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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...
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 (Rout 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 (OUCAl 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 hydrospholic microenvironment within the hydrophobic Sfh1 ligand-binding cavity. These altered dynamics facilitate confomational 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 sec14-1Δ 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* transformants and reintroduced into naive sec14-1Δ mutants by selection 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 Y109 and Y113, while the other highlights the sequence between residues I131 and T141 (Figure 1A; Supplemental Figure S1). The Y109Y113 hotspot involves an enzymatic 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 Q209R substitution was also identified in the SFH1* screen. As SFH1Q209R 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 sufficiency 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 enzymatic hydrophilic patch, we tested whether Sfh1E126A and Sfh1D111A (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 sec14A shuffle strains. Both Sfh1E126A and Sfh1D111A scored as Sfh1*. Sfh1E126A was particularly potent in this regard (Figure 2A). Sfh1D111A 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 sec14-1ts strain and its isogenic phospholipase D (PLD)-deficient derivative (sec14-1 spo14A) at the restrictive temperature of 37°C (Figure 2A). The ability of SFH1* expression to effect phenotypic rescue of sec14-1ts growth defects in the spo14A genetic background speaks to potency of the activation phenotype as PLD deficiency strongly exacerbates sec14-1ts-associated growth phenotypes. By comparison, SFH1 expression fails to rescue sec14-1ts 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 sec14-1ts genetically defective derivatives (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*Y113C-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 $SFH1^*$ and $SFH1^*$, 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 $SFH1^*$, $SFH1^*$, and $SFH1^*$, expression of $SFH1^*$ rescues growth of sec14-1ts yeast at 35°C (unpublished data) and rescues sec14Δ lethality when expressed from episomal expression plasmids (Figure 2A). The observation that $SFH1^*$ affects 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 Sh1 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 sec14-1ts mutants incubated at restrictive temperatures, and Sh1 expression failed to rescue this aberrant morphology (Figure 2B). By contrast, this morphological phenotype was alleviated in sec14-1ts mutants by $SFH1^*$, $SFH1^*$, or $SFH1^*$ 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 sec14-1ts 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 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 $SFH1^*$ expression, this fraction was reduced ~fivefold relative to that recorded in the face of ectopic Sh1 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.
an obvious (yet partial) improvement in dye trafficking from Sec14-deficient endosomes to the vacuole (Figure 3).

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 sec14A 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; Rout 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 Sh1Y109A, Sfh1Y113C, or Sh1E126A elevated PIP levels significantly (Figure 4A).

Similar conclusions were drawn from experiments in which the influences of Sfh1* genes in Sfh1 were compared in the isolated context of Stt4 PtdIns 4-OH kinase activity. To measure Sh1*-mediated potentiation of specific yeast PtdIns 4-OH kinase activity in a native intracellular environment, [3H]Ins radiolabeling experiments were performed in sec14A spo14 yeast strains expressing the Sh1* proteins of interest. The sec14A spo14 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 Sh1Y109A, Sfh1Y113C, or Sh1E126A expression in sec14A spo14 yeast mutants effectively reversed the Sh1* mutations observed in the parental negative control (Figure 4B). PtdIns-4-P levels were similar across all conditions described above (Figure 4B). PtdIns-4-P levels were indifferent to the Sfh1 expression constructs.

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 activity by Sh1*, “pinch-close” mutants that abrogate binding of all PLs were incorporated into the context of several SFH* alleles. Residues L179, I196, and V199 organize a region of the Sh1 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 Sh1 or Sec14 (Schaaf et al., 2008). As expected, recombinant Sh1Y113C, L179W, I196W was defective in

FIGURE 2: Functional characterization of Sh1* proteins. (A) Left and middle, isogenic sec14A-1ts and sec14A-1 spo14A yeast strains (as indicated on top) transformed with YCP(URA3) plasmids carrying either Sh1 or the designated Sh1* alleles were spotted onto YPD agar plates. Rescue at the restrictive temperature (37°C) reports Sec14-like activity. YCP(URA3) and YCP(SEC14, URA3) plasmids served as positive and negative controls, respectively. Right, an ade2 ade3 sec14A 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 Sh1 or the designated Sh1* 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 Sh1* product. YEp(URA3) alone and YEp(SEC14, URA3) plasmids served as negative and positive controls, respectively. (B) Thin-section electron microscopy. A sec14A-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 2 h. 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 sec14A-1ts yeast strain (CTY1-1A) carrying the indicated YCP expression plasmids was shifted to 37°C for 2 h 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 Sh1* are superior to Sh1 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). Sh1 expression did not rescue FM4-64 delivery from endosomal compartments to the vacuole, but Sh1Y109A, Sh1Y113C, Sh1Y109C, Y113C, or Sh1E126A expression supported
play important roles in coordinating the PtdCho headgroup phosphatase within the Sec13 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 Sec1113C (S175I, T177I) and Sec1E126A (S1E126A,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. Sec1113C,S175I,T177I exhibited a >25-fold reduction in PtdCho-transfer activity relative to Sec1113C and retained PtdIns transfer activity (Supplemental Figure S3, A and B). Expression of Sfh1113C,S175I,T177I or Sec1E126A,S175I,T177I from low-copy vectors failed to rescue growth of sec14-1 strains at restrictive temperatures (Figure 4C). Moreover, enhanced expression of Sec1113C,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 sec14-1-related growth defects translated to defects in regulation of PIP homeostasis. Stable variants of Sfh1* impaired for PL binding (Sfh1113C,L179W,V199W), specifically defective in either PtdCho binding (Sfh1113C,S175I,T177I), or specifically defective in PtdIns binding (Sfh1113C,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 ck1a yeast strain (Figure 4A). As expected, in every case, PtdIns and PtdCho binding were each individually required for Sfh1*-dependent rescue of CPY transis 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 Y109F, C, H, A; the Q111F, A; the Y113F, C; and the E125F 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 hydrophobic bonds by lack of the phenolic hydroxyl group. We therefore tested whether Sfh1* substitutions exhibited allele specificity 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 preserved) 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 Y109S are conserved between the two proteins. These conservations include Sfh1 (Sec14) residues: A106 (A104), K107 (K105), Y109 (Y107), P110 (P108), Q111 (Q109), E125 (E123), L128 (L126), M129 (M132), Y195 (Y193). Sfh1Y195S was of interest because the corresponding substitution interferes with cooperative interactions between residues Y195 and Y199—that is, interactions that organize polar contacts with ordered water in the hydrophilic patch. When tested in both high- and low-copy expression contexts, Sfh1Y195S 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 sec14-1Δ 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 Na3N/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.

PL-transfer activity (Supplemental Figure S3, A and B), and the triple mutant proteins were stable in vitro and in vivo (Supplemental Figure S3, C and D). Neither expression of Sfh1113C,L179W,V199W nor of Sec1113C,L179W,V199W rescued growth defects associated with the sec14-1Δ allele (Figure 4C). The Sec1E126A pinch-close derivative was also generated, but this triple mutant protein was unstable in vivo (unpublished data).

Incorporation of T238D into the Sfh1* context, that is, a substitution that specifically interferes with coordination of the PtdIns headgroup phosphate (Schaaf et al., 2008), evoked the expected abolition of PtdIns-transfer activity (Supplemental Figure S3A). Sfh1113C,T238D and Sec1E126A,T238D were both stable polypeptides in vivo and in vitro (Supplemental Figure S3, C and D), and expression of these proteins failed to rescue growth defects of sec14-1Δ yeast at restrictive temperatures (Figure 4C). Sfh1 residues S175 and T177

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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, I131 (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. I131 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 sec14-1Δ and the isogenic sec14-1Δ spo14Δ double mutant (Supplemental Figure S4A).

**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 Y1109 and Y1212, and Y1224 participates in an H-bond interaction with Q111 (Figure S8B). Atomic displacement parameters for these residues are 16.8, 17.2, and 16.9 Å^2 in the Sfh1::PtdEtn complex and 17.7, 17.9, and 16.9 Å^2 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 Y1113 and S1759 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 Y1109 and Y1212 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 Y1109 and Y195 displays atomic displacement parameters of 9.8 and 23.2 Å^2 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).

**Sfh1* and PtdCho binding/exchange**

While the structural data suggest E126A relieves the Y1109 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 resuscitates Sec14-like activities in Sfh1. One plausible mechanism is that Sfh1* are endowed with superior

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**FIGURE 4:** Potentiation of PtdIns 4-OH kinase activities. (A) The sec14-1Δ cki1 "bypass Sec14" yeast strain CTY303 was transformed with YCp(URA3) plasmids carrying SEC14, SFH1, or the designated SFH1* alleles. YCp(URA3) derivatives served as a negative control. Transformants were radiolabeled at 30°C for 12 h with 20 μCi/ml [3H]myo-inositol. Deacylated PIPs were separated by high-performance liquid chromatography and quantified (see Materials and Methods). PtdIns-3-P, PtdIns-4-P, and PtdIns(4,5)P2 are indicated. Average values and SD are presented (n = 4). (B) Strain CTY100 (sec14-1Δ spo14Δ) derivatives served as a negative control. Transformants were radiolabeled at 30°C for 12 h with 20 μCi/ml [3H]inositol (Schaaf et al., 2008; Ile et al., 2010). After a 3-h shift to 37°C, phospholipids were extracted and resolved by TLC (top). PtdIns-4-phosphate species is identified at left. The PtdIns-4-phosphate band intensities were measured by densitometry and expressed as a PtdIns-4-phosphate/PtdIns ratio for purposes of normalization. The normalized ratio was then compared with the URA3 control condition (set to 1.0 on the relative scale). The data represent the averages and SD obtained from at least three independent experiments. (C) Left and middle, derivatives of isogenic sec14-1Δ and sec14-1Δ spo14Δ yeast strains (as indicated on top) carrying designated YCp(URA3) plasmids were spotted in eightfold serial dilutions onto YPD agar plates. Complementation of growth defects at 37°C reports Sec14-like in vivo function. Right, an SEC14::PtdEn complex (Schaaf et al., 2008), the PtdEn-bound complex was analyzed. SEC14::PtdEn displays the canonical closed conformation that superimposes onto the SEC14::PtdEn structure with a root mean square deviation (rmsd) of 0.15 Å for all protein atoms. In the SEC14::PtdEn complex, the E126 side chain makes direct contacts with Y1109 and Y1212, and Y1224 participates in an H-bond interaction with Q111 (Figure S8B). Atomic displacement parameters for these residues are 16.8, 17.2, and 16.9 Å^2 in the SEC14::PtdEn complex and 17.7, 17.9, and 16.9 Å^2 in the SEC14E126A::PtdEn complex, respectively. As in the SEC14::PtdEn complex (Schaaf et al., 2008), PtdEn is stabilized by 14 van der Waals interactions and coordinated via hydrogen bonds by residues Y1113 and S1759 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 Y1109 and Y1212 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::PtdEn 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 Y1109 and Y195 displays atomic displacement parameters of 9.8 and 23.2 Å^2 in the Sfh1E126A::PtdEn and SEC14::PtdEn 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).

**Sfh1* and PtdCho binding/exchange**

While the structural data suggest E126A relieves the Y1109 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 resuscitates Sec14-like activities in Sfh1. One plausible mechanism is that Sfh1* are endowed with superior
The hydrophobic patch in (A) Sfh1E126A::PtdEtn and (B) Sfh1::PtdEtn (pdb 3B74). 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 *. (C) 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.

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 *. (C) 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$^{E126A}$, Sfh1$^{Y113C}$, and Sfh1$^{KPFLD}$. For the Sfh1$^{E126A}$ ::PL structures, 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 written 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 $\alpha_1T_3$ 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; 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 K29,KPFLD233 (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*.

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$_{b}$, and A$_{e}$ (corresponding Sec14 helices are A$_{0}$, A$_{b}$, and A$_{e}$) (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$_{0}$ element of the Sfh1 and Sfh1* helical gate (Figure 7). Likewise, the Sec14 A$_{e}$ helix exhibited increased fluctuations in both PL-bound structures as compared with the corresponding helix A$_{e}$ in Sfh1 and Sfh1*.—with the exception that the Sfh1$^{E126A}$::PtdCho complex also exhibited similarly enhanced fluctuations in the A$_{3}$ helix (Figure 7). Because

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

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.

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 multimamellar lipid dispersion after mixing with Sfh1 protein 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 n-doxyl-PtdCho prepared as multimamellar 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$^{E126A}$.

transfer relative to His$_{2}$-Sec14 (Supplemental Figure S3, A and B). All three Sfh1* tested (His$_{2}$-Sfh1$^{Y109A}$, His$_{2}$-Sfh1$^{Y113C}$, and His$_{2}$-Sfh1$^{E126A}$) presented increased PtdIns transfer activity relative to His$_{2}$-Sfh1. Similar outcomes were recorded for PtdCho-transfer activities of His$_{2}$-Sfh1$^{Y109A}$ and His$_{2}$-Sfh1$^{Y113C}$. Both Sfh1* proteins exhibited a > fivefold increase in PtdCho transfer relative to His$_{2}$-Sfh1. His$_{2}$-Sfh1$^{E126A}$ presented a twofold increase in PtdCho transfer activity relative to His$_{2}$-Sfh1 (Supplemental Figure S3, A and B). Taken together, these data demonstrate that Sfh1* exhibit significantly enhanced (i.e., Sec14-like) abilities to cycle PL from the lipid-binding pocket relative to Sfh1.
Sfh1 helices A8 and A9 (Sec14 A9 and A10) are in intimate contact in the PL-bound conformation, we speculate that increased fluctuations in Sfh1 Y109A helix A8 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 Y109A substitution (Figure 7).

The MD simulations also consistently identified common Sfh1*-specific and Sec14-like behaviors in key structural motifs. Sec14 residues 273-DES273 exhibit rmsf values of > 1.6 Å in Sec14::PtdCho simulations. Interestingly, all Sfh1*:PtdCho simulations, 48 residues in H-bond contact with the core Sfh1* residues (Supplemental Figure S6). Additional H-bond interactions are observed with residues of the A273 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 dramatic increase of H-bonding was observed for the interaction of the E126 backbone with the phenolic –OH group of Y113C (1.6 Å) to or within (Q111, Y113, and E126) the B1 helix, and these residues flank the 116VDKDGR121 component. To investigate whether the H-bonding network of residues comprising the hydrophilic path 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 exhibit rmsf values of Cx atoms of all protein residues were extracted from MD simulations of PtdCho (A) and PtdIns structures (B). Average Cx 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) (including the A127-K265 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 Y109C, Sfh1 Y113C, and Sfh1 Y126A simulations, respectively (Supplemental Table S2). The neighboring E127-K265 side chain interaction also increased 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 Y109C, Sfh1 Y113C, and Sfh1 Y126A simulations, 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 8; 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 D117 and R121 of the 115VDKDGR121 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 E250 and S270 (Figure 8, B and C; Supplemental Figure S6; Supplemental Table S2).

To address the functional relevance of the increased E126-Y266 interaction, the Y266F and Y266D substitutions were introduced into the context of Sfh1 Y113C. These substitutions were anticipated to ablate H-bonding by lack of the side chain donor (Y266F) for interaction with the E126 backbone, or to cause an electrostatic clash (Y266D) with the terminal carboxylate of the E127 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 A11LT4 is a consistent, and functionally important, feature of the mechanisms underlying Sfh1*.

Altered interactions between the B1LB2 and the A11LT4 substructures in Sfh1*

The Sec14 B1LB2 and A11LT4 substructures comprise a “gating module” that transduces conformational information to the A107T4A111 helical gate via an extensive H-bond network. The 114TKDGDR121 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 114VDKDGR121 component) and A11LT4. Strikingly, the core Sfh1* residues lie immediately C-terminal (Y109) to or within (Q111, Y113, and E126) the B1LB2 element, and these residues flank the 116VDKDGR121 component. To investigate whether the H-bonding network of residues comprising the hydrophilic path 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 exhibit rmsf values of Cx atoms of all protein residues were extracted from MD simulations of PtdCho (A) and PtdIns structures (B). Average Cx 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) (including the A127-K265 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 Y109C, Sfh1 Y113C, and Sfh1 Y126A simulations, respectively (Supplemental Table S2). The neighboring E127-K265 side chain interaction also increased 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 Y109C, Sfh1 Y113C, and Sfh1 Y126A simulations, 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 8; 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 D117 and R121 of the 115VDKDGR121 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 E250 and S270 (Figure 8, B and C; Supplemental Figure S6; Supplemental Table S2).

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and positive controls, respectively. 37°C reports Sec14-like activity. YCp( ), plasmids carrying either mutants (D). (E) Serial dilution spotting of the reduces interactions between the loop regions of the B strand and the T4 helix. Increased H-bonding between D 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 sec14-1ts yeast strain transformed with YCp(SEC14, URA3) 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.

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 sec14-1ts-associated growth defects by Sfh1* expression (Figure 8E), even though Sfh1* 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 Y109 (A9) and T225 (A6) were monitored in Sfh1*:PtdIns, Sfh1::PtdIns, and Sfh1*:PtdIns structures, as were interatomic distances between Cα atoms of K197 (A9) and F233 (A6; 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 A6. Interestingly, while rmsf values of A9 and A6 residues were indistinguishable between Sfh1 and Sfh1* (except for Sfh1*Y170F; Figure 7), average fluctuations in the interatomic Y190-T225 Cα distance were enhanced in all Sfh1*:PtdIns structures relative to the apo-Sec14 (Figure 9E), even though Sfh1*T124A expression (Figure 8E), even though Sfh1*T124A shows decreased stability in vivo (unpublished data).
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 (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, Sfh1Y109A, Sfh1Y113C, and Sfh1E126A considering internal energies (bonds, angle, 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 Shf1* (i.e., Y1109, Q1111, and E1268) 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-mod-ule; 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 Shf1 hydrophobic pocket. High-resolution structural data demonstrate the Shf1* substitutions effect a remarkably subtle rearrangement of the hydrophilic microenvironment. However, both structural analyses and MD simulations indicate Shf1* reconfigure interactions between side chains and ordered H2O molecules in this region. Analyses of the gain-of-function Shf1* substitutions demonstrate that the interaction between the C-terminal region of strand B2 (residues E1268 and E1272) with the T4 turn in the string motif (residues K265 and Y266) is critical for the Shf1* phenotype. By contrast, strong interactions between the loop regions of the B1LB2 (H1114, V1116, and D1119) and A11LT4 (Q255a and N2551) substructures are incompatible with Shf1*.

A role of buried water?

The fact that Shf1* substitutions involve Shf1 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 Shf1* demonstrate that strong polar interside chain interactions (i.e., E1268-Y124 and E1268-Y109) are incompatible with the conformational dynamics required for Sec14-like functions in the Shf1 context. The collective data suggest that interactions of these key side chains with water differ between Shf1 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/ Shf1. The internal water content of the hydrophobic pocket differs in the holo Shf1::PtdIns and Shf1::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, Q1111, Y124, and E1268 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 Shf1* residues within a 2-ns time window is < 51% and < 60% for Shf1::PtdCho and Shf1::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 Shf1* (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 Shf1* activation mechanisms. This remains an open question for future address.

Sec14 and Shf1*—like, yet unlike

Interestingly, Shf1* 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., Y266F and Y266D) are incompatible with Shf1*. 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 Shf1*. 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 Q255R Shf1* substitution. Although not characterized in this report, Shf1*Q255R involves a residue spatially removed from the physically clustered Shf1* residues upon which we focus here. The mechanism by which Sec14-like functions is resurrected in Shf1*Q255R is almost certainly distinct from the mechanisms that apply to Shf1* 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 keS1), CTY1079 (MATa ura3-52 lys2-801 Δhis3-200 sec14-1ts spo14Δ::HIS3), CTY1098 (MATa ura3-52 lys2-801 Δhis3-200 sec14-1ts keS1), CTY303 (MATa ura3-52 lys2-801 Δhis3-200 Δsec14, cki1::HIS3), CTY558 (MATa ade2 ade3 leu2 Δhis3 ura3-52 sec14A1::HIS3 YEp[SEC14, LEU2, ADE3]), and BY4741 (MATa his3Δ leu2Δ200 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; Schaal et al., 2008). PIPs were extracted from yeast radiolabeled to steady state with [3H]myo-inositol and decayed, 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-Ts 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 Sh₁₁₂₆₆₆-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::Sh₁₁₂₆₆ 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.

**Cry stallization 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 Beamline at Argonne National Laboratory (Argonne, IL) and processed and scaled with HKL2000 (Otwinowski, 1993). Initial phases were determined using the Sh₁₁₂₆₆-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 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, Sₑ, was calculated as described in Schorn and Marsh (1997):

\[
Sₑ = \frac{(A₀ - A₁)}{\sqrt{2}(A₀ + 2A₁)} ΔA
\]

where A₀ = Aₐₙₜ, that is, the outer hyperfine splitting, and A₁ is calculated from Aₙᵢ, a half of the inner hyperfine splitting expressed in Gauss:

\[
A₀ = (Aₙᵢ + 0.85) for Sₚₚₚ < 0.45
\]

\[
A₁ = Aₙᵢ + 1.82 + 1.86 log(1 - Sₚₚₚ) for Sₚₚₚ > 0.45
\]

\[
Sₚₚₚ = \frac{(Aₖₖₖ - A₀)}{ΔA}
\]

where A₀ is the isotropic nitrogen hyperfine coupling constant and ΔA is the maximum extent of the axial nitrogen hyperfine anisotropy.

Although previously we have shown that the values of ΔA 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 ΔA for all n-doxyl-PtdCho protein complexes. A₀ and ΔA were set to those measured for 5-doxyl-PtdCho in isopropanol (Smirnova 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 (Alouie and Smirnov, 2006).

**Supplemental material**

Supplemental material includes eight figures and three tables.

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**REFERENCES**


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