Regulation of Phosphoinositide Signalling by the Inositol Polyphosphate 5-Phosphatases

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Introduction

Phosphoinositides are ubiquitous components of eukaryotic cell membranes that act as signalling molecules, regulating many essential cellular functions. Stimulation of cells with growth factors leads to the activation of phosphoinositide 3-kinase (PI3K) which phosphorylates phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) at the D-3 position of the inositol ring to transiently generate phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (1,2). PtdIns(3,4,5)P_3 binds and recruits to the plasma membrane the pleckstrin homology domain of the AGC family protein kinases, including protein kinase B (PKB)/Akt, p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK) and protein kinase C (PKC), activating and allosterically regulating these kinases, thereby regulating growth, proliferation and survival, as depicted in **Fig. 1** (2). In insulin-sensitive tissues, PI3K-dependent activation of Akt stimulates metabolic pathways including translocation of the glucose transporter GLUT-4 to the plasma membrane, facilitating glucose uptake.

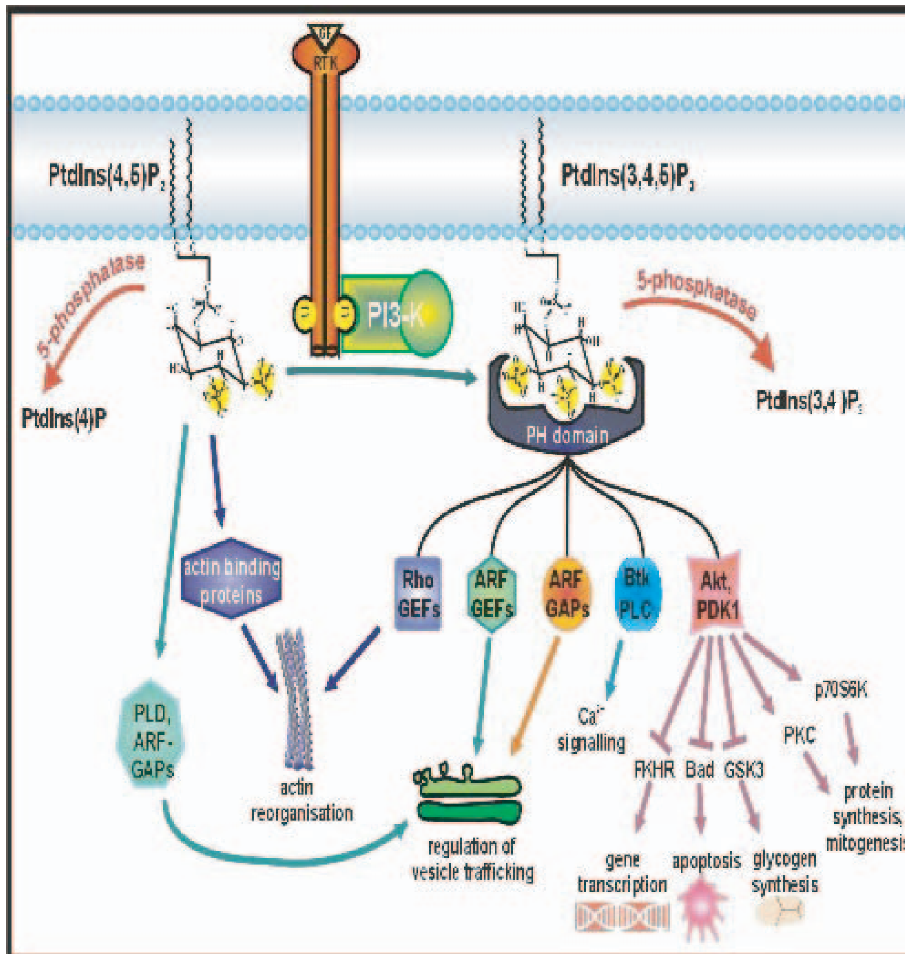

Fig. 1. Phosphoinositide 3-kinase signalling pathway.
Ligand binding by receptor tyrosine kinases results in auto-phosphorylation of receptor intracellular domains, allowing recruitment and activation of phosphoinositide 3-kinase (PI3K). This enzyme converts PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 by phosphorylating the D-3 position on the inositol ring. Following PtdIns(3,4,5)P_3 synthesis at the plasma membrane, pleckstrin homology (PH) domain-containing proteins are recruited and activated with varying downstream effects. Activation of Rho-GEFs can induce changes in actin dynamics, while ARF-GEFS and ARF-GAPs modulate vesicular trafficking, downstream of PtdIns(3,4,5)P_3 formation. The serine-threonine kinase Akt is activated upon phosphorylation by PDK1, resulting in effects on protein synthesis, metabolism, apoptosis and gene transcription via p70S6K and PKC, GSK3, Bad and the forkhead transcription factors (FKHR). The levels of both PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 are negatively regulated by the family of 5-phosphatases which remove the D-5 position phosphate from the inositol ring. PtdIns(4,5)P_2 regulates actin reorganisation via actin filament severing, capping and stabilising proteins and also affects vesicular trafficking via phospholipase D (PLD) and ARF-GAPs. 5-phosphatases negatively regulated the levels of PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 at the plasma membrane by hydrolysing the D-5 position phosphate and forming PtdIns(3,4)P_2 and PtdIns(4)P respectively.

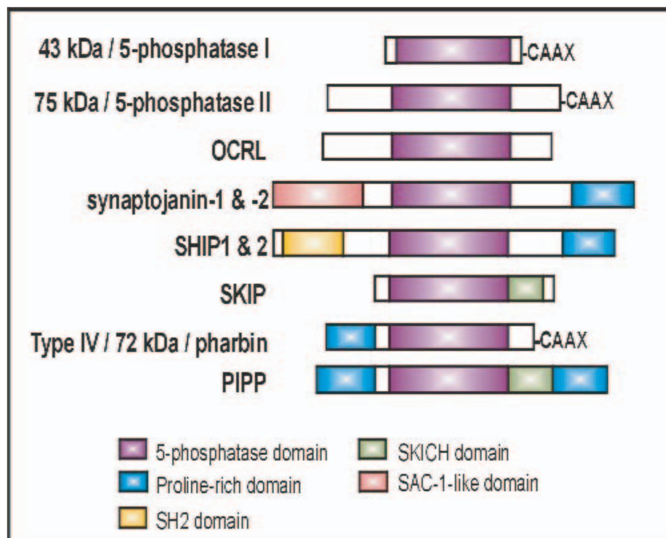

Fig. 2. Domain organisation of the mammalian 5-phosphatases.
Each of the 5-phosphatase family members contains a central conserved 5-phosphatase catalytic domain. The 43 kDa, 75 kDa and Type IV 5-phosphatases each contain a C-terminal CAAX motif, which can mediate plasma membrane targeting, while membrane localisation of PIPP and SKIP is mediated by their SKICH domains. The Src-homology-2 (SH2) domains of SHIP1 and SHIP2 mediate protein-protein interactions, also a function of the proline-rich domains of synaptojanin-1 and synaptojanin-2, SHIP1 and SHIP2, and although yet to be shown for the Type IV and PIPP. The Sac-1-like domains of synaptojanin-1 and -2 mediate hydrolysis of PtdIns(4)P.

PtdIns(4,5)P_2, the substrate for PI3K, is in its own right a signalling molecule regulating actin polymerisation, membrane trafficking and ion channel activity. PtdIns(4,5)P_2 promotes actin polymerisation at the plasma membrane or at membrane microdomains, whereas PtdIns(3,4,5)P_3 regulates the assembly of specialised actin filament structures, such as lamellipodia or filopodia, via activation of the Rho family of small GTPases Rac, Rho, and Cdc42. PtdIns(4,5)P_2 also promotes the formation of integrin-dependent focal adhesions and regulates cell adhesion (3).

Mutations that result in enhancement of PI3K activity, or PtdIns(3,4,5)P_3 signal duration, have been well described in a number of cancer types. In a normal cell PtdIns(3,4,5)P_3 is rapidly dephosphorylated by lipid phosphatases including the tumour suppressor PTEN and the inositol polyphosphate 5-phosphatases. PTEN hydrolyses PtdIns(3,4,5)P_3 forming PtdIns(4,5)P_2, whilst the 5-phosphatases dephosphorylate the 5-position phosphate from the inositol ring of PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 forming PtdIns(3,4)P_2 and phosphatidylinositol 4-phosphate (PtdIns(4)P) respectively (4). Loss of PTEN is associated with both inherited cancer predisposition syndromes and acquired sporadic cancers (2). However, the role of the 5-phosphatases as tumour suppressors has not been elucidated.

The inositol polyphosphate 5-phosphatases (5-phosphatases) are a large family of signal modifying enzymes comprising ten mammalian and four yeast members. The domain organisation of the mammalian 5-phosphatase family members is shown in **Fig. 2**. The crystal structure of an archetypal 5-phosphatase has revealed the 5-phosphatases belong to the AP endonuclease family with a His/Asp active site pair (5). Gene-targeted deletion of 5-phosphatases in mice has revealed these enzymes regulate hematopoietic cell proliferation (SHIP1), synaptic vesicle recycling (synaptojanin), insulin signalling (SHIP2 and SKIP), and vesicular trafficking (OCRL) (4).

This review will focus on the more recently identified mammalian 5-phosphatases including SHIP-2, OCRL, SKIP and PIPP, and their proposed role in cellular function and human disease. The first two 5-phosphatases to be discussed, SHIP2 and OCRL, have been linked to specific human diseases, whilst SKIP and PIPP have only recently been identified and remain partially characterised. These 5-phosphatases share common substrates and have wide tissue and cellular distributions. Despite this, the phenotypes associated with loss of function of discrete 5-phosphatases such as OCRL or SHIP2 are distinct, with seemingly little functional redundancy.

The *SH*2 Domain Containing *I*nositol *P*olyphosphate 5-Phosphatase-2: SHIP2

The SH2 domain-containing inositol polyphosphate 5-phosphatase-2 (SHIP2) is a widely expressed 142 kDa 5-phosphatase containing an N-terminal SH2 domain, a central catalytic 5-phosphatase domain and a C-terminal proline-rich domain. The catalytic activity of SHIP2 was initially proposed to be limited to the 5-position phosphate hydrolysis of PtdIns(3,4,5)P_3, with other studies suggesting an ability to hydrolyse PtdIns(4,5)P_2 and Ins(1,3,4,5)P_4. More extensive analysis of SHIP2 substrate specificity has revealed the rank order of SHIP2's substrates *in vitro* is Ins(1,2,3,4,5)P_4 > Ins(1,3,4,5)P_4 >PtdIns(3,4,5)P_3 ~ PtdIns(3,5)P_2 ~ Ins(1,4,5,6)P_4 ~ Ins(2,4,5,6)P_4. SHIP2 complexes with filamin, p130Cas and Shc, assisting in actin associated cytoskeletal activity (5).

SHIP2 studies have mainly focussed on investigating the cellular effects of PtdIns(3,4,5)P_3 hydrolysis in insulin-sensitive systems. Several SHIP2 over-expression studies in insulin-sensitive cells have provided evidence that SHIP2 regulates PI3K insulin signalling and trafficking of GLUT-4 to the plasma membrane (6). In support of these results *SHIP2* knockout mice were reported to develop fatal hypoglycaemia early after birth as a consequence of increased insulin sensitivity, mediated by enhanced

insulin-stimulated GLUT-4 translocation to the plasma membrane (7). The validity of these results has been questioned as the authors of this study recently reported their targeting construct left the first 18 exons encoding INPPL1 intact, generating an *INPPL1*$^{EX19\text{-}28-/-}$ mouse, and inadvertently deleted the third and final exon of another gene, *Phox2a* (8). This targeting construct should have deleted the key SHIP2 catalytic residues, however, the effect of *Phox2a* gene deletion is unknown.

More recently an additional *SHIP2* knockout (*Inppl1*$^{-/-}$) mouse has been described in which the first 18 exons of the gene were deleted. These mice are viable, have normal glucose and insulin levels and tolerances (9). Interestingly, these mice are highly resistant to weight gain when placed on a high-fat diet and show an increased metabolic rate with normal serum lipids, insulin and glucose levels. The liver and skeletal muscle of *Inppl1*$^{-/-}$ mice demonstrate evidence of enhanced activation of the Akt signalling pathway, however, the precise molecular mechanisms by which these mice remain lean on a high fat diet remain unresolved (9). The reason for the discrepancy between the phenotypes of the two knockout mouse models is not clearly understood, however, the difference in the sequences targeted to produce the knockouts may provide a possible explanation.

Separate genotypic analyses of diabetic rat and human subjects have found an association between *SHIP2* gene polymorphisms and Type 2 diabetes (5). Thus, although the precise role SHIP2 plays in the insulin signalling cascade, and perhaps other signalling cascades, is not thoroughly understood, many lines of evidence support its role in regulation of PI3K-dependent insulin signalling (5,6). While this remains to be clarified, inhibition of the 5-phosphatase may be an effective approach to the treatment of obesity and insulin-resistant states associated with Type 2 diabetes.

Oculocerebrorenal Syndrome of Lowe: OCRL

Oculocerebrorenal (OCRL) disease (Lowe's syndrome) is a rare X-linked disorder first recognised in 1952 by Lowe, characterised by renal failure, growth and mental retardation and cataracts. Positional cloning of X chromosome breakpoints in two Lowe's patients identified the gene *OCRL*, that codes for the 105 kDa 5-phosphatase, OCRL1. *In vitro*, recombinant OCRL1 hydrolyses the 5-position phosphate from Ins(1,4,5)P_3, Ins(1,3,4,5)$_4$ and PtdIns(4,5)P_2, with increased activity towards the latter phosphoinositide (10). It remains unclear how the clinical manifestations of Lowe's syndrome correlate with the enzyme's role in regulating cellular PtdIns(4,5)P_2 levels, however, recent studies suggest that this 5-phosphatase may regulate lysosomal vesicular trafficking.

The OCRL1 substrate PtdIns(4,5)P_2 regulates many cellular processes, including calcium signalling, actin dynamics and vesicle trafficking. Consistent with the role PtdIns(4,5)P_2 plays in the regulation of the actin cytoskeleton, cells derived from Lowe's syndrome patients display decreased actin stress fibres, increased actin punctae and heightened sensitivity to actin depolymerising reagents (11). Independent of PtdIns(4,5)P_2 hydrolysis, effects on actin dynamics are also mediated by the OCRL Rho-GAP domain, which binds active Rac-GTPases and stimulates their activity *in vitro*. Expression of the isolated recombinant OCRL RhoGAP domain inhibits Rac-dependent actin polymerisation in 3T3 cells (10).

Lowe's syndrome patients demonstrate increased lysosomal enzymes in their plasma. This 'leaky lysosome' phenotype, in conjunction with the observation that OCRL1 protein localises in some cell types to the lysosome, supports a role for this 5-phosphatase in lysosomal trafficking (10,12). Complex formation between OCRL1 and specific components of the vesicular trafficking pathway has recently been described and suggests a role for OCRL1 in the trafficking of clathrin-coated vesicles. A clathrin-box motif in OCRL1 that interacts with clathrin heavy chain has been identified, while a separate study demonstrated binding of OCRL1 to the adaptor protein AP-2 (10,12). Another PtdIns(4,5)P_2 specific 5-phosphatase, synaptojanin, functions in synaptic vesicle recycling by regulating the PtdIns(4,5)P_2-clathrin complex and thereby the assembly of clathrin lattices on plasma-membrane derived vesicles. Over- and/or under-expression of OCRL results in the cellular redistribution of AP-1 and the mannose 6-phosphate receptor, implicating OCRL1 as a key regulator of Golgi to endosome trafficking, or retrograde transport from the endosome to Golgi (12).

Further clarification is required to identify the molecular mechanisms mediating the phenotype of Lowe's syndrome. OCRL1 has a wide tissue and cellular distribution (13); however, the clinical manifestations of Lowe's syndrome are restricted to specific tissues. A possible explanation is that of a functional overlap between OCRL1 and other members of the 5-phosphatase family in particular organ systems. Surprisingly, OCRL1 knockout mice show no phenotype suggesting at least in mice the gene is functionally redundant (13). A double knockout mouse lacking both OCRL1 and the type II 5-phosphatase, *Inpp5b*, is embryonically lethal. In humans the type I, type II, and SHIP2 5-phosphatases are co-expressed in kidney proximal tubule cell lines of Lowe's syndrome patients, however, these 5-phosphatases appear unable to compensate for the loss of OCRL activity. The discrepancies between the human disease and mouse models highlight the functional complexity of the Lowe's syndrome.

*S*keletal Muscle and *K*idney Enriched *I*nositol *P*hosphatase: SKIP

SKIP is a recently identified 51 kDa inositol polyphosphate 5-phosphatase which is localised to a region on chromosome 17 frequently deleted or hyper-methylated in human cancers (17p13.3) (14). SKIP is ubiquitously expressed and found at high levels in heart, skeletal muscle and kidney (15). In resting cells, SKIP has a perinuclear distribution and translocates to the plasma membrane following growth factor

stimulation (16). The C-terminal region, designated the SKICH domain, is responsible for the membrane localisation of SKIP. This domain has also been identified in the 5-phosphatase PIPP, and targets this protein to the plasma membrane (16). Analysis of the *in vitro* substrate specificity of SKIP predicts that the enzyme hydrolyses both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, with kinetic studies using purified component enzyme assays demonstrating SKIP has a higher affinity for PtdIns(4,5)P_2 than PtdIns(3,4,5)P_3 (15,17). Over-expression of SKIP in both COS7 cells and myoblasts correlates with decreased actin stress fibres and actin polymerisation, perhaps by affecting PtdIns(4,5)P_2 levels (15,16).

Analysis of cell lines overexpressing or underexpressing SKIP have indicated that the 5-phosphatase shows significant activity towards PtdIns(3,4,5)P_3 *in vivo*, regulating PI3K signalling in insulin-sensitive tissues. Over-expression of SKIP inhibits insulin-induced cellular responses including membrane ruffling and GLUT-4 translocation to the plasma membrane. Decreases in glucose uptake and glycogen synthesis were also demonstrated in over-expressing cells (15). Furthermore, over- and under-expression experiments confirmed SKIP negatively regulates insulin-stimulated Akt phosphorylation (18). SKIP knockout mice are yet to be described, so the functional role of this 5-phosphatase remains unexplored.

*P*roline-Rich *I*nositol *P*olyphosphate 5-*P*hosphatase: PIPP

The relatively uncharacterised 108 kDa proline-rich inositol polyphosphate 5-phosphatase (PIPP) contains proline-rich domains at its N- and C-termini, a central 5-phosphatase domain and a C-terminal SKICH domain. Like SKIP, the SKICH domain of the recombinant enzyme mediates its localisation to plasma membrane ruffles (16,19).

PIPP was initially reported to hydrolyse the 5-position phosphate from its phosphoinositide substrates, PtdIns(4,5)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (19). However, PIPP has also recently been identified as a novel regulator of PtdIns(3,4,5)P_3 and, upon over-expression in COS1 cells, negatively regulates Akt activation following EGF stimulation (20). Despite a wide tissue distribution, including brain, heart, kidney, stomach, small intestine and lung, little is known of the *in vivo* function of this 5-phosphatase (19).

Studies of PIPP function in the neuronal-like rat pheochromocytoma PC12 cell have revealed a regulatory role for the 5-phosphatase in the PI3K-dependent process of neurite elongation (20). During neuritogenesis, PI3K is activated in the tips of extending neurites, resulting in the activation of Akt, inactivation of GSK-3β and subsequent promotion of microtubule polymerisation and neurite extension. In differentiated PC12 cells, PIPP localises constitutively to the plasma membrane of the cell body, and is enriched in the tips of extending neurites, specifically at the growth cone (20). Upon over-expression of wild type PIPP, but not the catalytically inactive enzyme, neurite elongation is inhibited. Conversely, targeted PIPP depletion, using RNA interference, results in the extension of hyper-elongated neurites that exhibit increased polymerised β-tubulin in the neurite shaft. Hyper-elongated neurites demonstrate an increased accumulation of PtdIns(3,4,5)P_3, phospho-Akt and phospho-GSK3β at the neurite growth cone (20). Therefore, PIPP is a negative regulator of PI3K-dependent neuronal differentiation, via the specific spatial regulation of PtdIns(3,4,5)P_3 and its downstream signalling cascades (20).

Summary

Members of the 5-phosphatase family have diverse functions in spatially and temporally regulating the levels of both PtdIns(3,4,5)P_3 and/or PtdIns(4,5)P_2 at the plasma membrane. Each of the 5-phosphatases discussed here, SKIP, PIPP, OCRL and SHIP2, have overlapping substrate specificities, yet have been implicated in distinct cellular processes. Protein-protein interactions mediated by N- and C-terminal protein interaction domains are likely to regulate 5-phosphatase catalytic activity at specific subcellular localisations, allowing individual family members non-redundant functions. Characterisation of PIPP and SKIP knockout mice will further our understanding of the functional role of these 5-phosphatases, and also of the complexity of phosphoinositide signalling.

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