, *DGK* DAG kinase, *LPP* lipid phosphate phosphatase, *PAK* PA kinase, *PAH* PA phosphohydrolase. Metabolites are abbreviated as follows: *DAG* diacylglycerol, *DAG-PPi* DAG pyrophosphate, *LPA* lyso-PA, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PS* phosphatidylserine, *PG* phosphatidylglycerol, PIP_2 phosphatidylinositol 4,5-bisphosphate

PA signaling have been a subject for several recent reviews dealing with plants, animals, and yeast [39, 45, 51, 79, 94, 100, 104]. Here, we will briefly review the knowledge of PA-mediated signaling and highlight some recent development in the field.

9.2 Cellular Production of Signaling PA

Cellular PA levels are highly dynamic and its production and removal are mediated by several complex families of enzymes (Fig. 9.1). PA constitutes approximately 1 % of total membrane glycerolipids in plants, but its level is increased greatly under various stresses, such as wounding, freezing, drought, and pathogen elicitation [104]. The production of signaling PA in plants and animals is thought to occur via the activity of two major sets of lipid reactions: phospholipase D (PLD) and diacylglycerol kinase (DGK) that is often coupled with the activation of phospholipase C (PLC) ([39, 79]; Fig. 9.1). On the other hand, lipid phosphate phosphatase (LPP), PA phosphohydrolase, PA-phospholipases, and PA kinases in plants can remove PA (Fig. 9.1). These enzymes are comprised of multiple forms that are under complex controls and control the spatial and temporal regulation of signaling PA production. PA is present in various membranes and its signaling roles have associated with events occurring on the plasma, endoplasmic reticulum, mitochondrial, nuclear, and vacuolar membranes [29, 30, 79, 96].

9.2.1 Phospholipase D Family

PLD hydrolyzes common membrane phospholipids, such as phosphatidylcholine (PtdCho), to produce PA and a free head group. Multiple forms of PLDs have been described in plants and animals (Fig. 9.2a). The *Arabidopsis* genome contains 12 PLDs that are grouped into PLDα(3), β(2), γ(3), δ, ϵ, and ζ(2) [104]. Based on protein domains structures, they are divided into two subfamilies: C2-PLDs (α, β, γ, δ, and ϵ) that contain the C2, a Ca^{2+}–dependent phospholipid binding domain, and PX/PH-PLDs (ζ) that have the N-terminal phox homology (PX) and pleckstrin homology (PH) domains. C2-PLDs are unique to plants, whereas PX/PH-PLDs are conserved in plants and animals (Fig. 9.2a). The activities of various plants PLDs from *Arabidopsis* have been characterized [104]. Some of them have different requirements for cofactors, such as Ca^{2+}, phosphatidylinositol 4,5-bisphopshate (PIP_2), free fatty acids, and G proteins. Thus, the enzymes are activated differently in the cell. In

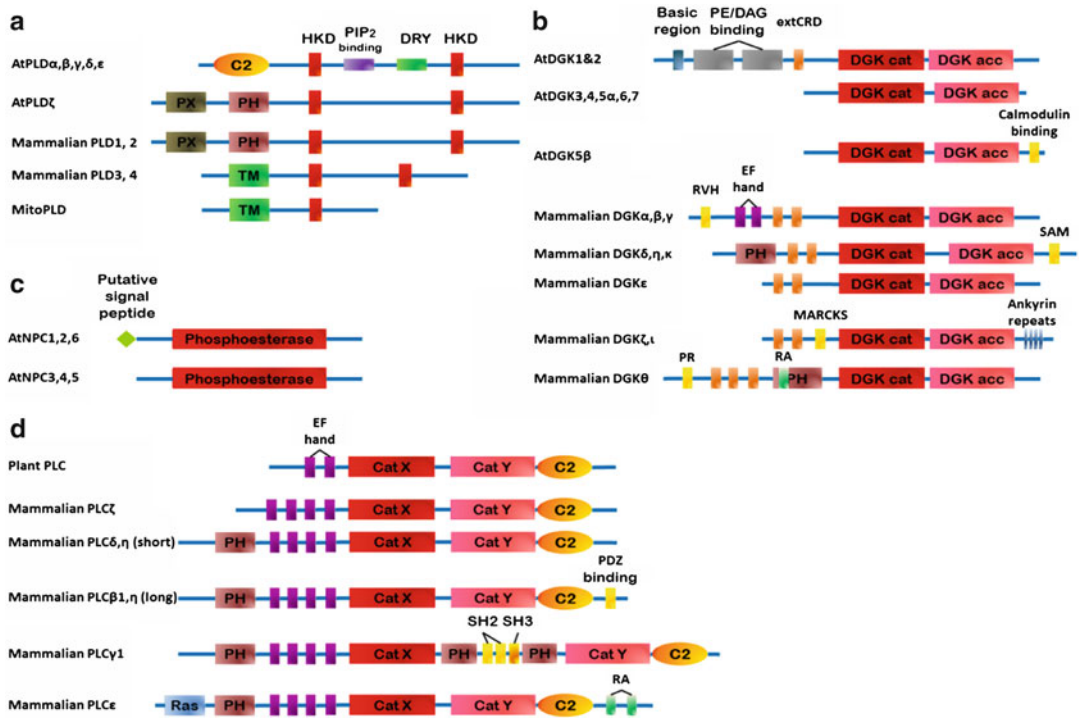

Fig. 9.2 Families of enzymes producing signaling PA and their domain structures. (**a**) The PLD families. (**b**) The DGK families. (**c**) The NPC (non-specific PLC) family in plants. (**d**) PI-PLC family. C2, Conserved two domain; HKD, PLD catalytic motif; *PX* phox domain, *PH* pleckstrin homology domain, *TM* transmembrane domain, *extCRD* extended cysteine-rich domain, *DGK cat* DGK catalytic domain, *DGK acc* DGK accessory domain, *RVH* recoverin homology-like domain, *SAM* sterile alpha motif, *MARCKS* full homology domain, *PR* proline-rich region, *RA* Ras-associated domain, *Cat X* PLC X catalytic domain, *Cat Y* PLC Y catalytic domain, *SH2* Src homology 2 domain, *SH3* Src homology 3 domain, *Ras* guanine-nucleotide-exchange factor domain for Ras

addition, several PLDs have distinctive subcellular localizations, thereby producing PA at specific cell membranes.

In addition to PLD1 and PLD2, MitoPLD is a new member of the PLD superfamily in mammalian cells [11]. MitoPLD has no sequence similarities to the above "typical" PLD family members, and instead, it is more similar to bacterial cardolipin synthase (Fig. 9.2a). It is associated with mitochondria via insertion of its N-terminal transmembrane domain into the outer mitochondria membrane. MitoPLD has a single PLD catalytic HxKxD motif and uses cardiolipin as substrate to produce PA [11]. Two other non-PtdCho hydrolyzing PLD family members, PLD3 and PLD4 (Fig. 9.2a), have been found in mammals, but their functions are not very well established [78, 113].

9.2.2 Diacylglycerol Kinase and Phospholipase C Families

DGK phosphorylates DAG, and multiple DGKs have been identified in mammals and plants ([2, 95]; Fig. 9.2b). *Arabidopsis* has seven DGKs that are grouped into three subgroups [25] whereas mammalian cells have ten DGKs that are grouped into five subgroups [26]. The domain structure of mammalian DGKs exhibits more structural diversity than plant DGKs (Fig. 9.2b). One DGK is found in bacteria and yeast [32, 33, 88]. Most DGKs use ATP as the phosphate donor to phosphorylate DAG to PA, however yeast DGK utilizes CTP [32].

The DGK phosphorylation of DAG is often coupled with the activation of PLC that produces DAG (Fig. 9.1). Plants have two distinctively

different PLC families: non-specific PLCs (NPCs) (Fig. 9.2c) and phosphoinositide (PI)-PLC (Fig. 9.2d). NPCs hydrolyze common membrane lipids, such as PtdCho and phosphatidylethanolamine (PtdEth), whereas PI-PLCs hydrolyze PIP_2 to DAG and inositol 1,4,5-trisphosphate. The *Arabidopsis* genome contains nine genes for PI-PLCs and six for NPCs (Fig. 9.2c and d; [80, 104]). The two PLC families share no sequence or domain similarities. DAG is a well-documented lipid messenger in animal cells, but its signaling function in plants is not well understood. A recent study on NPC4 indicates that DAG is involved in maintaining stomatal opening [80]. However, under drought or high salinity, DAG is phosphorylated to PA that acts as a signaling messenger.

9.3 Modes of PA Action as Cellular Mediators

Recent results have begun to shed light to how PA acts a messenger in the cells although many details in this regard remain to be elucidated. PA interaction with proteins has been identified as a main mode of PA action. The interaction may modulate the catalytic activity of target proteins, tether proteins to the membrane, or promote the formation and/or stability of protein complexes. Recent analyses have provided physicochemical evidence for how PA differs from other membrane lipids in its ability to interact with proteins (Fig. 9.3a). In addition, PA has an inverse cone packing and its accumulation increases the propensity of membrane structural perturbation (Fig. 9.3). The structural effect may affect membrane-protein interactions and membrane budding and fusion events (Fig. 9.3b).

9.3.1 PA Interaction with Proteins

PA has been reported to bind a number of proteins that are involved in a wide range of cellular processes in plants and animals (Tables 9.1 and 9.2). Different approaches have been used to identify and characterize PA-protein interactions,

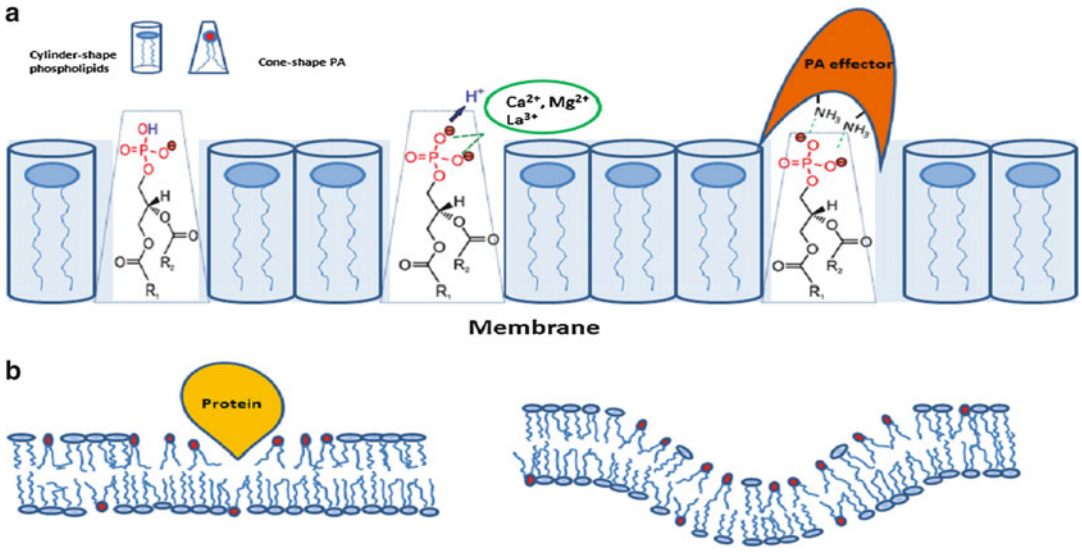

Fig. 9.3 PA charges and their effect on protein interaction and membrane structure. (**a**) Dual negative charges of PA due to loss of one or two protons. The negative one and two charges are influenced by cellular pH and the presence of cations. The electric static and hydrogen bonding switch facilitates PA interaction with basic amino acid residues on PA-effector proteins. (**b**) PA-mediated alterations of membrane structures that promote protein association with membranes (*left*) and lead to negative membrane curvature (*right*), potentially involved in membrane budding and fusion in vesicular trafficking. The *red head* molecules represent PA, whereas the *blue head* molecules are other phospholipids

Table 9.1 PA binding proteins in plants

PA-binding proteins	PA effect on proteins	Protein functions	Reference
14-3-3 protein	Inhibition	Protein interaction, activation of membrane H^+-ATPase	[9]
ABI1	Translocation	Protein phosphatase 2 family, ABA signaling	[117]
AGD7	Activation	Arf GTPase activation protein	[69]
AtPDK1	Activation	Phospholipid-binding 3-phosphoinositide-dependent protein kinase, pathogen response	[1]
CdeT11-24	Binding	The late embryogenesis abundant-like protein	[81]
CP	Inhibition	Heterodimeric capping protein, F-actin binding and inhibition	[40]
CTR1	Inhibition	Raf-like protein kinase	[99]
MAP65-1	Activation	Microtubule-associated protein	[116]
MGD1	Activation	MGDG synthase	[16]
MPK6	Activation	Mitogen-activated protein kinase, phosphorylates SOS1 in salt response	[115]
PEPC	Inhibition	Phosphoenolpyruvate carboxylase	[98]
PP1Cγ	Inhibition	Protein phosphatase 1 family	[46]
RbohD/F	Activation	NADPH oxidases	[118]
SnRK2.10/ SnRK2.4	Translocation	The sucrose non-fermenting-1-related protein kinases	[68]
SPHK1	Activation	Phytosphingosine kinase, ABA signaling	[28]
TGD2	Binding	PA transportation to chloroplast	[3]
TGD4	Binding	Lipid trafficking	[106]
ZmCPK11	Activation, stimulate transcription	Calcium-dependent protein kinase, plant wounding response	[50]

Table 9.2 PA binding proteins in animals

PA-binding proteins	PA effect on proteins	Protein functions	Reference
AGAP1	Activation	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	[75]
AGK	Produce	Acylglycerol kinase	[7]
ANXA3	Binding	Annexin A3	[18]
APOL1	Binding	Apolipoprotein L, 1	[103]
ARF1	Binding	ADP-ribosylation factor 1	[67]
ARF6	Binding	ADP-ribosylation factor 6	[67]
BAD	Translocation	BCL2-associated agonist of cell death	[34]
CDC42	Binding	Cell division cycle 42 (GTP binding protein)	[18]
COPB1	Binding	Coatomer protein complex, subunit beta 1	[67]
DDHD1	Activation	DDHD domain containing 1	[109]
DGKA	PA production	Diacylglycerol kinase, alpha 80 kDa	[36]
DNM1	Translocation	Dynamin 1	[8]
DOCK2	Translocation	Dedicator of cytokinesis 2	[76]
FER	Translocation	Fer (fps/fes related) tyrosine kinase	[44]
FGR	Activation	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	[93]
HMGB1	Binding	High-mobility group box 1	[90]
KIF5B	Binding	Kinesin family member 5B	[67]
MBP	Binding	Myelin basic protein	[73]
MTMR6	Binding	Myotubularin related protein 6	[121]

(continued)

Table 9.2 (continued)

PA-binding proteins	PA effect on proteins	Protein functions	Reference
mTOR	Activation	Mechanistic target of rapamycin	[17]
NCF1	Activation	Neutrophil cytosolic factor 1	[83]
NR5A1	Activation	Nuclear receptor subfamily 5A, member 1	[57]
NSF	Binding	N-ethylmaleimide-sensitive factor	[67]
OSBPL1A	Binding	Oxysterol binding protein-like 1A	[108]
OSBPL2	Binding	Oxysterol binding protein-like 2	[108]
PDE4A	Translocation	Phosphodiesterase 4A, cAMP-specific	[4]
PDE4D	Activation	Phosphodiesterase 4D, cAMP-specific	[27]
PIP5K1A	Activation	PI-4-phosphate 5-kinase, type Iα	[72]
PLCD1	Activation	Phospholipase C, δ1	[35]
PLD1	Binding	Phospholipase D1, phosphatidylcholine-specific	[97]
PPP1CB	Inhibition	Protein phosphatase 1, catalytic subunit, β	[42]
PPP1CC	Inhibition	Protein phosphatase 1, catalytic subunit, γ	[46, 47]
PRKCA	Activation	Protein kinase Cα	[23]
PRKCE	Translocation, Activation	Protein kinase C	[48]
PRKCZ	Activation	Protein kinase Cζ	[62]
PTPN6	Activation	Protein tyrosine phosphatase, non-receptor type 6	[21]
RAC1	Translocation	Ras-related C3 botulinum toxin substrate 1	[10]
RAC2	Binding	Ras-related C3 botulinum toxin substrate 2	[18]
RAF1	Translocation	v-raf-1 murine leukemia viral oncogene homolog 1	[86]
RGS4	Inhibition	Regulator of G-protein signaling 4	[77]
RhoG	Binding	ras homolog gene family, member G	[18]
RPS6KB1	Activation	Ribosomal protein p70S6 kinase	[56]
SOS1	Translocation	Son of sevenless homolog 1	[120]
SPHK1	Translocation	Sphingosine kinase 1	[14]
TP73	Binding	Tumor protein p73	[6]
TPPP	Activation	Tubulin polymerization promoting protein	[111]
UCHL1	Binding	Ubiquitin carboxyl-terminal esterase L1	[74]
VAT1	Translocation	Vesicle amine transport protein 1 homolog	[18]
VCL	Binding	Vinculin	[43]
VTN	Binding	Vitronectin	[112]

which include nitrocellulose filter binding (commonly referred to as lipid strips or fat-blotting), liposomal binding, and PA immobilized to beads or membranes followed by mass spectrometry. In some cases, the PA interaction has been quantitatively characterized using surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and other approaches [28, 70, 105]. Many PA-interacting proteins exhibit binding specificity to PA but not to other membrane phospholipid classes, such as PtdCho, PtdEth, phosphatidylglycerol (PG), phosphatidylinositol (PtdIns or PI), or phosphatidylserine (PtdSer). Moreover, PA is comprised of different molecular species due to the two fatty acid chains that may differ in the number of carbons and double bonds. Different PA molecular species may display different affinities to bind different proteins [28].

Unlike phosphoinositide-interacting proteins that have defined structural folds, such as PH and PX domains, the binding motifs of effector proteins with PA are not highly conserved among those that have been discovered so far. In general, positively charged amino acids such as arginine and lysine are involved in binding to the negatively charged phosphate head group of

PA (Fig. 9.3a). For example, PA binding to the mammalian protein kinase Raf-1 requires a motif of 35 amino acid residues [86]. A similar PA binding motif is found in the protein phosphatase 2C ABI1 (Abscisic Acid Insensitive 1) and protein kinase CTR1 (Constitutive Triple Response 1) in plants [99, 104, 117]. The motif contains polybasic amino acid residues followed by a stretch of hydrophobic amino acids, suggesting a specific structural fold, rather than a simple electrostatic interaction, is required for a PA-effector protein interaction. An "electrostatic/hydrophobic switch" could be a mechanism for PA interaction with some proteins, such as ABI1 and Raf1. In this model, the PA and protein interaction is initiated by electrostatic attraction followed by hydrophobic partitioning of the hydrophobic residues of the PA-binding region to membranes ([104]; Fig. 9.3b).

Recent physicochemical studies of PA structure and behavior have led to the proposition of an "electrostatic/hydrogen bond switch mechanism" for PA interaction with effector proteins ([54]; Fig. 9.3a). In this model, a PA-binding protein first binds to a membrane region with negative charges via electrostatic interactions. Then, the protein swings to the bilayer until it encounters a protonated PA which is −1 charged. A hydrogen bond will be formed between the side chain of the basic amino acids of the protein and the phosphomonoester head group of PA, which dissociates its remaining proton, switching the charge of PA from −1 to −2. This interaction leads to stronger electrostatic attraction between PA and effectors ([54, 94]; Fig. 9.3a). Besides hydrogen bonding with effector proteins, the deprotonation status of phosphomonoester head group of PA can be induced by bivalent cations, such as Ca^{2+} or Mg^{2+} (Fig. 9.3a) [54]. The electrostatic/hydrogen bond switch mechanism may explain the specificity of effector proteins for PA over other anionic membrane phospholipids, such as PS, with a maximum of −1 charge. Overall, the electrostatic/hydrogen bond and availability of hydrophobic area around the PA-binding region in a protein may determine the specificity of PA-protein binding [94, 104].

The ability of PA to change its negative charge numbers has also been proposed as a base for PA to act as a pH sensor in yeast cells. The charge number on PA head group is different from any other phospholipid and can change under physiological pH [94], which may render PA itself "active" or "inactive" under different environmental conditions. PA contains a phosphomonoester head group, and thus has a second pK_a within the physiological range (6.9–7.9; [53]). PA can be more or less deprotonated when the intracellular pH (pH_i) goes up or down, respectively. For example, Opi1p, a transcriptional suppressor for phospholipid biosynthesis, displays a higher affinity with deprotonated PA compared with protonated PA, and the binding is pH dependent [114]. The lower pH_i, caused by glucose starvation, alleviates PA-Opi1p binding. The released Opi1p enters the nucleus where it suppresses the expression of genes for phospholipid biosynthesis. Hence, PA acts as a pH_i sensor that connects glucose metabolism to membrane lipid biogenesis [64, 114].

9.3.2 PA Effect on Membrane Structure

Some of the PA's effect on protein interaction and cellular processes may result from its structural effect on cellular membranes. PA, with its smaller polar head and two relatively bulky acyl tails, is a hexagonal type II, cone-shape anionic lipid (Fig. 9.3a) [52, 101]. The cone-shape of PA can prevent tight packing with head groups from neighboring phospholipids, resulting in a loosened structure around PA and exposure of the hydrophobic zone to the effector proteins (Fig. 9.3b, *left*). PA derived from most abundant forms of PC in mammalian cells contains one saturated acyl chain and one unsaturated acyl chain. Most PAs in Arabidopsis contain two unsaturated acyl chains [104]. The unsaturated acyl chains expand the hydrophobic region in the planar bilayer compared with phospholipids containing two saturated chains. Thus, the aggregation of cone-shaped PA generated by PLD in the bilayer, confers a unique platform that attracts

proteins enriched with basic amino acid motifs and hydrophobic domains (Fig. 9.3b, *left*) [89]. The hydrophobic region of proteins can insert into the bilayer and bind to PA tightly. Although PE is also cone-shaped, it is a neutral lipid [101]. Whereas PS is negatively charged, it is a cylindrically shaped lipid [89].

The accumulation of the cone-shaped PA at a certain region in the lipid bilayer induces negative curvature, which is usually found at the neck of a vesicle during the process of fusing to an acceptor or budding from a donor membrane (Fig. 9.3b, *right*) [89]. In addition, the generation of DAG from PA by removal of the hydrophilic anionic phosphate group, increases the hydrophobicity of the membrane surface and enhances the stability of the membrane curvature. Such properties of PA and DAG reduce the free energy needed during membrane fission and fusion [89].

9.3.3 PA Modulation of Enzymatic Activity

One effect of PA-protein interaction is to modulate the protein catalytic activity, which can be inhibitory or stimulatory, depending on the effector protein. The PA modulation of enzyme activities could be an important cellular mechanism for coordinated regulation of a specific cellular process that involve a number of proteins. For example, the abscisic acid (ABA)-signaling in plants requires the function of many proteins and PA has been found to interact with several of them, including negative effector ABI1 and positive effectors NADPH oxidase and phytosphingosine kinase [28, 117, 118]. PA decreases ABI1 phosphatase activity, whereas it increases the oxidase and phytosphingosine kinase activity. Results from surface dilution kinetics analysis indicate that PA promotes the phytosphingosine activity by enhancing its binding to its substrate [28].

In the mammalian target of rapamycin (mTOR) signaling pathway, PA binds to mTOR at Arg2109 and activates its kinase activity. The activated mTOR then phosphorylates and activates its substrates such as S6K and Akt [17]. Meanwhile, PA can bind to the p70 ribosomal kinase S6K independently of mTOR and activates its kinase activity [56]. PA inhibits the activity of certain protein phosphatases, such as protein phosphatase 1Cγ (PP1Cγ) and PP2C [46]. It has been also proposed that PA-effector protein interaction acts as a coordinator in promoting protein phosphorylation in specific cellular responses [104].

9.3.4 PA-Tethering Proteins to Membranes

For some proteins, PA interaction may not directly affect their catalytic activity and instead, modulate their association with other proteins and intracellular location, such as tethering proteins to membranes. Signal transduction, vesicular trafficking, and many other critical cellular functions require targeting proteins to specific locations. The membrane tethering by PA helps to direct proteins to a specific membrane or to a membrane region.

In the PA-ABI1 interaction in ABA signaling, the PA binding, in addition to decreasing the PP2C activity, also tethers ABI1 to the plasma membrane, thus decreasing its translocation to the nucleus [117]. In *Saccharomyces cerevisiae*, increased PA tethers the transcriptional repressor Opi1p to the endoplasmic reticulum, thus keeping it from functioning in the nucleus [63]. The PA binding to Raf1 and sphingosine kinases regulates their specific functions associated with protein interactions associated with membranes [14, 86]. In addition to intracellular translocation of soluble proteins to the membranes, PA also interacts with regions of integral membrane proteins to modulate their functions. PA binds to a cytoplasmic region of the integral membrane protein NADPH oxidase to promote ROS production in plants [118].

The cellular levels of PA also support the hypothesis that membrane PA binds effector proteins. The basal level of PA in plant cells (50–150 μM) [117], is considerably above a phospholipid's critical micelle concentration in the sub-nanomolar range. Thus, the increase in signaling PA does not affect the concentration of

monomeric PA because the concentration of PA monomer in solution is constant when the level of PA is above its critical micelle concentration. Therefore, the membrane is likely the primary site for PA signaling and interaction with target proteins [104].

9.4 PA Involvement in Cellular Processes

The role of PA in various cellular processes has been documented in yeasts, plants, and animals using multiple approaches. The analyses of PA interaction with effector proteins involved in specific cellular processes have provided valuable mechanistic insights into PA functions (Tables 9.1 and 9.2). Genetic manipulations of genes encoding enzymes for PA production and removal have been particularly informative in revealing PA effects on cellular functions (Fig. 9.4). These studies are often supplemented by pharmacological manipulations of PA in the cells. Direct alterations of PA levels in the cell have been challenging because PA is water-insoluble and can be metabolized rapidly. Treatments of cells with primary alcohols, such as *n*-butanol, have been used to decrease PLD-mediated PA formation because of the enzyme's unique property of transphosphatidylation. However, cautions need to be excised for such treatment because butanol often activates, rather than inhibits its PLD activity. Recently, inhibitors for the PX/PH-PLD1 and PLD2 in mammalian cells have become available [92], but their efficacy on plant PLDs have not been determined. Increasing results indicate that PA plays a role in a wide range of cellular processes, including vesicular trafficking, cytoskeleton organization, membrane biogenesis, secretion, ionic flues, cell proliferation, apoptosis, and cell morphogenesis [45], some of which are highlighted below.

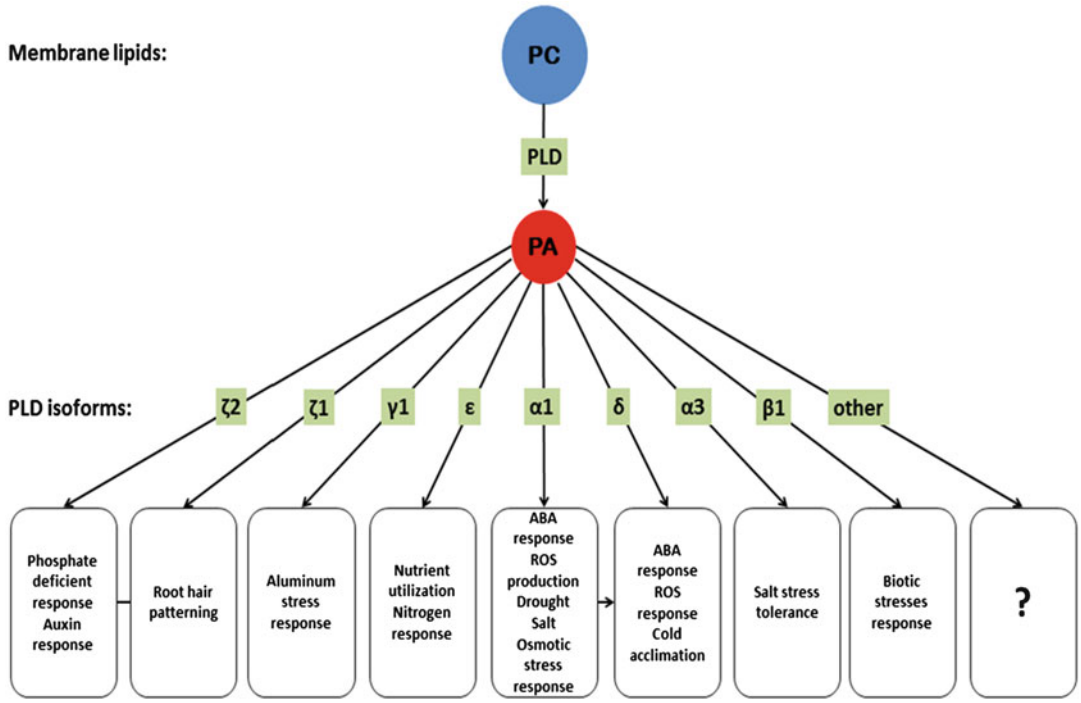

Fig. 9.4 PA produced by different PLDs has unique and overlapping physiological functions in plants. PA produced by different PLD isoforms has been shown to affect different physiological processes, as indicated by the analyses of genetically altered plants on specific PLDs. The *connected boxes* indicated documented interactions of two PLDs in specific signaling processes

9.4.1 Vesicular Trafficking

PA is a crucial regulator in vesicle formation and movements in mammals and yeast [45]. Mammalian PLDs are activated by ADP-ribosylation factor that regulates vesicle trafficking [85]. PLD is a key activator in various vesicle transport events including traffic of Glut4 glucose transporter and insulin secretion [41, 65, 66]. The phosphorylation and activation of PLD by CDK5 in pancreatic ß-cells is involved in secretion of insulin, whereas PA can recruit other proteins that are responsible for vesicle budding [45]. Furthermore, both PLD and PA have been found to bind to dynamin, a critical protein involved in vesicle budding from the Golgi network, and promote its GTPase activity [45, 89]. In addition, PA generated by PLD activation recruits phosphatidylinositol-4-phosphate 5-kinase (PIPK) which produces PIP_2, and is involved in vesicular trafficking [71]. Overexpression of MitoPLD causes mitochondria aggregation, a phenotype known to be associated with the presence of abundant fusion related proteins, whereas lacking MitoPLD made by RNAi resulted in fragmented mitochondria, suggesting that PA functions as a facilitator for membrane fusion [110]. Overall, PLD, PA, and their binding partners facilitate vesicle formation machinery in the vesicle budding and fusion processes that are critical for endocytosis and exocytosis. In plants, PLDζ2 and PA have been implicated in vesicle trafficking and regulating auxin polar transportation through the cycling of the auxin efflux carrier PIN-FORMED2 (PIN2) [58]. Auxin is a plant hormone that regulates plant growth, development and stress responses, and its polar transport is critical to its function [84, 119].

A general model for the role of PA in membrane trafficking may be summarized as follows: The activation of PLD generates PA at specific regions of a given membrane. Then, PA accelerates negative curvature of the membrane bilayer due to its anionic headgroup and hydrophobic conical shape [89]. Consequently, PA can recruit and activate proteins important for vesicle formation to the appropriate site in the membrane. In addition, PA-derived DAG participates in membrane trafficking. For instance, in the retrograde transport between Golgi and ER, DAG is required for COPI-coated vesicle formation [19].

9.4.2 Inter-organelle Lipid Transport and Membrane Biogenesis

In addition to the vesicle formation and trafficking, PA is involved in lipid production that is essential for membrane biogenesis and proliferation. In plants, genetic and biochemical evidence indicates that PA is involved in the transport of glycerolipids from the ER to the plastid [106]. Fatty acids in plants are synthesized exclusively in the plastid and then exported to the ER for the assembly of glycerolipids. Some of the glycerolipids in the form of PA are transported back to the plastid where they are used for the synthesis of galactolipids and sulfolipids, the abundant and essential components of photosynthetic membranes. The PA transport from the ER to the plastid is mediated by proteins at specific contact sites between the ER and plastids [105].

In yeast, PA has been documented to mediate the transcriptional regulation of phospholipid synthesis. PA binds to the transcriptional repressor Opi1p, serving as part of a lipid sensor complex in the ER [114]. The enriched PA in the ER keeps Opi1p out of the nucleus, leading to the increase in the transcription of genes encoding phospholipid-metabolizing enzymes. When the PA level at the ER membrane decreases, Opi1p translocates into the nucleus, where it represses a transcriptional activator complex and, consequently, represses the expression of genes for phospholipid metabolism [63, 114]. In addition, the hydrolysis of PA by the yeast PA phosphohydrolase PAH1p plays a role in phospholipid biosynthesis and nuclear structure. The abrogation of PAH1p leads to increased PA accumulation, ER/nuclear membrane proliferation, and nuclear expansion. On the other hand, the ablation of DGK1 decreases PA levels and alleviates nuclear

membrane expansion in *pah1*Δ cells. Increased DGK1 expression increases PA levels and accelerates nuclear growth [32]. These data indicate that PA plays a key role in membrane lipid synthesis and biogenesis.

9.4.3 Cytoskeletal Dynamics and Organization

Manipulating PA levels in the cell leads to alterations in cytoskeletal networks in plants. An increase in PA levels is generally associated with an increase in the density of actin filament arrays, possibly via actin filament polymerization, while a decrease in PA levels is associated with disassembly of actin filaments [59]. PA alters cytoskeletal organization by interacting with the specific effector protein, the heterodimeric capping protein (CP) [40]. CP is an abundant filament capper and it inhibits filament-filament annealing and filament elongation from free ends, thus reducing actin cytoskeletal dynamics. Both *in vitro* and *in vivo* evidence indicate that PA inhibits the end-capping activity of CP, thus promoting actin reorganization [59]. It has been proposed that CP is a PA biosensor that transduces cues from cell membranes into changes in actin cytoskeleton dynamics [59].

Arabidopsis PLDβ1 binds to actin via a specific actin-binding region. Whereas monomeric (G-) G-actin inhibits PLDβ activity, filamentous (F-) F-actin stimulates it [55]. Transient knockdown of PLDβ1 in tobacco results in pollen tube growth inhibition, which can be rescued by addition of PA [82]. These results indicate the importance of PLD-actin interaction and feedback activation in pollen tube growth. In addition, PLDδ was shown to bind to microtubules at membrane-cytoskeleton interface [24]. A recent report shows that PA enhances the binding of the microtubule-associated protein, MAP65-1, to microtubules. The PA-MAP65-1 binding and PLDα1 promote microtubule polymerization and bundling and is involved in plant response to salt stress [116]. The results indicate that PA and multiple PLDs are involved in both actin and microtubule cytoskeletal dynamics and rearrangements in plants.

In mammalian cells, PLDs have been found to interact with a number of effector proteins associated with the cytoskeletal organization (Table 9.2). For example, PA binds to type I PIPKI that generates the lipid messenger PIP_2, which has critical functions in many cellular processes such as cytoskeletal reorganization, membrane trafficking, and signal transduction. PA binding is involved in recruiting PIPKIγ to membranes, and the membrane localization of PIPKI is needed for its ability to induce actin cytoskeletal reorganization [87].

9.5 Role of PA in Physiological Processes

PA has been documented in a wide range of physiological processes. In animals, while most studies have used cellular models, increasing studies have implicated PA and PLD in a wide range of pathophysiological processes, such as inflammation, diabetes, neuronal cardiovascular disease, oncogenesis, metastasis, and reproductive processes [20, 22, 49, 79]. In cancer biology, PLD-derived PA is regarded as a tumor-promoting second messenger [20]. Recently, PLD and PA are shown to play an important role in spermatogenesis [79]. In yeast, PLD is required for sporulation [91]. In plants, PLD and PA are involved in regulating in pollen tube growth. These findings in plants, yeasts, and animals all point to a critical role of PLD and PA in reproduction. In plants, PA plays a role in plant responses to drought, salinity, nitrogen availability, phosphate deficiency, oxidative stress and microbial interaction, as well as seed germination, pollen tube growth, root growth and root hair patterning [60, 61, 100, 104] (Fig. 9.4). The impact of PA on physiological processes is broad and a number of reviews have covered various physiological processes in animals and plants [39, 45, 51, 79, 94, 100, 104]. The following discussion will concern three processes primarily associated with higher plants.

9.5.1 Plant Water Loss and Response to Drought

Water is a major-limiting environmental factor in crop production. Land plants lose most water via the pore of stomata on leaves. Stomatal aperture and thus the rate of water loss are highly regulated, and the plant hormone ABA promotes stomatal closure. PA and PLDα1 have also been shown to promote stomatal closure [70, 117]. PA interacts with and inhibits the protein phosphatase 2C ABI1, a negative effector in ABA response [117]. On the other hand, PA binds to NADPH oxidase to promote the production of the reactive oxygen species such as H_2O_2 [118]. PA and PLDα1 also promote nitric oxide (NO) production. NO and H_2O_2 are positive mediators in ABA responses. Recent studies show that PLDδ and its derived PA are involved in stomatal response to H_2O_2 and NO [15, 29]. The PLDδ/PA response to H_2O_2 is mediated through the interaction of PLDδ with cytosolic glyceraldehyde-3-phosphate dehydrogenases (GAPCs; [29]). H_2O_2 inactivates GAPC and enhances its binding to PLDδ to promote the production of signaling PA. Thus, PA produced by two different PLDs occupies two distinctive positions in the ABA signaling pathway (Fig. 9.4). PLDα1 and its generated PA promote ROS, whereas PLDδ and its derived PA mediate stomatal response to ROS. PLDα1 and PLDδ have different subcellular localizations, substrate preferences, and expression patterns in response to ABA. PLDα1 is constitutively expressed and the expression of PLDδ increases after ABA treatment [15]. These different properties of PLDα1 and PLDδ regulate the spatial and temporal patterns, as well as molecular species, of PA production. Loss of PLDα1, PLDδ, or GAPC function, compromises stomatal closure during water deficits, resulting in increased water loss [29].

In addition, recent results have revealed a connection of PLD/PA signaling with phytosphingosine kinase (SPHK) in the ABA signaling [28, 30, 31]. PA was found to bind and stimulate the activity of SPHKs to produce phytosphingosine-phosphate and stimulate stomata closure [12]. Genetic and biochemical data suggest that the PA stimulation is a feed-forward loop in which SPHK and phytosphingosine-phosphate act upstream, stimulating PLDα1 activity and PA production [30]. PA from NPC/DGK origin has also been shown to play a role in plant response to ABA [80].

9.5.2 PA in Plant Response to High Salinity and Hyperosmotic Stress

Salt stress is another major limiting factor for crop cultivation and agricultural productivity. Increases in PA have been reported under salt stresses in different plant systems using different approaches. Genetic ablation of PLDα1 and PLDδ compromises *Arabidopsis* seedling growth under high salinity [5, 116]. The loss of PLDα3 or PLDε also render plants more sensitive to salt, while plants overexpressing PLDα3 or PLDε show salt tolerance [5, 38]. High salinity results in hyperosmotic stress and ionic toxicity. PA has been reported to modulate the activity of ion channels/transporters on the membranes that remove excess ions such as Na^+. PA binds to and stimulates the MAP kinase MPK6, which regulates the Na^+ channel SOS1 [115]. Recently, PA binds to the sucrose non-fermenting-1-related protein kinase 2 (SnRK2), SnRK2.4 and SnRK2.10 [68]. Under high salinity, SnRK2 kinases were transiently increased, and the loss of SnRK2.4 reduced Arabidopsis primary root length, while the ablation of SnRK2.10 decreased lateral root number. It will be of interest to determine how PA affects the function of SnRK2 kinases in plant response to high salinity. The finding that PA binds to the microtubule-associated protein MAP65-1 and enhances microtubule bundling under high salinity [116] suggests that PA modulates plant response to high salinity via different modes of action, including protein phosphorylation, ionic fluxes, and cytoskeletal rearrangements.

PA from the PLC/DGK origin has also been shown to play a role in plant response to salt stress. NPC4-KO plants showed decreased sensitivity in ABA triggered seed dormancy, root growth arrest, and stomatal closure. In addition,

the PA/DAG/DGK inhibitor rescue studies also confirmed that PA is effective in this process rather than DAG, since the addition of DAG/DGK inhibitor could not rescue the phenotype while PA and DAG could [80]. Similarly, during salt and other osmatic stresses, PA generated by DGK pathway was observed, as indicated by the co-production of PIP_2 and DAG pyrophosphate [2].

9.5.3 PA in Nutrient Sensing

Nitrogen (N) is the most-limiting macronutrient on which plant growth depends. NO_3^- and NH_4^+ are the forms that plant can assimilate into amino acids for further metabolism and utilization. While the N assimilation pathway is well studied, how plants sense N availability is poorly understood [13, 102]. The analysis of the *Arabidopsis* PLD family indicates that PLDε and its product PA are involved in plant response to N availability. At sufficient N, PLDε promotes biomass accumulation and lateral root growth, whereas under N deficiency, PLDε promotes primary root elongation and root hair growth [37]. The promotion of root hair growth was not observed under N sufficient condition [37], and these results are in agreement with the notion that N uptake becomes crucial under N deficient conditions [13, 102]. The mechanism by which PA enhances plants' response to N remains to be elucidated.

The involvement of PLD and PA in plant N response may share some similarities in the mammalian mTOR, a serine/threonine kinase that integrates a wide range of signals such as nutrient, growth factors, and mitogen stimuli [17, 20]. PA competes with rapamycin to bind the FKBP12-rapamycin binding domain of mTOR [17]. The PA binding stabilizes mTOR complex formation and also activates the mTOR kinase activity [20]. On the other hand, PA seems to be necessary but not sufficient to activate mTOR, as extracellular stimuli such as glucose, amino acids, or growth factors, are needed in addition to PA [20]. Plants have the TOR kinase but the components in the TOR signaling complex are not well defined. In addition, the inhibition of plant TOR requires much higher concentrations of rapamycin [107]. It would be of interest to determine if PA modulates TOR function in regulating plant response to nutrient availability.

9.6 Perspectives

PA is a new class of lipid messengers and its signaling function has been documented in plants, animals, and yeast. The study of PA interaction with proteins has provided valuable insights into the processes that PA is involved and the mechanism by which PA functions in the cell. However, much remains to be understood as to how PA interacts with specific effector proteins and what the biochemical and cellular effects of the interaction are. The genetic manipulation of PA-producing enzymes has been highly insightful to learn PA's function in various cellular and physiological processes. Meanwhile, the complex families of multiple PLDs, PLCs, and DGKs in plants and animals make it challenging to define specific roles of PA in growth, development, and stress responses. Direct pharmacological manipulation of PA in the cell has been difficult because of its water insolubility and rapid metabolism in the cell. The current understanding of PA as a second messenger is still in its infancy. Further investigations of the PA signaling functions using a combination of different approaches have the potential to not only advance the current understanding of cell signaling but also help develop various biotechnological and pharmacological applications.

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Phosphatidylserine-Mediated Cellular Signaling

10

Jason G. Kay and Sergio Grinstein

Abstract

Phosphatidylserine (PS), a phospholipid with a negatively charged head group, is an important constituent of eukaryotic membranes. Rather than being a passive component of cellular membranes, PS plays an important role in a number of signaling pathways. Signaling is mediated by proteins that are recruited and/or activated by PS in one of two ways: via domains that stereospecifically recognize the head group, or by electrostatic interactions with membranes that are rich in PS and therefore display negative surface charge. Such interactions are key to both intracellular and extracellular signaling cascades. PS, exposed extracellularly, is instrumental in triggering blood clotting and also serves as an "eat me" signal for the clearance of apoptotic cells. Inside the cell, a number of pathways depend of PS; these include kinases, small GTPases and fusogenic proteins. This review will discuss the generation and distribution of PS, current methods of phospholipid visualization within live cells, as well as the current understanding of the role of PS in both extracellular and intracellular signaling events.

Keywords

Phosphatidylserine • Phosphatidylserine detection • Membrane signaling • Protein-lipid binding • Lipid dynamics

10.1 Phosphatidylserine Biosynthesis, Degradation and Cellular Distribution

Phosphatidylserine (PS) is a glycerophospholipid present in cellular membranes of all eukaryotic cells. Like other phospholipids, PS has a polar phosphate on *sn*-3 of the glycerol backbone; a serine attached to the phosphate gives PS its distinctive head-group, which has a net negative charge under physiological conditions (Fig. 10.1a). This is in contrast to the most abundant lipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which have a zwitterionic head group and therefore bear no net charge. When PS is present in large amounts, the net negativity of its head group can confer significant electrostatic charge to membranes, which can have important implications on the recruitment of soluble cations

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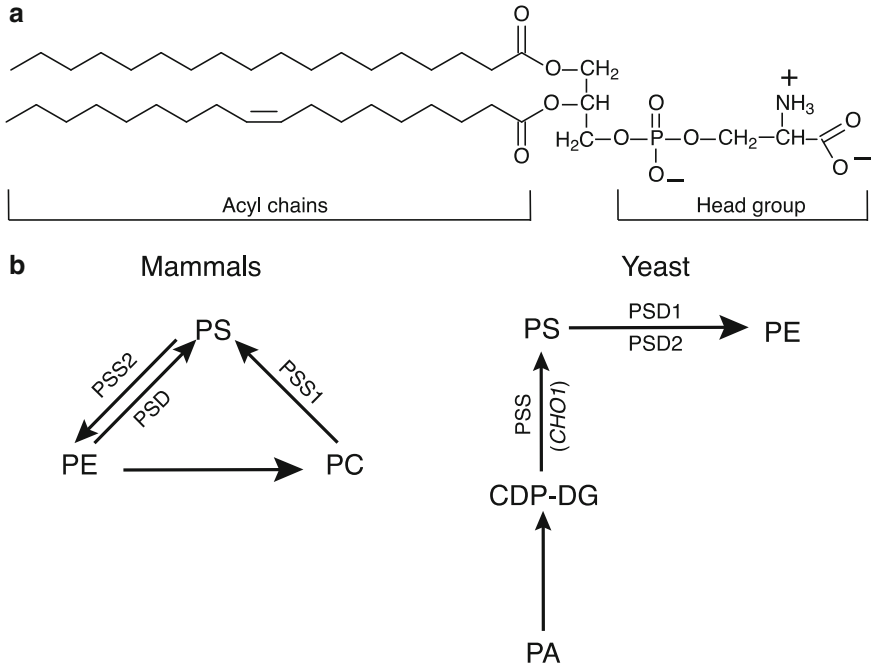

Fig. 10.1 Phosphatidylserine and its production and degradation pathways. (**a**) Diagram of the structure of PS, with one saturated and one unsaturated fatty acyl chains. Note that at physiological pH the head group bears one net negative charge. (**b**) Diagram showing pathways of production of PS in both mammalian and yeast cells. In mammalian cells PS can be produced from either phosphatidylcholine (PC) or phosphatidylethanolamine (PE) by PS synthase (PSS) 1 or 2, respectively, while in yeast phosphatidic acid (PA) is the only precursor that can be converted to PS, via CDP-diacylglycerol (CDP-DG). In both cases, PS is converted to PE by PS decarboxylases (PSDs)

and proteins; such recruitment, in turn, can have important signaling implications, as discussed below.

Both the *sn*-1 and *sn*-2 positions of PS, as with the majority of glycerophospholipids, have fatty acyl chains attached. A large variety of acyl chains are known to exist on PS, with the *sn*-1 position tending to have saturated chains of 16 carbons or longer, and the *sn*-2 position generally having unsaturated chains of 18 carbons or longer [84]. Interestingly, however, PS tends to display the greatest acyl chain composition diversity between tissues, with brain PS being especially enriched in long, poly-unsaturated fatty acyl chains, especially docosahexaenoic acid (22:6n-3); this has been speculated to have implications in neuronal development and function [60, 75, 161].

In mammalian cells, PS synthesis can occur via two pathways, using either PC or PE as a precursor (Fig. 10.1b). The enzyme PS synthase 1 (PSS1) exchanges the choline on PC for a serine, while the enzyme PSS2 can exchange the ethanolamine on PE for serine. These two synthesis pathways are at least partially redundant, as mice without either PSS1 or PSS2 are viable, though double-knockout of both synthases is lethal [8, 16], indicating that PS is an essential phospholipid in mammals. In yeast cells, a single PSS enzyme exists, encoded by *CHO1*, which converts phosphatidic acid (PA) to PS via cytidine diphosphate-diacylglycerol (CDP-DAG) (Fig. 10.1b). In contrast to mammals, yeast mutants without PSS expression – and thus no PS – are able to survive, albeit rather precariously, in medium supplemented with ethanolamine [112].

Degradation of PS occurs mainly by decarboxylation of the PS head group to produce PE, a reaction mediated by the enzyme PS decarboxylase (PSD). There is one PSD in mammals, encoded by the *Psd* gene, and two in yeast, psd1p and

Table 10.1 Phospholipid content of organellar membranes

Membrane	PC	PE	PI	PS	References
Endoplasmic reticulum	55[a]	30	15	3–5	[84, 160]
Golgi complex	50	15	10	5	
Early endosome	47	23	8	8.5	
Late endosome	48	18.5–20	4–7	2.5–3.9	
Mitochondria	40–45	30–35	5–10	1	
Plasma membrane	42	25	3	12	

[a]Numbers indicate percentage of total lipid present in mammalian cells

psd2p, encoded by the *PSD1* and *PSD2* genes, respectively (Fig. 10.1b) [84, 161]. Mammalian PSD and yeast Psd1p are found on the outer leaflet of the inner mitochondrial membrane [161], leading to production of a unique pool of PE from PS within the mitochondria. This mitochondrial production of PE is unable to be fully complemented by the CDP-ethanolamine pathway, as $Psd^{-/-}$ mice do not survive beyond embryonic day 9 [145]. Additio-nally, in mice missing three of four PSS alleles, where the catalytic levels of PSS are reduced to ~10 % of wild type mice and PS levels are consequently reduced, PE levels are concomitantly reduced [8]. Thus, PS is an important precursor for the production of mitochondrial PE, which is in turn important for the functioning of mitochondria. Finally, though a minor contributor to overall degradation, some phospholipases are additionally capable of degrading PS with some of the resulting products, especially lyso-PS, having important signaling effects [7, 65]; these will be discussed later in greater detail.

The intracellular (organellar) distribution of PS is not homogeneous, with the majority of the PS of the cell being on the plasma membrane (PM) (Table 10.1). The endoplasmic reticulum (ER) is the main site for lipid synthesis, and in mammals both PSS1 and PSS2 are localized and active in specialized areas of the ER, which are known as mitochondria-associated membranes due to their close relationship and tendency to be isolated with mitochondria [163]. Despite being produced at the ER, PS is not particularly abundant in this compartment (Table 10.1), suggesting that selective transport or removal of PS must occur. One means of removal of PS from the ER is by transport to the mitochondria where, as mentioned above, PS can be converted to PE. Transport from the ER to mitochondria appears to occur at the mitochondria-associated membranes. At this specialized junction PS delivery occurs by direct inter-membrane movement, possibly assisted by soluble or membrane-bound carrier proteins; vesicular-mediated transport is not involved [114, 140, 160, 162]. The other main method of PS removal from the ER is via anterograde vesicular transport to the Golgi apparatus, likely occurring via bulk flow, although specific transport mechanisms cannot be ruled out [84, 160]. How high PS levels are maintained at the PM without being returned to the Golgi apparatus via retrograde traffic, is not entirely clear.

Another important aspect of PS is its tendency to be asymmetrically distributed between leaflets of membranes. This is especially evident at the plasmalemma, where virtually all the PS is on the inner (cytoplasmic facing) leaflet in healthy cells, with none detectable on the outer leaflet. This asymmetry of PS at the surface membrane substantially increases the mole percentage of PS in the inner leaflet to 25–30 % of the total lipid. By contrast, the transmembrane distribution of PS in other organelles remains poorly defined; according to some reports the ER has little, if any, PS remaining on the outer (cytoplasmic-facing) leaflet, with the majority of detectable PS being on the inner (lumen-facing) monolayer [22, 42, 61, 74].

The distribution of PS across membrane leaflets is maintained by various flippases, floppases and scramblases. Flippase is a general name for lipid translocases that move lipid toward the cytoplasmic-facing leaflet of a bilayer. ATP-dependent flippases with selectivity for PS are known to function at the PM; these translocases belong to the family of P_4-ATPases [34, 135, 154]. Five

different P_4-type ATPases have been identified in yeast (DRS2, DNF1, DNF2, DNF3 and NEO1), and over a dozen have been deduced by sequence homology in humans; a number of these have been shown experimentally to have PS flippase activity [34, 51, 119, 134]. A number of the yeast proteins are located on intracellular membranes and their deletion has implications in membrane traffic [31, 66]. This implies that PS asymmetry occurs, and is important for, the function of intracellular organelles.

Floppases function to transport lipids away from the cytoplasm to the luminal-facing leaflet of organelles, or the topological equivalent of the PM, the extracellular-facing leaflet. Most belong to the ATP-binding cassette (ABC) superfamily but to date, only limited evidence exists for the ability of these proteins to move PS across membranes [34]. Scramblases, by contrast, are ATP-independent, bidirectional and function to randomize lipids across membranes [128]. Scramblases are important in the exposure of PS on the cell surface during apoptosis, discussed in more detail later.

The abundance of PS in the PM, together with its asymmetric distribution between leaflets and its negative charge, have important electrostatic implications. Soluble cations, polycations and proteins with cationic clusters can all be concentrated in the immediate vicinity of membranes, where they can influence biochemical and signaling events. Thus, the unique subcellular and transmembrane distribution of PS confers onto this lipid a special signaling connotation.

The importance of PS in cellular signaling and survival is highlighted by examination of cells lacking PS. Yeast cells lacking the PSS gene (*cho1Δ*) survive, but grow very poorly and have elevated phosphatidylinositol levels [9], possibly a compensatory attempt to increase levels of alternative negatively charged lipids. *Cho1Δ* cells do have normal fluid phase uptake, α-factor maturation, CPY trafficking and septin ring maturation [41, 109], but polarization of the yeast PM and recruitment of CDC42 are greatly inhibited, preventing proper formation of buds and mating projections [41]. The role of PS is even more prominent in higher organisms. In contrast to yeast, mammalian cells completely lacking both PSS genes are not viable [8], and cell lines that have greatly reduced PSS functionality are only viable when PS is added exogenously [129]. Although mice with only one of three PSS alleles remaining are viable (despite some tissues only having 10 % of wild type PS levels [8]), PS is clearly an important lipid for proper survival and growth and participates in many signaling cascades, as discussed below.

10.2 Detection of Phosphatidylserine

In order to determine the involvement of PS in signaling cascades, it is first necessary to determine the presence and localization of PS. Traditional methods of PS detection were biochemical in nature, such as the covalent reaction of its head group with amino-reactive chemicals such as 2,4,6-trinitrobenzenesulfonate, followed by extraction and thin layer chromatography or mass spectrometry. Such methods were successfully used to determine that PS resides exclusively on the cytoplasmic leaflet of the PM in healthy mammalian cells [92, 132], as well as to establish the sidedness of PS in a limited number of organelles [143, 158]. However, the usefulness of such probes is limited as they are generally slow to react, also label PE, and their use can compromise cellular viability [23].

Fluorescent analogues have also been successfully used to study the properties of PS within the cellular environment. Fluorescent PS analogues generally have a fluorescent moiety attached to a shortened *sn-2* acyl chain, where it is presumed to interfere minimally with the unique head group. The most commonly used fluorophore has been 7-nitro-2-1,3-benzoxadiazol-4-yl (NBD). Indeed, NBD-PS has been useful in measuring the activity of proteins involved in PS flipping [35, 93, 121]. However, NBD has a strong dipole moment which forces the attached acyl chain to loop towards the aqueous interface and distorts the PS-like molecule [29, 30, 104], reducing its hydrophobicity and allowing it to be removed easily from the membrane [74, 93]. More recently, a second

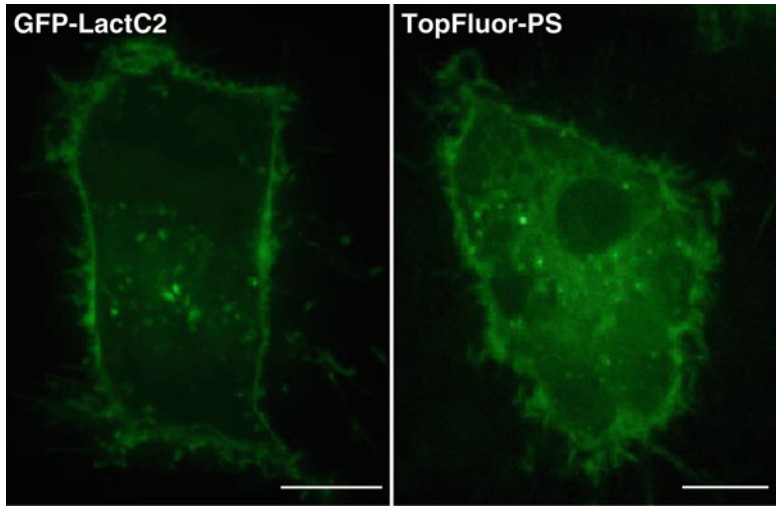

Fig. 10.2 Phosphatidylserine detection in cellular membranes of live cells. Two examples of PS detection in live HeLa cells. Cells were either transfected with a genetically encoded GFP-LactC2 (*left*), or were loaded with TopFluor-PS analogue (*right*). Both methods of PS visualization show fluorescence at the plasma membrane and internal (endosomal) membranes. Both probes have been used successfully for the detection and examination of live cell dynamics of PS

fluorescent PS analogue, TopFluor-PS, based on a BODIPY-derivative (1-palmitoyl-2-(dipyrrometheneboron difluoride) undecanoyl-*sn*-glycero-3-phospho-L-serine) has been successfully used to examine PS dynamics in cellular membranes; this analogue is perceived to have fewer drawbacks than NBD-PS [74] (Fig. 10.2).

Specific interactions of proteins with PS can also be used for detection of the phospholipid. There are two main means whereby proteins interact with PS. One of them is charge-based: a number of proteins that are prenylated or lipidated have in addition a stretch of cationic amino acids that can interact electrostatically with PS; however, the interaction is non-selective and such polycationic probes interact also with other anionic phospholipids. A number of Ras and Rho-family GTPases, as well as the tyrosine kinase Src have this property [43, 56, 141]. While these proteins do not interact with PS specifically, they do partition preferentially to PS-enriched membranes, which have a net negative surface charge [56, 141]. While these types of interactions can have important signaling consequences, they are not generally useful for specific detection of PS.

The second type of protein-PS interaction involves protein domains that recognize PS in a more specific manner, often by a stereospecific PS head group interaction domain. These include the calcium-dependent C2 domain of proteins such as annexin V, protein kinase C (PKC) α and PKCβ, the calcium-independent C2 discoidin-like domain of proteins such as lactadherin (also known as MFG-E8), and the γ-carboxyglutamic acid (Gla) domain on prothrombin (Table 10.2). Many of these interactions also have important signaling consequences, which will be discussed below, but some of these domains, or the entire proteins that contain them, can be used as probes for the specific detection of PS in cells. The best known example of this is annexin V, which has been used extensively to stain PS in apoptotic cells [80]. Annexin V works well for detecting PS that has appeared on the exofacial leaflet of the plasmalemma; however, due to its requirement for comparatively high (mM) concentrations of calcium [5], its usefulness for visualization of intracellular PS in live cells is limited. While the approach was reported [27], effective binding required artificial elevation of the cytosolic calcium concentration, which activates signaling cascades, degradative enzymes, etc. Furthermore, binding of annexin V to membranes is not strictly PS-dependent; it is instead sensitive to anionic

Table 10.2 Phosphatidylserine-binding domains

Class	Calcium dependency	Specificity	Examples	References
C2 domain (conventional)	Yes	Electrostatic, limited specificity for PS, but often nonspecific	Annexin V	[151]
			PKCα	[148]
			PKCβ	
Discoidin-like C2 domain	No	L-serine stereospecific binding	Coagulation factors V and VIII	[88]
			Lactadherin	[136]
γ-Carboxyglutamic acid (Gla)	Yes	Specific serine head group binding	Prothrombin	[68]
			Gas6	

lipids in general [98, 99]. Thus, while annexin V has been instrumental in furthering the understanding of PS exposure outside cells, its usefulness as an intracellular probe is questionable.

More recently, a new probe based on the discoidin-like C2 domain of the glycoprotein lactadherin has been developed [172]. Unlike conventional C2 domains, the discoidin-like C2 domain of lactadherin binds PS specifically in a calcium-independent manner (Table 10.2) [3, 86]. When the lactadherin C2 domain is linked to green fluorescence protein (GFP), the resulting chimera (GFP-LactC2) is highly specific for PS, has stereospecificity for the phospho-L-serine moiety over phospho-D-serine, has greater avidity for PS than does annexin V and, importantly, binds PS under intracellular physiological conditions [138, 139, 172]. Therefore, this probe provides a new tool to examine PS-mediated signaling events that occur within the cell. When combined with additional probes like TopFluor-PS, GFP-LactC2 can be used with minimal concern about possible effects on lipid scavenging by the probe itself [74]. Although much remains to be discovered, the new probes have been successfully used in a number of recent studies examining PS distribution, dynamics and PS-mediated signaling events in the intracellular environment (Fig. 10.2) [12, 41, 42, 74, 106, 156, 172, 173].

10.3 Phosphatidylserine-Mediated Extracellular Signaling Events

A great deal of attention has been paid to the appearance of PS on the cell surface. This reflects primarily the availability and convenience of annexin V for exofacial PS detection. A brief overview of what is currently known about extracellular signaling mediated by PS follows.

10.3.1 Hemostasis

As mentioned above, in healthy, unstimulated cells PS is normally present on the cytoplasmic leaflet of the cell, with virtually none on the exofacial leaflet. Changing this resting distribution of PS has a strong potential for initiating signaling. This was first recognized in the case of platelet activation. When stimulated, platelets expose PS on their surface, thereby triggering the coagulation cascade. The collagen receptor glycoprotein VI on platelets, which recognizes the underlying collagen upon breakage of a blood vessel, is a potent activator of platelets [17, 57, 58, 77, 177]. Platelet activation and PS exposure are also triggered by fibrin-binding receptors such as glycoprotein Ib-V-IX (vWF receptor) and integrin $\alpha_{IIb}\beta_3$ [20, 124]. Engagement of the receptors by their cognate ligands triggers a cascade of tyrosine and serine protein phosphorylation, with resultant activation of phospholipase Cγ2 and subsequent generation of inositol 1,4,5-*tris*phosphate. Inositol 1,4,5-*tris*phosphate is required for the release of calcium from intracellular stores and their depletion, in turn, activates calcium entry from the extracellular medium, amplifying the response [58]. While elevated calcium inhibits PS flippase activity, this is not enough on its own to cause the observed rapid appearance of exofacial PS required for efficient blood clotting to occur [85, 178]. Instead, the main effect of calcium is thought to be the activation of lipid scrambling. Scramblase activity

requires a sustained calcium elevation, and it can be inactivated simply by the removal of calcium [19, 167]. While the underlying molecular entity was elusive for many years, a bona fide calcium-dependent scramblase, TMEM16F, was identified recently and is thought to be involved in the translocation of PS in platelets [85, 149].

Once PS is exposed on the surface of the platelet, it functions as a scaffold for the coagulation cascade to occur in the correct location, which is in the vicinity of the activated platelet. Factor VIII, a co-factor for the factor Xa complex and factor V, a co-factor for the prothrombinase enzyme complex, both have discoidin-like C2 domains that specifically bind exposed PS by interacting with its unique head group [49, 88, 122, 126, 159]. In addition, factors X, VII, IX and II (prothrombin) contain 'Gla' domains, rich in glutamic acid residues, which are γ-carboxylated in a vitamin K-dependent reaction [171]. These domains also interact with PS specifically, but in a calcium-dependent manner [68], and serve to bring these additional factors to the activated platelet. The importance of the scaffolding function of PS in bringing together the required clotting factors is highlighted by patients with Scott syndrome. In these patients, platelets fail to scramble PS in response to activation, at least in some cases due to the lack a functional TMEM16F scramblase; as a result these patients present with a bleeding disorder [28, 125, 149, 165]. Further, it has been shown that the Xa and prothrombinase enzyme complexes depend indirectly on PS, as their enzymatic activity is completely lost or highly diminished by blocking the PS-binding capability of factor V and VIII with either antibodies, blocking peptides, or by mutations in the PS-binding sites [50, 69, 76, 137].

In addition to PS exposure on the surface of activated platelets, microvesicles formed by the evagination of the plasma membrane are released from platelets; these microvesicles also expose PS [33, 46]. The PS exposed on the surface of the microvesicles can also promote procoagulant activity, as highlighted by the fact that mice lacking serum lactadherin (which binds and promotes the clearance of microvesicles) suffer from excessive coagulation [37].

Finally, exofacial PS on activated platelets can be bound specifically in a calcium-dependent manner by the proteins Gas6 and protein S through their Gla domains [108]. When these proteins interact with their receptors – members of the TAM family (Tyro3, Axl and Mer) of tyrosine kinase receptors– under hemostatic conditions, they appear to stabilize thrombus formation by promoting platelet aggregation [6, 32].

10.3.2 Apoptosis

Cells turn over constantly, undergoing death and replacement. Apoptosis is the programmed death that allows for the removal of cells without the release of potentially harmful intracellular enzymes and antigens, and therefore without causing an inflammatory response. PS exposure on the surface of apoptotic cells is another highly studied area, as it is known to be the signal for the rapid uptake of cell corpses by phagocytes. Exposure of PS is therefore thought of as an 'eat-me' signal [40]. While the signaling cascades and cellular processes that result in a commitment to apoptosis are the subject of intense study and are quite well defined [39, 105], the determinants of PS exposure, other than a requirement for caspase activation, are not well known [94, 107]. In apoptosis, as during platelet activation, the movement of PS to the exofacial leaflet likely involves the activation of scramblases. However, the timescale for PS scrambling is vastly different, with PS exposure occurring in platelets on the scale of minutes, while PS exposure on apoptotic cells develops over hours [24, 168, 169, 178]. Additionally, while calcium has been implicated in upstream apoptotic signaling [1, 21, 70, 73, 96], whether calcium elevation is an absolute requirement for the scrambling of PS during apoptosis is not clearly defined. While most studies show there is no requirement for influx of extracellular calcium, the possible involvement of intracellular calcium storage sites is less clear [10, 13, 36, 53, 101, 102, 133, 168, 176]. What is evident, however, is that patients with Scott syndrome have normal apoptotic PS exposure despite having no calcium-induced PS exposure on activated platelets or lymphocytes [18, 125, 165, 168]; this

Table 10.3 Receptors recognizing phosphatidylserine on apoptotic cells

Phagocytic receptor	Direct PS recognition?	Accessory ligand	References
TIM-1, TIM-4	Yes	–	[79]
BAI1	Yes	–	[115]
Stabilin-2	Yes	–	[117]
Mer	No	Gas6, protein S	[4]
$\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins	No	Lactadherin	[54]
CD36	Oxidized lipid	?	[52]

suggests that a scramblase other than TMEM16F may be involved in exofacial exposure of PS during apoptosis. Possible candidates are the phospholipid scramblases 1–4, or members of the ABC transporter family, though recent evidence suggests that these are not efficient PS scramblases and that phospholipid scramblase proteins are in fact signaling proteins [19, 91, 128].

Once exposed on the surface of apoptotic cells, PS is one of the most potent 'eat me' signals, directing phagocytic cells to internalize and degrade the apoptotic cell. There are a number of receptors on the phagocyte membrane that recognize exofacial PS on apoptotic cells (Table 10.3), either by directly interacting with the lipid or through an intermediate protein. Gas6, protein S and lactadherin are proteins identified as PS-binding proteins that can act as bridging molecules that allow receptors to engage and take up apoptotic cells [4, 54, 108]. In addition to their role in hemostasis described above, protein S and Gas6 are important for phagocytic cells to recognize targets and signal through their TAM receptors. This applies not only to macrophages and dendritic cells, but also to Sertoli cells that clear the apoptotic cells formed during spermatogenesis, and to retinal pigment epithelial cells that take up sections of photoreceptor cells that are shed daily [83]. Lactadherin is expressed by macrophages and immature dendritic cells and, when interacting with PS via its C2 domain as described earlier, it can simultaneously associate with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins on phagocytic cells, thereby stimulating the engulfment of the apoptotic cell [54, 170].

Apoptotic receptors that have been reported to bind directly to PS include stabilin-2, brain specific angiogenesis inhibitor 1 (BAI1) and members of the T cell immunoglobulin mucin (TIM) family [79, 115, 117]. Stabilin-2 appears to interact with PS specifically, in a calcium-dependent manner [118], while BAI1 interacts with phosphatidylinositol 4-phosphate, PA and the mitochondrial lipid cardiolipin as well as PS [115]. Both TIM-1 and –4 bind PS in a calcium-independent manner, holding the head group of PS in a specific metal ion-dependent pocket [130]. Finally, a portion of the exposed PS appears to be oxidized during the apoptotic process [155], and CD36 has been shown to interact with oxidized lipids and with oxidized PS preferentially over other oxidized lipids, promoting the uptake of apoptotic bodies [52]. How all these receptors capable of PS binding interact and/or cooperate to allow for the recognition and uptake of apoptotic cells is still not clear, but PS is most certainly a signal from apoptotic cells that they need to be engulfed and eliminated [107, 123].

There is evidence to suggest that lyso-PS, a deacylated form of PS having increased aqueous solubility [111], may be involved *in vivo* in cellular signaling, especially in the context of the immune system [45, 90]. Lyso-PS was shown by mass spectrometry to be present in the serum of lipopo-lysaccharide-treated mice and in peritoneal lavages of casein- or zymosan-treated animals [44]. Because there is no known pathway for its *de novo* synthesis, lyso-PS is thought to be produced only by a deacylation reaction catalyzed by phospholipase A (PLA) enzymes. A secreted isoform that is PS-specific (PS-PLA_1), is massively upregulated by various inflammatory stimuli [65, 90] and was shown to be released from activated platelets [131]. When treated with lyso-PS, mast cells undergo enhanced degranulation [95, 142], T-cell growth is inhibited [15] and macrophage uptake of activated or apoptotic neutrophils is

enhanced [44]. Additionally, lyso-PS stimulates fibroblast migration [116] and may therefore play a role in tissue remodeling following injury. Thus, while there are tangible suggestions that lyso-PS may be playing important roles in both immune regulation and wound healing, much work is clearly required to understand the full consequences and significance of this more soluble and potentially more mobile form of PS.

10.4 Phosphatidylserine-Mediated Intracellular Signaling Events

While the detection and visualization of PS in live cells has until recently been difficult, there is still a fair amount known about the involvement and importance of PS in various intracellular signaling events. Recall that PS is heterogeneously and asymmetrically distributed throughout the cell (Table 10.1 and Fig. 10.2). It makes up to 30 % of the lipid on the inner leaflet of the PM; this, combined with its negatively charged head group, confers onto PS a nonpareil ability to direct the recruitment of both proteins containing polycationic stretches, as well as proteins that posses a specific PS-recognition site (Table 10.2).

10.4.1 Phosphatidylserine Charge-Based Interactions

A number of proteins contain lipidation or prenylation sites that drive their association with membranes; when a polycationic motif exists next to a lipidated site, the positive charge will promote the preferential partition and stabilization of the protein on membranes endowed with negative surface charge, e.g., membranes enriched in PS. One such example is the small GTPase K-Ras4B, which is essential for the signal transduction of a number of growth factors. The majority of Ras superfamily member proteins are geranylgeranylated or farnesylated at their C-terminal CAAX box [166], and while many are also palmitoylated or otherwise lipid-modified to allow their attachment to membranes strictly by hydrophobic means, K-Ras4B is not [55]. Instead, the polycationic stretch present near the C-terminus of K-Ras4B is an essential second signal (in addition to its farnesylation) for the PM association of K-Ras4B [56]. This highly charged polycationic stretch of K-Ras4B does not interact specifically with PS, but this lipid contributes significantly to the overall surface potential of the PM, aiding in the recruitment of Ras4B to the plasmalemma [56, 113, 172, 174].

Src, a tyrosine kinase and the first known oncogene, as well as Rac1, an additional member of the Ras superfamily, are both important for a number of signaling events [2, 25, 64]. Both Src and Rac1 are proteins whose membrane targeting requires a polycationic stretch in addition to lipid modifications [100, 141]. In fact, the distribution of Src within the cell parallels very closely that of PS (as determined by GFP-LactC2), being on the PM and endosomal system [172]. This is due to the fact that not only can the existence of a polycationic stretch of amino acids direct the association of proteins with the PM, but also other anionic membranes, depending on their charge. Thus, the polycationic stretch next to the farnesylation site of K-Ras4B, which has a net charge of +8, locates almost exclusively at the PM, but if the net charge of this stretch is varied, the resulting mutants are directed additionally to other membranes; constructs of intermediate charge (e.g., +5) localize to endosomal membranes [172, 174]. The same behavior was noted for Src, which has a polycationic stretch next to its myristoylated residue at the N-terminus. The cationic motif of Src has a net charge of +5, and the kinase was found to associate not only with the PM but also extensively with PS-enriched endosomal membranes [172, 173]. Further evidence that PS is important for the charge-based distribution of Src was obtained with phagocytosis. When certain pathogens cause depletion of PS from phagosomes, Src is also lost [173]. Rac1, which has a polybasic region with a net charge of +6, associates more strongly with the PM but is rapidly removed from a sealing phagosome, despite the persistence of PS [174], possibly through additional posttranslational modifications [110]. Overall,

we can conclude that the pattern of PS distribution among intracellular membranes (Table 10.1) plays an important role in determining their ability to interact with important signaling proteins, particularly those with cationic characteristics.

In some instances, cationic motifs on proteins are not sufficient to direct proteins to a membrane, but do influence their targeting. This is the case for other signaling proteins, such as with RhoB, TC10 and CDC42 [81, 100]. In such instances cationic motifs likely play a complementary role. Evidence to this effect comes from recent studies in yeast, where polarized PS is required for the recruitment of the signaling and polarity-regulating molecule CDC42 to the forming bud neck [41]. Without PS present, CDC42 remains mainly Golgi-associated and buds are very inefficiently formed, leading to poor growth [41].

Overall, then, there is an increasing body of evidence that the intracellular asymmetry of PS, and the resultant distribution of negative charge, is responsible for recruiting – through charge-based interactions – soluble or amphiphilic molecules to their proper location within the cell, where they serve crucial roles in signal transduction and propagation.

10.4.2 Phosphatidylserine-Specific Interactions: Head Group Recognition

In addition to the general charge-based interactions outlined above, there are a number of proteins that have domains that will stereospecifically interact with the PS head group (Table 10.2). Such interactions are often important for the localized initiation and propagation of various signaling cascades within the cell. Classical PKCs (α, βI, βII and γ) contain a conventional C2 domain, a calcium-dependent cysteine-rich region that recognizes PS and is responsible, in coordination with the C1 domain that binds to DAG, for activating and localizing the kinase to the PM of suitably stimulated cells [127, 152, 153]. The binding of PS is relatively specific, as the binding pocket of the C2 domain coordinates the binding of calcium and prefers L-PS [38, 164]. Without this calcium-dependent binding to PS, the classical PKC isoforms are not activated. By contrast, the novel PKC isoforms have a modified C2 domain that does not bind calcium and the atypical PKC isoforms lack a C2 domain entirely, making them independent of PS binding [164].

Synaptotagmins, a family of proteins whose members also mostly contain C2 domain-like regions that bind to PS, are important for calcium-mediated vesicle fusion, particularly for the fast, calcium-dependent release of neurotransmitters [26, 89, 147, 164]. The C2 domains of synaptotagmins tend to be fairly promiscuous, binding most acidic phospholipids [150]. However, as discussed earlier, PS is the most abundant acidic phospholipid at the PM, where neurotransmitter release occurs. During neurotransmitter secretion, when synaptotagmin binds calcium, in addition to increasing the affinity of synaptotagmin for acidic phospholipids 100–10,000 times, a hydrophobic loop of the protein is released which penetrates the membrane, stabilizing the protein on the lipid bilayer [11, 150]. Numerous *in vitro* studies document the importance of PS in synaptotagmin function during vesicle fusion, including the observation that the phospholipid needs to be present in both the plasmalemma as well as the incoming vesicles [146]. Moreover, recent *in vivo* studies have also shown that PS influences the opening and dilation of fusion pores during calcium-triggered vesicle fusion [175].

PS-specific binding is also important in the function of A-, B- and C-Raf kinases, important regulators of many signal transduction pathways. Raf kinases are generally downstream from the Ras GTPases, and transmit information to activate mitogen-activated protein kinase (MAPK) signaling [157]. When inactive, Raf kinases exist in the cytosol in a closed conformation, with their regulatory domain bound to, and inhibiting, the kinase domain. The protein 14-3-3 is additionally bound to the inactive Raf, stabilizing its inactive conformation. Activation of Raf occurs upon recruitment to the membrane by Ras, but a number of lipids have been found to interact with Raf and potentially play a role in its stimulation

[59, 72, 157]. The Raf proteins have a conserved N-terminal cysteine-rich domain that has some structural similarity to PKC-type C2 domains; this C2-like domain is responsible for PS binding to Raf in a calcium-independent manner [47, 48, 103]. In addition, there is a separate C-terminal domain that binds PA and a region also near the N-terminal that is involved in phosphoinositide binding [47, 59, 72]. While there is still much uncertainty about the functional role of all these lipid interactions [59, 157], the importance of PS binding for the recruitment of Raf to membranes, the release of 14-3-3, and the activation of the kinase has been demonstrated [47, 48, 97]. Thus, PS plays important roles in multiple steps of the cascades initiated by receptor tyrosine kinases that signal through Ras and Raf through to the MAPK system.

Sphingosine kinase (SK) 1 and SK2 are enzymes that convert sphingosine to sphingosine 1-phosphate, an important extracellular messenger involved in stimulating endothelial differentiation, migration and mitogenesis [62], as well as being implicated in intracellular signaling cascades leading to cytoskeletal changes, motility, release of intracellular calcium and protection from apoptosis [63, 82, 144]. SK is a cytosolic protein that can be phosphorylated and recruited to the PM upon stimulation of PKC [71, 120]. Current evidence suggests that SK displays a binding region that is specific for PS, and the binding affinity of SK for PS increases upon phosphorylation; this interaction with PS is essential for its proper recruitment and full enzymatic activity both *in vitro* and *in vivo* [144].

Finally, some signaling proteins with pleckstrin homology (PH) domains have been shown to have the binding of their cognate phosphoinositide enhanced in the presence of PS. For example Grp1, a guanine nucleotide-exchange factor (GEF) for Arf family GTPases, has greatly enhanced binding to phosphatidylinositol 3,4,5-trisphosphate-containing vesicles when PS is also present [78]. Similarly, the PH domain of Akt has been found to have basic residues nearby that are involved in binding of PS, which is required for its full activation [67]. Additionally, phosphoinositide-dependent kinase-1 (PDK1) is a serine/threonine kinase that functions upon binding to phosphatidylinositol-3,4,5-trisphosphate and phosphatidylinositol-3,4-bisphosphate to regulate a subgroup of 3-phosphoinositide-responsive protein kinase family members including Akt, p70 ribosomal S6 kinase, serum- and glucocorticoid-induced protein kinase, and atypical PKC [14]. PDK1 is partially bound to the PM in the absence of 3'-phosphoinositides and recent studies have shown that there is a site – separate from the canonical phosphoinositide-binding site – that specifically binds PS, is responsible for recruiting PDK1 to the PM and is important for its signaling function [87].

10.5 Conclusions

PS is an essential glycerophospholipid present in all mammalian cells with an asymmetric distribution throughout the cell. It is becoming clear from the research highlighted in this chapter that rather than being an inert component of biological membranes, PS, with its unique head group and combination of abundance and subcellular distribution, plays an important role in a number of signaling pathways. As detailed, signaling via PS is mediated by the interaction of proteins with PS in one of two ways: via domains that stereospecifically recognize the head group, or by electrostatic interactions with membranes that are rich in PS and therefore display negative surface charge, such as the PM. As outlined, such interactions are key to both intracellular and extracellular signaling cascades. Overall, then, there are a number of signaling proteins that depend upon the unique head group and cellular distribution of PS for their proper localization and activation. In closing, it is worth emphasizing that while much of the current understanding of PS involvement in signaling has been derived from *in vitro* studies, future studies will in all likelihood uncover additional signaling roles for PS in the physiological context, now that suitable tools to analyze in live cells are emerging.

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Cardiolipin-Mediated Cellular Signaling

11

Vinay A. Patil and Miriam L. Greenberg

Abstract

This review focuses on recent studies showing that cardiolipin (CL), a unique mitochondrial phospholipid, regulates many cellular functions and signaling pathways, both inside and outside the mitochondria. Inside the mitochondria, CL is a critical target of mitochondrial generated reactive oxygen species (ROS) and regulates signaling events related to apoptosis and aging. CL deficiency causes perturbation of signaling pathways outside the mitochondria, including the PKC-Slt2 cell integrity pathway and the high osmolarity glycerol (HOG) pathway, and is a key player in the cross-talk between the mitochondria and the vacuole. Understanding these connections may shed light on the pathology of Barth syndrome, a disorder of CL remodeling.

Keywords

Cardiolipin • Phosphatidylglycerol • Cellular signaling • Apoptosis • Cell wall biogenesis • Mitophagy • Mitochondria • Vacuolar function • Reactive oxygen species • Anionic phospholipids • Barth syndrome

11.1 Introduction

It is not unusual to find even current depictions of membranes as homogenous lipid matrices that function primarily to support the allegedly *important* protein molecules embedded within. This belies the fascinating discoveries in the past two decades of cellular and organelle-specific functions attributed to individual membrane lipids, and of the plethora of regulatory and signaling molecules derived from glycerolipids and sphingolipids. In this light, it is essential to elucidate the functions of specific membrane lipids and the cellular consequences of their depletion.

A phospholipid that has been the focus of considerable attention relatively recently – although it was first isolated and purified from beef heart in 1942 [1, 2], is cardiolipin (CL). CL is structurally unique. In contrast to the other membrane phospholipids, in which a single glycerol backbone is acylated to two fatty acid chains, CL contains two phosphatidyl groups (linked to a glycerol

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backbone) and four fatty acyl chains. It is enriched in energy harvesting membranes of mitochondria, chloroplasts, bacterial plasma membranes, and hydrogenosomes, underscoring the importance of this lipid in energy production [3–5]. CL is tightly associated with mitochondrial proteins and respiratory chain complexes and is essential for their optimal activity [6–10]. In the inner membrane, CL provides structural stability to membrane proteins through hydrophobic and electrostatic interactions.

In light of its association with the respiratory apparatus, the role of CL in mitochondrial bioenergetics was not entirely unexpected. Interestingly, however, recent studies carried out primarily in yeast indicate that CL is also required for cellular functions that are not directly associated with oxidative phosphorylation. In accordance with a broad definition of a 'bioactive lipid' as one in which changes in levels lead to functional consequences [11], perturbation of CL composition (including CL levels, acyl species, and degree of peroxidation) leads to dramatic cellular consequences: (1) Alterations in CL levels and acyl chain composition increases the recruitment to the mitochondria of cytosolic proteins that trigger apoptosis [12–14]. (2) Perturbation of CL synthesis or remodeling leads to increased production of reactive oxygen species (ROS), which induces aging [15–20]. (3) Blocking CL synthesis in yeast at the first step of the pathway deleteriously affects cell wall biogenesis and alters the response of two signaling pathways, the protein kinase C (PKC)-Slt2 mitogen activated protein kinase (MAPK) and the high osmolarity glycerol (HOG) pathways [21–23]. (4) The inability of yeast cells to synthesize CL leads to decreased vacuolar function and reduced V-ATPase activity, suggesting that CL mediates cross talk between mitochondria and the vacuole [24]. The current review focuses on the role of CL in regulating these cellular functions. We conclude with unanswered questions that remain exciting avenues for future studies, which may have implications for understanding the pathophysiology of Barth syndrome (BTHS), a genetic disorder of CL remodeling.

11.2 CL Biosynthesis and Remodeling

One of the most intriguing aspects of CL biosynthesis is that the lipid that is initially synthesized contains primarily saturated fatty acids, while the mature CL that is essential for normal cellular function contains unsaturated fatty acids. The distinct composition of acyl chains is achieved through a highly conserved pathway of synthesis and remodeling, as shown in Fig. 11.1. The first step is catalyzed by phosphatidylglycerolphosphate (PGP) synthase (Pgs1), which converts CDP-diacylglycerol (DAG) and glycerol-3-phosphate (G-3-P) to PGP [25, 33]. PGP is dephosphorylated to phosphatidylglycerol (PG) by PGP phosphatase (Gep4) [26, 34]. The mammalian homologue of the yeast *GEP4* gene was recently identified as protein tyrosine phosphatase localized in the mitochondrion (PTPMT1) [34]. CL synthase (Crd1) catalyzes an irreversible condensation reaction in which the phosphatidyl group of CDP-DAG is linked to PG via cleavage of a high-energy anhydride bond to form CL [27–30, 35–38]. CL synthase does not show strong preference for specific fatty acyl chains [38–40]. How, then, is acyl specificity achieved? The newly synthesized CL undergoes deacylation by a CL-specific deacylase (Cld1), which is homologous to the mammalian phospholipase A_2 [31, 41, 42]. Cld1 removes one saturated fatty acyl chain from CL to form monolysocardiolipin (MLCL) [31]. The transacylase tafazzin (Taz1) reacylates MLCL with an unsaturated fatty acid to form mature CL [32, 43, 44]. Taz1 carries out exchange of acyl chains between CL and phospholipids that primarily include phosphatidylcholine (PC), to sequentially replace the fatty acyl chains from all four acyl positions of CL [45, 46]. The end result of this exchange is molecular symmetry of CL molecules across the eukaryotic kingdom, from yeast to humans, which is characteristic of the organism and of specific tissues and organs [47]. For example, in yeast, the mature form of CL contains oleic acid, while CL in the normal human heart is primarily tetralinoleoyl-CL (L_4-CL) [47].

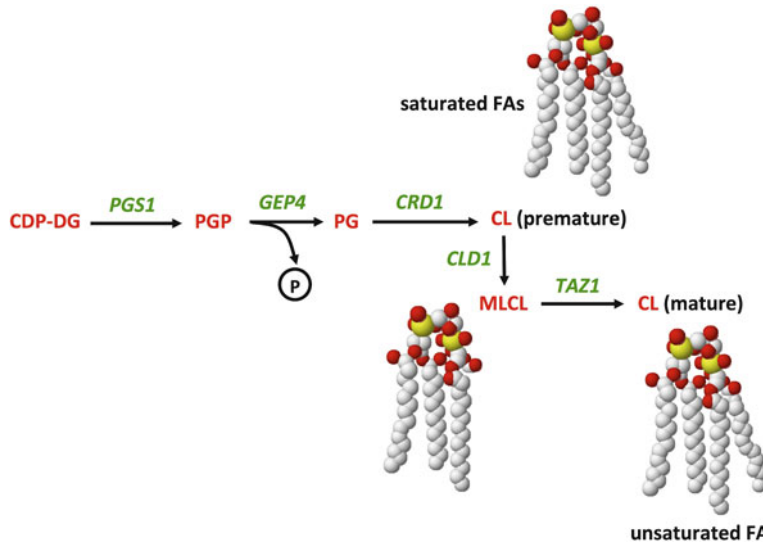

Fig. 11.1 Synthesis and remodeling of CL in yeast. CL synthesis begins with the conversion of CDP-diacylglycerol (CDP-DG) to phosphatidylglycerolphosphate (PGP) by PGP synthase (encoded by *PGS1*) [25]. PGP is dephosphorylated to phosphatidylgylcerol (PG) by *GEP4*-encoded PGP phosphatase [26]. CL synthase (encoded by *CRD1*) converts PG to premature CL containing primarily *saturated* fatty acids (FA) [27–30]. CL is deacylated by CL deacylase (encoded by *CLD1*) to monolyso-CL (MLCL) [31], which is reacylated by the *TAZ1*-encoded enzyme tafazzin to mature CL containing *unsaturated* fatty acids [32]. The yeast gene names are depicted in *green*, while phospholipids and their intermediates are shown in *red*

A deficiency of tafazzin in humans leads to a complete absence of L_4-CL, resulting in the severe cardiomyopathy observed in BTHS.

While tafazzin is the only known yeast enzyme that adds fatty acyl chains to MLCL, two other enzymes in addition to tafazzin remodel CL in mammalian cells. MLCL acyltransferase-1 (MLCLAT1), isolated and purified from pig liver mitochondria, shows specificity for linoleate [48]. Thus, over-expression of MLCLAT1 in tafazzin-deficient BTHS lymphoblasts increased incorporation of linoleic acid into CL, and RNAi knockdown of MLCLAT1 in HeLa cells showed reduced linoleic acid inclusion in CL [49]. The biological function of this enzyme is not clear. A second enzyme, acyl-CoA:lysoCL acyltransferase 1 (ALCAT1), identified in mouse, was initially thought to be located in the endoplasmic reticulum, but was subsequently determined to be present in the mitochondrial-associated membranes, where phospholipid traffic between the endoplasmic reticulum and the mitochondria takes place [17, 50]. In contrast to MLCLAT1, ALCAT1 shows no specificity for linoleic acid. ALCAT1 was shown to catalyze CL remodeling to incorporate long chain polyunsaturated fatty acyl chains such as docosahexaenoic acid (DHA) [17]. Enhanced incorporation of polyunsaturated fatty acyl chains in CL makes it more susceptible to oxidative damage by ROS, causing early peroxidation [51–53]. ALCAT1 null mutant mice exhibit elevated CL levels along with increased L_4-CL [16, 17], whereas overexpression of ALCAT1 has been shown to decrease total CL levels and increase incorporation of long chain polyunsaturated fatty acyl chains [54]. These findings suggest that ALCAT1 may negatively regulate CL biosynthesis.

In light of the importance of CL in cellular function, it is not surprising that perturbation of CL synthesis leads to serious illness. The most direct example of this link is seen in BTHS, a life-threatening illness characterized by dilated cardiomyopathy and sudden death from arrhythmia [55, 56]. BTHS results from mutation in the CL remodeling enzyme tafazzin [44, 57].

This leads to an abnormal CL profile characterized by decreased total CL, increased MLCL, and aberrant CL acylation, most notably the loss of the predominant CL species in normal myocardium, L_4-CL [58]. How these abnormalities cause the associated pathology in BTHS is not known [59].

CL abnormalities have also been observed in heart failure [60, 61]. Heart failure due to dilated cardiomyopathy is the primary cause of death in diabetic patients [62, 63]. Metabolic perturbations observed in diabetic cardiomyopathy include increased utilization of fatty acid substrates, decreased utilization of glucose, and mitochondrial dysfunction [64–66]. However, the molecular mechanism that leads to heart failure in diabetic patients is not known. Interestingly, a decrease in CL levels and alterations in CL acyl species were found in early stages of diabetes induced by streptozotocin in mice, suggesting that mitochondrial dysfunction and cardiomyopathy may be due to alterations in CL metabolism [67, 68]. The decrease in CL levels may result from remodeling of CL fatty acyl species with DHA, which is known to cause CL peroxidation by ROS [51–53]. In summary, depletion of CL content and alterations in CL fatty acyl species lead to BTHS, and may also contribute to pathological conditions and metabolic perturbations in other human disorders.

11.3 CL and Apoptosis

Perturbations in CL levels and acyl composition play a crucial role in regulating apoptosis, the complex process leading to programmed cell death. The role of CL in apoptosis derives from its interactions with cytochrome c (Cyt c) and with apoptotic proteins (Fig. 11.2).

11.3.1 CL and Cyt c

Interactions between CL and Cyt c are an important determinant of apoptosis. Cyt c, which transfers electrons from complex III to complex IV, is bound to the outer leaflet of the mitochondrion inner membrane through interactions with CL [69, 70]. The binding of Cyt c to CL is essential to anchor it to the inner membrane, and release of Cyt c to the cytosol serves as a signal to recruit apoptotic proteins to the mitochondria to initiate apoptosis [74, 75, 79, 80]. CL binds Cyt c in two different conformations – a loosely bound state that is facilitated by means of electrostatic interactions, and a tightly bound state that is mediated by hydrophobic interactions in which Cyt c is partially embedded in the inner membrane [69, 70]. Release of Cyt c from CL requires dissociation of both electrostatic and hydrophobic interactions [81]. The production of ROS may alter the CL-Cyt c association [80–82]. Alternatively, the peroxidation of CL by hydrogen peroxide generated in the mitochondria leads to the release of Cyt c from the tightly bound state into the intermembrane space [12, 76].

11.3.2 Recruitment of Apoptotic Proteins

An early trigger of apoptosis is the change in CL composition in the mitochondrial inner and outer membranes, followed by dissipation of the membrane potential and flipping of phosphatidyserine (PS) to the external surface of the plasma membrane [83, 84]. A diverse set of apoptotic proteins such as t-Bid, Bax, Bak, and caspase-8 are recruited to the mitochondrial surface of cells undergoing apoptosis in a CL-dependent manner [71–73]. Upon activation, caspase-8 migrates to the mitochondrial outer membrane in regions where CL is present. Caspase-8 is said to cleave Bid to its active form, tBid (truncated Bid). A significant amount of CL is translocated from the inner to the outer mitochondrial membrane, which likely serves as a signal for binding of the apoptotic proteins [14, 85, 86]. The binding of t-Bid to CL is thought to further increase CL transfer to the outer membranes. Alternatively, apoptotic proteins may be guided to the mitochondria by means of altering the outer membrane charge [87]. By increasing the CL content, the mitochondrial outer membrane may accrue a more

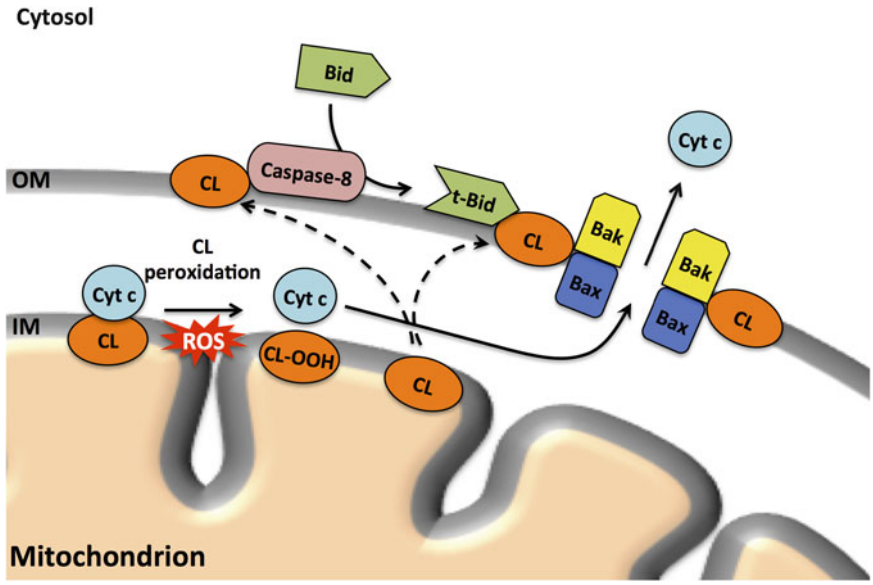

Fig. 11.2 Perturbation of CL metabolism triggers apoptosis. The binding of cytochrome c (Cyt c) to CL is essential to anchor it to the inner mitochondrial membrane, facing the intermembrane space [69, 70]. Peroxidation of CL (CL-OOH) by reactive oxygen species (ROS) leads to release of Cyt c to the cytosol, which serves as a signal to initiate apoptosis [71–73]. Caspase-8 cleaves Bid protein to its active form, truncated Bid (t-Bid) [74–76]. Binding of t-Bid to CL enhances translocation of CL to the outer mitochondrial membrane, which facilitates targeting of apoptotic proteins (Bak and Bax) to the outer membrane [77, 78]

negative charge, which serves as a targeting signal for recruiting polycationic apoptotic proteins to the mitochondria [77, 85]. Consistent with this, ectopic overexpression of a CL-binding protein masked the negative charge on the membrane and inhibited apoptosis [87]. The recruitment to and oligomerization of Bak-Bax in the outer mitochondrial membrane is a CL dependent process, which permeabilizes the outer mitochondria to trigger Cyt c release and progression of apoptosis [88, 89]. This suggests that CL-rich regions in the outer membrane serve as a key signal for targeting pro-apoptotic proteins of the Bcl2 family to bring about apoptosis [78, 85].

11.3.3 Translocation of CL

Early in apoptosis, CL translocation from the inner to the outer mitochondrial membrane may be carried out through several transport modes. First, the inner and outer membrane contact sites, which are enriched in CL through interactions with mitochondrial creatine kinase (MtCK), could facilitate the transfer of CL from the inner to outer membrane [90–95]. Second, phospholipid scramblase-3 (PLS-3) has been shown to translocate CL from the inner membrane to the outer membrane during the onset of apoptosis [96–98]. Consistent with this, cells overexpressing PLS-3 exhibit increased apoptosis, while inactivation of PLS-3 leads to increased resistance to UV-induced apoptosis [97]. CL and Bid interactions have been shown at the contact sites, which likely contribute to mitochondrial permeabilization to induce apoptosis [99]. Changes in CL content in the membrane may be mediated by Bid, as evidence suggests that Bid exhibits lipid transfer activity [100, 101]. Lymphoblastoid cells derived from BTHS and *TAZ* knockdown HeLa cells were more resistant to Fas-induced apoptosis [72]. Specifically, reduction of mature CL caused defective activation of caspase-8, suggesting that processing of caspase-8 on the mitochondrial membranes is

CL-dependent. To summarize, CL in the mitochondria is an important mediator of apoptosis, and apoptotic proteins are directed to the mitochondria in a CL-dependent manner.

11.4 CL in Bioenergetics and Mitochondrial Dysfunction

The relationship between CL and ROS is complex. CL physically interacts with proteins of the mitochondrial respiratory chain complexes and other components of the membrane and forms lipid scaffolds for tethering and stabilizing mitochondrial membrane proteins to enhance their enzymatic activities [7, 8, 102–105]. Consistent with the role of CL in bioenergetics, mitochondria deficient in CL exhibit decreased activity of respiratory complexes and carrier proteins [106]. The generation of ROS in mitochondria, which is a byproduct of oxidative phosphorylation [107–109], is enhanced upon CL deficiency [20]. ROS, in turn, damages CL by peroxidation of the unsaturated fatty acids.

11.4.1 CL and Supercomplexes

For efficient substrate channeling between the individual complexes, the mitochondrial respiratory chain components are organized in supramolecular structures called supercomplexes [110]. In mammalian cells, complex I is associated with two units of complex III and multiple units of complex IV. In *S. cerevisiae*, which lacks complex I, two copies of complex III are bound to either one or two units of complex IV. CL deficiency in yeast leads to destabilization of the respiratory supercomplexes, indicating that CL functions to stabilize these complexes [10, 111, 112]. Similarly, tafazzin deficient human fibroblasts exhibit destabilization of the supercomplexes [113]. For efficient ADP/ATP exchange, CL is also required for the association of the ADP/ATP carrier protein with the supercomplexes [114].

11.4.2 CL Deficiency and ROS Generation

The role of CL in the supercomplexes may be that of a proton trap, to avoid leakage of protons and enhance the membrane potential for efficient oxidative phosphorylation [115–117]. Not surprisingly, defective supercomplex formation and CL deficiency lead to increased ROS production [20, 118].

Among the respiratory chain complexes, complexes I and III are prime sites for ROS generation [119–122]. Because of the proximity of CL to these ROS generating centers, the unsaturated fatty acyl chains of CL are susceptible to damage by peroxidation. Superoxide generated by respiratory complex III causes peroxidation of CL and alters the activity of Cyt c oxidase [123–125]. Optimal function of Cyt c oxidase, the terminal enzyme complex of the respiratory chain, is dependent on CL [6, 126–128]. Reduced activity of Cyt c oxidase from reperfused heart was restored specifically by exogenous supplementation of CL, but not by peroxidized CL or other phospholipids [129]. In addition, reduced activity and increased ROS generation by complexes I and III were also rescued by CL supplementation [125, 130]. These studies indicate that peroxidized CL cannot effectively carry out mitochondrial functions that are dependent on normal CL.

Peroxidation of CL by ROS is seen as the primary cause of CL mobilization to the outer leaflet of the inner membrane. Human leukemia cells treated with the apoptosis-inducing drug staurosporine rapidly underwent apoptosis along with an increase in CL content in the outer mitochondrial membrane [83]. However, the change in CL content was preceded by increased ROS production and CL peroxidation, suggesting that perturbation of CL metabolism could be an early step in mitochondria-induced apoptosis. Due to the high content of unsaturated fatty acyl chains, CL is particularly susceptible to peroxidation [131, 132]. Peroxidation of CL alters the molecular conformation leading to formation of non-bilayer hexagonal structures, which could serve as a

marker for targeting the cytosolic apoptotic machinery to the mitochondria [77].

11.4.3 CL in Mitochondrial Dysfunction and Aging

Under normal physiological conditions, the damaged fatty acyl chains of CL may be replaced through the remodeling process [45]. Pathological remodeling of CL has been linked to mitochondrial dysfunction in human diseases [17, 18, 61, 67]. Recent studies have shown that ALCAT1 may be involved in the pathological remodeling of CL in cells undergoing oxidative stress. As mentioned earlier, *ALCAT1* overexpression leads to a decrease in CL levels and aberrant remodeling of CL with long chain polyunsaturated acyl chains such as DHA, which are highly susceptible to oxidation by ROS [17, 52, 53]. The close proximity of CL to respiratory complexes in the inner membrane where ROS is generated increases exposure of these long chain unsaturated fatty acyl chains to ROS. Aberrant CL remodeling resulting from increased *ALCAT1* expression leads to the mitochondrial dysfunction seen in pathological conditions such as hyperthyroid cardiomyopathy, diabetes, and diet-induced obesity in mice [15–17, 133]. ALCAT1 null mice exhibit increased expression of MLCAT1 along with elevated levels of CL containing linoleic acid. These findings underscore the significance of CL remodeling and the impact of this process on mitochondrial function and ROS generation.

A decline in CL levels appears to be a primary feature of aging [134–139]. In aging cells, CL is pathologically remodeled with polyunsaturated fatty acyl chains such as arachidonic and docosahexaenoic acids, which are more susceptible to peroxidation than linoleic acid in normal CL [18, 54]. Mitochondrial CL levels, along with oxidative capacity and ATP synthesis, decrease significantly with age [134, 140–143].

CL is required for the optimal function of several mitochondrial carrier proteins involved in the transport of essential metabolites into mitochondria [106]. In the heart, oxidation of pyruvate and β-oxidation of fatty acids are two major sources of ATP generation [144–146]. The transport of pyruvate into mitochondria by the pyruvate carrier and the exchange of carnitine esters by the carnitine:acylcarnitine translocase are, therefore, critical for energy metabolism. Studies have demonstrated that enzymatic activities of both the mitochondrial pyruvate carrier and carnitine:acylcarnitine translocase, which are dependent on CL [147, 148], are decreased in aging heart muscle [149, 150]. Interestingly, administration of acetyl-L-carnitine in aged rats restored decreased CL levels and the activities of the mitochondrial pyruvate carrier and carnitine:acylcarnitine translocase to levels found in young rats [149, 150]. Dietary supplementation of acetyl-L-carnitine also showed similar beneficial effects, increasing mitochondria membrane potential and, in turn, improving physical mobility in aged rats [141, 151]. These findings suggest that the supply of carnitine to the mitochondria may become limited during aging, hindering energy production through β-oxidation [150, 152]. Although acetyl-L-carnitine supplementation restored CL levels and improved mitochondrial metabolic functions in aged animals, the underlying molecular mechanism remains unresolved.

11.5 CL and the PKC-Slt2 Cell Integrity Pathway

Null mutants in yeast have been characterized for each step of the CL biosynthetic pathway, and mutants blocked earlier in the pathway have more severe phenotypes. Thus, the *pgs1*Δ mutant, which cannot synthesize CL or the precursor PG (Fig. 11.1), exhibits severe growth defects not only in non-fermentable carbon sources, which are metabolized by respiration, but also in fermentable carbon sources, in which respiration is not required [25, 153]. This observation indicated that PG and/or CL are required for cellular functions apart from mitochondrial bioenergetics [154]. Genetic studies to isolate spontaneous suppressors of the *pgs1*Δ mutant growth defect identified a loss of function mutation of *KRE5*, a gene involved in cell wall biogenesis [21].

Consistent with defective cell wall biogenesis, the *pgs1*Δ mutant exhibited enlarged cell size characteristic of cell wall mutants, reduced levels of β-1,3-glucan as a result of decreased activity of glucan synthase, and sensitivity to cell wall perturbing agents [155–157]. These defects were restored by disruption of *KRE5* in *pgs1*Δ, which increased expression of the genes *FKS1* and *FKS2* encoding glucan synthase [22]. These findings were in agreement with the identification of *PGS1* in a screen to identify genes involved in cell wall biogenesis [158].

Studies to gain insight into the mechanism linking CL to the cell wall focused on the PKC-Slt2 cell integrity pathway. Activation of the cell integrity pathway is triggered by signals generated from cell wall sensor proteins to Rom2, which, in turn, activates formation of the GTP-bound form of Rho1p. The activated Rho1 protein transmits a signal to Pkc1 to trigger the Mpk1/Slt2 MAPK signaling cascade, which results in dual phosphorylation of Slt2 [159, 160]. The dual phosphorylation of Slt2 is essential to activate transcription factors that up-regulate genes involved in cell wall remodeling, particularly in response to heat stress [156, 161, 162]. The *pgs1*Δ mutant exhibited defective activation of the PKC-Slt2 cell-integrity signaling cascade, indicated by decreased Slt2 phosphorylation levels [22]. Consistent with this, overexpression of individual genes in the PKC-Slt2 pathway rescued the growth defect of *pgs1*Δ at elevated temperature and improved resistance to the cell wall perturbing chemicals calcofluor white and caffeine. Interestingly, deletion of *KRE5* in *pgs1*Δ also led to increased activation of the PKC-Slt2 cell-integrity pathway.

A mitochondrial connection to the cell wall is not new. Genome-wide screens have identified several yeast genes required for mitochondrial function that, when mutated, affect chemical components of the cell wall [158, 163, 164]. Furthermore, mitochondrial respiratory defects negatively impact the synthesis of cell wall components [158, 164]. The underlying mechanism whereby CL regulates cell wall remodeling is not known. One possibility is that CL is required for activity of one or more proteins that exhibit dual localization in the cell wall/plasma membrane and mitochondria [165]. Interesting possibilities include three proteins of the PKC-Slt2 cell integrity pathway, Fks1, Zeo1 and Rho1, which are found both in the mitochondria and the plasma membrane [166–168]. Mitochondrial targeting of these proteins may be CL-dependent. Alternatively, their stability in the mitochondrial membrane may be decreased in the absence of CL.

The yeast cell wall also plays an important role in regulating replicative life span [169]. Consistent with this, the *pgs1*Δ mutant, which exhibits cell wall defects, also has a decreased replicative life span [21, 23]. Intriguingly, experiments to elucidate the mechanism linking PG/CL to defects in the cell wall, PKC/Slt2 signaling and aging led to another signaling pathway – the HOG stress response pathway.

11.6 CL and the HOG Stress Response Pathway

In response to stress, cells are regulated by the opposing actions of the PKC-Slt2 and HOG signaling pathways [170–172]. Heat or low osmolarity stress leads to activation of the PKC-Slt2 pathway, resulting in increased expression of the cell wall remodeling genes leading to a decrease in turgor pressure [173–175]. In contrast, activation of the HOG signaling pathway causes an increase in turgor pressure [175, 176]. Because the *pgs1*Δ mutant exhibited defective activation of the PKC-Slt2 signaling cascade, it was hypothesized that growth defects of the mutant resulted from increased turgor pressure, which may be rescued by down-regulation of the HOG pathway (Fig. 11.3) [23]. This hypothesis was supported by the finding that deletion of *SHO1*, an upstream activator of HOG signaling, rescued growth defects, increased the replicative life span, and alleviated sensitivity to cell wall perturbing agents in *pgs1*Δ [23]. Interestingly, the mutant did not exhibit increased activation of the HOG pathway. It is possible that, in the absence of PKC-Slt2 activation, even wild type levels of HOG activation lead to turgor pressure levels that affect growth. These findings suggest that homeostasis achieved

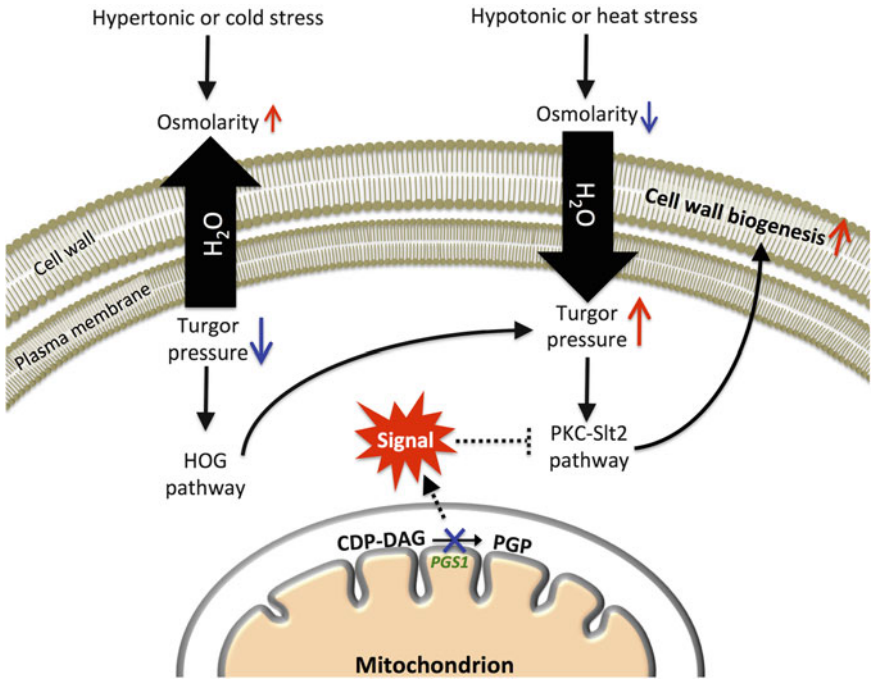

Fig. 11.3 CL deficiency leads to perturbation of PKC-Slt2 and HOG signaling pathways. The PKC-Slt2 and HOG signaling pathways coordinately regulate cell wall biogenesis and intracellular turgor pressure, respectively [173, 175]. Under hypertonic or cold stress conditions, extracellular osmolarity is increased, causing an efflux of intracellular water to reduce the turgor pressure on the cell wall. To counteract augmented extracellular osmolarity, the HOG pathway is activated, which leads to an increase in intracellular turgor pressure [170, 177]. In contrast, under heat or hypotonic stress, extracellular osmolarity is decreased, which causes an influx of water inside the cell to increase intracellular turgor pressure. To counteract the increased turgor pressure, the activated PKC-Slt2 pathway induces cell wall synthesis [172, 173]. We hypothesize that disruption of the CL pathway by mutation of *PGS1* generates a signal that is detected by regulators or components of the PKC-Slt2 pathway, which, in turn, down-regulates the pathway [21, 22]. Under these conditions, an increase in intracellular turgor pressure by activation of the HOG pathway is deleterious in *pgs1*Δ cells [23]

by these two signaling pathways is perturbed upon CL deficiency (Fig. 11.3).

11.7 CL Mediates Cross-Talk Between Mitochondria and Vacuole

The yeast *crd1*Δ mutant, which lacks CL, was shown to have defective vacuolar function [24]. CL deficiency caused decreased V-ATPase activity and proton pumping, reduced vacuolar acidification, and enlargement of the vacuole. The yeast vacuole plays a crucial role in adjusting to high external osmolarity and decreased turgor pressure, and in maintaining cytosolic ion concentrations [178, 179]. Consistent with perturbation of intracellular osmotic balance in the *crd1*Δ mutant, growth and vacuolar defects were rescued by supplementation of sorbitol [24].

In some genetic backgrounds, the *crd1*Δ mutant exhibits increased expression of *RTG2*, a critical sensor of mitochondrial dysfunction that relays metabolic defects to the nucleus via the retrograde signaling pathway [180, 181]. Consistent with overactivation of Rtg2, deletion of the *RTG2* gene restored vacuolar acidification and V-ATPase activity and rescued the growth defect of the *crd1*Δ mutant at elevated temperature. However, deletion of the retrograde pathway activator *RTG3* did not rescue the mutant, suggesting that the defects observed in *crd1*Δ resulted from Rtg2 functions unrelated to retrograde activation.

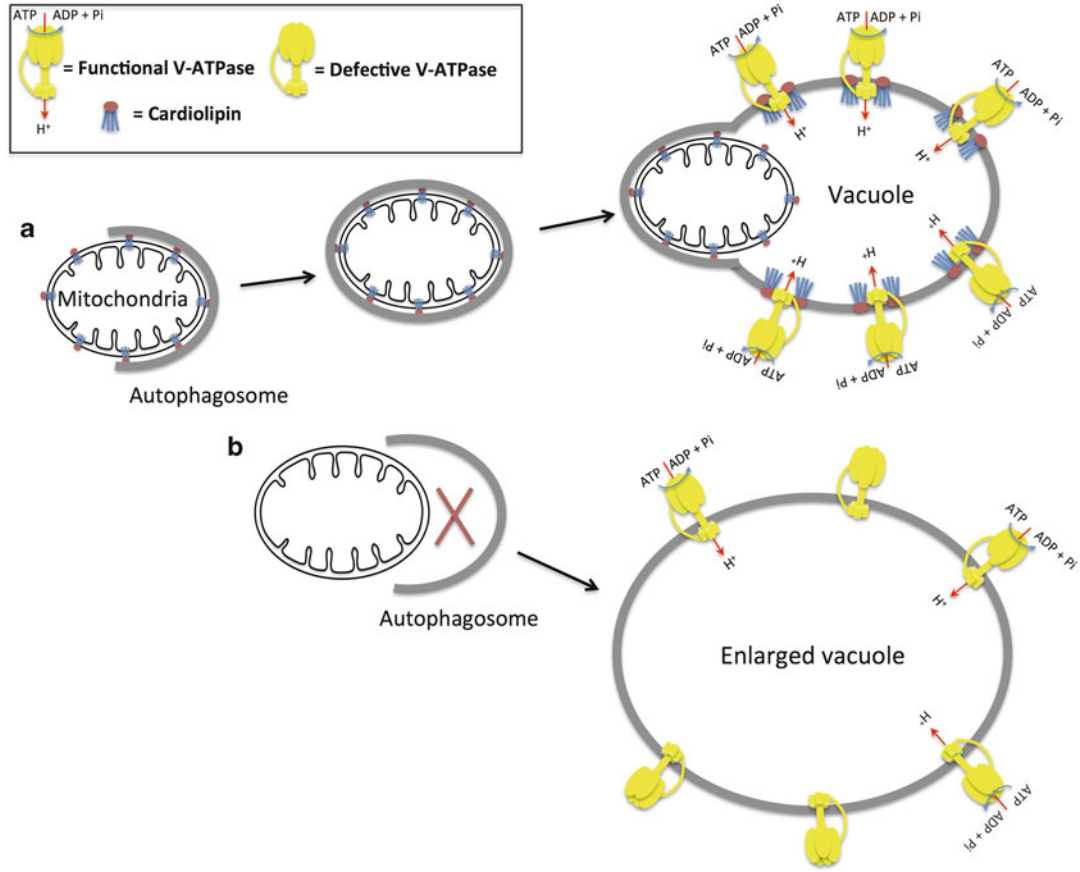

Fig. 11.4 Proposed models to explain the role of CL in vacuolar function. It is likely that CL is transported to the vacuole through mitophagy, the selective degradation of mitochondria via the autophagosome, which delivers its cargo to the vacuole. (**a**) Under normal physiological conditions, CL may provide stability to the V-ATPase, which is essential to maintain its activity [24]. (**b**) CL deficiency may lead to perturbation of mitophagy, which results in decreased delivery of CL to the vacuole and, subsequently, to destabilization of the V-ATPase, decreased ATPase activity, and enlargement of the vacuole

One possible explanation for the vacuolar defects in *crd1*Δ is that the loss of CL leads to intracellular osmotic imbalance, as suggested by the enlarged cell size of the mutant (Fig. 11.4). Consistent with this, deletion of the *NHX1* gene (but not any of the other vacuole ion transporters) in *crd1*Δ restored vacuolar morphology to wild type levels [24]. Nhx1 is the Na^+/H^+ exchanger located in late endosomal/prevacuolar membranes, and is involved in the export of protons in exchange for cytosolic Na^+ or K^+ [182, 183].

Another possibility is that CL may regulate vacuolar function by directly activating the V-ATPase (Fig. 11.4). While CL is predominantly found in the mitochondrial membranes, significant amounts are also detected in the vacuolar membrane, and the levels vary depending on the carbon source of the growth media [184]. How does CL, which is synthesized in the mitochondria, get to the vacuole? The most likely mechanism is via selective degradation of the mitochondria by the autophagic process known as mitophagy, which is strongly induced in yeast by nutrient starvation and during the stationary growth phase [185–190]. CL that has integrated into the vacuolar membrane as a result of mitophagy may directly activate the V-ATPase and/or stabilize the protein. This possibility is highly speculative at this stage, as such interactions have not yet been reported.

Cross-talk between mitochondria and vacuole is further supported by a recent finding, which showed that the vacuolar pH is a determinant of mitochondrial function and aging in yeast cells [191]. Aging yeast cells exhibit a decline in vacuolar acidity, which causes mitochondrial dysfunction and a decrease in replicative life-span [191]. Consistent with this, enhancing vacuolar acidity by overexpressing *VMA1* or *VPH2*, which encode proteins that regulate V-ATPase activity, suppressed mitochondrial dysfunction. The mechanisms underlying the interplay between vacuole and mitochondria, and the role of CL in this process, remain to be elucidated.

11.8 Unanswered Questions and Future Directions

The studies discussed here show that changes in the levels and species of CL affect not only mitochondrial function but also signaling pathways and other organelles. Elucidating the mechanisms whereby CL mediates these activities remains an exciting area for future investigation. In this regard, we pose the following questions.

During apoptosis, the CL content of the outer mitochondrial membrane increases [83, 87]. How is CL transferred from its site of synthesis in the inner mitochondrial membrane to the outer membrane?

Peroxidation of CL has been shown to be a major mechanism of free radical toxicity resulting from ischemia-reperfusion injury to cardiac myocytes [129, 192–194]. The degree of peroxidation is dependent upon the acyl composition of the lipid. What regulates the fatty acyl chain composition of CL? Is this regulation age dependent?

What is the mechanism whereby CL regulates vacuolar function and V-ATPase activity? Interestingly, enlargement of the lysosome (mammalian equivalent of the vacuole) was also observed in the mouse model of BTHS, suggesting that the role of CL in vacuole/lysosome function is highly conserved [195]. Are the vacuole/lysosome defects due to CL deficiency? Alternatively, are the defects an indirect consequence of perturbation of mitophagy?

How does CL regulate the PKC-Slt2 and HOG signaling pathways? The mammalian homolog of HOG1, p38, is also a signal relay protein that responds to osmotic stress. p38 is involved in the cardiac expression of proinflammatory cytokines and in the development of cardiac dysfunction relative to the inflammatory response [196]. A role for p38 in cardiomyopathy is suggested by the finding that depletion of p38α alleviates cardiomyopathy induced by overexpression of the α-adrenergic receptor [197]. However, the link between CL and p38 is speculative, as the effects of CL deficiency on mammalian p38 have not been studied. In addition to p38, members of the PKC family are also involved in maintaining cardiac structure and function [198, 199]. A recent study showed that PKCθ is expressed at significant levels in neonatal mouse ventricular myocytes and is specifically activated during stress [200]. Furthermore, PKCθ deficiency leads to dilation of heart muscle cells and decreased viability. Similarly, PKCε migrates to the mitochondria and plays a cardio-protective role in injuries arising from ischemia and reperfusion [201–203]. However, it should be noted that expression of only a few PKC isoforms exhibit beneficial effects in cardiac injury [204–206]. It would be interesting to determine if CL is involved in the modulation of PKC function in heart muscle.

Answering these questions will have important implications for understanding the pathophysiology of BTHS and other disorders in which CL deficiency plays a role. Although BTHS is a monogenic disorder, the clinical presentation is highly variable, even among patients with the same mutation, ranging from death in the newborn period to asymptomatic. This suggests that physiological modifiers may contribute to the clinical symptoms observed in BTHS patients. It is likely that additional deficiencies in cellular functions that require CL may exacerbate the symptoms of tafazzin deficiency in BTHS.

In conclusion, our understanding of the role of CL in essential cell functions and signaling networks has increased dramatically in recent years. However, it is probably safe to assume that we have only scratched the surface of this expanding field.

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