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# Structural Basis for Phosphoinositide Substrate Recognition, Catalysis, and Membrane Interactions in Human Inositol Polyphosphate 5-Phosphatases

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## SUMMARY

**SHIP2, OCRL, and INPP5B belong to inositol polyphosphate 5-phophatase subfamilies involved in insulin regulation and Lowes syndrome. The structural basis for membrane recognition, substrate specificity, and regulation of inositol polyphosphate 5-phophatases is still poorly understood. We determined the crystal structures of human SHIP2, OCRL, and INPP5B, the latter in complex with phosphoinositide substrate analogs, which revealed a membrane interaction patch likely to assist in sequestering substrates from the lipid bilayer. Residues recognizing the 1-phosphate of the substrates are highly conserved among human family members, suggesting similar substrate binding modes. However, 3- and 4-phosphate recognition varies and determines individual substrate specificity profiles. The high conservation of the environment of the scissile 5-phosphate suggests a common reaction geometry for all members of the human 5-phosphatase family.**

## INTRODUCTION

Membrane-bound phosphoinositides (PtdInsP) and the corresponding soluble inositol phosphates (InsP) regulate a multitude of cellular processes including cell proliferation, synaptic vesicle recycling, receptor signaling, and actin polymerization (Di Paolo and De Camilli, 2006; Michell, 2008). They consist of a glycerol-phospholipid linked through a phosphodiester bond to the hydroxyl in position 1 of a *myo*-inositol molecule. Hydroxyls in positions 3, 4, and 5 on the *myo*-inositol ring can be phosphorylated by kinases, thus generating the seven possible isoforms that have so far been identified in higher eukaryotes. These phosphorylation events can be reverted by the antagonist action of specific phosphatases. The consequence is that each phosphorylated isoform mediates specific signaling pathways while also being an intermediate in the production of other phosphorylated isoforms (reviewed in Hakim et al., 2012 and Maffucci, 2012). For example, PtdIns(4,5)-bisphosphate is involved in vesicle trafficking (Martin, 2001), ion-channel regulation (Gamper and Shapiro, 2007), endocytosis (Poccia and Larijani, 2009), exocytosis (Eberhard et al., 1990), actin polymerization (Hartwig et al., 1995; Tolias et al., 2000), and is the precursor of PtdIns(3,4,5)-triphosphate through the action of type I phosphoinositide 3-kinase (PI3K; Vanhaesebroeck et al., 1997).

The variety of processes regulated by phosphoinositides is illustrated by the large spectrum of diseases in which they have been implicated, such as cardiovascular diseases, diabetes, neurological diseases, ciliopathies, and cancers (Conduit et al., 2012; Hakim et al., 2012). Phosphatidylinositol 3-kinases (PI3Ks) are frequently mutated in human cancers and the PIK3 opposing phosphatase PTEN is the second most commonly mutated tumor suppressor after p53 (Ligresti et al., 2009; Liu et al., 2009; Samuels et al., 2004; Yin and Shen, 2008). Myotubularins, which catalyze the hydrolysis of the 3-phosphate on phosphoinositides, are mutated in X-linked centronuclear myopathy and demyelinating Charcot-Marie Tooth neuropathies and are also connected to cancers, epilepsy, and obesity (Amoasii et al., 2012; Azzedine et al., 2003; Bolino et al., 2000; Laporte et al., 1996; Senderek et al., 2003). Consequently, several proteins involved in phosphoinosite-based signaling constitute important targets for therapeutic intervention (Blunt and Ward, 2012; McCrea and De Camilli, 2009; Moses et al., 2009; Suwa et al., 2010a; Waugh, 2012).

The inositol polyphosphate 5-phosphatases (5-phosphatases) regulate phosphoinositide signaling by hydrolyzing the 5-phosphate position of the inositol ring of both soluble inositol phosphates and membrane-bound phosphoinositides (primarily PtdIns(3,4,5)P3, PtdIns(4,5)P2, and PtdIns(3,5)P2), with different substrate preferences (Astle et al., 2007). This family comprises ten members in mammals that share the catalytic 5-phosphatase domain. Five-phosphatases play important roles in human health (Ooms et al., 2009). The 5-phosphatase OCRL is responsible for the oculocerebrorenal syndrome of Lowe, which leads to neurological and renal defects (Attree et al., 1992). Mutations in OCRL also cause Dent disease, which, like Lowes syndrome,

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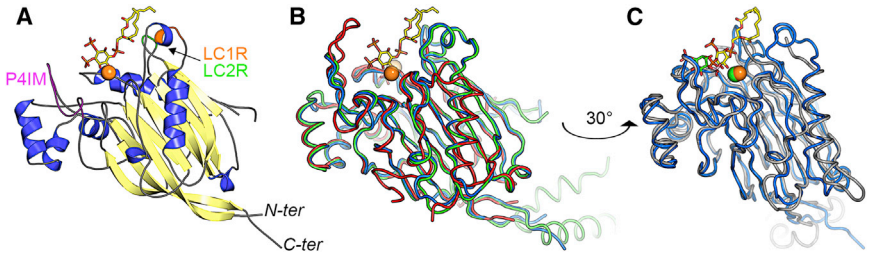


**Figure 1. Structure of Complex between INPP5B-cd and diC8PtdIns(3,4)P2: Overall View and Comparison with SHIP2, OCRL, and SPsynaptojanin**

(A) Ribbon diagram of INPP5B-cd bound to diC8PtdIns(3,4)P2. $Mg^{2+}$ ion is shown as an orange sphere. Motifs LC1R, LC2R, and P4IM are colored respectively orange, green and purple.

(B) Superimposition of INPP5B-cd/diC8PtdIns(3,4)P2 complex (blue), SHIP2-cd (red), and OCRL-cd (green) Cα traces.

(C) Superimposition of INPP5B-cd/diC8PtdIns(3,4)P2 complex (blue) and SPsynaptojanin-cd bound to inositol (1,4)-bisphosphate. Carbon atoms of diC8PtdIns(3,4)P2 complex and of inositol (1,4)-bisphosphate are yellow and green, respectively. $Mg^{2+}$ ion in INPP5B-cd is shown as an orange sphere, $Ca^{2+}$ ion in SPsynaptojanin-cd as a green sphere.

See also Figure S2 for the location of a putative $Ca^{2+}$ ion observed in the structure of apo-INPP5B-cd.

is an X-linked disorder characterized by kidney failure (Hoopes et al., 2005). To date, about 200 different mutations have been identified in OCRL, most of the missense mutations being located in the 5-phosphatase domain of the protein (Pirruccello and De Camilli, 2012; Zhang et al., 2013). OCRL shares 44% sequence identity with INPP5B and the function of INPP5B and OCRL is likely overlapping (Hellsten et al., 2001; Jänne et al., 1998). The 5-phosphatase SHIP2 negatively regulates insulin-mediated signaling (Dyson et al., 2005), and mice deficient in SHIP2 are resistant to diet-induced obesity (Sleeman et al., 2005). SHIP2 is therefore potentially an interesting drug target for obesity and type 2 diabetes. Mutations in the 5-phosphatase domain of INPP5E have also been identified in the Joubert syndrome type of ciliopathy (Bielas et al., 2009).

Despite many links to disease, the understanding of important aspects of phosphoinositide signaling is still limited. At the protein level, the molecular basis for how substrate recognition and membrane interactions control specificity and activity remains rudimentary for most of the enzyme families of these pathways. The only structure of a 5-phosphatase catalytic domain previously reported is that of *Schizosaccharomyces pombe* synaptojanin (SPsynaptojanin), which revealed a fold similar to that of $Mg^{2+}$-dependent endonucleases (Tsujishita et al., 2001). The structure was determined in complex with a soluble inositol(1,4)P2 product and thus did not provide any information on how the 5-phosphatases are able to accommodate the lipid moieties of their substrates. In addition, the orientation of inositol(1,4)P2 in the active site of SPsynaptojanin does not facilitate proper modeling of an intact substrate due to steric clashes with the region expected to accommodate the 5-phosphate. Thus, whereas the current model gives a rough estimation on the region that mediates the natural ligand, detailed information concerning substrate position and orientation, interactions between the protein and the fatty acid part of the phosphoinositides, and the catalytic mechanism in the 5-phosphatase family are not available. Understanding these aspects is essential to provide a link between the missense mutations that have been identified in the 5-phosphatase catalytic domains and the diseases they are responsible for. This is exemplified by the phenotypical differences that exist between Lowe syndrome and the milder Dent disease that can be both caused by mutations targeting the catalytic domain of OCRL. Currently, the incomplete model of protein/ligand interactions in the 5-phosphatase family does not allow discrimination between effects caused by a destabilizing mutation and effects due to alterations either in the catalytic machinery or in the ligand-binding site.

To answer these points as well as to understand the molecular basis for membrane interaction in the human 5-phosphatase family, we have determined structures of the catalytic domains of the human 5-phosphatases SHIP2, OCRL, and INPP5B, the latter in complex with two PtdInsP products. These studies give a detailed view of the catalytic machinery and structural features involved in substrate recognition for key members of the human 5-phosphatase family and revealed striking differences in the substrate binding-mode compared to what has previously been suggested from the SPsynaptojanin structure.

## RESULTS

### Overall Structures of OCRL and INPP5B Catalytic Domains

Useful crystals of the INPP5B and OCRL catalytic domains (INPP5B-cd and OCRL-cd) were obtained after extensive screening of constructs expressing soluble and stable protein using the strategy described by Gräslund et al. (2008). These constructs lack their C-terminal Rho-GAP domains and their N-terminal PH domains, the latter being responsible for the targeting of OCRL to endocytic clathrin-coated pits (Erdmann et al., 2007) mediated through interaction with both clathrin and endocytic clathrin adaptor AP-2 (Ungewickell et al., 2004), whereas its function remains unknown in INPP5B (Mao et al., 2009). The purified catalytic domains were active toward phosphoinositide substrates in vitro (see below). First a 2.65 Å resolution structure of INPP5B-cd was determined in the presence of high $Mg^{2+}$ concentration (200 mM $Mg^{2+}$), revealing an overall fold similar to the SPsynaptojanin catalytic domain (Protein Data Bank [PDB] number 1I9Y, root-mean-square deviation [rmsd] 1.2 Å for 273 superimposed residues, sequence identity of 34%; for sequence alignment, see Figure S1 available online). The fold is composed of a β sandwich, which is lined by several α helices. Subsequently, structures of INPP5B in complex with product analogs were determined (Figure 1A). A tentative catalytic $Mg^{2+}$ ion was found in the active site of all INPP5B structures. A residual density that could not be attributed to either protein or water molecules was observed in apo-INPP5B-cd maps at Asp414, ca 15 Å from the catalytic $Mg^{2+}$ ion. Its size and coordination environment looked similar to what can be expected of a $Ca^{2+}$ ion (Figure S2; this is the identity in the apo-INPP5B-cd model deposited to the

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