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Allosteric Activation of the Phosphoinositide Phosphatase Sac1 by Anionic Phospholipids

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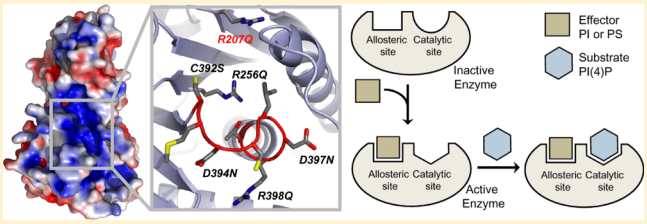
Supporting Information

ABSTRACT: Sac family phosphoinositide phosphatases comprise an evolutionarily conserved family of enzymes in eukaryotes. Our recently determined crystal structure of the Sac phosphatase domain of yeast Sac1, the founding member of the Sac family proteins, revealed a unique conformation of the catalytic P-loop and a large positively charged groove at the catalytic site. We now report a unique mechanism for the regulation of its phosphatase activity. Sac1 is an allosteric enzyme that can be activated by its product phosphatidylinositol or anionic phospholipid phosphatidylserine. The activation of Sac1 may involve conformational changes of the catalytic P-loop induced by direct binding with the regulatory anionic phospholipids in the large cationic catalytic groove. These findings highlight the fact that lipid composition of the substrate membrane plays an important role in the control of Sac1 function.



Phosphoinositides (PIs), a collection of seven biologically active and interconvertable phosphatidylinositol derivatives, are minor yet essential components of the cytosolic leaflet of eukaryotic cell membranes. In response to acute signaling inputs, phosphorylation or dephosphorylation of PIs provides spatial and temporal cues within the cell for PI binding proteins, which in turn promote the assembly of macromolecular complexes and initiate physiological events, including membrane trafficking and actin dynamics. Moreover, in response to extracellular stimuli, PIs can serve as precursors for several types of second messengers in multiple intracellular signaling pathways.[1−3] The acute conversion between different PI derivatives is mediated by a large number of PI kinases and phosphatases.

The Sac domain-containing proteins make up an essential family of PI phosphatases. Members of this family include the transmembrane protein Sac1, as well as the cytoplasmic proteins Sac2/Inpp5f, Sac3/Fig4, and several synaptojanin homologues. The founding member of this PI phosphatase family, yeast Sac1p, an integral membrane protein, was identified more than 20 years ago.[4,5] Since then, considerable progress on this enzyme has been made from yeast to mammals, as well as plants.[6−12] Sac1 is an ER/Golgi-localized PI phosphatase that plays a major role in regulating phosphatidylinositol 4-phosphate [PI(4)P] metabolism.[6,13] It has been shown that Sac1 is essential for the development of multicellular organisms,[9,10,14] and the deletion of Sac1 in yeast results in a broad spectrum of functional defects, including impaired membrane trafficking, an abnormal actin cytoskeleton, and altered lipid metabolism.[15]

Sac1 is conserved in all eukaryotes with an N-terminal cytoplasmic Sac homology domain, a central unstructured linker region, and two C-terminal transmembrane motifs. Recently, the crystal structure of the Sac1 cytoplasmic domain has been determined.[16] The structure revealed that the Sac homology domain is comprised of two closely packed subdomains: a novel N-terminal subdomain and the catalytic phosphoinositide phosphatase subdomain. Unlike other CX_5RT/S-based PI phosphatases, such as PTEN[17] or MTM,[18] Sac1 has a large positively charged groove at the catalytic site. In addition, the catalytic P-loop of Sac1 has a striking conformation in that the catalytic cysteine is far from the conserved arginine that functions to bind the releasing phosphate group.[16] These structural findings suggest that a conformational change of the catalytic P-loop may be required to achieve a functional arrangement of the catalytic residues. It is likely that other protein factors or lipid components are needed for the activation of Sac1. Recent studies have shown that a group of oxysterol binding homology (Osh) proteins activates Sac1 at the ER−plasma membrane contact sites.[19] However, it is not known whether Sac1 phosphatase activity can be regulated by the lipid environment of its targeting membrane bilayer.

To understand the intrinsic enzymatic properties and regulation of Sac1, we performed in vitro kinetic studies of the phosphatase domain of Sac1. We found that Sac1 is an

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allosteric enzyme. The activity of Sac1 can be stimulated by anionic phospholipids, phosphatidylinositol (PtdIns) and phosphatidylserine (PS). We propose that the PI phosphatase activity of Sac1 is intrinsically regulated by the lipid composition of the membrane bilayer where Sac1 acts.

MATERIALS AND METHODS

Materials. diC_{16} PI(4)P, diC_{16} PI(4,5)P_2, diC_{16} PtdIns, diC_{16} PA, and diC_8 PS were obtained from Avanti Polar Lipids, Inc. POPC and POPS were from NOF America Corp. All other diC_8 lipids and inositol phosphates were purchased from Cellsignals Inc. (Columbus, OH).

Mutagenesis, Recombinant Protein Expression, and Purification. DNA sequence corresponding to amino acids 1−511 of yeast Sac1 was amplified via polymerase chain reaction and cloned into a pET28-based bacterial expression vector in frame with an N-terminal six-His-sumo tag. The recombinant fusion protein was expressed in BL21 cells. Cells were harvested and lysed by sonication in phosphate-buffered saline (PBS). The lysate was clarified by centrifugation, and the supernatant was collected and passed through a column packed with cobalt affinity beads (Clontech). The column was extensively washed with PBS and eluted with 200 mM imidazole in PBS. The tag was removed by Ulp1 sumo specific protease, and the recombinant protein was further purified by size exclusion chromatography. The purified protein was stored at −80 °C in storage buffer [50 mM Tris (pH 6.8), 150 mM NaCl, 2 mM DTT, and 50% glycerol]. Sac1 mutants were generated by site-directed mutagenesis, and the constructs were verified by sequencing. The mutant proteins were expressed and purified as described above.

Preparation of Phospholipid Vesicles. Long fatty acid chain lipids were dissolved in a solution containing 90% chloroform and 10% methanol. An appropriate amount of lipids solution was mixed in a glass vial according to the composition of the liposome. The lipids were then dried under a nitrogen stream and rehydrated with enzyme reaction buffer [50 mM Tris (pH 6.8), 150 mM NaCl, and 2 mM DTT] at 37 °C for 1 h. The resulting multilamellar liposome was extruded through a 30 nm pore diameter polycarbonate filter (WaterMan) using a Mini-Extruder device (Avanti) to obtain unilamellar vesicles.

Enzymatic Assays. Because the short acyl chain lipids can form micelles at high concentrations, it is critical to ensure the substrate or the activators are below their critical micelle concentration (CMC) in our assay with water-soluble lipids. It has been shown that diC_8 PS has a CMC of 2.28 mM.[20] The CMC of PtdIns was reported to be ∼0.5 mM.[21] Although the CMC of diC_8 PI(4)P is still unknown, the CMC of diC_8 PI(3)P is ∼0.7 mM;[21] thus, it is safe to estimate that the CMC of diC_8 PI(4)P should also be ∼0.7 mM. Other phospholipids, such as PI(4,5)P_2, should have a CMC value of >0.7 mM because of the larger and highly charged headgroup. The concentrations of the lipids used in our enzymatic assays are all significantly below the CMC values. We expect that the lipid substrates or activators should be monodisperse and do not form micelles in our assays.

Before the enzymatic assay, Sac1 proteins from −80 °C storage were first diluted with reaction buffer to make an enzyme reaction stock (0.1 mg/mL or 1.8 μM). For all the reactions, except for the experiments designed to test the effects of enzyme concentration, the enzyme was further diluted 50 times to a final concentration of 36 nM. All the reaction mixtures were incubated at room temperature. An aliquot of the reaction mixture was taken out and mixed with 50 mM *N*-ethylmaleimide (NEM) to stop the reaction. Phosphate release was measured by the addition of malachite green reagent and absorbance at OD_{620}. To obtain the initial velocities of the reactions, a series of reaction progress curves was recorded at different substrate concentrations. Initial velocities were derived using Dynafit[22] and then plotted against the substrate concentrations using GraphPad Prism version 5 (http://www.graphpad.com/prism).

Analytical Ultracentrifugation. Sedimentation velocity analytical ultracentrifugation experiments were performed on a Beckman Proteomelab XL-A analytical ultracentrifuge at 10 °C. Protein samples were diluted with 20 mM HEPES, 20 mM NaCl, and 2 mM TCEP to a concentration of 1 or 0.1 mg/mL; 300 scans were collected at 50,000 rpm (200,000 *g*) and 230 nm with the time interval between scans set to zero. Sedimentation boundaries were analyzed by the continuous distribution method [$c(S)$] using SEDFIT.[23] SEDNTERP[24] was used to correct experimental S values (s) to standard conditions at 20 °C in water ($S_{20,w}$) and to calculate the partial specific volume of Sac1.

Yeast Strains, Fluorescence Microscopy, and PI Analysis. Yeast strains used in this study are described in Table S1 of the Supporting Information. Plasmid pRS415-p*GPD*-mCherry-2xPH^{Osh2}, which expresses mCherry-labeled tandem PH domains from the yeast Osh2 protein, was used as an in vivo PI(4)P biosensor reporter.[19] Microscopy was performed on midlog cultures at room temperature. Images were obtained with a DeltaVision system and were deconvolved with softWoRx. For in vivo PI level analysis, yeast strains incubated at the indicated temperatures were labeled with [^{3}H]-*myo*-inositol. Lipids were extracted and deacylated for analysis by high-performance liquid chromatography (HPLC) as described previously.[25]

RESULTS

Sac1 Is an Allosteric Enzyme. The phosphatase domain of Sac1 has promiscuous substrate specificity in vitro. In agreement with previous results,[7,26,27] we found that Sac1 prefers to act on PI(5)P, PI(4)P, PI(3)P, with low activity on PI(3,5)P_2, and with no activity on other phosphoinositides, such as PI(4,5)P_2, PI(3,4)P_2, and PI(3,4,5)P_3, or inositol polyphosphates (Figure S1A of the Supporting Information). Furthermore, unlike other inositol polyphosphate phosphatases,[28] divalent ions, such as Mg^{2+}, Ca^{2+}, and Mn^{2+}, have no obvious effect on the activity of Sac1 (Figure S1B of the Supporting Information). Because the major substrate of Sac1 in vivo is PI(4)P,[6,8,29] we used water-soluble diC_8 PI(4)P, which has two C8 fatty acid chains, or liposome-embedded brain diC_{16} PI(4)P (Avanti) as the substrate in all of the following kinetic experiments.

The kinetics of hydrolysis of diC_8 PI(4)P by Sac1 were evaluated by an end point chromogenic assay based on a modified version of the malachite green method as described previously.[30] Initial velocities were derived from series of reaction progress curves at different substrate concentrations with a fixed amount of enzyme (36 nM) and plotted against PI(4)P concentration (Figure 1A). Interestingly, the reaction does not follow simple Michaelis−Menten kinetics; instead, the velocity data assumed a sigmoidally shaped curve, and thus, the data were fit to the Hill equation: $v = V_{max}{}^h[S]/[S]^h + K_{0.5}{}^h$ (where v is the initial velocity, h is the Hill coefficient, and $K_{0.5}$ is the substrate concentration at which v is half-maximal). The

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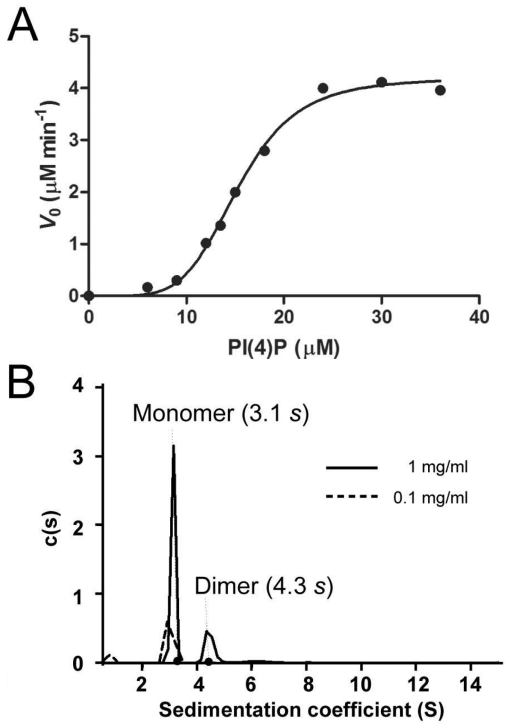

Figure 1. Sac1 is an allosteric enzyme. (A) Concentration dependence of PI(4)P hydrolysis. Initial velocities were determined from reaction progress curves and were plotted vs substrate concentration (*x*-axis). The data were fit to the Hill equation. The Hill coefficient *h* is 4.5 ± 0.3, and the $K_{0.5}$ is 16 ± 2 μM. (B) Sedimentation velocity AUC measurements of Sac1 in solution at different concentrations [1 (—) and 0.1 mg/mL (---)]. The differential sedimentation coefficient distribution *c*(*s*) is plotted vs the sedimentation coefficient. Sac1 is predominantly in the monomeric form (95%) at a protein concentration of 0.1 mg/mL (---).

calculated Hill coefficient *h* is 4.5 ± 0.3, and the $K_{0.5}$ is ~16 ± 2 μM. Sigmoidal kinetics can arise from many possibilities, such as the oligomeric state of the enzyme, multiple catalytic sites, or slow conformational changes.[31,32]

Enzyme Oligomerization Is Not Responsible for the Sigmoidal Kinetic Behavior of Sac1. To test whether Sac1 can form oligomers in solution, we used sedimentation velocity analytic ultracentrifugation (AUC) to examine the solution behavior of Sac1. At 1 mg/mL (18 μM), the enzyme exhibited two peaks with sedimentation coefficients of 3.1 S (3.2 $S_{20,w}$, 55 kDa) and 4.3 S (4.4 $S_{20,w}$, 96 kDa), corresponding to monomeric (80%) and dimeric (20%) forms of the protein, respectively (Figure 1B, solid line). However, 95% of the protein is monomeric when the protein concentration is reduced to 0.1 mg/mL (1.8 μM) (Figure 1B, dashed line). In our enzymatic assays, the final enzyme concentration is only 2 μg/mL (36 nM). These data suggest that Sac1 predominantly exists as a monomer under our assay conditions. We further investigated the correlation between the enzyme activity and its concentration. Sac1 enzymatic assays were conducted with the same amount of substrate but different enzyme concentrations (Figure S2A,B of the Supporting Information). Plotting of the initial velocities versus the enzyme concentration resulted in a linear line, indicating that the catalytic activity of Sac1 has no sigmoidal dependence on enzyme concentration. This result further suggests that the sigmoidal kinetics observed for Sac1 is not due to its oligomerization.

Allosteric Activation of Sac1 by Anionic Phospholipids. It has been reported that allosteric enzymes can be activated by their products to form a positive feedback loop.[33,34] In our reaction system, the product is phosphatidylinositol (PtdIns). We asked whether PtdIns is an activator for Sac1. To examine the effect of PtdIns, the PI(4)P substrate concentration was held constant at 20 μM while the concentration of PtdIns was varied. A series of progress curves were recorded, and the initial velocities extracted from each progress curve were plotted versus PtdIns concentration (Figure 2A). Indeed, the activity of Sac1 was stimulated by the addition of PtdIns, and the activation reached saturation at ~50 μM PtdIns. Furthermore, the activation of Sac1 was not due to enzyme oligomerization because Sac1 remained

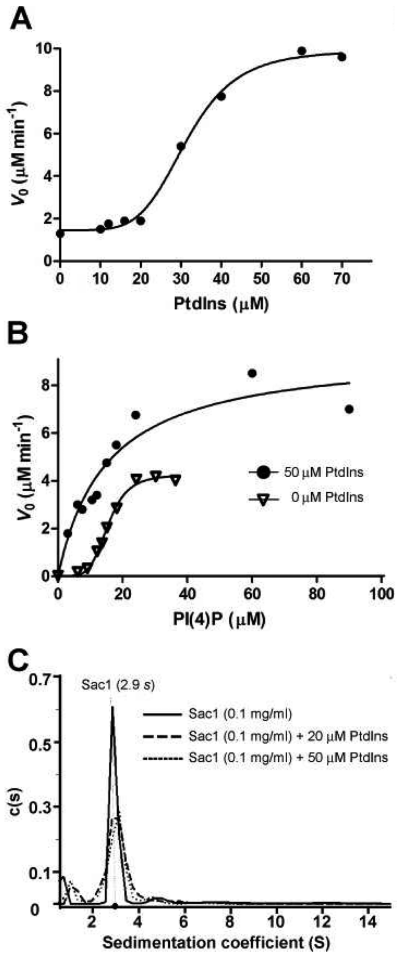

Figure 2. Allosteric activation of Sac1 by its product PtdIns. (A) Concentration-dependent activation of Sac1 by PtdIns. The reactions were conducted with a fixed amount of substrate PI(4)P and varying amounts of PtdIns. Initial velocities obtained from each progress curve were plotted vs PtdIns concentration. (B) PI(4)P hydrolysis in the presence of 50 μM diC_8 PtdIns (●). The initial velocity data were plotted and fit to the Michaelis–Menten equation. Full activation of Sac1 by PtdIns results in a hyperbolic kinetic curve. For comparison, the kinetic curve without an activator (▽, the same curve shown in Figure 1A) is also plotted on the same scale. (C) Sedimentation velocity AUC measurements of Sac1 in solution. Differential sedimentation coefficient distribution *c*(*s*) plotted vs sedimentation coefficient. Experiments were conducted with a protein concentration of 0.1 mg/mL in the absence of PtdIns (—) or in the presence of 20 (– – –) or 50 μM diC_8 PtdIns (···).

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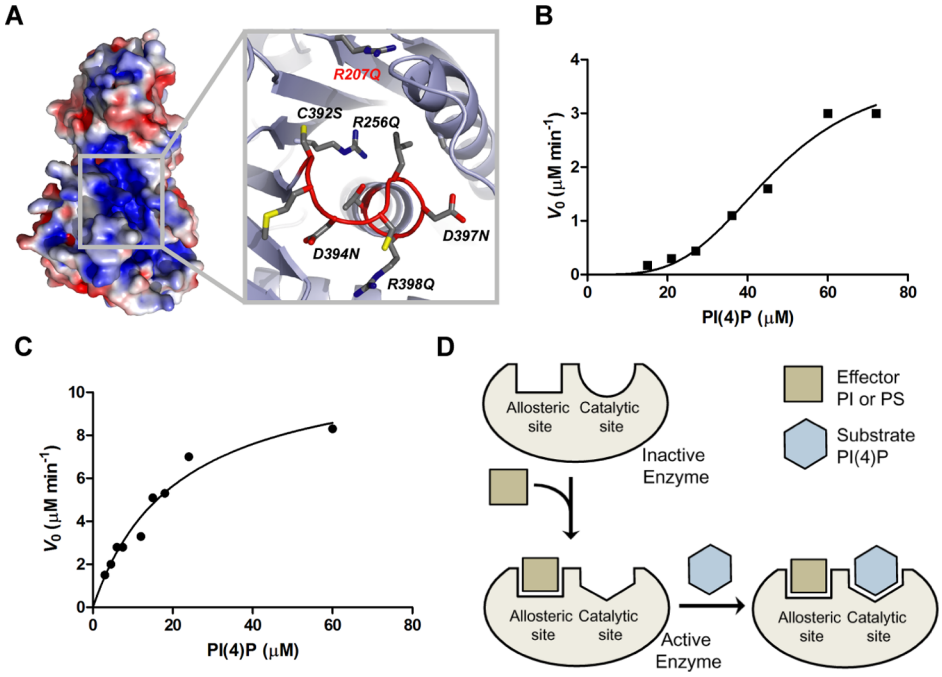

Figure 3. Sac1 mutants exhibit altered kinetics. (A) Surface representative of Sac1 phosphatase. The surfaces were colored on the basis of electrostatic potential with positively charged regions colored blue (+3 kcal/electron) and negatively charged surfaces red (−3 kcal/electron). Sac1 has a deep cationic cleft where the catalytic site resides (left). The catalytic P-loop with the unique conformation is shown as a red tube. Residues selected for mutagenesis are labeled and shown as sticks (close-up on the right). (B) Plot of the kinetics of the R207Q mutant. The initial velocities were plotted vs substrate concentration. Fitting with the Hill equation revealed a significant increase in the $K_{0.5}$ to ~46 μM. (C) Hydrolysis of PI(4)P by the Sac1 R207Q mutant in the presence of 20 μM diC$_8$ PS. The initial velocity data were plotted and fit to the Michaelis−Menten equation. (D) Schematic diagram for the allosteric activation of Sac1. When allosteric activators such as PtdIns or PS bind, the catalytic P-loop may change its conformation, allowing substrate binding and active catalysis by Sac1.

predominantly as a monomer in the presence of PtdIns, as shown by sedimentation velocity measurements (Figure 2C). These results suggest that PtdIns may function as an allosteric activator. The data further predict that if all the enzyme molecules are fully activated by the addition of PtdIns, the reaction should follow simple Michaelis−Menten kinetics. Indeed, when PtdIns was kept at a fixed concentration (50 μM) in our reaction system, the plot of initial velocity versus PI(4)P substrate concentration changed to a hyperbolic curve (Figure 2B) that is distinct from the sigmoidal curve observed in the absence of an activator. These kinetic results, together with previous structure information, suggest that the enzyme may have heterogeneous conformations in solution and the unique catalytic P-loop may be involved in a slow conformational change sampling between an inactive form (observed in our crystal structure) and the active form (seen in other lipid phosphatases, such as PTEN).[16] When activators bind, the balance is favored toward the active form.

Allosteric activation of Sac1 by PtdIns raised the question about the specificity of activators. PtdIns is an anionic lipid with a net charge of −1. We asked whether other negatively charged phospholipids could also activate the enzyme. We performed enzymatic assays in the presence of other water-soluble phospholipids and a fixed amount of PI(4)P. We found that short chain phosphoinositol derivatives, such as PI(4,5)P$_2$ and PI(3,4)P$_2$, did not activate the enzyme. However, Sac1 is strongly activated by phosphatidylserine (PS) (Figure S3A,B of the Supporting Information). To further investigate the activation of Sac1 by PS, we performed kinetic assays similar to the PtdIns activation experiments described above. Interestingly, a sigmoidal PS concentration-dependent rate curve was observed (Figure S4A of the Supporting Information), and the enzyme can be fully activated at a PS concentration of 20 μM. When the enzyme was fully activated by 20 μM PS, a hyperbolic rate curve of initial velocitie versus PI(4)P concentration was obtained (Figure S4B of the Supporting Information). These kinetic data suggest that the activity of Sac1 can be stimulated by anionic phospholipids PtdIns and PS. The biological significance of this activation will be further discussed below.

Conserved Residues That Affect the Kinetics of Sac1. To further investigate the allosteric mechanism of Sac1, we utilized structure information about Sac1 and created substitutions to perturb the catalytic behavior of Sac1. The surface of Sac1 that contains the catalytic site has a deep cationic groove (Figure 3A). The catalytic P-loop assumes a unique conformation.[16] In that conformation, the catalytic C392 is far from R398, which holds the releasing phosphate group on substrates. A conformational change of the P-loop seems to be required to bring C392 and R398 close enough to form a functional catalytic site. We introduced substitutions of several conserved residues, including R207Q, R256Q, C392S, D394N, D397N, and R398Q, that align inside the cationic groove (Figure 3A). R207 and R256 are the major residues that contribute to the cationic properties of the groove. The other residues are components of the catalytic P-loop. Recombinant proteins were expressed and purified as described for the wild-type protein. All mutant proteins were stable and displayed a profile similar to that of the wild-type protein as determined by gel filtration chromatography. Although most of the mutants have no detectable activity in our in vitro enzymatic assays, R207Q is active but with reduced allosteric kinetics compared

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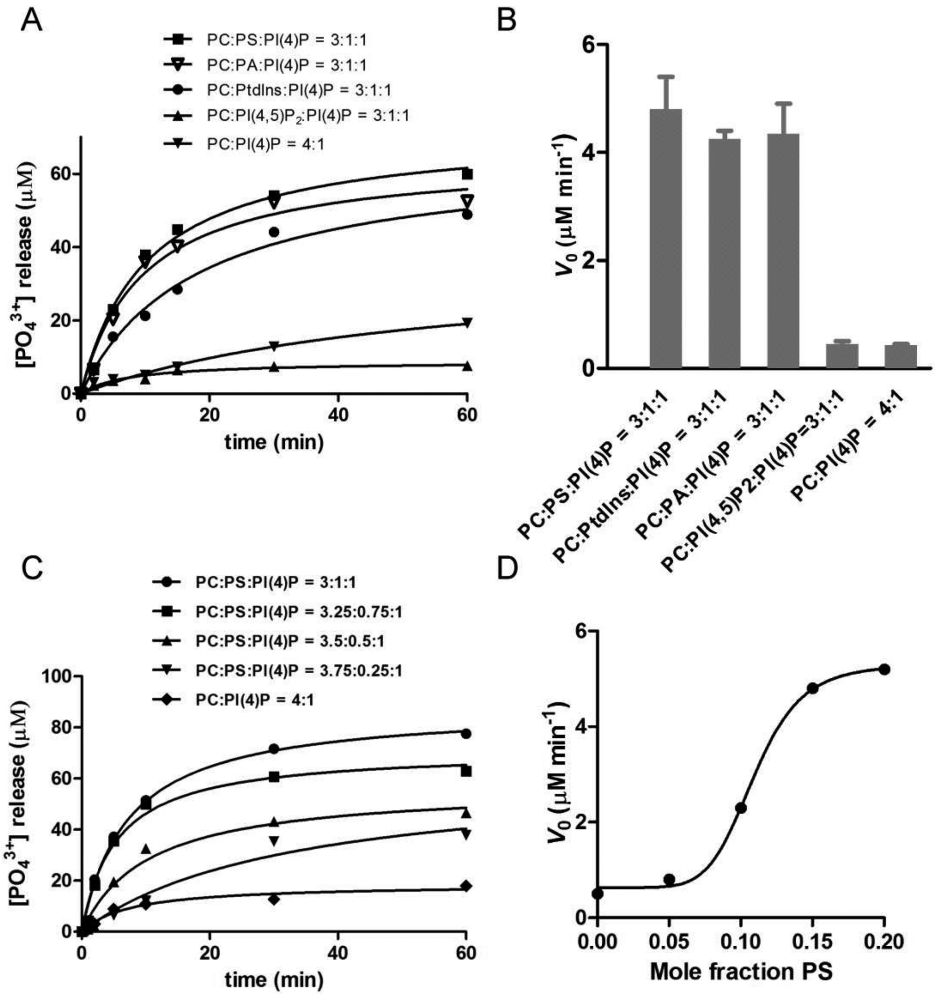

Figure 4. Hydrolysis of diC_{16} PI(4)P substrates presented in liposomes by Sac1. (A) Representative reaction progress curves of the hydrolysis of diC_{16} PI(4)P in liposomes with different lipid compositions by Sac1. (B) Activity of Sac1 toward liposomes with different lipid compositions ($n = 2$). The initial velocity data were extracted from progress curves shown in panel A. (C) Reaction progress curves of the hydrolysis of diC_{16} PI(4)P in liposomes with a fixed amount of PI(4)P substrate but with a different molar fraction of PS. (D) Initial velocities obtained from panel C plotted vs the mole fraction of PS.

to that of the wild-type enzyme. Plots of the initial velocity versus PI(4)P concentration were sigmoidal with a Hill coefficient h of 3.6 ± 0.3, and the $K_{0.5}$ was ~46 ± 5 μM (Figure 3B). The structure of Sac1 revealed that R207 is ~11 Å from the catalytic cysteine and ~20 Å from the arginine (Figure 3A). This large distance suggests that the R207 site cannot be the substrate binding site; instead, it may be an allosteric site. In agreement with this idea, the R207Q mutant can be fully activated by a high concentration (20 μM) of PS (Figure 3C), suggesting R207 does not affect substrate binding. The reduced allosteric effect of the R207Q mutation is likely due to the reduced affinity for allosteric activators. Taken together, our structural observation and kinetic data suggest that R207 is an allosteric site, through which the binding of potential activators induces conformational changes in the catalytic P-loop to activate the enzyme. We thus propose a general mechanism for the kinetic behavior of Sac1 (Figure 3D). Sac1 is an allosteric enzyme and is predominantly in its inactive form in the absence of allosteric regulators. Upon binding of activators, the catalytic P-loop changes its conformation to allow the catalytic cysteine and arginine residues to be positioned in its proximity as seen in other active PI phosphatases, such as PTEN, leading to active catalysis.[17]

Activation of Sac1 on the Surface of Membrane Vesicles. The physiological substrates of Sac1 are embedded in the membrane bilayer. We then asked whether the observed activation of Sac1 by PtdIns or PS applies to membrane-embedded substrates. To answer this question, liposomes were prepared as described (see Materials and Methods). Reaction progress curves were evaluated using liposomes with different lipid compositions (Figure 4A,B). When liposomes with a PC:PI(4)P lipid ratio of 4:1 or a PC:PI(4,5)P_2:PI(4)P lipid ratio of 3:1:1 were used in the assay, the hydrolysis of PI(4)P was barely detectable. However, when liposomes with a PC:PtdIns:PI(4)P lipid ratio of 3:1:1, a PC:PS:PI(4)P lipid ratio of 3:1:1, or a PC:PA:PI(4)P lipid ratio of 3:1:1 were tested, significant PI(4)P hydrolysis was detected in comparison to liposomes lacking PtdIns, PS, or PA (Figure 4A,B). Because PS and PI are relatively abundant phospholipids in eukaryotic cells, we further investigated the dependency of the enzyme activity on the mole fraction of PS and PtdIns in liposomes. Liposomes were prepared with a fixed molar ratio of diC_{16} PI(4)P. The amount of PS or PtdIns was sampled from 0 to 20%, and the neutral lipid PC was adjusted accordingly to make the rest of the fraction. The progress of the reaction was recorded, and initial velocities were plotted (Figure 4C,D and

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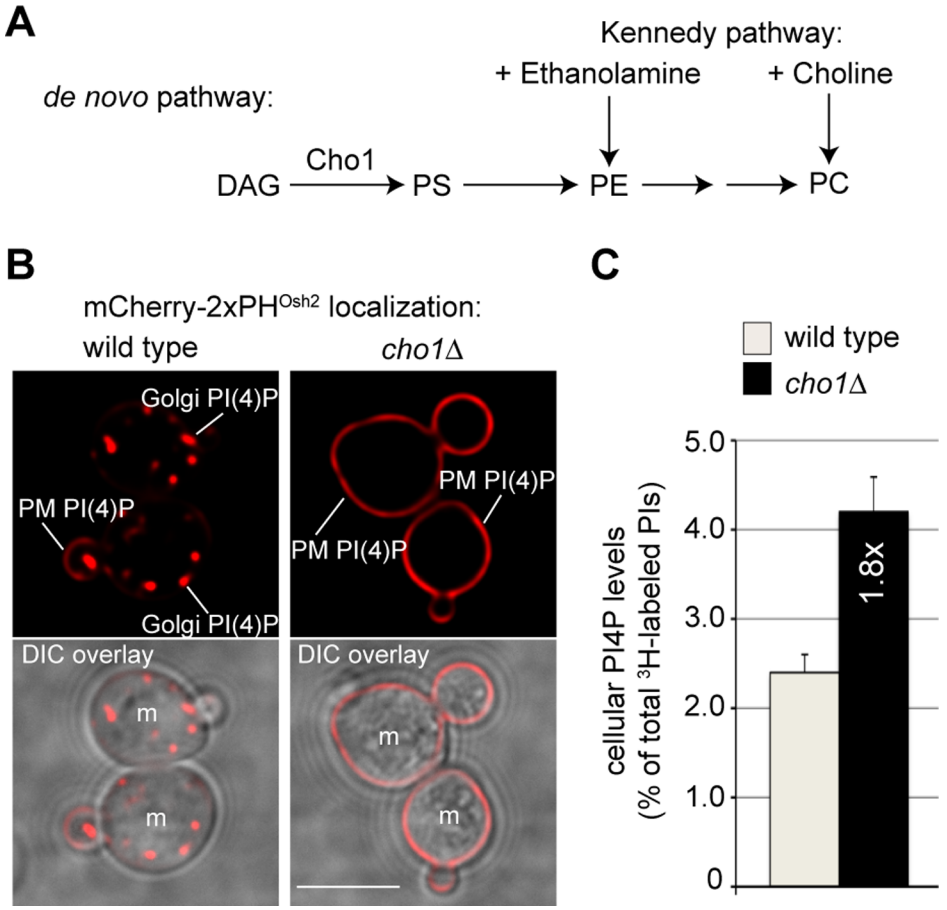

Figure 5. Elevated PI(4)P levels in *cho1*Δ yeast cells. (A) Metabolic pathway of PS. Cho1 is a phosphatidylserine synthase that catalyzes the synthesis of PS from CDP-diaclyglycerol and L-serine. PS can be used to generate PE. *CHO1*-deficient yeast cells are viable, provided the growth medium is supplemented with ethanolamine that can be used to synthesize PE through the Kennedy pathway.[45] (B) PI(4)P localization in wild-type and *cho1*Δ cells. Cells expressing mCherry-2xPH^{Osh2} were observed by fluorescence microscopy. Mother cells are indicated (m). The scale bar is 5 μm. (C) Cellular PI(4)P levels in wild-type and *cho1*Δ cells were measured by [^{3}H]-*myo*-inositol labeling. Lipids from wild-type (gray) and *cho1*Δ (black) cells were extracted and deacylated for analysis by HPLC. Error bars denote the standard deviation of three experiments.

Figure S5 of the Supporting Information). Interestingly, Sac1 activity on PI(4)P-containing liposomes can be stimulated in a PS or PtdIns mole fraction-dependent fashion. These data demonstrate that the activity of Sac1 can be modulated by the lipid composition of the membrane bilayer.

PI(4)P Levels Are Elevated in Phosphatidylserine (PS)-Depleted Cells. The allosteric activation of Sac1 by anionic lipids, particularly by PS, led us to predict that PI(4)P levels should increase in cells with reduced levels of PS. We took the advantage of the available yeast W303-1A strain, which carries the *cho1* gene deletion.[35] Yeast Cho1 is a CDP-diglyceride serine phosphatidyltransferase and is responsible for the de novo synthesis of PS (Figure 5A). We first used the PI(4)P FLARE (fluorecent lipid-associated reporter) mCherry-2xPH^{Osh2} to examine the localization of PI(4)P in vivo. In wild-type cells, the PI(4)P FLARE was found at the Golgi apparatus and the inner plasma membrane surface. However, in *cho1*Δ yeast cells, which lack PS,[35] the amount of PI(4)P FLARE was increased at the PM (Figure 5B), similar to the localization of the PI(4)P FLARE in cells lacking Sac1.[19,36] Furthermore, analysis of [^{3}H]inositol-labeled phospholipids indicated that PI(4)P levels in *cho1*Δ cells are nearly 2-fold higher than in wild-type cells (Figure 5C). This effect was specific for PI(4)P, as levels of other phosphoinositide isoforms were reduced in *cho1*Δ cells (Table S2 of the Supporting Information). Taken together, our results suggest that the anionic lipid PS modulates the localization and levels of PI(4)P in vivo, likely through the regulation of the phosphatase activity of Sac1.

DISCUSSION

In this study, we report that Sac1 is an allosteric enzyme. Although sigmoidal kinetics often indicate multimeric enzymes with several interacting active sites, monomeric enzymes have also been reported to display allosteric regulation.[37] In the case of Sac1, equilibrium between the dimer and monomer was observed in our AUC experiment at a relatively high concentration. However, at the concentration of our enzymatic assays, Sac1 did not exhibit any sigmoidal correlation between its activity and concentration. Although dimerization of Sac1 may provide a regulatory mechanism,[11] the allosteric kinetics of Sac1 that we recorded is likely not due to the oligomerization of the enzyme. On the basis of our mutagenesis studies and the previously published Sac1 structure,[16] we propose that Sac1 is an allosteric enzyme. Binding of activators in the deep cationic groove induces a conformational switch of the catalytic P-loop, thereby activating Sac1. This intrinsic structural switch is reflected macroscopically by the sigmoidal kinetic behavior of the enzyme. Our studies further revealed that Sac1 can be activated by anionic phospholipids PtdIns and PS. The

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activation of Sac1 seems to be specific as other lipids with negatively charged headgroups, such as PI(4,5)P_2 and PI(3,4)-P_2, did not activate Sac1.

Activation of an enzyme by its product has been observed in other lipid phosphatases, including PTEN,[33,38] myotubularin phosphatases,[34] and a secreted salivary inositol polyphosphate 5-phosphatase.[28] PtdIns is most enriched in the endoplasmic reticulum (ER), up to 15% of total phospholipids in ER.[39] Moreover, PtdIns synthesis occurs in the ER where Sac1 resides. This may provide a mechanism for efficiently maintaining low PI(4)P levels at the ER. PA is a minor component of cell membranes. Production of PA by receptor-stimulated phospholipase D has been shown to activate PIP 5-kinase that converts PI(4)P to PI(4,5)P_2 at the plasma membrane.[40−42] Our results showed that PA can activate Sac1 in vitro. However, whether PA can also stimulate Sac1 activity in vivo and the biological consequence of this potential regulation by PA remains to be investigated. The allosteric activation of Sac1 by PS may have interesting biological implications. The requirement of PS for Sac1 to hydrolyze its substrates presented on liposomes suggests that the lipid composition of the membrane on which Sac1 acts is critical for its enzyme activity. Consistent with this, we found that PI(4)P levels are increased in *cho1*Δ mutant cells that are defective in PS synthesis. This further implies that Sac1 may prefer to act in a membrane environment enriched with PS. While PS is an abundant negatively charged phospholipid in eukaryotic cells, it is distributed unevenly within cellular membrane-bound organelles. PS is found preferentially in the inner leaflet of the plasma membrane and less abundant in other membranes, such as the ER and Golgi compartments.[39,43] Genetic and biochemical data have shown that Sac1 regulates a pool of PI(4)P on the plasma membrane.[6,13] The crystal structure of Sac1 revealed a long flexible linker between the catalytic domain and the transmembrane domains. This long linker physically allows the ER-anchored Sac1 to function in trans on the plasma membrane.[16] Our in vitro kinetic results support the idea that the PS-enriched plasma membrane would be a favorable membrane environment in which Sac1 can function. The regulation of Sac1 activity by the anionic phospholipid PS may also be part of a homeostatic regulatory loop that controls the overall surface charge of the plasma membrane. In agreement with this notion, we found that *cho1*Δ cells with reduced PS levels in turn have increased levels of PI(4)P on the inner leaflet of the plasma membrane. Interestingly, cells with impaired Sac1 activity accumulate PI(4)P and in turn have reduced levels of PS.[8,44]

Allosteric enzymes possess a physiological advantage in their ability to respond to changes in the concentration of their substrates and products. The sigmoidal behavior of Sac1 suggests an intrinsic mechanism for the tight and acute control of PI(4)P levels in vivo. However, control of Sac1 activity also requires additional regulatory protein factors. Recent data have shown that a group of oxysterol binding homology (Osh) proteins activate Sac1 at ER−plasma membrane contact sites.[19] Our finding of the activation of Sac1 by anionic lipids adds another layer of regulation to the maintenance of PI(4)P homeostasis in vivo.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting materials, including Figures S1−S5 and Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

PA, phosphatidic acid; PI, phosphoinositide; PtdIns, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; POPC or PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS or PS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; DAG, diacylglycerol; PE, phosphatidylethanolamine.

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