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Resurrection of a functional phosphatidylinositol transfer protein from a pseudo-Sec14 scaffold by directed evolution


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ABSTRACT Sec14-superfamily proteins integrate the lipid metabolome with phosphoinositide synthesis and signaling via primed presentation of phosphatidylinositol (PtdIns) to PtdIns kinases. Sec14 action as a PtdIns-presentation scaffold requires heterotypic exchange of phosphatidylcholine (PtdCho) for PtdIns, or vice versa, in a poorly understood progression of regulated conformational transitions. We identify mutations that confer Sec14-like activities to a functionally inert pseudo-Sec14 (Sfh1), which seemingly conserves all of the structural requirements for Sec14 function. Unexpectedly, the “activation” phenotype results from alteration of residues conserved between Sfh1 and Sec14. Using biochemical and biophysical, structural, and computational approaches, we find the activation mechanism reconfigures atomic interactions between amino acid side chains and internal water in an unusual hydrophilic microenvironment within the hydrophobic Sfh1 ligand-binding cavity. These altered dynamics reconstitute a functional “gating module” that propagates conformational energy from within the hydrophobic pocket to the helical unit that gates pocket access. The net effect is enhanced rates of phospholipid-cycling into and out of the Sfh1* hydrophobic pocket. Taken together, the directed evolution approach reveals an unexpectedly flexible functional engineering of a Sec14-like PtdIns transfer protein—an engineering invisible to standard bioinformatic, crystallographic, and rational mutagenesis approaches.

INTRODUCTION

Phosphorylated derivatives of phosphatidylinositol (PtdIns), or phosphoinositides (PIPs), are elementary components of membrane-associated signaling systems in eukaryotes (Majerus, 1997; Fruman et al., 1998; McLaughlin and Murray, 2005; Ile et al., 2006; Strahl and Thorner, 2007). The remarkable diversity of PIP signaling is established by two major factors. First, enzymes that produce PIPs are differentially localized within cells, thereby facilitating compartment-specific enrichment of individual PIP species. Second, PIP chemical heterogeneities are interpreted by proteins harboring headgroup-binding domains with appropriate positional specificities. The diversity in PIP signaling promoted by chemical heterogeneity is subject to further amplification by combinatorial mechanisms that couple biological recognition of a PIP-dependent event to a second molecular

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Abbreviations used: CPY, carboxypeptidase Y; CW, continuous wave; EPR, electron paramagnetic resonance; MD, molecular dynamics; PIP, phosphoinositide; PITP, PtdIns-transfer protein; PL, phospholipid; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; rmsd, root mean square deviation; rmsf, root mean square fluctuation; TGN, trans-Golgi network; YPD, yeast peptone dextrose.

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interaction (Balla, 2005; Lemmon, 2008). Such coincidence detection is termed a “back-loaded” strategy on the principle that diversification occurs once the PIP has been generated (Bankaitis et al., 2010).

Recent studies demonstrate that production of an individual PIP species by a specific Ptdlns kinase evokes diverse biological outcomes in a single cell (Routt et al., 2005). Those findings are difficult to explain by back-loaded mechanisms alone. Rather, front-loaded strategies also contribute to diversification of PIP signaling outcomes (Schaff et al., 2008; Bankaitis et al., 2010). The principle that underlies front-loaded mechanisms highlights an intrinsic biological inadequacy of Ptdlns kinases (particularly Ptdlns 4-OH kinases), which are insufficient interfacial enzymes when confronted with the dual issues of 1) substrate Ptdlns presented in liposomal contexts and 2) competing activities that degrade PIPs. Productive action of these enzymes is subject to what we term “intrusive” activation (Bankaitis et al., 2010). Sec14-like Ptdlns-transfer proteins (PITPs) are the instructive components in front-loaded regulation of Ptdlns 4-OH kinase activities in yeast by virtue of their roles as primeable “Ptdlns-presentation” modules. For Sec14, effective presentation requires heterotypic Ptdlns/phosphatidyicholine (PtdCho) exchange (Schaff et al., 2008; Bankaitis et al., 2010). The Sec14-protein superfamily is diverse, and a Ptdlns-binding “bar code” is recognized in nearly all members of the superfamily. PtdCho-binding signatures are not narrowly conserved, however, forecasting that lipids other than PtdCho are sensed by Sec14-like proteins and that metabolism of these lipids might also be coupled to PIP synthesis by Sec14-like proteins. Thus the Sec14 superfamily might link diverse territories of the lipid metabolism with PIP signaling (Bankaitis et al., 2003; D’Angelo et al., 2006).

Understanding the mechanics of how Sec14-like PITPs execute phospholipid (PL) exchange is of central importance given heterotypic PL-exchange reactions lie at the heart of the mechanisms by which these proteins integrate lipid metabolism with PIP signaling (Bankaitis et al., 1990, 2010; Cleves et al., 1991). To interrogate the functional engineering of Sec14-like proteins, we used a directed evolution approach to resurrect Sec14-like activities in a protein (Sfh1) that substantially lacks them. We find single residue substitutions, involving conserved residues, are sufficient to endow the (Sfh1) that substantially lacks them. We find single residue substitutions, involving conserved residues, are sufficient to endow the Sfh1 “pseudo-Sec14” with Sec14-like activities in yeast (Sfh1*).

These properties are associated with enhanced abilities of Sfh1* to cycle PL into and out of the protein interior in vitro and stimulate Ptdlns kinase activities in vivo. The resurrected Sec14-like activities result from altered atomic interactions involving an unusual hydrophilic microenvironment within the hydrophobic Sfh1 ligand-binding cavity. These altered dynamics facilitate conformational transitions in core structural elements that control PL exchange from the Sfh1 hydrophobic pocket. Taken together, these studies provide new mechanistic insights into the functional design of a Sec14-like PITP.

RESULTS
Isolation of SFH1* alleles that endow Sfh1 with Sec14-like activities
Enhanced expression of Sfh1, the protein most closely related to the essential yeast Sec14, fails to rescue phenotypes associated with Sec14 defects. Moreover, Sfh1 does not catalyze robust Ptdlns- or PtdCho-transfer activity in vitro (Li et al., 2000). Yet Sfh1 shares 64% primary sequence identity with Sec14 and conserves all of the obvi-ous Sec14 structural motifs critical for Ptdlns and PtdCho binding (Schaff et al., 2008). These findings suggest either that Sfh1 is a pseudo-Sec14 that is unable to effectively undergo the conforma-tional dynamics that accompany PL transfer reactions in Sec14 or that these transitions fundamentally differ from those of Sec14. Our description of Sfh1 as a pseudo-Sec14 holds no implied evolutionary connotation. This convenient descriptor simply reflects that Sfh1, while sharing high primary sequence similarity with Sec14, is not functionally an active Sec14.

To better understand the mechanics of how Sec14-like PITPs function at the single molecule level, we sought to resurrect Sec14-like activities in Sfh1. To that end, missense mutations were incorporated into the SFH1 gene by error-prone PCR and in vivo gap repair, and reconstituted plasmids driving expression of mutagenized SFH1 genes were introduced into a sec14-1ts tsr-32 yeast strain. Transformants were coselected for growth at 37°C and uracil prototrophy. From an estimated 1 × 10^6 potential Ura* transformants, 486 Ura*Ts* transformants were recovered (~0.05% of potential Ura* transformants). Of these, 100 were saved for detailed analysis. In all cases, the Ts* growth phenotype was plasmid linked on the basis of two criteria. First, plasmid loss induced by URA3 counterselection with 5-fluoro-orotic acid was accompanied by loss of the Ts* phenotype. Reciprocally, SFH1 plasmids were recovered from each of the parental Ura*Ts* transformants and reintroduced into naive sec14-1ts mutants by selec-tion for Ura*. In each case, the Ts* phenotype was coherited with plasmid. We refer to these SFH1 activation alleles as SFH1*.

Single substitutions activate Sec14-like functions in Sfh1
Of the 100 SFH1* isolates, complete nucleotide sequences were determined for 40 clones. These analyses demonstrated the SFH1* isolates contained an average of 4.4 nucleotide and 3.1 amino acid substitutions per gene and gene product, respectively (Figure 1A, Supplemental Figure S1). Two hot spots for SFH1* activation alleles were identified. One hot spot includes Sfh1 residues Y_{109} and Y_{113}, while the other highlights the sequence between residues I_{131} and T_{141} (Figure 1A; Supplemental Figure S1). The Y_{109}Y_{113} hotspot involves an enigmatic hydrophilic patch on the floor of the Sfh1 hydrophobic cavity (Sminov et al., 2006; Schaff et al., 2008). This patch is configured in close proximity to the sn-2 acyl chain of bound PtdCho (Figure 1B) and Ptdlns (Figure 1C). The hydrophilic patch surface is organized by residues Y_{109}, Q_{111}, Y_{124}, and E_{125} and involves several coordinated H_2O molecules, one of which resides in close proximity to Y_{113}. The Q_{204}R substitution was also identified in the SFH1* screen. As SFH1{Q204R} is unique among the SFH1*, in that it is far removed from the hydrophilic patch, details of its characterization will be presented elsewhere.

Incorporation of individual Y_{109}C, Y_{109}H, Y_{109}F, and Y_{113}C substitutions into an otherwise wild-type Sfh1 protein demonstrated sufficiency of these substitutions for SFH1* (Figure 2A). Because several substitutions at residue Y_{109} yielded Sfh1*, the analyses were extended by determining whether the Y_{109}A substitution (a side chain deletion) had the same effect. Indeed, Y_{109}A conferred Sfh1* properties to an otherwise unadulterated Sfh1 (Figure 2A).

Because the Y_{109} → F, C, H, A, and Y_{113} → C all represent Sfh1* substitutions, and all affect the enigmatic hydrophilic patch, we tested whether Sfh1{E126A} and Sfh1{Q111A} (i.e., substitutions not encountered in the original activation screen) similarly manifest Sfh1* properties when expressed from centromeric and episomal plasmids in sec14-1ts and sec14A shuffle strains. Both Sfh1{E126A} and Sfh1{Q111A} scored as Sfh1*. Sfh1{E126A} was particularly potent in this regard (Figure 2A). Sfh1{Q111A} was less so—primarily due to reduced stability of this protein (discussed later in this article).
SFH1* protein expression from low-copy plasmids supports protein accumulation to levels that do not exceed those of endogenous Sec14 (Supplemental Figure S2). Moreover, SFH1 and SFH1* gene products generally accumulated to comparable steady state levels, demonstrating the SFH1* phenotype is not a trivial result of increased protein expression or stability. Exceptions included Sfh1Y109A and Sfh1Q111A, both of which exhibited reduced protein stability at 37°C relative to Sfh1 (Supplemental Figure S2). This property is reflected in the modest Sfh1* phenotypes associated with Sfh1Y109A, Q111A. However, expression of Sfh1Q111A rescues growth of sec14-1 yeast at 35°C (unpublished data) and rescues sec14Δ lethality when expressed from episomal expression plasmids (Figure 2A). The observation that Y109A effects a strong Sfh1* phenotype, despite reduced expression levels, testifies to its potency.

**SFH1** expression and trans-Golgi network (TGN)/endosomal membrane trafficking

Phenotypic rescue of Sec14 insufficiencies translates to enhanced ability of Sfh1* to execute Sec14-like functions in protein trafficking through the TGN/endosomal system. The accumulation of cytoplasmic toroid structures that represent defective cargo-laden TGN/endosomal compartments was readily apparent in thin-section electron micrographs of sec14-1* mutants incubated at restrictive temperatures, and Sfh1 expression failed to rescue this aberrant morphology (Figure 2B). By contrast, this morphological phenotype was alleviated in sec14-1* mutants by Sfh1I109A, I113C, or Sfh1I109A, I113C expression. Moreover, introduction of the T238D or S175I, T177I missense substitutions (which specifically compromise PtdIns and PtdCho binding, respectively; discussed later in this article) functionally ablated the Sec14-like functions of these Sfh1* proteins.

Pulse-radiolabeling experiments demonstrate that transit of carboxypeptidase Y (CPY) through the yeast secretory pathway to the vacuole is restored in Sec14-deficient cells by Sfh1* (Figure 2C). In a sec14-1 strain ectopically expressing Sfh1, pulse-radiolabeled CPY was recovered in approximately equimolar fractions as TGN/endosomal p2-CPY and mature vacuolar mCPY forms after a 25-min chase at 37°C. The persistence of p2-CPY postchase reports a trafficking defect from TGN/endosomes to the vacuole. Cells reconstituted with physiological levels of Sec14 expression presented a single radiolabeled CPY species, the mCPY, under the same experimental conditions. Even modest Sfh1* expression was sufficient to rescues growth of sec14-1 yeast at 35°C (unpublished data) and rescues sec14Δ lethality when expressed from episomal expression plasmids (Figure 2A). The observation that Y109A effects a strong Sfh1* phenotype, despite reduced expression levels, testifies to its potency.

**Characterization of Sec14-like activities in Sfh1* proteins**

Expression of any one of these SFH1* gene products rescued robust growth of both a sec14-1Δ strain and its isogenic phospholipase D (PLD)–deficient derivative (sec14-1 spo14Δ) at the restrictive temperature of 37°C (Figure 2A). The ability of SFH1* expression to effect phenotypic rescue of sec14-1Δ growth defects in the spo14Δ genetic background speaks to potency of the activation phenotype as PLD deficiency strongly exacerbates sec14-1Δ–associated growth phenotypes. By comparison, SFH1 expression fails to rescue sec14-1Δ growth defects at 37°C—even after prolonged incubation (Figure 2A). The potencies of SFH1* alleles were also apparent in plasmid shuffle assays that score their ability to restore viability to sec14Δ strains (Phillips et al., 1999; see Materials and Methods). Expression of SFH1* from low-copy plasmids failed to support shuffle of YEp(SEC14), even when SFH1* gene products with two independent activation substitutions were expressed (e.g., Sfh1Y109C,Y113C). We tested 14 double mutant and 2 triple mutant combinations in this regard, and in no case did we observe intragenic synergy between SFH1* mutations (unpublished data). While SFH1 expression failed to support plasmid shuffle under any condition, shuffle of the YEp(SEC14) plasmid was observed when SFH1* expression was driven under control of the powerful plasma membrane ATPase (PMA1) promoter from episomal vectors (Figure 2A).

**FIGURE 1:** SFH1* activation alleles. (A) Protein alignment of Sfh1* mutants obtained in the primary directed evolution screen. Depicted are amino acid residues 100–119. The sequences of Sec14 and the Sfh1 wild-type protein are presented for comparison. (B and C) Ribbon diagrams illustrating the hydrophilic patch at the floor of the hydrophobic PL-binding pocket. Sfh1 residues that are substituted by missense mutations in Sfh1* are shown as sticks. Water molecules are presented as red spheres. PtdCho is rendered in cyan as sticks and transparent spheres in the Sfh1-PtdIns structure (pdb 3B7N, C), while PtdIns is rendered in magenta as sticks and transparent spheres in the Sfh1-PtdCho structure (pdb 3B7Q, B), while PtdIns is rendered in magenta as sticks and transparent spheres in the Sfh1-PtdCho structure (pdb 3B7N, C).

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Sfh1* mutations increase Sfh1-mediated stimulation of PIP synthesis

Sfh1* competence in stimulating PIP production in vivo was quantified by reconstituting SFH1* expression from low-copy vectors in a sec14Δ cki1 “bypass Sec14” yeast strain (see Materials and Methods). This strain exhibits low basal PIP levels as a consequence of sec14 nullizygosity (Phillips et al., 1999; Rott et al., 2005; Schaaf et al., 2008). Reconstitution of Sec14 expression elevates PtdIns-3-P, PtdIns-4-P, and PtdIns-4,5-P2 levels by ~twofold relative to the parental negative control (Figure 4A). While Sfh1 expression was ineffectual in this regard, even modest expression of Sfh1Y109A, Sfh1Y113C, or Sfh1E126A elevated PIP levels significantly (Figure 4A).

Similar conclusions were drawn from experiments in which the influences of Sfh1* proteins and Sfh1 were compared in the isoleted context of Stt4 PtdIns 4-OH kinase activity. To measure Sfh1*-mediated potential of specific yeast PtdIns 4-OH kinase activity in a native intracellular environment, [3H]Ins radiolabeling experiments were performed in sec14Δ sac1 yeast strains expressing the Sfh1 proteins of interest. The sec14Δ sac1 double mutants are defective in Sac1 PIP phosphatase activity and exhibit a dramatic and selective expansion of the PtdIns-4-P pool generated by the Stt4 PtdIns 4-OH kinase (Guo et al., 1999; Rivas et al., 1999; Nemoto et al., 2000; Foti et al., 2001). Reconstitution of Sfh1Y109A, Sfh1Y113C, or Sfh1E126A expression in sec14Δ sac1 mutants effected significant enhancements in PtdIns-4-P levels at 37°C (Figure 4B). PtdIns-4-P levels were indifferent to Sfh1 expression.

PtdIns and PtdCho binding are required for manifestation of SFH1* phenotypes

Individual PtdIns- and PtdCho-binding activities are essential for productive Sec14 biological activity, and these cooperate in stimulating PtdIns 4-OH kinase activity in vivo via a mechanism proposed to involve Sec14-mediated PtdIns-presentation to lipid kinase (Schaaf et al., 2008; Bankaitis et al., 2010). To determine whether PL binding in general is required for elaboration of Sec14-like activities by Sfh1*, “pinch-close” mutants that abrogate binding of all PLs were incorporated into the context of several SFH1* alleles. Residues L179, I196, and V199 organize a region of the Sfh1 hydrophobic cavity that defines PtdCho and PtdIns acyl chain–binding space. Thus introduction of bulkier amino acids (e.g., W) at these positions results in steric incompatibility with PL binding by Sfh1 or Sec14 (Schaaf et al., 2008).

As expected, recombinant Sfh1Y113C, Sfh1Y113C, Sfh1Y113C, Sfh1E126A expression supported an obvious (yet partial) improvement in dye trafficking from Sec14-deficient endosomes to the vacuole (Figure 3).
play important roles in coordinating the PtdCho headgroup phosphate within the Sfh1 binding pocket, and combinatorial substitution of these residues with bulky aliphatic amino acids (e.g., I) is nonpermissive for PtdCho binding (Schaaf et al., 2008). The PtdCho-binding-deficient derivatives of both Sfh1<sup>Y113C</sup> (Sfh1<sup>Y113C,S175I,T177I</sup>) and Sfh1<sup>E126A</sup> (Sfh1<sup>E126A,S175I,T177I</sup>) were stable polypeptides (Supplemental Figure S3, C and D), and in vitro PL-transfer assays confirmed the expected biochemical defects for these two proteins. Sfh1<sup>Y113C,S175I,T177I</sup> exhibited a >25-fold reduction in PtdCho-transfer activity relative to Sfh1<sup>Y113C</sup> and retained PtdIns transfer activity (Supplemental Figure S3, A and B). Expression of Sfh1<sup>Y113C,S175I,T177I</sup> or Sfh1<sup>E126A,S175I,T177I</sup> from low-copy vectors failed to rescue growth of sec14<sup>ts</sup>-strains at restrictive temperatures (Figure 4C). Moreover, enhanced expression of Sfh1<sup>Y113C,S175I,T177I</sup> or Sfh1<sup>E126A,S175I,T177I</sup> driven by high-copy vectors failed to rescue lethality associated with the sec14Δ allele (Figure 4C).

The failures of the various PL-binding-defective variants of SFH1* to rescue sec14<sup>ts</sup>-associated growth defects translated to defects in regulation of PIP homeostasis. Stable variants of Sfh1<sup>*</sup> impaired for PL binding (Sfh1<sup>Y113C,L179W,I196W</sup>), specifically defective in either PtdCho binding (Sfh1<sup>Y113C,S175I,T177I</sup> and Sfh1<sup>E126A,S175I,T177I</sup> or specifically defective in PtdIns binding (Sfh1<sup>Y113C,T238D</sup> and Sfh1<sup>E126A,T238D</sup>), were all incompetent for augmentation of PtdIns-3-P, PtdIns-4-P, or PtdIns-5-P production in the sec14Δ cl/1A yeast strain (Figure 4A). As expected, in every case, PtdIns and PtdCho binding were each individually required for Sfh1*-dependent rescue of CPY transit through the TGN/endosomal system (unpublished data).

### SFH1* alleles and polar interactions in the hydrophilic microenvironment

The data suggest a relationship between Sfh1* mechanisms and the functional status of the enigmatic hydrophilic patch. The Y<sub>109</sub>→F, C, H, A; the Q<sub>111</sub>→A; the Y<sub>113</sub>→C; and the E<sub>126</sub>→A Sfh1* substitutions are all predicted to weaken polar contacts within this polar motif. In this regard, the Y<sub>109</sub>F<sub>126</sub>A<sub>127</sub>F<sub>129</sub> substitution maintains the shape and size of the side chain but abolishes the ability to establish side chain hydrogen bonds by lack of the phenolic hydroxyl group. We therefore tested whether Sfh1* substitutions exhibited allele specificities consistent with weakened polar interactions within this motif. Indeed, in contrast to the cognate Sfh1<sup>E126A</sup> example, Sfh1<sup>E126Q</sup> (i.e., protein in which polar contacts within the hydrophilic patch are presumably conserved) presented severely attenuated Sfh1* phenotypes—despite its in vivo stability (Supplemental Figure S4, A and B). The residual Sfh1* character associated with the E<sub>126</sub>D substitution suggests that the length of the side chain (and thus spacing within the hydrophilic patch) contributes to these polar interactions.

Sfh1 and Sec14 share substantial primary sequence conservation within the hydrophilic patch. Indeed, most residues within a 4-Å radius of Sfh1 residue Y<sub>105</sub> are conserved between the two proteins. These conservations include Sfh1 (Sec14) residues: A<sub>106</sub> (A<sub>104</sub>, K<sub>107</sub> (K<sub>103</sub>), Y<sub>109</sub> (Y<sub>107</sub), P<sub>110</sub> (P<sub>108</sub>, Q<sub>111</sub> (Q<sub>109</sub>, E<sub>126</sub> (E<sub>124</sub), E<sub>127</sub> (E<sub>125</sub), L<sub>128</sub> (L<sub>126</sub), M<sub>129</sub> (M<sub>127</sub>, and Y<sub>195</sub> (Y<sub>193</sub). Sfh1<sup>Y195A</sup> was of interest because it corresponded to the substrate substitution interferes with cooperative interactions between residues Y<sub>195</sub> and Y<sub>197</sub>—that is, interactions that organize polar contacts with ordered water in the hydrophilic patch. When tested in both high- and low-copy expression contexts, Sfh1<sup>Y195A</sup> exhibited Sfh1* properties (unpublished data). These results are congruent with the idea that strong polar interactions within the hydrophilic patch are incompatible with Sfh1*.

Primary sequence divergences between Sfh1 and Sec14 in the hydrophilic region include L<sub>105</sub> (I<sub>103</sub>, M<sub>108</sub> (F<sub>106</sub>, and I<sub>131</sub> (V<sub>129</sub>). M<sub>108

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**FIGURE 3:** Sfh1* and endocytic membrane trafficking from the plasma membrane to the vacuole. A sec14<sup>ts</sup>-yeast strain (CTY1-1A) carrying the indicated YCp expression plasmids (top) were cultured at 25°C to early logarithmic growth phase in uracil-free minimal medium and shifted to 37°C for 2 h. Cells were then pulsed with FM4-64 (10 μM for 10 min), rapidly sedimented, and reconstituted in fresh medium without FM4-64 to initiate chase. Aliquots were removed at the indicated times of chase, poisoned with a NaN<sub>3</sub>/NaF cocktail (final concentration of 10 mM each), and kept on ice in the dark before visualization. FM4-64 profiles were imaged using a Nikon E600 fluorescence microscope. The YCp(URA3) and YCp(Sec14) conditions represent negative and positive controls, respectively. (A) Representative images are shown. Bar = 5 μm. (B) Distributions of imaging profiles (key shown at left) are quantified as % of total cells analyzed (150 for each condition). Quantitative chase of FM4-64 from the plasma membrane into the terminal vacuolar compartment is depicted in white, partial delivery in gray, and strictly endosomal localization of tracer in black.
was excluded from analysis because structural models indicate the side chain protrudes away from the hydrophilic surface and is not involved in the polar interactions of interest. By contrast, 113C (a residue identified as an Sfh1* hotspot; discussed previously) lies close to the C11 and C18 positions of the PtdCho and PtdIns sn-2 acyl chains, respectively. 113C extends the hydrophilic patch boundary toward the headgroup-distal ends of bound PtdCho or PtdIns sn-1 and sn-2 acyl chains (Supplemental Figure S4C). Sfh1E126A expression rescued growth defects associated with sec14-1 and the isogenic sec14-1 spo14Δ double mutant (Supplemental Figure S4A).

Sfh1* and PtdCho binding/exchange

While the structural data suggest E126A relieves the Y109 side chain from its normally strong interaction with E126 in favor of an interaction with this newly positioned H2O molecule, the nearly complete structural identity between Sfh1 and Sfh1E126A otherwise offers little insight into why the E126A relieves Sec14-like activities in Sfh1. One plausible mechanism is that Sfh1* are endowed with superior

Sfh1E126A crystal structure

The crystal structure of Sfh1E126A in complex with a bound 16:0/18:0 phosphatidylethanolamine (PtdEtn) was determined to 1.8 Å resolution (Supplemental Table S1). A structure was also solved for an Sfh1E126A::PtdCho complex, but the resolution was inferior (2.8 Å). For this reason, and because both amino-PLs occupy the same site within the Sfh1 hydrophobic pocket (Schaaf et al., 2008), the PtdEtn-bound complex was analyzed. Sfh1E126A::PtdEtn displays the canonical closed conformation that superimposes onto the Sfh1::PtdEtn structure with a root mean square deviation (rmsd) of 0.15 Å for all protein atoms. In the Sfh1::PtdEtn complex, the E126 side chain makes direct contacts with Y110 and Y124, and Y124 participates in an H-bond interaction with Q111 (Figure S8). Atomic displacement parameters for these residues are 16.8, 17.2, and 16.9 Å2 in the Sfh1::PtdEtn complex and 17.7, 17.9, and 16.9 Å2 in the Sfh1E126A::PtdEtn complex, respectively. As in the Sfh1::PtdEtn complex (Schaaf et al., 2008), PtdEtn is stabilized by 14 van der Waals interactions and coordinated via hydrogen bonds by residues Y113 and S175 and by one H2O molecule (Figure 5C).

Inspection of the ligand-binding pocket reveals that the primary difference between Sfh1 and Sfh1E126A is the configuration of ordered H2O that fills the hydrophilic patch directly underneath the bound PL. In Sfh1, the E126 side chain carboxylate moiety engages in strong H-bond interactions with residues Y109 and Y124 and is part of a larger H-bond network that extends to residue Y195 and involves another conserved H2O. The E126 side chain carboxylate interaction is replaced by an H2O in the Sfh1E126A::PtdEtn complex (Figure 5, A and B), a configuration that preserves the H-bonding network and hydrophilic character of this region of the hydrophobic pocket. The H2O molecule coordinated by residues Y109 and Y195 displays atomic displacement parameters of 9.8 and 23.2 Å2 in the Sfh1E126A::PtdEtn and Sfh1::PtdEtn complexes, respectively. These data report that E126A causes a remarkably subtle destabilization of the hydrophilic patch and suggest altered water behavior in this region is a primary contributor to Sfh1* phenotypes (discussed later in this article).
FIGURE 5: Crystal structure of Sfh1E126A bound to PtdEtn. The hydrophilic patch in (A) Sfh1E126A::PtdEtn and (B) Sfh1::PtdEtn (pdb 3874). The patch is formed by residues Y109, Q111, Y113, Y124, and E126. The protein atoms are rendered as green sticks, bound PtdEtn is depicted as purple sticks, ordered waters are depicted as red spheres, and H-bonds are shown as dashed lines. The sn-2 acyl chain C5 position is highlighted by *.

2Fo-Fc composite omit electron density (contoured at 1σ) for the bound PtdEtn. Residues within 4.2 Å of the bound PL are shown as white spheres, and waters are shown as red spheres. Residues that coordinate the headgroup moieties are shown as sticks, with H-bonds represented as dashed lines.
model how Sfh1* conformational dynamics may differ from those of Sfh1 and more closely resemble those of Sec14. Unrestrained MD simulations were carried out with PL-bound Sfh1 and models of PL-bound Sec14, Sfh1\(^{Y109A}\), Sfh1\(^{Y113C}\), and Sfh1\(^{E126A}\). For the Sfh1\(^{E126A}\)::PL structure, PtdIns and PtdCho were modeled into the crystal structure of Sfh1\(^{E126A}\):PtdEtn (see Supplemental Materials). Surveys of the rmsd values of backbone atoms in snapshots every 2 ps, during the course of three independent simulations, indicated that all systems consistently reached equilibrium within the 14-ns production run (Supplemental Figure S5). All independent simulations exhibited fluctuations (Δrmsd) < 0.8 Å within the last 2 ns (12–14 ns). Unless otherwise noted, this interval was analyzed.

The primary difference between Sfh1::PL structures and a previously crystallized apo-Sec14 is the repositioning of the Sfh1 A\(_{\beta T3}\) structural element, which controls access of PL to the hydrophobic pocket (A\(_{10}/T_{4}\) in Sec14; Sha et al., 1998; Ryan et al., 2007; Schaar et al., 2008). The large motions of this helical gate, which occur in the transitions between open and closed Sec14 conformers, are essential for PL exchange and in vivo activity (Ryan et al., 2007). We were therefore interested in simulating the conformational transitions that occur when PL-bound Sec14, Sfh1, or Sfh1* are used as starting structures. To this end, root mean square fluctuation (rmsf) of Ca atoms of all protein residues were extracted from the MD simulations and analyzed. In the case where dynamics of the open PL-free Sec14 starting structure were simulated, rmsf values exceeded 5 Å for helical gate residues 229KFPFLD233 (Ryan et al., 2007). The corresponding rmsf values for all PL-bound starting structures remained < 2.5 Å, however (Figure 7). Thus the large conformational transitions required for PL exchange do not occur in PL-bound Sec14, Sfh1, or Sfh1* within the time frame of our MD simulations. This is not surprising given that the simulations were performed in a virtual aqueous environment with no membrane system to promote completion of the closed to open transition and to encourage egress of PL from the hydrophobic pocket.

Overall, Sec14-PL bound structures exhibited fluctuation profiles similar to those of their Sfh1 counterparts with rigid body motions of Sfh1 helices A\(_{0}\), A\(_{8}\), and A\(_{9}\) (corresponding Sec14 helices are A\(_{2}\), A\(_{8}\), and A\(_{9}\)) (Figure 7). Fluctuations of the A\(_{10}\) element of the Sec14 helical gate were obviously larger in both the PtdIns and the PtdCho structures relative to fluctuations in the corresponding A\(_{8}\) element of the Sfh1 and Sfh1* helical gate (Figure 7). Likewise, the Sec14 A\(_{9}\) helix exhibited increased fluctuations in both PL-bound structures as compared with the corresponding A\(_{8}\) helix in Sfh1 and Sfh1*—with the exception that the Sfh1\(^{Y109A}\):PtdCho complex also exhibited similarly enhanced fluctuations in the A\(_{3}\) helix (Figure 7). Because

FIGURE 6: Spin-labeled PtdCho-binding parameters for Sfh1* proteins. (A) Room temperature (T = 23°C) X-band CW EPR spectra from n-doxyl-PtdCho prepared as multilamellar lipid dispersion after mixing with Sfh1 and its mutants in ~2:1 lipid-to-protein molar ratio (left, Sfh1; center, Sfh1\(^{E126A}\); right, Sfh1\(^{Y113C}\)). Modulation amplitude is 0.5 G, and spectra are intensity normalized using double integration. (B) Binding of n-doxyl-PtdCho to protein as a function of the label position along the sn-2 acyl chain after mixing n-doxyl-PtdCho prepared as multilamellar lipid dispersion with proteins in ~2:1 lipid-to-protein molar ratio. Fraction of protein containing n-doxyl-PtdCho bound: circles, n-doxyl-PtdCho bound to Sec14; diamonds, n-doxyl-PtdCho bound to Sfh1; triangles, n-doxyl-PtdCho bound to Sfh1\(^{Y113C}\); squares, n-doxyl-PtdCho bound to Sfh1\(^{E126A}\).

Molecular dynamics (MD) simulations

Because the transition pathway between “closed” and “open” forms of the Sec14/Sfh1 fold that accompanies PL exchange cannot be directly monitored experimentally, MD simulations were used to
Sfh1 helices $A_9$ and $A_9$ (Sec14 $A_9$ and $A_1$) are in intimate contact in the PL-bound conformation, we speculate that increased fluctuations in Sfh1$^{1109A}$ helix $A_9$ reflect enhancements in the initial motions of the conformational transition that results in opening of the helical gate, thereby accounting for the strong Sfh1* phenotype associated with the Y$_{109A}$ substitution (Figure 7).

The MD simulations also consistently identified common Sfh1*–specific and Sec14-like behaviors in key structural motifs. Sec14 residues $273^{\text{DESD}}$ and $273^{\text{HNP}}$ motif and corresponding Sfh1$^{273^{\text{HNP}}}$ motif are depicted in green (A).

**FIGURE 7:** Conformational transitions of PL-bound structures. (A and B) Root mean square fluctuation (rmsf) values of $C_{\alpha}$ atoms of all protein residues were extracted from MD simulations of PtdCho (A) and PtdIns structures (B). Average $C_{\alpha}$ atomic position fluctuations from three simulations are plotted as a function of residue number (N to C terminus) for Sec14-PL, Sfh1-PL, and Sfh1*-PL (colors as indicated). Selected regions of high mobility are highlighted in black letters (Sfh1 and Sfh1*) or gray letters (Sec14). Sec14 residue G$_{266}$ and corresponding Sfh1 residue G$_{268}$ are depicted in red, and the Sec14 $273^{\text{DESD}}$ motif and corresponding Sfh1 $273^{\text{HNP}}$ motif are depicted in green (A).

Altered interactions between the B$_1$LB$_2$ and the A$_{11}$LT$_4$ substructures in Sfh1*

The Sec14 B$_1$LB$_2$ and A$_{11}$LT$_5$ substructures comprise a "gating module" that transduces conformational information to the A$_{111}$D$_{111}$ helical gate via an extensive H-bond network. The $114^{\text{DKGDGR}}$ component of the B$_1$LB$_2$ element plays a particularly important role in that conformational coupling (Ryan et al., 2007). The corresponding gating module elements in Sfh1 are B$_1$LB$_2$ (including the $114^{\text{VDKGDGR}}$ component) and A$_{11}$LT$_4$. Strikingly, the core Sfh1* residues lie immediately C-terminal (Y$_{1109}$ to or within (Q$_{1111},$ Y$_{1113},$ and E$_{1126}$) the B$_1$LB$_2$ element, and these residues flank the $114^{\text{VDKGDGR}}$ component. To investigate whether the H-bonding network of residues comprising the hydrophilic patch is functionally involved in the Sfh1* phenotype, we searched for altered H-bond interactions in this network in the context of Sfh1*. Alterations of interest were defined by consistent absolute values of change of > 10% occupancy (i.e., the percentage of time an individual hydrogen bond is present) in all Sfh1* mutants relative to Sfh1. In the Sfh1::PtdCho complex, 48 residues are in H-bond contact with the core Sfh1* residues (Supplemental Figure S6). Additional H-bond interactions are observed with residues of the A$_2$LA$_9$ helical motif and residues of the C-terminal string motif (Supplemental Figure S6).

Increased H-bonding occupancy was consistently recorded in all Sfh1*::PtdCho simulations for five H-bond pairs. The most significant increase of H-bonding was observed for the interaction of the E$_{126}$ backbone with the phenolic –OH group of Y$_{266}$. While the occupancy in Sfh1::PtdCho simulations was < 5.5%, it increased to 48% and 49% in the Sfh1$^{1109C}$ and Sfh1$^{1113C}$ contexts, respectively. The backbone H-bonding interaction between residue A$_{126}$ and Y$_{266}$ in Sfh1$^{1126A}$ likewise exhibited a significant increase to 21% occupancy (Supplemental Table S2). The neighboring E$_{127}$–K$_{265}$ side chain interaction also increased robustly in all Sfh1* simulations. While the side chain interaction showed an occupancy of 75% in the Sfh1::PtdCho context, this value increased to 95%, 88%, and 97% in the corresponding Sfh1$^{1109C}$, Sfh1$^{1113C}$, and Sfh1$^{1126A}$ contexts, respectively (Supplemental Table S2). These data signify increased interactions between the C-terminal region of B$_2$ and the T$_{14}$ in the Sfh1*::PtdCho structures (Figure 8A; Supplemental Figure S6). Two potential consequences of those increased interactions include the following: 1) The loop region between the B$_1$ and the B$_2$ strands undergoes conformational changes (as evidenced by increased H-bonding between loop residues D$_{117}$ and R$_{121}$ of the $114^{\text{VDKGDGR}}$ component of the gating module; see Figure 8B and Supplemental Figure S6), and 2) the unstructured regions that flank the T$_{14}$ are brought into closer proximity to each other as indicated by increased side chain interactions between E$_{250}$ and S$_{270}$ (Figure 8, B and C; Supplemental Figure S6; Supplemental Table S2).

To address the functional relevance of the increased E$_{126}$–Y$_{266}$ interaction, the Y$_{266}^{F}$ and Y$_{266}^{D}$ substitutions were introduced into the context of Sfh1$^{1113C}$. These substitutions were anticipated to ablate H-bonding by lack of the side chain H-donor (Y$_{266}^{F}$) for interaction with the E$_{126}$ backbone, or to cause an electrostatic clash (Y$_{266}^{D}$) with the terminal carboxylate of the E$_{127}$ side chain. Neither double mutant protein rescued sec14-1–associated growth defects, even though the in vivo stability of each mutant polypeptide was preserved (Figure 8E). Thus increased interaction between the N-terminal part of β-strand B$_2$ with the T$_{14}$ turn of substructure A$_{11}$LT$_4$ is a consistent, and functionally important, feature of the mechanisms underlying Sfh1*.
Consistent alterations in the H-bond network between the B1LB2 and A11LT4 structural elements were also recorded in Sfh1*:PtdIns simulations. Those alterations were characterized by reduced H-bonding occupancy between the loop regions of the B1LB2 and A11LT4 (Figure 8D; Supplemental Figure S7). Both elements strongly interact with each other in Sfh1 via 4 H-bonds: 1) the imidazole NH of H114 interacting with the N261 carbonyl, 2) a Q256–D119 side chain interaction, 3) a Q256–D117 side chain interaction, and 4) interaction of the N261 side chain with the V116 carbonyl. Three of these four interactions (H114–N261; Q256–D119; V116–N261) are significantly reduced in Sfh1* (Supplemental Tables S2 and S3). The latter two involve the 116VDKDGR121 component of the gating module—again emphasizing enhanced B1LB2 dynamics as a mechanistic feature of the Sfh1* phenotype.

A consistent Sfh1*-dependent decrease in H-bond occupancy was also recorded for the interaction of the side chain carboxylate of activation residue E126 with the phenolic hydroxyl group of Y124 (Supplemental Table S2). That the reduced H-bonding between these two residues contributes to the enhanced Sec14-like activities of Sfh1* is demonstrated by rescue of sec14-1 ts-associated growth defects by Sfh1Y124A expression (Figure 8E), even though Sfh1Y124A shows decreased stability in vivo (unpublished data).

Sfh1* enhance conformational fluctuations of the helical gate
To assess the effects of Sfh1*-dependent alterations in the H-bond network (which connects the B1LB2 and the A11LT4 substructures) on the dynamics of the helical gate, the interatomic distances between the Cα atoms of Y190 (Aα) and T225 (Aα) were monitored in Sec14::PtdIns, Sfh1::PtdIns, and Sfh1*:PtdIns structures, as were interatomic distances between Cα atoms of K197 (Aα) and F233 (Aα) (Figure 9A). Variations in this latter interatomic distance are useful for distance-monitoring analyses that report opening and closing of the helical gate in the apo-Sec14 molecule (residues K195 and F231 in that context; Ryan et al., 2007). In agreement with the rmsf plots (Figure 7), Y190–T225 Cα distance fluctuations were larger than those recorded for K197–F233 (Figure 9, B and C), suggesting that initial helical gate movements in the PtdIns-occupied polypeptides are restricted mostly to the C-terminal regions of helices Aα and Aβ. Interestingly, while rmsf values of Aα and Aβ residues were indistinguishable between Sfh1 and Sfh1* (except for Sfh1Y170KA; Figure 7), average fluctuations in the interatomic Y190–T225 Cα distance were enhanced in all Sfh1*:PtdIns structures relative to the

FIGURE 8: Model of Sfh1*-dependent alterations in H-bonding. (A–D) Ribbon diagrams illustrate H-bonds that are altered consistently in Sfh1* mutants relative to Sfh1 (see Materials and Methods). The A9T3A10 gating module is depicted in red, T5 is depicted in magenta, and all other α-helices are presented in blue. Unstructured regions are in gray, and β-strands are presented in yellow. PtdCho is shown as cyan stick (A and B) while PtdIns is rendered in magenta (D). Relevant H-bonds are presented as dashed lines. The B1LB2 substructure consists of β1- and β2-β-strands and the intervening loop region. The second substructure A11LT4 includes helix A11 and extends to helix T4. Sfh1* mutants show increased interaction between the C-terminal part of the β2-strand and the T4 turn (mediated by H-bonding pairs E126–Y266 and E127–K265). Increased H-bonding between D117 and R121, and between E260 and S270, are possible consequences of this interaction (B). Decreased H-bonding between I103 and Y281 provide a rational for increased fluctuations of the immediately N-terminally positioned 273HNP275 motif in the MD simulations (C). Decreased H-bonding of A11LT4 loop residue N261 with H114 and V116 reduces interactions between the loop regions of the B1LB2 and A11LT4 substructures in Sfh1* mutants (D). (E) Serial dilution spotting of the sec14-1 ts mutant strain transformed with YCp(URA3) plasmids carrying either Sfh1* or the designated sfh1* alleles on YPD agar plates. Rescue at 37°C reports Sec14-like activity. YCp(URA3) and YCp(SEC14, URA3) plasmids served as negative and positive controls, respectively.

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used in this study yielded gain-of-function Sfh1 derivatives. These proteins. Herein, we describe how Sec14-like functions are activated in the natural pseudo-Sec14 (Sfh1). The directed evolution approach rules that govern the functional dynamics of Sec14-superfamily proteins. At present, we have no clear appreciation of the significance given the broad conservation, across the Sec14 superfamily, of the structural elements that control ligand cycling into and out of the protein interior. 5) MD simulations project that Sfh1* facilitate a network of conformational transitions that enhance the dynamics of the helical substructure that gates the hydrophobic pocket. The collective results indicate Sfh1 is a pseudo-Sec14 because it cannot efficiently propagate the intramolecular conformational transitions that support the robust heterotypic PL exchange required for potentiation of PtdIns 4-OH kinase activity in vivo. These data also reveal mechanisms for how such transitions can be restored.

**Kinetic basis for mechanism of Sfh1**

Because no intragenic synergies in potency of SFH1* phenotypes were observed (i.e., in combinatorial arrangements of the mutations studied in this report), we suggest a common activation mechanism is involved in the Sfh1* examples highlighted here. Furthermore, the data point to a kinetic basis for the Sfh1* activation mechanism rather than a thermodynamic one. Our previous experiments demonstrated that Sfh1 is able to quantitatively load with PtdIns or PtdCho under thermostatically controlled conditions (Schaaf et al., 2008). Yet we demonstrate enhanced PL cycling is a feature of Sfh1* examples highlighted here. The available data suggest that the dynamics that govern the intramolecular motions (and biological activities) of Sec14-like proteins share general mechanistic principles. These dynamics are complex and involve coordination of chemical interactions on the protein surface (e.g., membrane binding), with those in the protein interior (e.g., with bound PL), and with the conformational transitions that gate access to the ligand-binding pocket. At present, we have no clear appreciation of the rules that govern the functional dynamics of Sec14-superfamily proteins. Herein, we describe how Sec14-like functions are activated in the natural pseudo-Sec14 (Sfh1). The directed evolution approach used in this study yielded gain-of-function Sfh1 derivatives. These Sfh1* provide a novel prism through which the functional engineering of a Sec14-like protein can be observed. The concepts culled from these analyses are invisible to standard bioinformatic, crystallographic, and rational mutagenesis approaches.

We reach five major conclusions in this study: 1) Single residue substitutions are sufficient to endow Sfh1 with significantly enhanced abilities for promoting Sec14-dependent membrane trafficking events and for stimulation of PtdIns 4-OH kinase activities in vivo. These substitutions show an informative allele specificity. 2) Missense substitutions that produce Sfh1* involve residues conserved between Sec14 and Sfh1. Most of these residues cluster in an enigmatic hydrophilic microenvironment buried deep within the Sfh1 (and Sec14) hydrophobic pocket. 3) Both PtdIns and PtdCho binding are required for Sfh1* mediated potentiation of PtdIns 4-OH kinase activity in vivo, indicating that Sfh1* must execute heterotypic PtdIns/PtdCho-exchange reactions for productive biological function. 4) Sfh1* are associated with increased cycling of PL into and out of the protein interior. 5) MD simulations project that Sfh1* facilitate a network of conformational transitions that enhance the dynamics of the helical substructure that gates the hydrophobic pocket. The collective results indicate Sfh1 is a pseudo-Sec14 because it cannot efficiently propagate the intramolecular conformational transitions that support the robust heterotypic PL exchange required for potentiation of PtdIns 4-OH kinase activity in vivo. These data also reveal mechanisms for how such transitions can be restored.

**DISCUSSION**

An understanding of the mechanisms by which Sec14-like PTPs integrate lipid metabolism with PIP signaling requires a detailed description of the mechanics by which these proteins execute heterotypic PL exchange. This information is of central importance given the broad conservation, across the Sec14 superfamily, of the structural elements that control ligand cycling into and out of the protein interior, including cases of Sec14-like proteins whose dysfunction results in human disease (Ryan et al., 2007; Schaaf et al., 2008; Bankaitis et al., 2010). The available data suggest that the dynamics that govern the intramolecular motions (and biological activities) of Sec14-like proteins share general mechanistic principles. These dynamics are complex and involve coordination of chemical interactions on the protein surface (e.g., membrane binding), with those in the protein interior (e.g., with bound PL), and with the conformational transitions that gate access to the ligand-binding pocket. At present, we have no clear appreciation of the rules that govern the functional dynamics of Sec14-superfamily proteins. Herein, we describe how Sec14-like functions are activated in the natural pseudo-Sec14 (Sfh1). The directed evolution approach used in this study yielded gain-of-function Sfh1 derivatives. These Sfh1* provide a novel prism through which the functional engineering of a Sec14-like protein can be observed. The concepts culled from these analyses are invisible to standard bioinformatic, crystallographic, and rational mutagenesis approaches.

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Because no intragenic synergies in potency of SFH1* phenotypes were observed (i.e., in combinatorial arrangements of the mutations studied in this report), we suggest a common activation mechanism is involved in the Sfh1* examples highlighted here. Furthermore, the data point to a kinetic basis for the Sfh1* activation mechanism rather than a thermodynamic one. Our previous experiments demonstrated that Sfh1 is able to quantitatively load with PtdIns or PtdCho under thermodynamically controlled conditions (Schaaf et al., 2008). Yet we demonstrate enhanced PL cycling is a feature of the Sfh1* mechanism, and MD simulations indicate increased frequencies of helical gate opening (Figure 9). These independent analyses are both consistent with models in which PL exchange is kinetically favored in Sfh1* contexts. Finally, we calculated free binding enthalpies for Sec14, Sfh1, Sfh1*Y110A, Sfh1*Y113C, and Sfh1*E126A considering internal energies (bonds, angles, and torsion), van der Waals and electrostatic energies, nonpolar and electrostatic contributions to the solvation free energy, and contributions from the gas phase (see Supplemental Material and Methods). The relative protein-PL binding enthalpies calculated from MD simulations indicated the binding affinities of Sfh1, Sfh1*, and Sec14 for PtdIns were significantly stronger than for PtdCho (Supplemental Figure S8A). However, no significant Sfh1*-specific differences were discerned between binding enthalpies for the PtdIns or PtdCho structures.
Functional connectivity between the hydrophilic microenvironment and the helical gate

The hydrophilic patch residues whose alteration generates Sfh1* (i.e., Y109, Q111, Y113, and E126) reside in, or lie immediately adjacent to, the B1LB2—that is, one of the two substructures that define a critical conformational element termed the gating module (G-module; Ryan et al., 2007). As such, these directly influence the very elements that conduct propagation of conformational energy between the hydrophobic pocket floor and the helical substructure that gates access to the Sfh1 hydrophobic pocket. High-resolution structural data demonstrate the Sfh1* substitutions effect a remarkably subtle rearrangement of the hydrophilic microenvironment. However, both structural analyses and MD simulations indicate Sfh1* reconfigure interactions between side chains and ordered H2O molecules in this region. Analyses of the gain-of-function Sfh1* substitutions demonstrate that the interaction between the C-terminal region of strand B2 (residues E126 and E127) with the T24 turn in the string motif (residues K265 and Y266) is critical for the Sfh* phenotype. By contrast, strong interactions between the loop regions of the B1LB2 (H114, V116, and D119) and A11LT4 (Q256, and N261) substructures are incompatible with Sfh1*.

A role of buried water?

The fact that Sfh1* substitutions involve Sfh1 residues that are conserved with Sec14 highlights the limitations of primary sequence alignments, with or without high-resolution structural information, in annotating protein function or in determining dynamic aspects of protein function. The Sfh1* demonstrate that strong polar interside chain interactions (i.e., E126-Y124 and E126-Y109) are incompatible with the conformational dynamics required for Sec14-like functions in the Sfh1 context. The collective data suggest that interactions of these key side chains with water differ between Sfh1 and Sec14 and that internal waters play an important functional role in the regulation of H-bond interactions within the hydrophobic pocket.

While the hydrophobic PL-binding cavity would seem an unlikely environment for active water flux, fatty acid–binding proteins exhibit significant internal water rearrangements coincident with ligand binding and release (Lücke et al., 2002; Modig et al., 2003). The available evidence indicates this is also the case for Sec14/ Sfh1. The internal water content of the hydrophobic pocket differs in the homolog Sfh1::PtdIns and Sfh1::PtdCho complexes as a direct result of the differential PtdIns- and PtdCho-binding strategies, and water flux is suggested to contribute to the energetics that drive heterotypic PL exchange (Schaaf et al., 2008). The hydrophilic microenvironment plays a central role in this flux as it organizes a network of coordinated waters with the Y109, Q111, Y124, and E126 side chains playing primary roles (Schaaf et al., 2008; Figure 5B). Indeed, we find a surprisingly robust turnover of H2O in this region (as defined in the Supplemental Materials and Methods and Supplemental Figure S8)—even after equilibrium conditions are reached (Supplemental Figure S8C). For instance, the average residence of H2O molecules in the proximity of core Sfh1* residues within a 2-ns time window is <51% and <60% for Sfh1::PtdCho and Sfh1::PtdIns, respectively (Supplemental Figure S8C). These residence values are reduced to 25% for the complete course of the 14-ns MD simulation (unpublished data). Of note, the variability of simulated H2O turnover is both significantly and consistently increased in Sfh1* (Supplemental Figure S8, C and D). While a functional role for internal water is an attractive idea, it remains to be determined whether increased fluctuations in water turnover are essential features of Sfh1* activation mechanisms. This remains an open question for future address.

Sec14 and Sfh1*—like, yet unlike

Interestingly, Sfh1* do not precisely follow the Sec14 engineering blueprint for regulating their acquired Sec14-like conformational dynamics and activities. For example, decreased interactions of the T32 turn with β-strand B2 (i.e., Y266F and Y266D) are incompatible with Sfh1*. Yet the corresponding Sec14 residue is F264—not Y264. The F264 residue seemingly disqualifies Sec14 from coupling the conformational transitions of these two conserved substructures through the specific H-bonding mechanism utilized by Sfh1*. Use of an alternative conformational coupling mechanism for biologically sufficient rates of PL exchange reports a significant plasticity for how functional reactivation can be achieved in operationally inactive Sec14-like proteins. Another line of evidence to that effect is provided by the Q256R Sfh1* substitution. Although not characterized in this report, Sfh1*Q256R involves a residue spatially removed from the physically clustered Sfh1* residues upon which we focus here. The mechanism by which Sec14-like functions is resurrected in Sfh1*Q256R is almost certainly distinct from the mechanisms that apply to Sfh1* substitutions that cluster in the hydrophilic microenvironment.

Finally, the results described in this report hold out the prospect that plasticity in conformational coupling can be used for the purpose of bypassing the normal requirements for specific structural elements in Sec14 (or a model Sec14-like protein). This capability might have interesting applications. A number of inherited human disease mutations in proteins of the Sec14 superfamily compromise the G-module, that is, the structural unit that transmits conformational information to the helical gate (Ryan et al., 2007). It might be feasible to “reactivate” such mutant proteins with small molecules that reprogram the conformational transitions upon which the biological activity of the Sec14-like protein depends.

MATERIALS AND METHODS

Yeast strains

Strains CTY182 (MATa ura3-52 lys2-801 Δhis3-200), CTY1-1A (MATa ura3-52 lys2-801 Δhis3-200 sec14-1ts), CTY 100 (MATa ura3-52 lys2-801 Δhis3-200 sec14-1ts sac1-26), CTY159 (MATa ura3-52 lys2-801 Δhis3-200 sec14-1ts ke11), CTY1079 (MATa ura3-52 lys2-801 Δhis3-200 sec14-1ts spo14Δ::HIS3), CTY1098 (MATa ura3-52 lys2-801 Δhis3-200 sec14-1ts ke11), CTY303 (MATa ura3-52 lys2-801 Δhis3-200 Δsec14, cki1::HIS3), CTY558 (MATa ade2 ade3 leu2Δ his3 ura3-52 sec14A1::HIS3, YEP3C14, LEU2, ADE3), and BY4741 (MATa his3Δ leu2Δ20 met15Δ0 ura3Δ0) have been described previously (Cleves et al., 1991; Fang et al., 1996; Xie et al., 1998; Phillips et al., 1999; Li et al., 2000; EUROSCARF: http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html).

Reagents

Standard reagents were purchased from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA). All PLs—including nitroxide-labeled PtdCho species [1-acyl-2-(n-4,4-dimethyloxazolidine-N-oxyl)(stearyl)]-sn-glycero-3-phosphocholines; n-doxyl-PtdCho], with the doxyl label incorporated at n = 5, 7, 10, 12, or 16 of the sn-2 acyl chain—were purchased from Avanti (Alabaster, AL). Monoclonal Anti-V5 antibodies were obtained from Invitrogen (Carlsbad, CA); secondary antibodies were from Bio-Rad Laboratories (Hercules, CA).

Media, genetic techniques, and PL-transfer assays

Genetic methods, media, CPY pulse chase experiments, and PL-transfer assays have been described (Kearns et al., 1998; Phillips et al., 1999; Li et al., 2000; Yanagisawa et al., 2002; Schaaf et al., 2008). PIPs were extracted from yeast radiolabeled to steady state with [3H]myo-inositol and dec酰ylated, and glycerol-inositol
phosphate derivatives were resolved and quantified by anion-exchange chromatography (Guo et al., 1999; Rivas et al., 1999). Measurements of PtdIns-4-P produced specifically by the Stt4 PtdIns 4-OH kinase were performed using sec14-Tps sac1Δ strains radiolabeled to steady state with [3H]myo-inositol as previously described (Phillips et al., 1999; Schaaf et al., 2008; Ile et al., 2010).

**Protein expression, purification, and loading with PL**

His8-Sfh1 \(^{1126A}\) was purified from *Escherichia coli* as described previously (Schaaf et al., 2006; Schaaf et al., 2008). To obtain Sh1 \(^{126A}\):PtdCho complexes, 16.0/18.1 PtdCho dissolved in CHCl\(_3\) was dried under N\(_2\) in glass tubes. Lipid films were resuspended in 150 mM NaCl and 20 mM Tris (pH 7.5) and sonicated until clear. Liposomes were incubated with 40:1 M ratio of PtdCho::Sh1 \(^{1126A}\) for 1 h at 37°C. Complexes were repurified by binding and extensive washing on Talon cobalt-affinity resin (BD Biosciences, Clontech, Palo Alto, CA) followed by dialysis and gel filtration chromatography.

**Cryrstallization and structure determination**

His8-Sfh1 \(^{1126A}:\)PtdEtn crystals were grown by sitting-drop vapor diffusion at 22°C from solutions containing 1.5 μl protein at -2.5 mg/ml (1.8 mg/ml) and 1.5 μl crystallant: 25.5% (wt/vol) PEG 4000, 11.9% (vol/vol) glycerol, and 170 mM sodium acetate and 85 mM Tris, pH 7.5. Crystals were grown in cryoprotectant and flash cooled in liquid nitrogen. Data to 1.8 Å resolution were collected at 100 K at the South East Regional Collaborative Access Team beamline at Argonne National Laboratory (Argonne, IL) and processed and scaled with HKL2000 (Otwinowski, 1993). Initial phases were determined using the Sh1:1::PtdEtn structure (3B74) as a molecular replacement search model (Schaaf et al., 2008). Model building and refinement were performed using standard methods in Coot (Emsley and Cowtan, 2004) and CCP4 (1994, Oxford, UK), respectively. The final model contains one His8-Sfh1 \(^{1126A}\) monomer (residues 4–309) and one bound PtdEtn in the asymmetric unit.

**Preparation of aqueous dispersion of n-doxyl-PtdCho and protein binding**

Multilamellar aqueous dispersions of n-doxyl-PtdChos were prepared in a phosphate pH 6.8 buffer as previously described (Srinivona et al., 2007). Loading of Sh1 proteins with n-doxyl-PtdCho was achieved by mixing a 130-μM protein solution with twofold molar excess of 100% spin-labeled multilamellar liposomes and incubating at room temperature until no changes in EPR spectra were observed. In case of Sh1, the EPR signals were also recorded 2 d after the mixing to verify the absence of any slow binding events. Effective order parameter, \(S^\text{eff}\), was calculated as described in Schorn and Marsh (1997):

\[
S^\text{eff} = \frac{1}{3} \left( \frac{A_0 - A_x}{A_0 + 2A_x} \right) \Delta A
\]

where \(A_0\) is \(A_{out}\), that is, the outer hyperfine splitting, and \(A_x\) is calculated from \(A_{in}\), a half of the inner hyperfine splitting expressed in Gauss:

\[
A_0 = (A_0 + 0.85) \text{ for } S^{\text{mea}} < 0.45
\]

\[
A_x = A_0 + 1.32 + 1.86 \log(1 - S^{\text{mea}}) \text{ for } S^{\text{mea}} > 0.45
\]

\[
S^{\text{mea}} = \frac{(A_{out} - A_{in})}{\Delta A}
\]

where \(A_0\) is the isotropic nitrogen hyperfine coupling constant and \(\Delta A\) is the maximum extent of the axial nitrogen hyperfine anisotropy.

Although previously we have shown that the values of \(\Delta A\) and \(A_0\) can vary with position of the label along the sn-2 acyl chain for Sec14p-bound n-doxyl-PtdCho, we used the same values \(A_0\) and \(\Delta A\) for all n-doxyl-PtdCho protein complexes. \(A_0\) and \(\Delta A\) were set to those measured for 5-doxyl-PtdCho in isopropanol (Srinivona et al., 2007).

**EPR spectroscopy and spectral analysis**

CW X-band (9.0–9.5 GHz) EPR spectra were acquired with a Century Series Varian E-109 (Varian Associates, Palo Alto, CA) EPR spectrometer and digitized to 2048 data points per spectrum. Spectrometer settings were as follows: Microwave power was 2 mW, magnetic field modulation frequency was 100 kHz with < 1 G amplitude to avoid overmodulation, and magnetic field scan was 160 G. Sample temperature was 23.0 ± 0.1°C controlled by a home-built variable temperature system (Alaouie and Srinivona, 2006).

**Supplemental material**

Supplemental material includes eight figures and three tables.

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**REFERENCES**


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Molecular Biology of the Cell
Emsley P, Cowtan K (2004). Coot, model-building tools for molecular graph-
Fang M, Kearns BG, Gedvilaite A, Kagiwada S, Kearns M, Fung MKY, 
Bankaitis VA (1996). Kes1 shares homology with human oxysterol 
binding protein and participates in a novel regulatory pathway 
for yeast Golgi-derived transport vesicle biogenesis. EMBO J 15, 
6447–6459.
Foti M, Audhya A, Emt SR (2001). Sac1 lipid phosphatase and Stt4 
phosphatidylinositol-4-kinase regulate a pool of phosphatidylinositol 
4-phosphate that functions in control of the actin cytoskeleton and 
Sac1, INPS2, and INPS3 and of human synaptopain encode polyphos-
Ile KE et al. (2010). The zebrafish class 1 phosphatidylinositol transfer pro-
tein family, PITPF isomers and double cone cell outer segment integrity 
and cellular nanoreactors for lipid signaling. Nature Chem Biol 2, 
576–583.
Kearns MA et al. (1998). Novel developmentally regulated phosphoinositide 
binding proteins from soybean whose expression bypasses the require-
ment for an essential phosphatidylinositol transfer protein in yeast. 
EMBO J 17, 4004–4017.
Lemmon MA (2008). Membrane recognition by phospholipid-binding 
Li X, Routt S, Xie Z, Cui X, Fang M, Kearns MA, Bard M, Kirsch D, 
Bankaitis VA (2000). Identification of a novel family of nonclassi-

cal yeast PITPs whose function modulates activation of phospho-

lipase D and Sec14p-independent cell growth. Mol Biol Cell 11, 
intracellular lipid binding proteins: the role of buried water. Protein Sci 
11, 2382–2392.
FASEB J 11, A1297.
McLaughlin S, Murray D (2005). Plasma membrane phosphoinositide orga-
molecular basis of vitamin E retention: structure of human tocopherol 
Modig K, Rademacher M, Lücke C, Halle B (2003). Water dynamics in the 
large cavity of three lipid-binding proteins monitored by 17O magnetic 
Nemoto Y, Kearns BG, Wenc MR, Chen H, Mori K, Alb JG Jr, De Camilli P, 
Bankaitis VA (2000). Functional characterization of a mammalian Sac1 
and mutants exhibiting substrate specific defects in PIP phosphatase 
CCP4 Study Weekend, Sawyer L, Isaacs N, and Bailey S, eds., 
Daresbury, UK: SERC Daresbury Laboratory, 56–62.
carried by mutations in the α-tocopherol transfer protein. Nature Genet-
ics 9, 141–145.
Phillips SE et al. (1999). Yeast Sec14p deficient in phosphatidylinositol trans-
Rivas MP, Kearns BG, Xie Z, Guo S, Sekar MC, Hosaka K, Kagiwada S, York 
JD, Bankaitis VA (1999). pleiotropic alterations in lipid metabolism in 
yeast sac1 mutants: relationship to “bypass Sec14p” and inositol aux-
Routt SM, Ryan MM, Tyerar K, Rizzieri K, Roumanie O, Brennwald PJ, 
Bankaitis VA (2005). Nonclassical PITPs activate phospholipase D via an 
Stt4p-dependent pathway and modulate function of late stages of the 
secretory pathway in vegetative yeast cells. Traffic 6, 1157–1172.
dynamics of the major yeast phosphatidylinositol transfer protein Sec14: 
insights into the mechanisms of PL exchange and diseases of Sec14-like 
Schaaf G, Betts L, Garrett TA, Raetz CRH, Bankaitis VA (2006). Crystalliza-
tion and preliminary X-ray diffraction analysis of phospholipid-bound 
Sac1p: a member of the Saccharomyces cerevisiae Sec14p-like phos-
phatidylinositol transfer protein family. Acta Crystallographica F 62, 
1156–1160.
CRH, Redinbo M, Bankaitis VA (2008). The functional anatomy of phos-
pholipid binding and regulation of phosphoinositol homeostasis by 
Schon K, Marsh D (1997). Extracting order parameters from powder EPR 
ilineshapes for spin-labelled lipids in membranes. Spectrochimica Acta A 
53, 2235–2240.
charomyces cerevisiae phosphatidylinositol transfer protein Sec14p. 
Smirnova T, Chadwick TG, MacArthur R, Poluekov O, Song L, Ryan M, 
Schaaf G, Bankaitis VA (2006). The chemistry of PL binding by the Sac-
charomyces cerevisiae phosphatidylinositol transfer protein Sec14 as 
determined by electron paramagnetic resonance spectroscopy. J Biol 
Chem 281, 34987–34908.
Smirnova T, Chadwick TG, van Tol J, Ozarowski A, Poluektov O, Schaaf G, 
Ryan MM, Bankaitis VA (2007). Local polarity and hydrogen bonding 
inside the Sec14 PL-binding cavity: high-field multielement studies. 
Biophys J 92, 3686–3695.
Strahl T, Thonier J (2007). Synthesis and function of membrane phospho-
inositides in budding yeast, Saccharomyces cerevisiae. Biochim Biophys 
Xie Z, Fang M, Rivas MP, Faulkner A, Sternweis PC, Engebrecht J, Bankaitis 
phosphatidylinositol transfer protein defects. Proc Natl Acad Sci USA 95, 
12346–12351.
Yanagisawa L, Marchena J, Xie Z, Li X, Poon PP, Singer R, Johnston G, 
Randazzo PA, Bankaitis VA (2002). Activity of specific lipid-regulated 
ARFGAPs is required for Sec14p-dependent Golgi secretory function in 