A blueprint for functional engineering

Single point mutations reconstitute phosphatidylinositol presentation in a pseudo-Sec14 protein

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Phosphoinositides, phosphorylated species of phosphatidylinositol (PtdIns), are critical regulatory lipids in all eukaryotic cells. The molecular mechanisms that lead to the phosphorylation of an individual PtdIns- or phosphoinositide molecule remain largely unknown even though lipid kinases and phosphatases involved in these processes have been studied in detail. The observation by us and others that liposomal PtdIns (and phosphoinositide) molecules are poor in vitro substrates for kinases and phosphatases raises the question of how these enzymes execute their function in living cells. Recent work indicates that Sec14, the founding member of a large superfamily of eukaryotic proteins, is crucial for the process of PtdIns phosphorylation. The collective data suggest that Sec14 mediates a heterotypic phospholipid exchange reaction of PtdIns with phosphatidylcholine (PtdCho) during which PtdIns becomes vulnerable for kinase attack and thereby promotes the generation of phosphoinositides.1,2 In a recent paper we address the molecular mechanism of this phospholipid (PL) exchange reaction in a pseudo-Sec14 protein (Sfh1) that we rendered functional by a directed evolution approach. We find that enhanced PL-cycling into and out of the hydrophobic pocket of these activated Sfh1 mutants depends on the reconfiguration of interactions between a C-terminal string motif and the floor of the hydrophobic pocket that results in increased oscillations in a helical gate that controls pocket access. Here we further discuss our findings and propose molecular dynamics simulations as a tool to approach energetically unfavorable transition states and to identify novel protein-ligand interactions invisible to X-ray crystallography.

Sec14 is an essential protein required for the efficient biosynthesis of distinct pools of phosphatidylinositol-4-P (PtdIns-4-P) in yeast.3 Inactivation of Sec14 causes severe trafficking defects4,5 in agreement with an essential role of PtdIns 4-P to recruit and activate regulatory proteins involved in the formation of secretory vesicles at the yeast trans-Golgi network (TGN).6,7

Structural Appreciation of Phospholipid Binding by Sec14 Proteins Suggests a Role in PtdIns Presentation

In a previous study addressing the mechanism of Sec14-stimulated PtdIns-4-P production, we crystallized and functionally characterized Sec14 homolog Sfh1, in complex with PtdEtn and Sec14’s physiological lipid ligands phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho).8,9 Our structural and functional analyses suggest that (i) the hydrophobic cavity of Sfh1 and (Sec14) is large enough to entirely accommodate a single phospholipid (PL) molecule; (ii) PtdIns and PtdCho bind at distinct yet overlapping sites; (iii) binding of both PtdIns and PtdCho are essential activities of Sec14; and (iv) both individual...
In an attempt to elucidate the topology of productive Sec14-like heterotypic PL exchange and to understand the inefficiency of Sfh1 in stimulating PtdIns-4-P production, we randomly mutagenized Sfh1 and screened for Sfh1 mutants (named Sfh1*) that were now able to rescue growth defects associated with Sec14 deficiency in yeast. To our surprise, single missense substitutions identified in this screen that endow Sfh1* with Sec14-like activities affect residues conserved between Sec14 and Sfh1 (Fig. 1A and B). These Sfh1* mutations reside in an enigmatic hydrophilic patch at the floor of the hydrophobic PL-binding pocket (Fig. 1C).

PtdIns and PtdCho binding activities have to reside in cis to reconstitute a functional Sec14 protein that allows stimulation of PtdIns-4-P production in vivo. Our collective data suggest that Sec14 mediates a PL exchange reaction that results in presentation of PtdIns to PtdIns 4-OH kinases. The requirement for heterotypic PL exchange (PtdCho for PtdIns, or vice versa) to efficiently stimulate PtdIns 4-P kinase activity suggests a high complexity of PtdIns-4-P biosynthesis. Surprisingly, Sfh1, which shares 64% primary sequence identity with Sec14 and conserves all of the structural motifs critical for PtdIns and PtdCho binding, does not substitute for Sec14 in vivo even under conditions of high protein expression.9,10

**Figure 1.** Functional characterization of Sfh1* activation mutants. (A) The sec14-1ts yeast strain was transformed with YCp(URA3) plasmids carrying either SFH1 or the designated SFH1* alleles and transformants were spotted in serial dilutions onto YPD agar plates. Rescue at restrictive temperatures (35.5°C and 37°C, middle and right part, respectively) reports Sec14-like activity. Centromeric plasmids YCp(URA3) and YCp(SEC14, URA3) were employed as negative and positive controls, respectively. (B) Sequence alignment of Sec14 and Sfh1. Shown is a selected region that includes the hydrophilic patch and residues substituted by missense mutations in Sfh1* (as indicated). Conserved amino acids are depicted in black, similar amino acids depicted in gray. (C and D) Ribbon diagrams illustrating the hydrophilic patch at the floor of the hydrophobic PL-binding pocket. Sfh1 residues that are substituted by missense mutations in Sfh1* (except Y113) are shown as sticks. PtdEtN is rendered in cyan, H-bonds are shown as dashed lines. Structures of the Sfh1::PtdEtN complex (C), pdb ID 3B74, and the Sfh1126A::PtdEtN complex (D), pdb ID 3Q8G are shown. Note that an intricate H-bonding network remains intact in the Sfh1126A::PtdEtN complex by virtue of an immobilized H2O molecule that occupies the space of the missing E126 carboxy group (D).
mean square deviation (rmsd) below 0.2 Å suggesting that the E126A substitution does not cause any major conformational changes. This finding was further corroborated by investigating the immediate environment of the missense substitution. The carboxy group of E126 connects the phenolic hydroxyl of Y124 with the side chains of Y109/Q111 via strong hydrogen bonding that also involves several immobilized H₂O molecules and therefore seems to play a key role in maintaining the integrity of the hydrophilic patch. Surprisingly, patch residues Y109, Q111 and Y124 appear unaltered in the Sfh1E126A structure with respect to position and side chain orientation. Furthermore, the strong interactions observed within the hydrophilic patch of the wild type structure appear to be intact in Sfh1E126A::PtdEtN (Fig. 1C and D). Interestingly, the hydrogen bonding network of the hydrophilic patch stays intact by immobilization of an additional H₂O molecule that occupies the space filled by the E126 carboxy group in the wild type structure (Fig. 1C and D).

From these structural analyses, the strong activation phenotype of Sfh1E126A remains largely elusive and suggests that activation of Sfh1 does not require conformational alterations in the thermodynamically most stable PL bound state that we crystallized here. Rather, it suggests that the E126A mutation alters energy levels and/or the topology of PL exchange intermediates.

Reconfiguration of Atomic Interactions in Sfh1* Correlate with Increased Phospholipid Cycling

Since energetically unfavorable transitional states generally escape conventional crystallographic methods, we decided to approach exchange intermediates by molecular dynamics (MD) simulations. Computational simulations of protein dynamics provide high-resolution information when complemented by laboratory experiments. MD simulations were successfully applied to investigate solute transport through membrane-spanning channels, and helped to identify transient conformational states of signaling proteins. We performed MD-simulations for PtdIns- and PtdCho-bound complexes of Sfh1, various Sfh1* (including Sfh1E126A) and Sec14. Two major conclusions could be drawn from these analyses: (i) Sfh1* activation mutants consistently reconfigure an H-bonding network between the BₐL₇B₂ substructure at the floor of the hydrophobic pocket and residues at the C-terminal string motif (Fig. 2A), (ii) these reconfigured atomic interactions result in conformational coupling that transduces conformational energy from the C-terminus and hydrophobic pocket to the A₉T₃A₁₀ helical motif that gates hydrophobic pocket access (Fig. 2A and see also ref. 9). In particular, we find consistently increased hydrogen bonding in all Sfh1*:PtdCho complexes between the C-terminal half of the B₁ strand with the C-terminal T₉ turn and consistently decreased hydrogen bonding in all Sfh1*:PtdIns complexes between the loop regions of the B₁L₂ and the C-terminal A₁₁LT₄ substructures (Fig. 2A). These consistent alterations correlate with increased fluctuations of the helical gate, suggesting that they increase cycling of PL into and out of the hydrophobic PL binding pocket. To test whether this is indeed the case, we performed experimental assays that monitor protein-mediated PL transfer between two distinct liposomal populations and provide a sensitive readout of the protein’s ability to extract and release a PL molecule from/into a liposomal membrane. In these experiments, Sfh1* activation mutants consistently showed increased transfer (and thus cycling) for both PtdCho and PtdIns (Fig. 2B and see also ref. 9), supporting the predictions from our MD simulations.

Atomic Interactions between Critical Sfh1/Sec14 Substructures: Mechanistic Insights in Sec14 Dysfunction Causing Human Disease?

Interestingly, the structural motifs identified in our work are also present in human Sec14 proteins including α-tocopherol transfer protein (αTTP), SNPF, cellular retinaldehyde binding protein (CRALBP) and the neurofibromin 1 (NF1) ras GTPase activating protein, for which no physiological ligand of the Sec14-domain has been identified yet. Strikingly, dysfunctions in these proteins cause human disease and many of the disease causing mutations lie within the B₁L₂ substructure and the helical gate of these proteins, suggesting a general significance of these motifs for Sec14 function.

Molecular Dynamics Simulations as a Tool to Predict Transient Sec14::PL Exchange Intermediates

MD-simulations employed in our directed evolution approach provided a rationale for the Sfh1* activation mechanism. To further deepen the structural appreciation of exchange intermediates that escape conventional structural and biochemical analyses, we used here MD-simulations to identify transient H-bonds that are absent in high resolution crystal structures but might be of functional importance to mediate conformational transitions independent of the Sfh1* activation mechanism. Initial coordinates for Sfh1::PtdCho were obtained from the Protein Databank (PDB ID: 3B7Q), and a structural model as described previously in reference 9, provided coordinates for the Sec14::PtdCho complex. Unconstraint MD simulations were performed for 14 ns in triplicate essentially as described in reference 9, and structural snapshots that were written every 2 ps during the last 2 ns of the simulation (12 to 14 ns) employed to identify H-bonds coordinating the PtdCho ligand. Our high resolution crystal structure suggested that polar interactions of Sfh1 with PtdCho is mediated by strong hydrogen bonding of S175 and T177 with the phosphate oxygens of the PL headgroup. Evaluation of our MD simulations supported this observation with respect to T177 with a predicted H-bonding occupancy of 64% (Fig. 3). However, only a minor contribution (23% H-bonding occupancy) of S175-mediated PtdCho coordination was observed in these MD simulations (Fig. 3). To our surprise, MD simulations suggested a novel robust H-bonding interaction of the S203 side chain hydroxyl group with the headgroup acyl oxygens that is completely invisible in the high resolution Sfh1::PtdCho structure. While simulations predict 74% and 8% occupancy of
S203-mediated H-bonding of the PtdCho sn-2 and sn-1 acyl oxygen, respectively (i.e., 82% occupancy of S203 mediated PtdCho-coordination), distances of the S203 hydroxyl group to both acyl oxygens exceed 5 Å in the high resolution crystal structure and hence in theory should cause negligible polar interactions. A similar situation is observed in the context of the Sec14::PtdCho complex, where all three hydroxyl amino acids (here S173, T175 and S201) are conserved and where MD simulation likewise predicted a very robust interaction of S201 with the sn-2 acyl oxygen of PtdCho (74% H-bonding occupancy), while predicting no interaction of S173 with the phosphate group at all (Fig. 3).

In agreement with a less important role of S173 in PtdCho headgroup coordination as predicted by MD simulations, Ile, Val and Trp substitutions at Sec14 position 173 did not cause any reduction in complementation efficiency of sec14Δ-associated lethality. It remains to be shown by mutational analyses and biochemical characterization whether S1f1 residue S203 (and likewise Sec14 residue S201) is indeed involved in PtdCho-headgroup coordination and whether this interaction is physiologically important. Our preliminary data at this point support the idea that MD-simulations are powerful to describe Sec14-ligand interactions that are invisible to X-ray crystallography.

Acknowledgements
We would like to thank Kristina E. Ile for critically reading this manuscript. We acknowledge bwGrid, member of the German D-Grid initiative, founded by the Ministry for Education and Research and the Ministry of Science, Research and Arts Baden-Württemberg, for providing computational resources (http://www.bw-grid.de). Contact information for MD simulation data, Marek.Dynowski@rz.uni-freiburg.de. This work was supported by Emmy Noether grant 1274/2-1 from the Deutsche Forschungsgemeinschaft.

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Figure 2. Biochemical properties of Sfh1* and model of Sfh1* dependent alterations in H-bonding and helical gate oscillations. (A) Ribbon diagram illustrates H-bonds that are altered consistently in PtdIns bound Sfh1* mutants as deduced from MD simulations. The A T A gating module is depicted in red; all other α-helices are presented in blue. Unstructured regions are in gray and β-strands are presented in yellow. PtdIns is rendered in magenta. Relevant H-bonds are presented as dashed lines. The B.LB, substructure consists of β1- and β2-β-strands and the intervening loop region. Sfh1* mutants show decreased H-bonding of A.L, loop residue N261 with both H114 and V116 and decreased H-bonding of A1 residue Q256 with B.LB, loop residue D119. Altered H-bonding occupancies coincide with increased helical gate oscillations (depicted by red arrow). The topology of PtdIns exit (purple arrow) and the coordinates of kinase attack (asterisk) remain elusive. (B) Biochemical properties of Sfh1* with defects in PL-binding. Purified re-combinant Sec14, Sfh1 and mutant Sfh1 proteins were assayed for PtdIns-(upper part) and PtdCho-(lower part) transfer activity. Proteins were assayed at 1 μg/500 μL reaction volume as described in reference 9. Average values and standard deviations are given (n = 3).
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Figure 3. MD simulation analysis predicts a novel Protein/PtdCho interaction in Sfh1 and Sec14. H-bonding occupancies of PtdCho by Sfh1 and Sec14 residues (values for Sec14 are given in parenthesis) as predicted by MD-simulations. Note that the strong interaction of S203 to the acyl oxygen of sn-2 is absent in the high resolution structure obtained by X-ray crystallography.