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

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\textbf{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\textsuperscript{*} 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.

\textbf{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
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 PtdIns 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 (Schaaf et al., 2008; Bankaitis et al., 2010). The principle that underlies front-loaded mechanisms highlights an intrinsic biological inadequacy of PtdIns kinases (particularly PtdIns 4-OH kinases), which are insufficient interfacial enzymes when confronted with the dual issues of 1) substrate PtdIns presented in liposomal contexts and 2) competing activities that degrade PIPs. Productive action of these enzymes is subject to what we term "instructive" activation (Bankaitis et al., 2010). Sec14-like PtdIns-transfer proteins (PITPs) are the instructive components in front-loaded regulation of PtdIns 4-OH kinase activities in yeast by virtue of their roles as primeable "PtdIns-presentation" modules. For Sec14, effective presentation requires heterotypic PtdIns/phosphatidylincholine (PtdCho) exchange (Schaaf et al., 2008; Bankaitis et al., 2010). The Sec14-protein superfamily is diverse, and a PtdIns-binding "bar code" is recognized in nearly all members of the superfamily. PtdCho-binding signatures are not broadly 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 metabolome with instructed PIP signaling (Schaaf et al., 2008; Bankaitis et al., 2010). This interface is of interest given the causal links between Sec14-like protein deficiencies and human disease (Ouachi et al., 1995; Benomar et al., 2002; Bomar et al., 2003; Meier 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 “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 PtdIns 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 PtdIns- 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 PtdIns and PtdCho binding (Schaaf et al., 2008). These findings suggest either that Sfh1 is a pseudo-Sec14 that is unable to effectively undergo the conformational 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 sec14-1 ura3-52 yeast strain. Transformants were coselected for growth at 37°C and uracil prototrophy. From an estimated 1 x 10⁶ 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-fluoroorotic 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 naïve sec14-1 Δ mutants by selection for Ura*. In each case, the Ts+ phenotype was coinherited 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 Y109 and Y113, while the other highlights the sequence between residues I131 and T141 (Figure 1A; Supplemental Figure S1). The Y109Y113 hotspot involves an enigmatic hydrophilic patch on the floor of the Sfh1 hydrophobic cavity (Smirnova et al., 2006; Schaaf et al., 2008). This patch is configured in close proximity to the sn-2 acyl chain of bound PtdCho (Figure 1B) and PtdIns (Figure 1C). The hydrophilic patch surface is organized by residues Y109, Q111, Y124, and E125, and involves several coordinated H₂O molecules, one of which resides in close proximity to Y113. The Q204R substitution was also identified in the SFH1* screen. As Sfh1Q204R 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 Y109C, Y109H, Y109F, and Y113C substitutions into an otherwise wild-type Sfh1 protein demonstrated sufficient of these substitutions for Sfh1* (Figure 2A). Because several substitutions at residue Y109 yielded Sfh1*, the analyses were extended by determining whether the Y109A substitution (a side chain deletion) had the same effect. Indeed, Y109A conferred Sfh1* properties to an otherwise unadulterated Sfh1 (Figure 2A).

Because the Y109 → F, C, H, A, and Y113 → C all represent Sfh1* substitutions, and all affect the enigmatic hydrophilic patch, we tested whether Sfh1E126A and Sfh1G111A (i.e., substitutions not encountered in the original activation screen) similarly manifest Sfh1* properties when expressed from centromeric and episomal plasmids in sec14-1Δ and sec14Δ shuffle strains. Both Sfh1E126A and Sfh1G111A scored as Sfh1*. Sfh1E126A was particularly potent in this regard (Figure 2A). Sfh1G111A was less so—primarily due to reduced stability of this protein (discussed later in this article).
Characterization of Sec14-like activities in Sfh1* proteins

Expression of any one of these SFH1* gene products rescued robust growth of both a sec14Δ ts strain and its isogenic phospholipase D (PLD)-deficient derivative (sec14Δ spo14Δ) at the restrictive temperature of 37°C (Figure 2A). The ability of SFH1* expression to effect phenotypic rescue of sec14Δ ts growth defects in the spo14Δ genetic background speaks to potency of the activation phenotype as PLD deficiency strongly exacerbates sec14Δ ts–associated growth phenotypes. By comparison, SFH1 expression fails to rescue sec14Δ ts 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 Δ 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., SFH1[Y109A,Q111A]). 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).

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 SFH1[Y109A] and SFH1[Y109A,Q111A], both of which exhibited reduced protein stability at 37°C relative to Sec14 (Supplemental Figure S2). This property is reflected in the modest SFH1* phenotypes associated with SFH1[Y109A,Q111A]. However, expression of SFH1[Y109A,Q111A] rescues growth of sec14Δ ts 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 Shh1* 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 sec14Δ ts mutants incubated at restrictive temperatures, and Shh1 expression failed to rescue this aberrant morphology (Figure 2B). By contrast, this morphological phenotype was alleviated in sec14Δ ts mutants by SFH1[Y109A, Y113C], or SFH1[Y109A,E126A] 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 Shh1* (Figure 2C). In a sec14Δ ts strain ectopically expressing Shh1, pulse-radiolabeled CPY was recovered in approximately equimolar fractions as TGN/endosomal p2-CPY and mature vacuolar mCPY. 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 Shh1* expression was sufficient to yield CPY profiles that largely recapitulated those recorded for the Sec14-proficient condition. While a persistent p2-CPY fraction remained detectable in the face of Shh1* expression, this fraction was reduced ~fivefold relative to that recorded in the face of ectopic Shh1 expression (Figure 2C). The persistence of p2-CPY under conditions of low-copy plasmid-driven SFH1* expression indicates the cognate gene products remain suboptimal Sec14 surrogates.
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; Routt et al., 2005; Schaaf et al., 2008). Reconstitution of Sec14 expression elevates PtdIns-3-P, PtdIns-4-P, and PtdIns-4,5-P_2 levels by twofold relative to the parental negative control (Figure 4A). While Sfh1 expression was ineffectual in this regard, even modest expression of Sfh1Y_{109A}, Sfh1Y_{113C}, or Sfh1E_{126A} 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 isolated context of Stt4 PtdIns 4-OH kinase activity. To measure Sfh1*-mediated potentiation of specific yeast PtdIns 4-OH kinase activity in a native intracellular environment, [14C]InsP_3 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 Sfh1Y_{109A}, Sfh1Y_{113C}, or Sfh1E_{126A} 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 L_{179}, I_{196}, and V_{199} 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 Sfh1Y_{113C}L_{179W}I_{196W} was defective in

FIGURE 2: Functional characterization of Sfh1* proteins. (A) Left and middle, isogenic sec14-1ts and sec14-1ts spo14Δ yeast strains (as indicated on top) transformed with YCp(URA3) plasmids carrying either Sfh1 or the designated SFH1* alleles were spotted in eightfold serial dilutions onto yeast peptone dextrose (YPD) agar plates. Rescue at the restrictive temperature (37°C) reports Sec14-like activity. YCp(URA3) and YCp(SEC14, URA3) plasmids served as negative and positive controls, respectively. Right, an ade2 ade3 sec14Δ yeast strain carrying a parental YEp(SEC14, LEU2, ADE3) plasmid (strain CTY558; see Materials and Methods) was transformed with high-copy YEp(URA3) plasmids harboring SFH1 or the designated SFH1* alleles and dilution spotted onto YPD agar plates. Segregation with appearance of white colonies that harbor the indicated YCp* product. YEp(SEC14, LEU2, ADE3) plasmid (strain CTY558; see Materials and Methods) was transformed with high-copy YEp(URA3) plasmids harboring SFH1 or the designated SFH1* alleles and dilution spotted onto YPD agar plates. Segregation with appearance of white colonies that acquire leucine and histidine auxotrophies reports loss of parental YEp(SEC14, LEU2, ADE3) and hence functionality of the mutant SFH1* product. YEp(URA3) alone and YEp(SEC14, URA3) plasmids served as negative and positive controls, respectively. (B) Thin-section electron microscopy. A sec14-1ts yeast strain (CTY1-1A) expressing the indicated gene (or allele) from a YCp vector was cultured in YPD medium at 30°C and subsequently shifted to 37°C for two hours. Cells were fixed, embedded in Spurr’s resin, stained with uranyl acetate, and imaged by transmission electron microscopy. Representative images are shown (bar = 1 μm). (C) A sec14-1ts yeast strain (CTY1-1A) carrying the indicated YCp expression plasmids was shifted to 37°C for two hours and radiolabeled with [35S]amino acids for 35 min followed by a 25-min chase. Immunoprecipitated CPY forms were separated by SDS-PAGE and visualized by autoradiography. Core glycosylated p1 CPY (diagnosing ER and early Golgi pools), TGN p2 CPY, and vacuolar mCPY are identified at right.

FM4-64 tracer experiments similarly demonstrated that Sfh1* are superior to Sfh1 in potentiating membrane flow through a Sec14-deficient endosomal system. Whereas Sec14-deficient cells internalize the lipophilic dye efficiently, these accumulate FM4-64 in punctate endosomal compartments and fail to chase the tracer into the vacuole (Figure 3). Reconstitution of Sec14-deficient cells with Sec14 rescued these defects. Significant delivery of FM4-64 to vacuolar compartments was already apparent by 7.5 min of chase, and the endosomal FM4-64 pool quantitatively trafficked to vacuoles during the 30-min chase (Figure 3). Sfh1 expression did not rescue FM4-64 delivery from endosomal compartments to the vacuole, but Sfh1Y_{109A}, Sfh1Y_{113C}, Sfh1E_{126A}, or Sfh1Y_{109A}Y_{113C} 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 (Schaafl et al., 2008). The PtdCho-binding-deficient derivatives of both Sfh1Y113C (Sfh1Y113C,S175I,T177I) and Sfh1E126A (Sfh1E126A,S175I,T177I) were stable polypeptides (Supplemental Figure S3, C and D), and in vitro PL-transfer assays confirmed the expected biochemical defects for these two proteins. Sfh1Y113C,S175I,T177I exhibited a >25-fold reduction in PtdCho-transfer activity relative to Sfh1Y113C and retained PtdIns transfer activity (Supplemental Figure S3, A and B). Expression of Sfh1Y113C,S175I,T177I or Sfh1E126A,S175I,T177I from low-copy vectors failed to rescue growth of sec14Δ strains at restrictive temperatures (Figure 4C). Moreover, enhanced expression of Sfh1Y113C,S175I,T177I or Sfh1E126A,S175I,T177I 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 sec14Δ-associated growth defects translated to defects in regulation of PIP homeostasis. Stable variants of Sfh1* impaired for PL binding (Sfh1Y113C,L179W,V199W), specifically defective in either PtdCho binding (Sfh1Y113C,S175I,T177I) or specifically defective in PtdIns binding (Sfh1Y113C,T238D and Sfh1E126A,T238D), were all incompetent for augmentation of PtdIns-3-P, PtdIns-4-P, or PtdIns-4,5-P2 production in the sec14Δ ck1α 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 Y109→F, C, H, A; the Q111→A; the Y113→C; and the E125→A Sfh1* substitutions are all predicted to weaken polar contacts within this polar motif. In this regard, the Y109F 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 Sfh1E126A example, Sfh1E126D (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 E125D 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 Y109 are conserved between the two proteins. These conservations include Sfh1 (Sec14) residues: A106 (A104), K107 (K105), Y109 (Y107), P110 (P108), Q111 (Q109), E122 (E124), E127 (E125), L128 (L126), M129 (M124), and Y195 (Y193). Sfh1Y195A was of interest because the corresponding substitution interferes with cooperative interactions between residues Y195 and Y197—that is, interactions that organize polar contacts with ordered water in the hydrophilic patch. When tested in both high- and low-copy expression contexts, Sfh1Y195A 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 L105 (I103), M108 (F106), and I131 (V129). M108

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**FIGURE 3:** Sfh1* and endocytic membrane trafficking from the plasma membrane to the vacuole. A sec14ΔΔ 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 NaN3/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.
One plausible mechanism is that Sfh1* are endowed with superior insight into why the E126A* gene product. While the structural data suggest E126A* retains the H-bond network that extends to residue Y113 of the hydrophilic patch of Sfh1, the E126A* derivative does not engage in strong H-bond interactions with E126A* residues Y113 and Y119 because of the altered side chain carboxylate moiety.

Sfh1* and PtdCho binding/exchange

While the structural data suggest E126A* retains the Y119 side chain from its normally strong interaction with E126A* in favor of an interaction with this newly positioned H2O molecule, the nearly complete structural identity between Sfh1 and Sfh1* otherwise offers little insight into why the E126A* resuscitates Sec14-like activities in Sfh1. One plausible mechanism is that Sfh1* are endowed with superior this region is a primary contributor to Sfh1* phenotypes (discussed later in this article).
capacities to cycle PL into and out of the hydrophobic pocket. To monitor protein::PL interactions directly, continuous wave (CW) electron paramagnetic resonance (EPR) spectroscopy was used to compare the parameters of PtdCho binding by Sfh1 and Sfh1*. Spin-labeled n-doxyl-PtdCho molecules were used to survey protein::PtdCho interactions across the lipid molecule. In these experiments, the EPR signal from liposomal n-doxyl-PtdCho species appears as a broad single line of ∼25–27 G peak-to-peak width. Such a spectrum is readily modeled by a Lorentzian function and reflects the strong dipole–dipole and exchange interactions that occur when nitroxide moieties are packed in close proximity. On sequestration of n-doxyl-PtdCho within the Sfh1 or Sfh1* lipid-binding cavity, spin–spin interactions between n-doxyl-PtdCho molecules are eliminated with the result that a sharper EPR spectrum is produced.

Figure 6A displays the CW X-band spectra recorded when individual n-doxyl-PtdCho species were incubated with Sfh1, Sfh1Y113C, or Sfh1E126A. For all spin probe positions analyzed, the contribution of liposomal n-doxyl-PtdCho registered as a broad line most readily observed at the wings. Contributions from the liposomal n-doxyl-PtdCho were subtracted from the experimental spectra, yielding the component corresponding to protein-bound n-doxyl-PtdCho. Relative contributions from protein-bound and liposomal forms were quantified by a double integration of the corresponding spectral components. These analyses demonstrate Sfh1 fails to incorporate 5- and 7-doxyl-PtdCho, while only poor binding was measured for the 10- and 12-doxyl-PtdCho; only ∼1–2% of the Sfh1 molecules incorporated those spin-labeled species (Figure 6A, left; Figure 6B). 16-Doxyl-PtdCho was also inefficiently incorporated by Sfh1. Some 20% of total Sfh1 loaded with this spin-labeled PtdCho. These X-band CW EPR data demonstrate Sfh1 exhibits poor loading capacities for all n-doxyl-PtdCho–binding substrates.

By contrast, both Sfh1* proteins showed enhanced capacities for n-doxyl-PtdCho incorporation. For Sfh1Y113C, weak incorporation was observed for 5- and 7-doxyl-PtdCho (10–15% of total protein loaded), moderate incorporation was measured for 10- and 12-doxyl-PtdCho (30–40% of total protein loaded), and Sfh1E126A was quantitatively loaded with 16-doxyl-PtdCho (Figure 6A, right; Figure 6B). Sfh1E126A was more impressive with regard to its capacity for n-doxyl-PtdCho incorporation. This protein was quantitatively occupied with 5-doxyl-PtdCho and demonstrated significant incorporation of 7-, 10-, 12-, and 16-doxyl-PtdCho as well (40–50% of total protein loaded across the range of probe positions; Figure 6A, center; Figure 6B). By comparison, Sec14 is quantitatively loaded with each of the n-doxyl-PtdCho species under these experimental conditions (Figure 6B; Smirnova et al., 2006; Smirnova et al., 2007). The acquired capability of Sfh1* to load with n-doxyl-PtdCho into the hydrophobic pocket is not likely due to relief of steric problems associated with accommodating the spin label within the hydrophobic pocket. The effects were scored even when the spin-label position was physically distant from the operant Sfh1* substitution.

The EPR data project that Sfh1* substitutions are associated with enhanced cycling of PL into and out of the protein interior. We therefore expected that Sfh1* proteins would exhibit increased specific activities of PtdIns and/or PtdCho transfer relative to Sfh1. This prediction was tested by comparing the PtdIns- and PtdCho-transfer activities of Sfh1* to those of purified Sec14 and Sfh1. All proteins were stable during the course of the PL-transfer experiments (Supplemental Figure S3C), thereby permitting quantitative comparisons. His8-Sec14 exhibited robust PtdIns- and PtdCho-transfer activities, whereas His8-Sfh1 showed > fivefold reductions in specific activities for PtdIns and PtdCho.
transfer relative to His$_{S}$-Sec14 (Supplemental Figure S3, A and B). All three Sfh* tested (His$_{S}$-Sfh$^{Y109A}$, His$_{S}$-Sfh$^{Y113C}$, and His$_{S}$-Sfh$^{E126A}$) presented increased PtdIns transfer activity relative to His$_{S}$-Sfh. Similar outcomes were recorded for PtdCho-transfer activities of His$_{S}$-Sfh$^{Y109A}$ and His$_{S}$-Sfh$^{Y113C}$. Both Sfh* proteins exhibited a > fivefold increase in PtdCho transfer relative to His$_{S}$-Sfh. His$_{S}$-Sfh$^{E126A}$ presented a twofold increase in PtdCho transfer activity relative to His$_{S}$-Sfh (Supplemental Figure S3, A and B). Taken together, these data demonstrate that Sfh* exhibit significantly enhanced (i.e., Sec14-like) abilities to cycle PL from within the lipid-binding pocket relative to Sfh.

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 model how Sfh* 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, Sfh$^{Y109A}$, Sfh$^{Y113C}$, and Sfh$^{E126A}$. For the Sfh$^{E126A}$::PL structures, PtdIns and PtdCho were modeled into the crystal structure of Sfh$^{E126A}$::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 (Δ rmsd) < 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 Ap/T$_{3}$ 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; Schaaf et al., 2008). The large motions of this helical gate, which occur in the transition 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 Sfh* 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 229KPFLD233 (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 Sfh* 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$_{2}$, and A$_{7}$ (corresponding Sec14 helices are A$_{0}$, A$_{6}$, and A$_{10}$) (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$_{7}$ element of the Sfh1 and Sfh* helical gate (Figure 7). Likewise, the Sec14 A$_{7}$ helix exhibited increased fluctuations in both PL-bound structures as compared with the corresponding helix A$_{7}$ in Sfh1 and Sfh1*—with the exception that the Sfh$^{Y109A}$::PtdCho complex also exhibited similarly enhanced fluctuations in the A$_{3}$ helix (Figure 7).
Altered interactions between the B₁LB₂ and the A₁LT₄ substructures in Sfh1*

The Sec14 B₁LB₂ and A₁LT₄ substructures comprise a "gating module" that transduces conformational information to the A₁LT₄ helical gate via an extensive H-bond network. The 113TKDGDR₄₁ component of the B₁LB₂ element plays a particularly important role in that conformational coupling (Ryan et al., 2007). The corresponding gating module elements in Sfh1 are B₁LB₂ (including the 113VDKDGDR₂₁ component) and A₁LT₄. Strikingly, the core Sfh1* residues lie immediately C-terminal (Y₁₁₀₉) to or within (Q₁₁₁, Y₁₁₁₃, and E₁₁₂₆) the B₁LB₂ element, and these residues flank the 113VDKDGDR₂₁ 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 A₁LA₉ 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 E₁₁₂₆ backbone with the phenolic –OH group of Y₁₁₁₃. While the backbone H-bonding interaction between residue A₁₁₂₆ and Y₁₁₁₃ in Sfh1* likewise exhibited a significant increase to 21% occupancy (Supplemental Table S2). The neighboring E₁₁₂₇–K₁₁₂₅ 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*::PtdCho, Sfh1*::PtdCho, and Sfh1*::PtdCho contexts, respectively (Supplemental Table S2). These data signify increased interactions between the C-terminal region of B₂ and the T₄ 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 B₁ and the B₂ strands undergoes conformational changes (as evidenced by increased H-bonding between loop residues D₁₁₁₇ and R₁₂₁₂ of the 113VDKDGDR₂₁ component of the gating module; see Figure 8B and Supplemental Figure S6), and 2) the unstructured regions that flank the T₄ are brought into closer proximity to each other as indicated by increased side chain interactions between E₁₂₅₀ and S₁₂₇₀ (Figure 8, B and C; Supplemental Figure S₆; Supplemental Table S2).

To address the functional relevance of the increased E₁₁₂₆–Y₁₁₁₃ interaction, the Y₁₁₁₃F and Y₁₁₁₃D substitutions were introduced into the context of Sfh1*::PtdCho. These substitutions were anticipated to ablate H-bonding by lack of the side chain H-donor (Y₁₁₁₃F) for interaction with the E₁₁₂₆ backbone, or to cause an electrostatic clash (Y₁₁₁₃D) with the terminal carboxylate of the E₁₁₂₇ 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 B₂ with the T₄ turn of substructure A₁₁₁LT₄ 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-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 (A9) and T225 (A9) were monitored in Sec14::PtdIns, Sfh1::PtdIns, and Sfh1*::PtdIns structures, as were interatomic distances between Cα atoms of K197 (A9) and F233 (A9; 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 A9 and A8. Interestingly, while rmsf values of A9 and A8 residues were indistinguishable between Sfh1 and Sfh1* (except for Sfh1Y170A; Figure 7), average fluctuations in the interatomic Y190-T225 Cα distance were enhanced in all Sfh1*:PtdIns structures relative to the vector control.

**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; A). 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-ts reporter 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.

**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; A). 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-ts reporter 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.
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.

**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 thermodynamically controlled (i.e., equilibrium) conditions (Schaf et al., 2008). Yet we demonstrate enhanced PL cycling is a feature of Sfh1*-mediated potentiation of PtdIns 4-OH kinase activity in vivo, corresponding to the closed-to-open transition necessary to facilitate PL exchange.

**DISCUSSION**

An understanding of the mechanisms by which Sec14-like PITPs 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; Schaf 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
Functional connectivity between the hydrophilic microenvironment and the helical gate

The hydrophilic patch residues whose alteration generates Sfh1* (i.e., Y109, Q111, Y124, and E126) reside in, or lie immediately adjacent to, the B1-LB2—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 T4 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 B1-LB2 (H114, V116, and D119) and A11 LT4 (Q256, and N251) 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 holos 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 S8B)—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 T4 turn with β-strand B2 (i.e., Y262F and Y262D) 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 Q32R Sfh1* substitution. Although not characterized in this report, Sfh1*Q32R 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*Q32R 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 ), CTY100 (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 spo14::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 leu2 his3-1 ura3-52 sec14A1::HIS3 YEp[SCEC14, LEU2, ADE3]), and BY4741 (MATa his3Δ1 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)(stearoyl)sn-glycerol-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 decacylated, 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 Ptd-Chol exchange were performed using sec14-T² 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^{E126A} was purified from *Escherichia coli* as described previously (Schaaf et al., 2006; Schaaf et al., 2008). To obtain Sfh₁^{E126A}:PtdCho complexes, 16.0:18.1 PtdCho dissolved in CHCl₃ was dried under N₂ 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:Sfh₁^{E126A} 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.

Crystralization and structure determination

His₈-Sfh₁^{E126A}: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 N₂. 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 (Otwinski, 1993). Initial phases were determined using the Sfh₁::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, Oxon, UK), respectively. The final model contains one His₈-Sfh₁^{E126A} 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 (Smirnova et al., 2007). Loading of Sfh1 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 Sfh1, the EPR signals were also recorded 2 d after the mixing to verify the absence of any slow binding events. Effective order parameter, \( S^{eff} \), was calculated as described in Schorn and Marsh (1997):

\[
S^{eff} = \frac{(A_1 - A_0)A_2}{3(A_1 + 2A_0)} \Delta A
\]

where \( A_0 = A_{out} \) that is, the outer hyperfine splitting, and \( A_1 \) is calculated from \( A_{in} \), a half of the inner hyperfine splitting expressed in Gaus:

\[
A_1 = (A_0 + 0.85) \text{ for } S^{mm} < 0.45
\]

\[
A_2 = A_0 + 1.42 + 1.86 \log(1 - S^{mm}) \text{ for } S^{pp} > 0.45
\]

\[
S^{pp} = (A_{out} - A_0) / \Delta A
\]

where A₀ 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 A₀ can vary with position of the label along the sn-2 acyl chain for Sec14p-bound n-doxyl-PtdCho, we used the same values A₀ and \( \Delta A \) for all n-doxyl-PtdCho protein complexes. A₀ and \( \Delta A \) were set to those measured for 5-doxyl-PtdCho in isopropanol (Smirnova et al., 2007).

EPR spectroscopy and spectral analysis

CWX-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 Smirnov, 2006).

Supplemental material

Supplemental material includes eight figures and three tables.

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