SAC1-like Domains of Yeast SAC1, INP52, and INP53 and of Human Synaptojanin Encode Polyphosphoinositide Phosphatases*

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The SAC1 gene product has been implicated in the regulation of actin cytoskeleton, secretion from the Golgi, and microsomal ATP transport; yet its function is unknown. Within SAC1 is an evolutionarily conserved 300-amino acid region, designated a SAC1-like domain, that is also present at the amino termini of the inositol polyphosphate 5-phosphatases, mammalian synaptojanin, and certain yeast INP5 gene products. Here we report that SAC1-like domains have intrinsic enzymatic activity that defines a new class of polyphosphoinositide phosphatase (PPIPase). Purified recombinant SAC1-like domains convert yeast lipids phosphatidylinositol (PI) 3-phosphate, PI 4-phosphate, and PI 3,5-bisphosphate to PI, whereas PI 4,5-bisphosphate is not a substrate. Yeast lacking Sac1p exhibit 10-, 2.5-, and 2-fold increases in the cellular levels of PI 4-phosphate, PI 3,5-bisphosphate, and PI 3-phosphate, respectively. The 5-phosphatase domains of synaptojanin, Inp52p, and Inp53p are also catalytic, thus representing the first examples of an inositol signaling protein with two distinct lipid phosphatase active sites within a single polypeptide chain. Together, our data provide a long sought mechanism as to how defects in Sac1p overcome certain actin mutants and bypass the requirement for yeast phosphatidylinositol/ phosphatidylcholine transfer protein, Sec14p. We demonstrate that PPIPase activity is a key regulator of membrane trafficking and actin cytoskeleton organization and suggest signaling roles for phosphoinositides other than PI 4,5-bisphosphate in these processes. Additionally, the tethering of PPIPase and 5-phosphatase activities indicate a novel mechanism by which concerted phosphoinositide hydrolysis participates in membrane trafficking.

Phosphoinositides are essential components of eukaryotic membranes and are key regulators of membrane trafficking and actin cytoskeleton (1–3). Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) serves as a precursor to second messengers and as a signaling molecule itself by regulating protein activities and through interactions with protein modules (3–5). Additionally, roles for PI(3)P, PI(3,5)P2 and PI(3,4,5)P3 in membrane movement have been defined (1, 6–10). Homeostasis of phosphoinositides occurs both spatially and temporally via a plethora of lipase, kinase, and phosphatase activities, thereby providing several unique points of regulation (for reviews see Refs. 11–17).

A role for inositol lipid phosphatases in membrane trafficking has come from the characterization and cloning of synaptojanin, a mammalian neuronal inositol polyphosphate 5-phosphatase (5-ptase) involved in synaptic vesicle recycling (18). Additionally, studies of three yeast INP5 gene products (also known as SJLs) demonstrate that although they are collectively essential, the individual proteins also have nonredundant roles in regulating membrane trafficking, cell wall synthesis, osmo-sensitivity, and actin cytoskeleton structure (19–22). Synaptojanin and the Inp5ps are members of a large 5-phosphate gene family, conserved from yeast to humans, that specifically remove the D-5 position phosphate from certain phosphoinositides and/or inositol polyphosphates (15–21). Of interest, the domain structures of Inp52p and Inp53p, and to a lesser extent Inp51p, are analogous to synaptojanin, having a 300-amino acid 5-paste domain that is flank by amino- and carboxyl-terminal SAC1-like and proline-rich domains. Although the function of SAC1-like domains in synaptojanin, the Inp5ps, and Sac1p is unknown, genetic studies have shown that mutations in Sac1p overcome certain actin defects and bypass the requirement for Sec14p, a phosphatidylinositol/ phosphatidylcholine transfer protein required for generation of secretory vesicles from Golgi (23–26). In addition, Sac1p function is required for proper transport of ATP into the endoplasmic reticulum (27).

Here we report studies aimed at elucidating the unique and overlapping roles of the INP5 gene products. We demonstrate that Inp52p, Inp53p, and synaptojanin have two lipid phosphatase activities, one as a novel polyphosphoinositide phosphatase (PPIPase) and the other as a PI(4,5)P2 5-paste. Remarkably, we show that these activities are encoded by two autonomous active sites provided by the SAC1-like and 5-paste domains, respectively. PPIPase activity is biologically relevant because loss of this activity in cells results in significant changes in the levels of certain phosphoinositides. The unique substrate selectivity and regulatory properties of each activity suggests novel hypotheses regarding how individual and/or combinatorial control of PI(4)P, PI(3)P, PI(3,5)P2 and PI(4,5)P2 are intimately involved in the regulation of vesicle trafficking and actin cytoskeleton organization.

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was constructed by digestion of pGEX53 with restriction enzymes to yield pGEX53–5pt. The INP53 coding region was inserted in-frame into pGEX-3X vector (Amersham Pharmaclon Science Products) or prepared from steady-state inositol-labeled cells grown in CM containing 200 μCi/ml of [3H]myo-inositol over 10 doublings, harvested at log-phase (1 liter of LB medium was used for a 24-h induction at 23 °C, and cells were disrupted by French press). Fusion proteins were purified by glutathione-Sepharose 4B chromatography according to manufacturer's directions (Amersham Pharmacia Biotech).

Expression of Recombinant Protein in Bacteria—Production of INP5p Proteins—Overexpression of Inp51p, Inp52p, and Inp53p in yeast was achieved on a triple knockout background. Construction of triple mutant strains required deletion of INP54 in previously reported double mutants (20). The INP54 coding region was deleted and replaced in a W303 diploid strain with the LEU2 gene by a PCR-based method (20). Haploid inp54 and appropriate haploid double mutants were mated, sporulated, and dissected to yield inp51 inp52 inp54, inp51 inp53 inp54, and inp52 inp53 inp54 triple mutants.

Overexpression of Inp5ps was accomplished in triple mutant yeast harboring plasmids containing the coding sequence of each Inp5 under control of a galactose-inducible promoter. Plasmids were constructed using a PCR-based strategy in which the coding sequences of Inp5s were amplified by PCR. Cells harboring plasmids containing the coding sequence of each Inp5 were grown in CM/ura– containing 2% raffinose as a galactose (2%) and 0.2% glucose for 6 h and harvested. Soluble extracts were prepared from 1 × 10^8 cells by bead beating in 200 μl of 250 mM Tris-HCl, pH 7.5, supplemented with 25 mM EDTA, 5 mM sodium pyrophosphate, 750 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO_4, and 25 mM EDTA, 5 mM sodium pyrophosphate, 750 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO_4. Mixed vesicle substrates were prepared by drying total cell or purified lipids under a N_2 stream and briefly sonicating in buffer containing 50 mM HEPES, pH 7.4, 50 mM KCl, 3 mM EGTA. Detergent mutant strains were grown in CM containing 0.2% Triton X-100. Enzymatic assays were performed in 2 ml of buffer containing 0.2% Triton X-100 (21). Lipids were deacylated with methylamine reagent essentially as described (21).

RESULTS AND DISCUSSION

Sequence of the Saccharomyces cerevisiae genome reveals four gene products, designated INP5ps, that have the canonical catalytic motifs present in all inositol polyphosphate 5-phosphatases. Previously, we reported that recombinant Inp51p functions as a lipid-selective 5-ptase that converts PI(4,5)P_2 to PI(4)P but does not hydrolyze Ins(1,4,5)P_3 (21). We have recently found that Inp54p is also a PI(4,5)P_2 lipid selective 5-ptase. These data coupled with published reports that yeast extracts do not have detectable Ins(1,4,5)P_3 5-ptase activity (21, 30) suggest that the Inp5ps utilize lipid substrates. Candidate D-5 phosphorylated lipid substrates present in yeast include PI(4,5)P_2 and the newly discovered osmotic stress-induced PI(3,5)P_2 (31, 32). To help explain the individual and overlapping cellular functions of the Inp5ps, we characterized their substrate selectivities.

**Polyphosphoinositide Phosphatase and 5-Phosphate Activities of the Inp5ps**—In our initial biochemical assays, we used native Inp5 proteins and presented lipid substrate in two distinct contexts: mixed vesicles and detergent micelles. Individual native Inp5ps were overproduced in a yeast strain that lacked the other three 5-phosphatases (GAL51 in inp52 inp53 inp54, GAL52 in inp51 inp53 inp54, and GAL53 in inp51 inp52 inp54). Total cellular lipids harvested from a yeast strain labeled with [3H]-inositol-labeled yeast were used to prepare the substrates as a means to approximate the phospholipid stoichiometry and composition encountered in vivo. The abundance of PI(4,5)P_2 and PI(3,5)P_2 candidate substrates was increased 2- and 10-fold by preparing lipids from stressed inp51 mutant strains, resulting in readily detectable 3H-PIP_1, 3H-PIP(3)P, 3H-PIP(4)P, 3H-PIP(3,5)P_2, and 3H-PIP(4,5)P_2 (Fig. 1).

Individual Inp5ps show distinct substrate selectivities and sensitivity to detergent (Fig. 1). Treatment of mixed vesicle substrate (Fig. 1A) with buffer control or pGAL extract (not

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**TABLE I**

**Yeast strains used in this study**

| Strain       | Genotype                                      | Reference |
|--------------|-----------------------------------------------|-----------|
| W303         | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 | a         |
| JYY3         | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 insp51::LEU2 | 20        |
| LSY70        | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 insp51::LEU2 insp52::HIS3 | 21        |
| LSY105       | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 insp51::LEU2 insp53::HIS3 | 21        |
| LSY132       | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 insp54::LEU2 | This study |
| LSY169       | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 insp53::LEU2 insp54::LEU2 | This study |
| LSY172       | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 insp53::LEU2 insp54::LEU2 | This study |
| LSY294       | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 insp52::LEU2 insp53::HIS3 insp54::LEU2 leu2::pBJINP53 | This study |
| LSY302       | MATa ade2–1 ura3–1 his3–11,15 trp1–1 leu2–3, 112 can–100 insp52::LEU2 insp53::HIS3 insp54::LEU2 leu2::pBJINP52 | This study |
| CTY182       | MATa ura3–52 his3–200 lys2–801                | 25        |
| CTY244       | MATa ura3–52 his3–200 lys2–801 sac1::296::HIS3 | 23        |

a Strain W303 was kindly provided by S. Wente (Washington University, St. Louis, MO).
resulted in a 100% decrease in PI(4,5)P₂ and a quantitative increase in PI(4)P (not shown). These data are similar to those obtained with native Inp53p and demonstrate that both the PPIPase and 5-ptase activities are intrinsic properties of Inp53p and presumably Inp52p.

The PPIPase Activity Is Attributable to SAC1-like Domains—The unique biochemical properties and substrate selectivity of PPIPase and 5-phosphatase activities suggest that they are separable. Therefore, we separated partially proteolyzed recombinant Inp53p by size exclusion chromatography and assayed individual fractions for PPIPase and 5-ptase activities. This analysis confirmed that these activities are separable (not shown). Furthermore, the PPIPase and 5-ptase activities were detected in fractions eluting as low as 30 and 25 kDa, respectively (not shown). This was intriguing because multiple sequence alignments predict that the SAC1-like and 5-ptase domains are approximately this size. With this in mind, the 5-ptase and SAC1-like domains of INP53 were independently expressed as GST fusion proteins and analyzed (Fig. 2A). Treatment of mixed vesicle substrate with the 5-ptase domain resulted in a 40% decrease in PI(4,5)P₂ and a quantitative increase in PI(4)P (GST53-5pt), demonstrating that it encodes a 5-ptase but not PPIPase activity. Remarkably, treatment of mixed vesicles with the SAC1-like domain resulted in >70% reduction in the levels of PI(3,5)P₂, PI(4)P, and PI(3)P, whereas PI(4,5)P₂ levels did not change (GST53-Sac). Using detergent micellar substrate we found that the PPIPase activity of GST53-Sac was ablated and that the 5-ptase activity of GST53-5pt was stimulated (not shown), recapitulating data observed for the full-length GST53 protein. The PPIPase activity was found to be Mg²⁺-independent and inhibited by 2 mM Cu²⁺ and Zn²⁺ (80%) and by Mg²⁺ (20%) (not shown). Addition of DTT markedly increased activity. Together these data demonstrate that both the SAC1-like and 5-ptase domains of Inp53p autonomously fold and are sufficient to encode the intrinsic PPIPase and 5-ptase activities found in Inp53p.

The product(s) formed by the PPIPase activity characterized using purified PIP₂ and PIP mixed vesicle substrates (Fig. 2B and C). Treatment of either lipid substrate with GST protein alone did not produce detectable hydrolytic activity. Full-length GST53 quantitatively hydrolyzed PIP₂ to form PI, and no PIP intermediates were detected. Treatment of PIP₂ with GST53-5pt protein converted PI(4,5)P₂ but not PI(3,5)P₂ to PI(4)P. In contrast, GST53-Sac protein hydrolyzed PI(3,5)P₂ to PI without producing detectable intermediates and did not utilize PI(4,5)P₂. Treatment of PIP with GST alone results in no hydrolytic activity. Addition of GST53-Sac results in conversion of both PI(3)P and PI(4)P to PI. In contrast, PIP is not hydrolyzed by GST53–5pt protein (not shown).

To examine whether the SAC1-like domain of human synaptotagmin harbors similar PPIPase activity, we expressed this domain as a GST fusion protein. Of interest, although the majority of reports of the biochemical activity of synaptotagmin show that it is restricted to a type II 5-ptase, Chung et al. (33) reported that purified rat brain synaptotagmin exhibits 4-5-phosphatase activity, which converts PI(4,5)P₂ to PI, although it was not determined whether this activity was intrinsic or a co-purifying contaminant such as the polyphosphoinositidase activity reported by Hope and Pike (34). Treatment of total cell substrate with purified GSTSyn-Sac resulted in >50% decreases in levels of PI(3)P, PI(4)P, and PI(3,5)P₂ (not shown). Again, PI(4,5)P₂ was not a substrate, consistent with the results obtained using GST53-Sac protein. Treatment of purified ³H-PIP with GSTSyn-Sac results in 50% conversion of both isoformes to PI (Fig. 2C). The activity is time- and dose-dependent and has similar biochemical properties to GST53-Sac.
steady-state levels of PI(4,5)P$_2$ did not change in \textit{inp52 inp53} mutant strains. Previously, we reported that steady-state levels of PI(4,5)P$_2$ did not change in \textit{inp52 inp53} mutants (20), whereas two-fold increases were detected in \textit{inp51} null cells (21). However, these studies were performed using thin layer chromatography system not capable of resolving PI(4,5)P$_2$ and PI(3,5)P$_2$. We therefore analyzed \textit{H}-inositol lipid levels in wild type and \textit{inp52 inp53} mutant strains using a deacylation and HPLC strategy. The levels of PI(4,5)P$_2$, PI(3,5)P$_2$, and PI(4)P were similar in both strains (not shown). However, at early log phase ($3 \times 10^6$) the levels of PI(3,5)P$_2$ increased 5–7-fold in \textit{inp52} mutant cells (Fig. 3A). It is noteworthy that even at the increased levels, the amount of PI(3,5)P$_2$ was only 30% of the PI(4,5)P$_2$, thereby explaining why this may have been missed in our previous study. Additionally, this difference was not observed at late log and stationary phase. These data are consistent with a role for \textit{Sac1p} in regulating PI(3,5)P$_2$. This is most likely to be maintained by feedback mechanisms controlling phosphatase, lipase, and/or kinase activities. It is also noteworthy that the pool of PI(4,5)P$_2$ utilized by \textit{Sac1p} is different or sequestered from a pool used to make PI(4,5)P$_2$. This may occur through spatial sequestration or by a protein masking as was recently proposed by Stevenson et al. (35). Alternatively, the levels of PI(4,5)P$_2$ may be maintained by feedback mechanisms controlling phosphatase, lipase, and/or kinase activities. It is also noteworthy that upon deletion of \textit{Fig4p}, another yeast \textit{Sac1}-like domain-containing protein, only small changes were observed in steady-state phosphoinositide levels (not shown). One explanation may be that the pool of lipid that \textit{Fig4p} accesses is relatively small or tightly regulated.

**Conservation of a Mg$^{2+}$-independent Catalytic Motif within Inositol and Protein Phosphatases—\textit{Sac1}-like domains contain a highly conserved sequence motif, RTNCLDCLDRTNX (Fig. 4A). Within this sequence is the CX$_2$R/T/S motif found in other metal-independent protein and inositol polyphosphate phosphatases including the tyrosine and dual function protein PIPases PTP1B and VHR; the inositol polyphosphate 4-phosphatase family; and the PI(3,4,5)P$_3$-phosphatase encoded by the tumor suppressor PTEN (36–38). The observed PIPase in terms of metal inhibition and DTT stimulation (not shown).

The inability of any \textit{Sac1}-like domains tested to hydrolyze PI(4,5)P$_2$ is surprising because they are able to function as 3-, 4-, and 5-phosphatases. Attempts to detect PI(4,5)P$_2$ hydrolytic activity under a variety of conditions, including using commercially available PI(4,5)P$_2$, have reproducibly failed (not shown). Thus, the mechanism by which \textit{Sac1}-like domains discern their substrates remains uncertain and will be of significant future interest.

**\textit{Sac1}-like Domain PIPase Activity Is Functional in the Cell**—To ascertain if PIPase activity of \textit{Sac1}-like domains was functional \textit{in vivo}, we examined the phosphoinositide levels in \textit{inp52 inp53} mutant strains. Previously, we reported that steady-state levels of PI(4,5)P$_2$ did not change in \textit{inp52 inp53} mutants (20), whereas two-fold increases were detected in \textit{inp51} null cells (21). However, these studies were performed using thin layer chromatography system not capable of resolving PI(4,5)P$_2$ and PI(3,5)P$_2$. We therefore analyzed \textit{H}-inositol lipid levels in wild type and \textit{inp52 inp53} mutant strains using a deacylation and HPLC strategy. The levels of PI(4,5)P$_2$, PI(3,5)P$_2$, and PI(4)P were similar in both strains (not shown). However, at early log phase ($3 \times 10^6$) the levels of PI(3,5)P$_2$ increased 5–7-fold in \textit{inp52} mutant cells (Fig. 3A). It is noteworthy that even at the increased levels, the amount of PI(3,5)P$_2$ was only 30% of the PI(4,5)P$_2$, thereby explaining why this may have been missed in our previous study. Additionally, this difference was not observed at late log and stationary phase. These data are consistent with a role for \textit{Fig4p} and \textit{Inp53p} in regulating levels of PI(3,5)P$_2$.

Previous work of Kearns et al. (25) suggested that \textit{Sac1} mutant strains had large increases in mannosyl di-inositol diphosphorylceramide (MIP$_2$C). Our biochemical studies and the observation that TLC systems, such as those used in the Kearns study, may not effectively resolve PIP$_2$s from MIP$_2$C were regulated by \textit{Sac1p} PIPase activity. The dramatic accumulation of PI(4)P in \textit{Sac1} mutant strains suggests that \textit{Sac1p} regulates a major pool of PI(4)P, possibly at the Golgi. Remarkably, despite the 10-fold increases in PI(4)P, the levels of PI(4,5)P$_2$ did not increase, if anything they appear to decrease slightly (Fig. 3B). It is therefore possible that the pool of PI(4)P utilized by \textit{Sac1p} is different or sequestered from a pool used to make PI(4,5)P$_2$. This may occur through spatial sequestration or by a protein masking as was recently proposed by Stevenson et al. (35). Alternatively, the levels of PI(4,5)P$_2$ may be maintained by feedback mechanisms controlling phosphatase, lipase, and/or kinase activities. It is also noteworthy that upon deletion of \textit{Fig4p}, another yeast \textit{Sac1}-like domain-containing protein, only small changes were observed in steady-state phosphoinositide levels (not shown). One explanation may be that the pool of lipid that \textit{Fig4p} accesses is relatively small or tightly regulated.
effects of metal and DTT on PPIPase activity are consistent with the role of this motif in catalysis. These biochemical and sequence similarities indicate a possible evolutionary relationship among these proteins. Additionally, the lack of PPIPase activity in both native and recombinant Inp5p is likely explained by the presence of extensive mutations within this motif to RisafDsiekpN. Most notable are the mutations in the consensus residues C → A, R → K, and (T/S) → P.

Concerted and Independent Action of PPIPase Activity—The assignment of PPIPase activity to SAC1-like domains represents a significant step forward in understanding how its substrates or products may regulate vesicle trafficking, actin organization, and other cellular events as summarized in Fig. 4B. Loss of PPIPase activity in sac1 mutants results in profound changes in the cellular levels of phosphoinositides, especially PI(4)P. In the case of all the "sec14 bypass" mutants, it is thought that the common downstream component is expansion of the diacylglycerol pool (25). The accumulation of phosphoinositides in sac1 mutants may expand the pool of diacylglycerol directly through P1c1p-mediated turnover of PI(4)P or indirectly through alterations in novel phosphoinositide-regulated lipid metabolic pathways that lead to increased diacylglycerol production. Although the largest metabolic changes are observed in PI(4)P, significant changes are also found in PI(3)P and PI(3,5)P2. Thus, it is possible that these lipid-regulated events related to diacylglycerol production may be linked to the ability of defects in Sac1p and presumably PPIPase activity to overcome defects in act1-1 mutants. Our work has been focused on PI(4,5)P2 in regulating actin cytoskeleton; however, we cannot rule out that changes in this lipid do not significantly change in sac1 mutants. Thus we suggest a novel role for PI(4)P, PI(3)P, and/or PI(3,5)P2 in regulating actin organization. Furthermore, our work provides insight into possible roles for PPIPase activity and phosphoinositides in the regulation of ATP transport into the lumen of the endoplasmic reticulum (27).

The fact that Inp52p, Inp53p, and synaptojanin possess both PPIPase and 5-ptase activities is novel and necessitates re-evaluation of the relative roles of PI(4,5)P2 versus PI(4)P, PI(3)P, and PI(3,5)P2 in synaptic and general vesicle trafficking. It is intriguing to speculate as to why the two catalytic domains are found in nature to be tethered or alone (Fig. 4B). One possible reason may be that tethering enables localized substrate hydrolysis. In a concerted model, the 5-ptase domain may hydrolyze PI(4,5)P2 to produce PI(4)P, signal event one, which is then sequentially converted to PI by the SAC1-like domain, signal event two. These events may occur rapidly or slowly awaiting the appropriate regulatory queues. Alternatively, the lipid substrates for 5-ptase and PPIPase activities may be independent and/or localized at different membranes. To this end, each activity may be involved at different steps in vesicle trafficking, for example one during vesicle formation and the other during vesicle docking or fusion. Perhaps PPIPase activity functions to reduce polyphosphoinositides, thereby enabling combinatorial signaling modes for proteins such as Inp52p, Inp53p, and synaptojanin. Clearly, both 5-ptase and PPIPase activities are important in regulating membrane trafficking. It is interesting that Inp51p and certain alternative splice variants of synaptojanin have lost the PPIPase functional domain.

Lastly, our report represents the first description at the molecular level of a PI(3)P-3-phosphatase, a PI(4)P-4-phosphatase, a PI(3,5)P2-3-/5-phosphatase, and collectively a PPIPase. Of significant recent interest is the regulation of PI(3,5)P2. PI(3,5)P2 is transiently present during osmotic stress (32), and its synthesis by Fab1p, a PI(3)P 5-kinase, may be involved in delivering vacuolar proteins via endosome pathway, especially the formation of multi-vesicular bodies (MVB) (7–10). The osmo-sensitive phenotype and abnormal vacuolar structure of certain inp5 mutants suggests their involvement in the regulation of PI(3,5)P2 (19–21). Our data demonstrate that the PPIPase activity of Inp52p, Inp53p, and synaptojanin, and Sac1p are biologically relevant regulators of PI(3,5)P2. Whether or not any of these proteins and PPIPase activity plays a role in regulation of MVB formation or MVB recycling remains to be established.

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