Conformational Dynamics of the Major Yeast Phosphatidylinositol Transfer Protein Sec14p: Insight into the Mechanisms of Phospholipid Exchange and Diseases of Sec14p-Like Protein Deficiencies

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Molecular dynamics simulations coupled with functional analyses of the major yeast phosphatidylinositol/phosphatidylcholine transfer protein Sec14p identify structural elements involved in regulating the ability of Sec14p to execute phospholipid exchange. The molecular dynamics simulations suggest large rigid body motions within the Sec14p molecule accompany closing and opening of an A₁₋₁₋₁ helical gate, and that “state-of-closure” of this helical gate determines access to the Sec14p phospholipid binding cavity. The data also project that conformational dynamics of the helical gate are controlled by a hinge unit (residues F₂₁₂, Y₂₁₃, K₂₃₉, T₂₄₀, and I₂₄₂) that links to the N- and C-terminal ends of the helical gate, and by a novel gating module (composed of the B₁LB₂ and A₁₂LT₅ substructures) through which conformational information is transduced to the hinge. The Y₁₁₇TDKDGR₁₁₉ motif of B₁LB₂ plays an important role in that transduction process. These simulations offer new mechanistic possibilities for an important half-reaction of the Sec14p phospholipid exchange cycle that occurs on membrane surfaces after Sec14p has ejected bound ligand, and is reloading with another phospholipid molecule. These conformational transitions further suggest structural rationales for known disease missense mutations that functionally compromise mammalian members of the Sec14-protein superfamily.

INTRODUCTION

Phosphatidylinositol transfer proteins (PITPs) play important biological roles in defining the identities of individual lipid signaling pools that regulate specific biological processes. It is in this capacity that PITPs are suggested to represent components of lipid metabolic nanoreactors that promote specific signaling reactions (Ile et al., 2006). How PITPs couple exchange of phosphatidylinositol (PtdIns) or phosphatidylcholine (PtdCho) monomers to physiological function remains unresolved (Phillips et al., 2006). PITPs fall into two families (i.e., the Sec14 family and the metazoan PITP family) on the basis of primary sequence homology and structural fold. Members of one family share no primary homology or structural similarity with members of the other family (Bankaitis et al., 1989; Dickeson et al., 1989; Sha et al., 1998; Yoder et al., 2001).

The SEC14 gene product (Sec14p) is the major PITP of budding yeast, and it is required for efficient transport of secretory cargo from the yeast trans-Golgi network (TGN; Bankaitis et al., 1989, 1990; Cleves et al., 1991a). Genetic and biochemical evidence demonstrates Sec14p coordinates lipid metabolism with activities of proteins required for biogenesis of transport vesicles on yeast TGN membranes (Cleves et al., 1991b; McGee et al., 1994; Xie et al., 1998; Li et al., 2002; Yanagisawa et al., 2002; Phillips et al., 2006). Sec14p is of additional interest in that it is the prototype for a eukaryotic protein superfamily of Sec14-like proteins consisting of >500 known members distributed across the Eukaryota (Phillips et al., 2006). Sec14-like proteins regulate membrane trafficking, membrane biogenetic pathways, or both, that include polarized membrane trafficking in yeast (Carmen-Lopez et al., 1994; Nakase et al., 2001; Rudge et al., 2004) and in higher plants (Vincent et al., 2005). Other Sec14-like proteins are implicated in control of specific stress responses (Kearns et al., 1998; Monks et al., 2001). Finally, loss-of-function mutations in members of the Sec14 superfamily are associated with inherited human disorders (Aravind and Koonin, 1999; Boram et al., 2003; Meier et al., 2003; Panagabko et al., 2003; Stocker and Baumann, 2003).

Although not formally proven, phospholipid binding and exchange are presumed to be of relevance to Sec14p function in vivo (Ile et al., 2006; Phillips et al., 2006). This reaction is of biochemical interest in that Sec14p ejects bound phospholipid upon encountering a membrane surface and reloads...
with another phospholipid molecule before disengaging from that surface. Studies in purified systems using defined liposomes and recombinant Sec14p demonstrate the exchange reaction involves efficient abstraction of phospholipid from a stable membrane bilayer and that Sec14p executes this interfacial reaction in the absence of cofactors or ATP (Phillips et al., 1999). Thus, Sec14p couples conformational change to a cycle of phospholipid exchange. The crystal structure of detergent-bound Sec14p suggests how this may occur. The Sec14p hydrophobic cavity is bounded by a hybrid α/β-helix proposed to function as a gate (Figure 1). The conformational status of this gate is proposed to determine whether Sec14p is in an "open" or a "closed" conformation (Sha et al., 1998; Phillips et al., 1999).

Herein, we report the results obtained from unrestrained molecular dynamics (MD) simulations and biochemical analyses of Sec14p conformational dynamics. These analyses simulate oscillations between open and closed Sec14p conformers, and they implicate three Sec14p structural elements in control of this conformational dynamics pathway. Finally, the data suggest the Sec14p conformational circuitry described here is broadly conserved across the Sec14 protein superfamily and that defects in this circuitry may represent the molecular basis for some disease-causing mutations in mammalian Sec14-like proteins.

MATERIALS AND METHODS

Preparation of Starting Structures

The 2.5-Å resolution coordinates of the open Sec14p conformation (PDB 1AUA; Sha et al., 1998) was obtained from the Protein Data Bank (Berman et al., 2000). The two bound β-octylglucoside molecules were removed, and crystallographic waters were retained. Hydrogen atoms to both amino acid residues and water molecules were added using the LEaP module of AMBER 8.0 (Case et al., 2004). LEaP was also used to place eight sodium ions at positions of minimum electrostatic potential to neutralize the protein system. The system was solvated in 10,414 TIP3P water molecules (Jorgensen et al., 1983) with a buffer distance of 12.5 Å around the solute. In this manner, an octahedral box with initial dimensions of 81.5 × 81.5 × 81.5 Å, and containing a total of 36,156 atoms, was generated as input to the MD simulations. For purpose of simplicity, we loosely refer to this starting Sec14p structure as apo-Sec14p to reflect it is not a phospholipid-bound conformer.

Glycine 266 was replaced with an aspartate while selecting the lowest energy rotamer by using the Biopolymer module of Insight II (www.accelrys.com) to generate a starting structure for simulations of Sec14pG266D. This protein system was neutralized with nine sodium ions and solvated in 10,420 TIP3P molecules in a manner similar to the wild-type structure. The final Sec14pG266D system contained a total of 36,180 atoms in an octahedral box with initial dimensions similar to the Sec14p system.

Molecular Dynamics Simulation

MD simulations were carried out using the SANDER module of AMBER version 8.0 (Case et al., 2004) with the ff03 forcefield of Duan et al. (2003) as defined in parm99.dat and frcmod.803 parameter files (Cornell et al., 1995). Long-range electrostatic interactions were estimated using the particle mesh Ewald method (Toukmaji et al., 2000), whereas bonds involving protons were constrained using the SHAKE algorithm (tolerance = 0.0001 Å). A 2-fs time step was used throughout the simulation. A 10-Å cutoff was applied to Lennard–Jones interactions, and the nonbonded list was updated every 25 steps. Periodic boundary conditions were applied. All MD production runs were carried out at constant temperature and pressure.

Protein systems were minimized for 5000 steps. The first 2000 steps were of steepest descent followed by 3000 steps of conjugate gradient minimization. The minimization was followed by 20-ps constant volume dynamics with heating from 50 K to 300 K. Pressure and temperature were then held constant for 100 ps for the system to reach a density of 1 g/cm³. This was followed by a 40-ps regime at constant volume with reheating from 200 to 300 K, yielding a total equilibration time of 160 ps. The production run was continued for more than 32 ns, and snapshots of the coordinates were written out every 1 ps.

Molecular Dynamics Data Analyses

The PTRAJ module of AMBER 8.0 was used for trajectory processing and analyses of root mean square deviation (rmsd) values, interatomic distances, hydrogen bonding, and atomic positional fluctuations. The φ and ψ dihedral angles measuring residue backbone conformations were also reported using PTRAJ. Unless otherwise stated, data analyses are reported for the 15- to 32-ns
time frames of production simulations so that analyses could be focused on a
time window where large conformational changes were evident in the Sec14p
molecular dynamics simulation.

Correlation coefficients (Cohen, 1988; Blaikie, 2003) were calculated be-
tween local backbone angular conformations and Sec14p “state-of-closure”
according to the following equation:

$$\text{where } x_i \text{ represents the local backbone angular conformation (}\phi_i \text{ or } \psi_i \text{) of}
\text{a given residue at time } t. \text{ The variable } y_i \text{ represents state-of-closure at time } t \text{ as}
\text{measured by distance between the } C\alpha \text{ atoms of residues } K_{116} \text{ and } F_{231}. \text{ The}
summations are over all snapshots included in the analysis. Snapshots be-
tween 15 and 32 ns were used for the Sec14p analysis, because conformational
changes of a gate-like movement (including both opening and closing) oc-
curred during this entire period. For Sec14pG266D, only snapshots between 2.5
and 4 ns were included in the analysis, because no “gating-like” motion
occurred after this period. In addition, the 2.5- to 4.0-ns period included a
closing motion without a subsequent opening motion. Correlation coefficients
were calculated, and they are reported for every backbone dihedral angle
for all residues.

Yeast Strains and Methods

Standard media, genetic techniques, and plasmid shuttle assays were de-
scribed previously (Ito et al., 1983; Rothstein, 1983; Sherman et al., 1983; Cleves
et al., 1991b; Phillips et al., 1999; Li et al., 2002). Strains included CTY182
[MATa ade2 leu2 his3-200, Δhis3-200, lys2-801, SEC14], CTY1079 [MATa ade2 leu2
his3-200, lys2-801, sec14-14F spo14Δ-HIS3], CTY558 [MATa ade2 leu2 his3
ura3-52 sec14Δ131::HIS1 Ycp(SEC14, URA3)], and CTY303 [MATa ade2
his3-200, exki, sec14Δp::his3]. Experiments were performed at 30 and 37°C.
All transformants were selected based on acquisition of uracil prototrophy.

Protein Stability

Appropriate derivatives of yeast strain CTY303 carrying SEC14 expression
plasmids of interest were grown to mid-logarithmic phase at 25°C and shifted
to 37°C for 1, 3, and 5 h. Cell densities were normalized to an OD600 = 1.5,
protein extracts were prepared by cell disruption with glass beads, and
extracts were subsequently analyzed by SDS-polyacrylamide gel electro-
phoresis (PAGE) and immunoblotting.

Site-directed Mutagenesis

The various mutagenic primers and their corresponding reverse complemen-
tary sequences are available from us by request. Mutagenesis of SEC14
sequences was performed on a 2.6-kb EcoRI-1spl SEC14 fragment resident in
either plasmid pRE246 (Alb et al., 1995) or the pQE31-based pMN85 SEC14 plas-
mid pRE526 (Skinner et al., 1995). Mutagenized SEC14 sequences were con-
formed by DNA sequence analysis and correctly mutagenized gene cassettes
were individually subcloned into the centromeric and episomal yeast–Esche-
richia coli shuttle vectors YCPlac33 and YEpLac195, respectively (Geitz and
Sugino, 1988).

Sec14p Purification

Recombinant Sec14p and selected mutant derivatives were purified from E.
coli as described previously (Phillips et al., 1999) with modification. Briefly,
cultures of E. coli strain KK2186 were grown aerobically at room temperature
to an OD600 of 0.5 in superbroth medium supplemented with 60 μg/ml
ampicillin. Sec14p expression was induced by addition of isopropyl β-thio-
galactoside to 1 mM final concentration, and cultures were incubated for an
additional 5 h with aeration. Cells were harvested by centrifugation at 7000
× g (10 min), and resuspended in lysis buffer (50 mM sodium phosphate, pH 7.1,
300 mM sodium chloride, 1 mM NaN3, and 0.2 mM phenylmethanesulfonyl
fluoride). Lysozyme was added, and the suspension was incubated at room
temperature for 10 min with occasional agitation. Cells were disrupted using
0.1- to 0.3-mm glass beads and a bead beater (Biospec Products, Bartlesville,
OK), and cell-free lysate was clarified by centrifugation at 7000 and
130,000 × g, respectively. The supernatant was applied to Talon Sepharose
gel and incubated for 5 min

PtdCho Transfer Assays

[14C]PtdCho transfer from liposomes to bovine heart mitochondria by using
either recombinant protein or yeast cytosol was assayed as described previ-
ously (Phillips et al., 1999; Li et al., 2000). When yeast cytosol was used, Sec14p
species were expressed in strain CTY303 from the episomal pDR195 vector
where Sec14p expression was driven by the PMA1 promoter (Rentsch et al.,
1995). This system drives significant overexpression of Sec14p species and

Figure 2. Mobile regions of the Sec14p molecule. (A) All atom
RMSD plot for Sec14p (black) and Sec14p G266D (gray) simulations as
a function of simulation time (nanoseconds). (B) Average Ca atomic
position fluctuation plot for Sec14p (black) and Sec14p G266D (gray)
simulations as a function of residue (plotted from N to C terminus). Two
major areas of distinction between the Sec14p and Sec14p G266D
plots are centered around residues K116 and F231. The position of
G266 is identified with the arrow for reference.
such mobile Sec14p structural elements, the atomic positional fluctuation (apf) for all Sec14p Cα atoms were monitored during the 15- to 32-ns simulation time window. In all references to amino acid residues herein, we define the initiator Met as residue 1. Plots of the root mean square fluctuation (rmsf) data indicated the largest atomic positional motions of Sec14p occurred within helix A10/T4 around F231 (Figure 2B). Residue F231 is located at the C terminus of helix A10/T4 and exhibited the greatest rmsf value (5.4 Å). The rmsf values that averaged in excess of 2.0 Å included residues 223–238 that span helices A10/T4 and A11 (referred to as the A10/T4/A11 helical gate; see below). Similarly, the 271DESK274 cluster positioned immediately C terminal to helix T5 in the string motif also showed average rmsf values in excess of 2.0 Å. The significance of these string motif dynamics is discussed below.

Evidence to indicate helix A10/T4/A11 was closing during the simulation was obtained from monitoring of interatomic distances between positional references on helix A10/T4 and on helix A9, i.e., the structural element that lies opposite from helix A10/T4 across the opening to the Sec14p hydrophobic cavity. Distance-monitoring plots demonstrated that the Cα atoms of residues R195 (helix A9) and F231 (helix A10/T4) approached to within 10.9 Å of each other and then separated to distances in excess of 25 Å during the simulation (Figure 3, A and B). These motions proceeded in an oscillating manner characterized by periods of proximity followed by periods of distance. Periods of proximity were characterized by a stepwise progression with interatomic distances of 18.7, 14.0, and 10.9 Å measured at 3.6, 8.9, and 18.1 ns of the simulation, respectively (snapshots 3616, 8937, and 18130). The lifetime of each period of proximity increased progressively (0.3, 0.6, and 0.85 ns). The smallest R195-F231 interatomic Cα distance (10.9 Å) was recorded first at 18.1 ns of the simulation. It was recorded again during the 4-ns period of proximity attained after helix A10/T4 closed at 24 ns of the simulation, and this 10.9-Å distance was maintained throughout that entire 4-ns period of proximity. The R195-F231 interatomic Cα distances increased again at 28 ns.

The apf plots suggest the motion was concentrated in the gate with helix A10/T4/A11 moving toward helix A9. As indicated above, the largest rmsf value (5.4 Å) for any Sec14p residue in the simulation was recorded for the gate residue F231. In contrast, the rmsf values for helix A9 residue R195 and for residues on helix A12 were only 0.8 and 1.0 Å, respectively. These data suggest helices A9 and A12 undergo only small positional fluctuations during the simulation, whereas helix A10/T4/A11 engaged in much larger motions relative to these two structural elements. Distance monitor-
ing plots recorded for the Ca atoms of residues K229 and F230 as well as R195 and F230 are also consistent with this conclusion (Supplemental Figure S1, A and B). K229 marks the C terminus of the A9 element of helix A10/T4 and is an invariant residue among Sec14-domain proteins, whereas F230 resides in helix A12. The interatomic distances between Ca atoms of K229 and F230 fluctuated inversely as a function of time relative to fluctuations recorded between R195 and F231. In addition, distances on average of 29 Å are maintained between helix A9 residue R195 and helix A12 residue F230 throughout the simulation.

Together, unrestrained MD simulations of apo-Sec14p in explicit solvent reveal an oscillatory pattern for Sec14p between open and closed conformations. The data further suggest that conformational oscillations are associated with "gate-like" motions of the A10/T4/A11 helices. For purposes of simplicity, we refer to the Sec14p conformation characterized by R195-F231 interatomic Ca distance of 10.9 Å as closed, although it is likely this represents a "partially closed" conformation (see Discussion).

**Biochemical Evidence for Gate Closure**

We sought direct validation that 1) the trajectory of gate closure for Sec14p described by the MD simulation models a relevant conformational pathway and 2) that gate-mediated closure of the Sec14p hydrophobic pocket occurs by constriction of distances between the A10/T4/A11 helical gate and helix A9. To that end, opposing pairs of Cys residues were positioned on helices A9 and A10/T4, and each pair was evaluated for competence to form a disulfide bond. The double Cys mutant proteins were subsequently purified and assayed in parallel for PtdCho transfer activity under nonreducing and reducing conditions.

Sec14p contains four endogenous Cys residues, all of which are buried within the interior of the protein and are not solvent accessible (Sha et al., 1998). This is reflected by the robust PtdCho transfer activity of Sec14p in both nonreducing and reducing conditions (Figure 3C) and by our inability to modify Sec14p with N-ethylmaleimide without first denaturing the protein (our unpublished data). In the open apo-Sec14p conformation, residues R195 and F230 are 23.82 Å (Sha et al., 1998). When recombinant Sec14p(R195C,F231C) was assayed under nonreducing conditions, a 72% decrease in specific PtdCho transfer activity was recorded for the mutant protein relative to Sec14p (Figure 3C). This diminution of activity was not the result of some trivial and undefined protein denaturation. Sec14p(R195C,F231C) PtdCho transfer activity was substantially reconstituted upon introduction of β-mercaptoethanol into the transfer assay. These results demonstrate Sec14p tolerates the R195C,F231C substitutions so long as Sec14p(R195C,F231C) is in a reducing environment.

We interpret these data to indicate that the R195C and F231C side chains efficiently form disulfide bonds in nonreducing environments and that the resultant "forced closure" of the Sec14p hydrophobic pocket is inconsistent with phospholipid transfer activity. That inhibition of Sec14p(R195C,F231C) activity under nonreducing conditions was quantitatively constant over a 40-fold range of protein concentration in the assay, strongly argues against formation of intermolecular disulfide bonds (our unpublished data). Such intermolecular reactions are expected to be exquisitely sensitive to protein concentration, whereas formation of intramolecular disulfide bonds should not be so sensitive. As significant distance constraints govern disulfide bond formation (i.e., participating sulfhydryl groups must be within ~3 Å of each other; Careaga and Falke, 1992), these data indicate the side chains of helix A9 residue R195 and A10/T4 residue F231 must lie in proximity to each other in the closed Sec14p conformation. In support, a mutant Sec14p(Y188C,F231C) (where the interatomic distance for the Ca atoms of these helix A9-helix A10/T4 Cys pairs is 16 Å in the closed Sec14p conformation defined by MD simulation; compared with 10.9 Å in the case of Sec14p(R195C,F231C)) exhibited wild-type PtdCho transfer activities in both oxidative and reducing environments (Figure 3C).

Limited proteolysis was used as an independent probe for changes in the proximity of helices A9 and A10/T4/A11 as Sec14p transitions from closed to open conformations. Opening of the A10/T4/A11 helical gate evokes large conformational changes, and we sought to detect proteolytic events unique to open Sec14p conformations. These experiments were aided by the fact that nondenatured Sec14p adopts a compact fold and is highly resistant to proteolytic challenge with trypsin (Figure 3D) and a variety of other proteases (our unpublished data). In the presence of SDS micelles, however, trypsin rapidly and quantitatively cleaves Sec14p to generate two significant degradation products (Sec14p and 'Sec14p; Figure 3D). In-gel trypsinolysis of each species coupled with MALDI-TOF mass spectrometry unambiguously identified the more rapidly migrating 'Sec14p species as being derived from the C-terminal 109 residues of Sec14p. The N terminus of 'Sec14p is defined by the 19h,EASYIYSQNYYP ER208 tryptic peptide, and all subsequent major tryptic fragments C-terminal to this peptide were identified. Only the short 209MGK211, 250ELLK253 and 264FGGK267 peptides were not detected in these analyses (our unpublished data).

The more slowly migrating Sec14p' represents the N-terminal Sec14p fragment and the most C-terminal tryptic peptide identified from this fragment was 146NLVWEYES-VVQYR LPACSR164. In the intervening 31 residues between Sec14p and 'Sec14p, the only possible site(s) of trypsinolysis are limited to the amide linkages between helix A9 residues K180/G181 and/or R195/E196, and A8 residues R164/A165. Although we have not yet been able to distinguish several viable possibilities for pattern(s) of cleavage (i.e., whether only one or several of these positions define the precise site(s) of cleavage; we know similar trypsin cleavage patterns are obtained for Sec14p and Sec14p(R195C,F231C), we note all three candidate positions surround the opening of the hydrophobic pocket in the open Sec14p conformer. We interpret these data to suggest SDS micelles induce transition of Sec14p from the closed to the open conformation with the result that the K180/R195 and/or R164 amide bonds are rendered exquisitely susceptible to trypsin cleavage. Together, the Cys-scanning and trypsinolysis data independently indicate that transition from the open to the closed Sec14p conformation involves closure of the A10/T4/A11 helical gate with respect to helix A9.

**A Hinge for Rigid Body Motions of the A9/T4/A11 Helical Gate**

Distance monitoring between the Ca atoms of the highly conserved A10/T4 residue F231 and the B4 residue I214 suggests restricted motion of the N-terminal region of the helix A9/T4 with respect to the β-strands. Similarly, interatomic Ca distance monitoring between F221 and helix A9 residue A187 suggest that relatively constant distances averaging ca. 14.9 Å are maintained between these A9 and A10/T4 residues in Sec14p throughout the simulation (Supplemental Figure S2, A and B). A superimposition of average structures from different time frames of the MD simulation is depicted in Supplemental Figure S3. This superimposition illustrates the
displacement of helix A$_{10}$/T$_4$ in the transition of Sec14p from an open conformation to a closed conformation during the simulation and that the N-terminal aspect of A$_{10}$/T$_4$ remains relatively fixed during this transition.

To identify candidate structural elements that might be involved in supporting the transitions between open and closed Sec14p conformations, a set of correlation coefficients between Sec14p state-of-closure parameters and backbone (φ, ψ) angle variations were calculated for each residue along the polypeptide chain (Supplemental Figure S4). Distances between the R$_{195}$ and F$_{231}$ Ca atoms in each snapshot reported the state-of-closure parameter, whereas (φ, ψ) angles in each snapshot reported the backbone conformation for each residue. Changes in the (φ, ψ) angles of F$_{212}$ and Y$_{213}$ (both reside within the B$_4$ strand) as well as K$_{239}$ (immediately precedes B$_5$), I$_{240}$ (N-terminal residue of B$_5$), and I$_{242}$ (immediately follows B$_3$) correlated most strongly with the state-of-closure for the A$_{10}$/T$_4$/A$_{11}$ helical gate (less than −0.34 or >0.34). Interestingly, the B$_4$ and B$_5$ structural elements flank the A$_{10}$/T$_4$ and A$_{11}$ helices, i.e., these residues occupy both the appropriate positions and exhibit the appropriate conformational motions to hinge the A$_{10}$/T$_4$/A$_{11}$ gate (Figure 4A). For purposes of definition, we identify the overall gating region as residues G$_{210}$-I$_{242}$ and we refer to residues F$_{212}$, Y$_{213}$ as the FY component of the hinge unit and residues K$_{239}$, I$_{240}$, I$_{242}$ as the KII component, respectively.

The hinge residue F$_{212}$ orients its side chain toward the interior of the hydrophobic pocket, whereas the side chain of Y$_{213}$ is disposed toward the posterior of the Sec14p molecule. The orientation of Y$_{213}$ allows the hinge to be supported on the exterior of the phospholipid binding pocket by permitting the side chain of Y$_{213}$ to participate in π-stacking interactions with B$_3$ residue F$_{234}$ (Supplemental Figure S5A). In addition, the Y$_{213}$ side chain packs neatly into a pocket formed by the side chains of helix A$_{12}$ residues L$_{251}$ and Q$_{254}$. Structural support for the hinge unit (and contribution to rigidity of the N-terminal aspect of helix A$_{10}$/T$_4$ within the Sec14p) is construed from interactions on the pocket surface where I$_{242}$ sits in a cleft between F$_{221}$ and S$_{222}$ of helix A$_{10}$/T$_4$ (Supplemental Figure S5B).

Together, the MD simulations suggest the structural elements that undergo large-scale motions during gating of the Sec14p hydrophobic pocket move as a rigid body. The data further suggest that such rigid-body conformational motion is facilitated by “hinge-like” local backbone angular changes in the KII component of the hinge that interdigitates with the N terminus of the A$_{10}$/T$_4$/A$_{11}$ helical gate and the FY component of the hinge that resides within the phospholipid binding pocket itself.

**Hinge Function and Efficient Sec14p Activity**

To assess whether the hinge unit is a functionally important element, the ability of Sec14p derivatives compromised for this element to catalyze PtdCho transfer was assessed in vitro. With regard to the KII hinge component, Sec14p$_{F221A,S222A}$ is defective in interdigitation of hinge residue I$_{242}$ with the N terminus of the A$_{10}$/T$_4$/A$_{11}$ helical gate and exhibits an ~80% reduction in PtdCho transfer activity relative to Sec14p when the corresponding purified recombinant proteins are assayed (Figure 4B). With regard to the FY component of the hinge, the single mutant Sec14p$_{V231A}$ protein similarly exhibits a 40% reduction in PtdCho activity in vitro (Figure 4C), but combination of individual defects in the KII and FY hinge components levies more dramatic defects in general hinge function than those observed for either individual defect alone.
were observed for Sec14p. This is reflected in the reduced rmsf value (1.5 Å) for residue F231 in Sec14pG266D relative to the value of 5.4 Å calculated for F231 in Sec14p MD simulations (Figure 2B). Distance-monitoring analyses indicate that, after 3.4 ns, the interatomic distances between Ca atoms of R195 and F231 converged to an average value of 10.5 Å. This interatomic distance was then maintained throughout the remainder of the 32-ns simulation (Figure 3A). These measurements suggest apo-Sec14pG266D collapses to a closed structural conformation and cannot efficiently reopen. Closure of the helical gate in the Sec14pG266D simulation recapitulated the trajectory observed in the unrestrained Sec14p MD simulation. That is, helix A10/T4 residue K229 receded from residue E250 on helix A12 at the same time that helix A10/T4 residue F231 approached residue R195 on helix A9 (Supplemental Figure S1C).

The rmsf values in the immediate vicinity of the G266D missense substitution were altered in the Sec14pG266D simulation relative to those observed for that region in Sec14p. An additional region of Ca fluctuation also became apparent in the Sec14pG266D simulation. This new region of conformational mobility involved residues D115-A116 that reside within the loop element between β-strands B1 and B2. This unique conformational mobility was maximal for residue K116 which was displaced 1.7 Å in Sec14pG266D compared with 0.8 Å for Sec14p. The significance of these observations is discussed below.

### Hinge Function and Gate Closure in Sec14pG266D

The unrestrained MD simulations of apo-Sec14pG266D suggested this mutant protein is defective in gate opening. We therefore inspected the conformational dynamics of the FY and KII hinge components to assess their functionality in the context of Sec14pG266D. As for Sec14p, distance monitoring plots of the F231 and I214 Ca atoms suggest that rigidity of the N-terminal region of helix A10/T4 with respect to the β-strands is maintained (Supplemental Figure S2). However, in contrast to Sec14p, distance monitoring of F231 and A107 Ca atoms reveal the N-terminal region of helix A10/T4 approaches helix A9 during the first 7.0 ns of simulation (Supplemental Figure S2). An average interatomic distance of 8.5 Å is then maintained throughout the remainder of the Sec14pG266D MD production run. This is in contrast to the average interatomic distance of 15 Å for the F231 and A107 Ca atoms maintained in the Sec14p simulation. The contrast between the conformational dynamics of Sec14p and Sec14pG266D is highlighted by superimposition of the two average structures (Supplemental Figure S3).

Insight into the mechanism of the gate opening defect was gleaned from correlations of backbone (φ, ψ) angle variations with R195-F231 distance monitoring plots that report state-of-closure of the A10/T4/A11 helical gate. These analyses identified candidate bonds, rotations around which permit conformational changes of the helical gate. The strong correlations between (φ, ψ) angle changes and gate closure we observed in the Sec14p MD simulations were preserved in the Sec14pG266D context for hinge residues K229 and I242 (−0.48 and −0.59, respectively; Supplemental Figure S4). Unlike the case of Sec14p, however, changes in (φ, ψ) angles for hinge residues F212 and Y213 were no longer correlated with closure of the A10/T4/A11 helical gate. These collective data suggest the FY component of the hinge functions primarily to open the Sec14p A10/T4/A11 helical gate, whereas the KII hinge component functions in gate closure.

### Table 1. Functional evaluation of mutant sec14 proteins

<table>
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<tr>
<th>Sec14p species</th>
<th>Structural element</th>
<th>Rescue of sec14Δ</th>
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<tr>
<td>WT</td>
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<tr>
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<td>D115G</td>
<td>B1, B2</td>
<td>++</td>
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<tr>
<td>D115A,K116P</td>
<td>B1, B2</td>
<td>—**</td>
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<tr>
<td>D115A,K116G</td>
<td>B1, B2</td>
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A plasmid shuffle assay that reports whether physiological levels of expression of the indicated mutant sec14 protein rescues the lethality associated with sec14 nullizygosity (sec14Δ). This plasmid shuffle assay employs CRY58 as parental yeast strain (see Materials and Methods) and has been described (Phillips et al., 1999; Li et al., 2000). Expression of wild-type Sec14p (WT) served as positive control, and ability to rescue identifies proteins that retain at least some function. The structural element disturbed for each mutant sec14 protein is identified. Robustness of plasmid shuffle was scored after 72 h of incubation at 26°C on YPD medium, and scores are assigned as qualitative comparisons relative to the WT control. Scores were assigned in a range from +++ (best) to + (poor) to — (failure to rescue). ** indicates relevant sec14 proteins are stable under the conditions of the experiment.

Sec14pY213A,F221A,S222A exhibits no measurable PtdCho transfer activity in vitro (Figure 4C) and is nonfunctional as a Sec14p in vivo (Table 1), even though it is a stable protein both in vivo and in cytosol fractions used in transfer assays.

### Altered Dynamics of the A10/T4/A11 Helical Gate in Sec14pG266D

The rmsf values plotted for apo-Sec14p in the 15- to 32-ns window of the MD simulation suggested a relationship between A10/T4/A11 motion and the dynamics of the C-terminal string motif as rmsf values in excess of 2 Å were recorded for residues 271DESK274 of the Sec14p string motif (Figure 2B). In that regard, the sec14-l^a gene product (Sec14pG266D) harbors a G266D missense substitution that resides in the string motif immediately N-terminal to the 271DESK274 cluster (Cleves et al., 1989; Sha et al., 1998). To explore this issue further, an unrestrained 32-ns MD simulation was run for Sec14pG266D after a starting structure for the mutant protein was calculated (see Materials and Methods). Conformational equilibration of Sec14pG266D occurred rapidly in the simulation as evidenced by the structural transition that occurs between 2.9 and 3.8 ns in the rmsd plot (Figure 2A). Similar to Sec14p, the rmsf values between the average Sec14pG266D structure recorded in the last 20 ns of the simulation, and the Sec14p reference structure, were large (4.0 Å). Monitoring of atomic positional fluctuations and interatomic distances between helix A10 residue R195 and helix A10/T4 residue F231 were consistent with the large rmsf values reporting closure of the Sec14pG266D A10/T4/A11 helical gate.

In contrast to the oscillations between open and closed conformations evident in the Sec14p simulation, however, rmsf calculations indicated the A10/T4/A11 helical gate failed to undergo the large-scale conformational motions throughout the 15- to 32-ns simulation time window that...
A Novel Gating Module That Regulates Opening and Closing of the A10/T4/A11 Helical Gate

How does the G$_{266}$D missense substitution, which resides in the string motif, compromise C-terminal hinge function given these two structural elements are not in proximity to each other? As described below, the 3$_{10}$-helix T$_5$ (i.e., the structural element directly impacted by G$_{266}$D) is one component of a novel gating module that we posit regulates opening and closing of the A$_{10}$/T$_4$/A$_{11}$ helical gate (overall distance monitoring schematic is illustrated in Figure 5).

This gating module, designated the Sec14G-module, is made up of two independent substructures that are themselves interdigitated via a hydrogen bond (H-bond) network. The first substructure (A$_{12}$/LT$_5$) includes helix A$_{12}$, extends slightly beyond 3$_{10}$-helix T$_5$ and encompasses residues Q$_{248}$–S$_{268}$. The second substructure is made up of the B$_1$ and B$_2$-strands along with the intervening loop region. It includes residues P$_{198}$–E$_{225}$ and is designated B$_1$LB$_2$. Sec14p residues 114TDKDGR$_{119}$ of the B$_1$LB$_2$ motif cluster with Q$_{254}$ of the A$_{12}$/LT$_5$ substructure to form a polar, solvent-exposed patch (Figure 5A). In addition to H-bonding interactions with components of helix T$_5$, V$_{262}$ also bridges the helical and post-T$_5$ regions of the A$_{12}$/LT$_5$ substructure by forming a 6 H-bond with S$_{268}$. Although the integrity of the V$_{262}$–S$_{268}$ interaction fluctuates during the MD simulation, the S$_{268}$ amide engages in a H-bond with the L$_{260}$ carbonyl (Supplemental Figure S6, C and D). An additional H-bond interaction exists between the K$_{267}$ amide and the A$_{257}$ carbonyl (Supplemental Figure S6E), and these residues flank the T$_5$ 3$_{10}$-helix. The last H-bond interaction within this unit engages the T$_5$-proximal N$_{259}$ amide with the carbonyl of the helix A$_{12}$ proximal residue P$_{256}$ (Supplemental Figure S6F). MD simulation data project five of these six H-bonds are stable in the Sec14p context, because these persist in >90% of the snapshots collected during the 32-ns simulation time. The remaining H-bond (V$_{262}$–S$_{268}$) is present in >50% of the snapshots collected.

Backbone H-Bond Interactions and the A$_{12}$/LT$_5$ Component of the Sec14G-Module

The A$_{12}$/LT$_5$ composite is stabilized by a series of H-bond interactions within the substructure itself. Helix T$_5$ is made up of three core and two flanking residues (261PVKFG$_{265}$). P$_{261}$ and G$_{265}$ reside at the two terminal turns of 3$_{10}$-helix T$_5$ and cap it at its N and C terminus (Figure 6A). These capping residues engage in the 4 → 1 H-bond interactions typical of 3$_{10}$-helices (Richardson and Richardson, 1988; Penel et al., 1999a,b; Pal et al., 2002; Pal et al., 2003, 2005). The P$_{261}$ carbonyl also engages in a strong H-bond interaction with the F$_{264}$ amide. F$_{264}$ exhibits (δ, γ) angles of −104 and 14°, respectively, and these angles are again typical for C-terminal residues of 3$_{10}$-helical helices. The backbone angular conformation of F$_{264}$ orients both the G$_{265}$ amide and the V$_{262}$ carbonyl in a geometrically favorable configuration for a stable H-bond interaction (Figure 6A).

Helix T$_5$ residues engage in contacts with both the intervening loop region between helix A$_{12}$ and T$_5$ as well as with the region downstream of T$_5$ in the A$_{12}$/LT$_5$ substructure (Figure 6A). The F$_{264}$–P$_{261}$ and G$_{265}$–V$_{262}$ interactions that involve the capping residue for helix T$_5$ are preserved throughout the simulation (Supplemental Figure S6, A and B). In addition to H-bonding interactions with components of helix T$_5$, V$_{262}$ also bridges the helical and post-T$_5$ regions of the A$_{12}$/LT$_5$ substructure by forming a 6 → 1 H-bond with S$_{268}$. Although the integrity of the V$_{262}$–S$_{268}$ interaction fluctuates during the MD simulation, the S$_{268}$ amide engages in a stable H-bond interaction with the L$_{260}$ carbonyl (Supplemental Figure S6, C and D). An additional H-bond interaction exists between the K$_{267}$ amide and the A$_{257}$ carbonyl (Supplemental Figure S6E), and these residues flank the T$_5$ 3$_{10}$-helix. The last H-bond interaction within this unit engages the T$_5$-proximal N$_{259}$ amide with the carbonyl of the helix A$_{12}$ proximal residue P$_{256}$ (Supplemental Figure S6F). MD simulation data project five of these six H-bonds are stable in the Sec14p context, because these persist in >90% of the snapshots collected during the 32-ns simulation time. The remaining H-bond (V$_{262}$–S$_{268}$) is present in >50% of the snapshots collected.

Interface between A$_{12}$/LT$_5$ and B$_1$LB$_2$ in the Sec14G-Module

Several categories of H-bond interactions are evident between the A$_{12}$/LT$_5$ and B$_1$LB$_2$ substructures in Sec14p MD simulations (Figure 5). B$_1$LB$_2$ residues T$_{114}$, D$_{115}$, and K$_{116}$ interact with A$_{13}$/LT$_5$ residues Q$_{254}$ and N$_{259}$ to stabilize the Sec14G-module. R$_{119}$ is a candidate for several intra- and interstructural side chain solvent interactions as well. The panels displayed in Supplemental Figure S7 overlay distance-monitoring plots for the indicated residue pairs within the Sec14G-module. These results suggest helix A$_{12}$ residue Q$_{254}$ engages in two H-bond interactions; the backbone interaction with K$_{116}$ and a side chain interaction with D$_{115}$. A side chain to main chain H-bond interaction between residues N$_{259}$ and T$_{114}$ further stabilizes this configuration. As detailed below, rearrangements in this H-bond network correlate with dynamics of the A$_{10}$/T$_4$/A$_{11}$ helical gate.
Sec14G-Module Conductance Correlates with Motions of the A_{10}/T_{4}/A_{11} Helical Gate

Gate motions (as reported by distance monitoring between the Cα atoms of R_{195} and F_{231}) correlate with the atom-to-atom H-bond switch involving the Q_{254}-D_{115} side chains. Although the side chain interactions are maintained throughout the Sec14p MD simulation, the interactions alternate between the two H-bond acceptor atoms of the D_{115} side chain (OD1 and OD2) (Figure 7A). In the open conformation, H-bonds from Q_{254} NE2 form with either D_{115} OD1 or OD2. When Sec14p transitions to closed conformations, only the Q_{254} NE2:D_{115} OD1 H-bond is evident. A Q_{254} NE2 H-bond switch from D_{115} OD1 to D_{115} OD2 is also registered at the onset of every gate-opening event that occurs once the R_{195} and F_{231} Cα atoms approach to within 14 Å of each other.

Although the S_{268} carbonyl interaction with the V_{262} amide fluctuates throughout the Sec14p simulation, the S_{268} amide maintains an H-bond interaction with the L_{260} carbonyl throughout (Supplemental Figure S6, C and D). These fluctuations in V_{262}-S_{268} interaction coincide with discreet clusters in opening and closing of the A_{10}/T_{4}/A_{11} gate. The V_{262}-S_{268} fluctuations also coincide with the H-bond switch between Q_{254} NE2 and the D_{115} OD1 and OD2 acceptors (Figure 7B). Finally, S_{268} lies immediately N terminal to a region of significant conformational mobility (residues 271–
274) as indicated by the Sec14p atomic fluctuation plot (Figure 2B). These data suggest that the conformational dynamics in this region are linked to conformational events within the Sec14G-module.

**Rearrangements in the T₅ Helical Component of Sec14pG₂₆₆D**

The closed Sec14pG₂₆₆D conformation is suggested to exhibit significant rearrangements within the helix T₅ backbone conformation. These rearrangements minimize the steric problems otherwise imposed by the G₂₂₆D missense substitution, while maximizing favorable interactions of the G₂₆₆D side chain with solvent. The projected consequence of the G₂₂₆D substitution for the string motif is loss of four H-bonds normally found within the Sec14p T₅ helical component, and formation of a new H-bond not present in Sec14p (Figure 6B). The G₂₆₆D-V₂₆₆ interaction is weakened, and the V₂₆₆-S₂₆₈ and S₂₆₈-L₂₆₀ H-bonding interactions are subsequently lost in Sec14pG₂₆₆D (Supplemental Figure S6, B–D).

In Sec14p, the K₂₆₇ carbonyl engages in an H-bond with the A₂₅₇ amide. By contrast, that specific H-bond interaction breaks early in the Sec14pG₂₆₆D simulation and is replaced by an alternate interaction of the A₂₅₇ amide with the backbone carbonyl of the mutant D₂₆₆ residue (Supplemental Figure S6G). This alternate A₂₅₇-D₂₆₆ interaction is quite long-lived; it persists for almost 13 ns. The F₂₆₄-P₂₆₁ and N₂₅₉-P₂₆₁ interactions observed for Sec14p are preserved in the Sec14pG₂₆₆D MD simulations (Supplemental Figure S6, A and F).

**Disengagement of Sec14G-Module Structural Elements in Sec14pG₂₆₆D and Closure of the A₁₀/T₄ Helical Gate**

The loops of the two Sec14G-module substructures move apart during the simulated transition of Sec14pG₂₆₆D to the closed conformation, and all H-bonds that link A₁₂L₅₅ and B₁L₉₀ of the Sec14G-module are broken (Supplemental Figure S7, A–D). The transition from open to closed conformations occurs early (between 3 and 4 ns) in the Sec14pG₂₆₆D simulations. During this transition period, side chain distances between Q₂₅₄ and D₁₁₅ increase in a manner inconsistent with maintenance of H-bond interactions. The distance monitoring plot between these two specific amino acids then remains erratic throughout the remaining 28 ns of the MD simulation (Supplemental Figure S7, B and C). The N₂₅₉ side chain to T₁₁₄ main chain interaction breaks first (at 0.882 ns of the simulation; snapshot 882), but the interaction forms again (Figure 7C). The next dissociation is the backbone H-bond interaction between K₁₁₆ and Q₂₅₄, and this bond is not recovered again in the simulation. Loss of this H-bond interaction suggests a rationale for the unique site of conformational flexibility that involves residues of the 114TDKDGR119 motif (particularly K₁₁₆) that is apparent in the Sec14pG₂₆₆D apt plot (Figure 2B). The K₁₁₆–Q₂₅₄ dissociation is accompanied by strong correlation between changes in (θ, Ψ) angles of B₁ residues Y₁₁₁ and H₁₁₂ and closure of the A₁₀/T₄/A₁₁ helical gate (Supplemental Figure S4). These specific correlations uniquely occur in Sec14pG₂₆₆D simulations and not in Sec14p MD production runs.

Sec14pG₂₆₆D MD simulation of the status of three hydrogen bond interactions between the B₁L₉₀ and A₁₀L₅₅ substructures during the transition from the open-to-closed Sec14p conformers at ~3 ns. The T₁₁₄–N₂₅₄ interaction breaks at 0.882 ns but reforms again. Main chain hydrogen bonding interaction (blue) between the K₁₁₆ amide and the Q₂₅₄ carbonyl is lost during the transition and fails to reestablish during the simulation.

Figure 7. Sec14G-module transitions and gate dynamics. (A) Interatomic distances between the Ca atom of residues R₁₉₅ and F₂₃₁ report status of A₁₀/T₄/A₁₁ gating helix closure (gray). Distances between the Q₂₅₄ side chain amine and D₁₁₅ OD₁ (red) and D₁₁₅ OD₂ (black) are also plotted. A hydrogen bond interaction between the side chains of Q₂₅₄ and D₁₁₅ persists throughout the simulation, but this interaction switches between OD₁ and OD₂ of residue D₁₁₅ when Sec14p is in the open conformation, and it is restricted to OD₁ when Sec14p is in the closed conformation. (B) Correlation of Sec14G-module to the fluctuating interaction between residues V₂₆₂ and S₂₆₈. Segments of time exist (3.5–8.9, 8.9–17.2, and 17.2–22.9 ns) when V₂₆₂ and S₂₆₈ (green) are bound or unbound, defining clusters that show transitions occurring at times when the side chains of Q₂₅₄ and D₁₁₅ (black) switch between the OD₁ and OD₂ atoms of D₁₁₅ as well as transitions in the opening and closing of the A₁₀/T₄/A₁₁ helical gate (R₁₉₅–F₂₃₁; gray). (C) First 5 ns of unrestrained Sec14pG₂₆₆D MD simulation of the status of three hydrogen bond interactions between the B₁L₉₀ and A₁₀L₅₅ substructures during the transition from the open-to-closed Sec14p conformers at ~3 ns. The T₁₁₄–N₂₅₄ interaction breaks at 0.882 ns but reforms again. Main chain hydrogen bonding interaction (blue) between the K₁₁₆ amide and the Q₂₅₄ carbonyl is lost during the transition and fails to reestablish during the simulation.
The MD simulation data implicate residue 114 TDKDGR119 of the B1LB2 substructure as playing an important role in activity of the Sec14G-module. Specifically, the data suggest 114 TDKDGR119 helps maintain the association of the B1LB2 and A12LT5 substructures and that this component of the B1LB2 motif serves as an inflection point, or swivel, that transduces B1LB2 conformational motions to helix A10/T4/A11 gating function (see Figure 8A for relative positions of K116 and G266). The MD simulations suggest the backbone H-bond between K116 amide (B1LB2) and the Q254 carbonyl (A12LT5), and that side chain interactions between D115 (B1LB2) and Q254 play important roles in execution of these functions. One prediction of this hypothesis is that removal of the backbone H-bond formed by K116 to the Q254 carbonyl and side chain H-bond interactions between residues D115 and Q254 will be of functional consequence. This prediction was tested in silico with a Sec14p K116P mutant. The K116P missense substitution obviates formation of the relevant backbone H-bond with the Q254 carbonyl. A 32-ns Sec14pK116P MD simulation reproduced key features of the Sec14pG266D simulations described above. Distance monitoring plots for interatomic R195-F231 Cα distances show Sec14pK116P begins to transition toward the closed state at 10 ns of simulation and that a closed state is achieved at 14 ns (average interatomic R195-F231 Cα distance of 10.5 Å; Figure 8B). Sec14p K116P remains closed for the remainder of the simulation.

Biochemical and in vivo assays reinforce the collective importance of the K116-Q254 main chain and D115-Q254 side chain H-bond interactions in Sec14p function. PtdCho transfer activities for Sec14pK116P and Sec14pK116G were assessed in vitro as a biochemical test of the prediction that Sec14pK116P will exhibit functional defects, whereas Sec14pK116G will not. The latter alteration mimics the secondary structural disruptions associated with the proline substitution, while maintaining both the relevant backbone H-bond with the Q254 carbonyl and the side chain H-bond interactions between D115 and Q254. The biochemical data indicate Sec14pK116P exhibits a ~50% reduction in PtdCho transfer activity in vitro, whereas Sec14pK116G is as active as Sec14p in those assays (Figure 8C). Similarly, both Sec14pD115A and Sec14pD115G (ablated for the side chain H-bond interactions between D115 and Q254) but retain the backbone H-bond between the K116 amide and the Q254 carbonyl) exhibited significant PtdCho transfer activity in vitro when assays were performed at 30°C to limit destabilization of the mutant proteins (Figure 8C; see Materials and Methods). Thus, K116-Q254 backbone and D115-Q254 side chain H-bond interactions are individually sufficient to maintain a reasonably functional interface between the B1LB2 and A12LT5 substructures. We also note that the PtdCho transfer activity of Sec14pK116P was more strongly reduced (but nonetheless detectable) under the con-
Conformational Dynamics of Sec14 Protein

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DISCUSSION

Herein, are described a series of conformational transitions that we propose accompany phospholipid exchange by the major yeast PITP Sec14p. These findings were obtained from a combination of unrestrained MD simulations of Sec14p and Sec14pG266D conformational dynamics and functional analyses that test the predictive power of the simulations. Because simulations derive from a starting structure that is strongly supported by three lines of experimental evidence. First, trypsinolysis and Cys pair cross-linking experiments demonstrate that holo-Sec14p exists in a closed conformation in solution and that open and closed Sec14p conformers are distinguished by the distances between helix $\alpha_9$ and the $\alpha_{10}/T_4/\alpha_{11}$ helical gate. The Cys pair experiments further demonstrate that covalent forced closure of the $\alpha_{10}/T_4/\alpha_{11}$ helical gate is incompatible with phospholipid exchange. These forced closure data provide a powerful first demonstration that mobility of the $\alpha_{10}/T_4/\alpha_{11}$ helical gate is required for phospholipid exchange by Sec14p. Third, both in vitro and in vivo data demonstrate that a functional hinge unit is essential for Sec14p to catalyze phospholipid transfer in vitro, and for Sec14p functionality in cells.

The MD simulations suggest state-of-closure of the $\alpha_{10}/T_4/\alpha_{11}$ helical gate is controlled by N- and C-terminal hinges that flank the gate itself. The N-terminal aspect of the helical gate is projected to remain reasonably fixed during transitions between open and closed conformations, whereas the C-terminal region is simulated to swing open and shut, much like a windshield wiper blade. We find it most interesting that the hinge residue F231 contacts the acyl chain of $\beta$OG in the detergent-bound Sec14p structure, which we loosely refer to as the apo-Sec14p conformer, whereas a combination of genetic, biochemical, and crystallographic data identify an involvement of the hinge residue K239 in binding of the inositol headgroup of PtdIns (Phillips et al., 1999; our unpublished data). These physical interactions raise the attractive possibility that activities of the hinge subcomponents are instructed not only by intramolecular transitions within the Sec14p molecule but also by the phospholipid ligand itself. Given that the MD simulation data suggest a primary role for the KH component of the hinge in gate closure, and residue K239 resides within this component, the ligand-dependent regulation of conformational transitions may restrain the helical gate from reopening. Such a restraint could thereby direct apo-Sec14p from a "shallow breathing" conformational dynamics regime down a trajectory to a stable closed conformer. In that regard, the unrestrained MD simulation for Sec14p almost certainly approximates a partially closed" structure. The closest approach of the helix $\alpha_{10}/T_4$ residue F231 Ca atom to the residue R195 Ca atom on helix $\alpha_9$ in the Sec14p MD simulation is 10.21 Å (snapshot 27653). The corresponding interatomic distance in the closed phospholipid-bound conformer of a yeast Sec14-like protein must be smaller given our ability to induce disulfide bond formation in Sec14p.$^{19,21}$

An unanticipated outcome from comparisons of MD simulations of Sec14p and Sec14pG266D was identification of a novel gating module, the Sec14G-module, that we propose...
transduces conformational information to the hinges, and, ultimately, to the $A_{30}/T_3/A_{11}$ helical gate. This Sec14G-module includes the $A_{30}LT_3$ and $B_{12}LB_2$ composites, and these are linked by an intricate H-bond network. The data suggest a primary function of the Sec14G-module is conductance of conformational change to the hinges to open the $A_{30}/T_3/A_{11}$ helical gate. A movie depicting the defects associated with the $sec14^{1-10}$ mutation, and of the role of the Sec14p string motif itself. Originally, it was suggested the string motif functions merely to stabilize the Sec14-fold and that the $sec14^{1-10}$ mutation results in structural destabilization of the protein (Sha et al., 1998). Although the stability of the $sec14^{1-10}$ gene product (Sec14p$^{C266D}$) is clearly affected, the mutant protein is functionally defective even under conditions where stability is not yet compromised. The Sec14p$^{C266D}$ MD simulations suggest this intrinsic flaw reflects an inability of Sec14p$^{C266D}$ to efficiently open the $A_{30}/T_3/A_{11}$ helical gate because of a defect in the $B_{12}LB_2$ substructure of the Sec14G-module. In this regard, the MD simulations reported herein make a strong prediction with respect to the types of conformational differences we expect to discern in the G-modules of open and closed conformers of Sec14p. Specifically, the twist of the $\beta_3$ strand, and, subsequently, the $\beta_3$ strand around the $D_{112}/K_{116}/Q_{254}$ swivel depicted in the Supplemental Figure S9B, is expected in the closed phospholipid-bound form of Sec14p. This transition from open to closed conformations will also alter the relative orientations of the loops of the $B_{12}LB_2$ and $A_{12}LT_5$ substructures to each other in a specific way. Determination of whether these key predictions are fulfilled is forthcoming given recent progress in the crystallization of phospholipid-bound forms of the yeast Sec14p homologue Sh1p (Schaat et al., 2006).

With regard to the $B_{12}LB_2$ substructure of the Sec14G-module, we are compelled to comment on the recent work of Griac and coworkers. They reported the $D_{115}G$ missense substitution ablates Sec14p PtdCho transfer activity and simultaneously effects a strong reduction in PtdIns transfer activity (Tahotna et al., 2007). Although we concur that Sec14p$^{D115G}$ reduces Sec14p PtdCho and PtdIns transfer activity, and now provide the first mechanistic explanation for why this missense substitution exerts these effects, we cannot agree with the claim of Griac and coworkers that Sec14p$^{D115G}$ is devoid of PtdCho transfer activity (Tahotna et al., 2007). In our hands, Sec14p$^{D115G}$ exhibits readily detectable PtdCho transfer activity, particularly when precautions are taken to ensure that levels of this polypeptide are detectable PtdCho transfer activity, particularly when precautions are taken to ensure that levels of the PtdCho activating protein, and cellular retinaldehyde binding protein (CRALBP). Either crystal structures, or structural models, are available for each (Stocker et al., 2002; Min et al., 2003; Liu et al., 2005; D’Angelo et al., 2006). We find the structural elements geraine to this study (i.e., the helical gate, the hinge, and the $A_{12}LT_5$ and $B_{12}LB_2$ substructures of the Sec14G-module) are represented in each of these proteins. In Sec14p, $\alpha$TTP, NF1, and SNPF, the loop between cognate $\beta_1$ and $\beta_2$ elements (B$_{12}$LB$_2$) orients itself such that the respective $\beta$-strands bend away from the interior of the hydrophobic pocket and toward the posterior of the protein. A backbone H-bond (K$_{116}$Q$_{254}$ in Sec14p) is observed between the cognate B$_{12}$LB$_2$ and A$_{12}$LT$_5$ substructures in three of the four structures. With regard to that H-bond interaction, $\alpha$TTP is the single exception where the cognate K$_{116}$ is substituted with a proline. Thus, $\alpha$TTP represents a natural version of the K$_{116}$P context that partially compromises Sec14p. Why is $\alpha$TTP tolerant of this K $\rightarrow$ P substitution, but Sec14p is less.

### Figure 9.

**BIL2B (P108-E125)**

Sec14C 108 POYHNTDGRGPRVFEELG
ATTP 100 GBPVRSGRPTGSKVLRIAR
CRALBP 144 P+WLS8KGYGRVMDFNIE
NF1 1571 IFYQAGT-SKAGN-eyFVVRK

**GATE (G210-I242)**

Sec14C 208 RMGKHLINAPFP8DFAFLKFPFLD-PVTW8HIF
ATTP 190 KTLHILINEVPHAVFMSKPFKL-EXIKR8HNM
CRALBP 233 FRAIPAHPQHPQTTLTYNVKVKPLK-SKLLERVVF
NF1 1652 MAVAVYXCNCSWREYTKYHERLLTGLGSKRLVF

**A12LT5 (Q248-S268)**

Sec14C 248 QKELKQIFARNLVPSKFGC8S
ATTP 230 KQSLHQQFF-DILPLEYGG8S
CRALBP 275 S-GFYQREDNLSDFSFGGT
NF1 1691 PGKLAHIEHIEQ----------

Figure 9. Relationship between disease mutations in Sec14-like proteins and structural elements involved in conformational transitions. Primary sequences of Sec14p, human $\alpha$TTP, and human NF1 were aligned on the basis of primary sequence homology and structural criteria. CRALBP was aligned according to an $\alpha$TTP-based structural homology search (Liu et al., 2005). Relevant secondary structures for each conformational element are indicated at top. Key Sec14p residues in this study and disease-causing mutations in the indicated Sec14-like proteins are highlighted.
tolerant? Inspection of the αTTP crystal structure suggests the C-terminal helix of this protein provides structural support to the atypical αTTP G-module, thereby obviating the necessity of a backbone hydrogen bond between the cognate αTTP B₁L₂B₂ and A₂L₂T₃ motifs (data not shown).

Are the cognate helical gate, hinge and A₂L₂T₃/B₁L₂ substructures of the G-module of functional consequence for these distant members of the Sec14 superfamily? It seems so. αTTP, NF1 and SNPF, and CRALBP insufficiencies cause various human diseases, and the responsible disease mutations have been identified at the primary sequence level. Strikingly, a number of these mutations lie either within the hinge regions that flank the helical gate or within the B₁LB₂ substructure of the cognate G-module itself (Figure 9). Given the small size of the B₁LB₂ element (~17 residues), it is impressive how well represented this motif is with regard to incidence of mutation. We suggest the disease mutations highlighted in Figure 9 resemble Sec14pG266D in functional crippling of the G-modules of their respective proteins. These defects in turn hinder opening of the corresponding helical gates that control access of the individual binding substrates to the hydrophobic ligand-binding cavities of each of these Sec14-like proteins. With regard to the atypical G-module of αTTP, a common truncation mutation found in families affected by severe ataxia with vitamin E deficiency is caused by a single base-pair insertion (Ouachi et al., 1995; Benomar et al., 2002). This insertion results in a frame shift that alters the last 30 residues of αTTP (Figure 9), thereby compromising the C-terminal αTTP helix we posit stabilizes the atypical G-module of this Sec14-like protein.

Although the experiments reported here project a pathway for conformational change in what is most likely a transition from an apo-Sec14p to a phospholipid-bound holo-Sec14p, it is virtually certain that the motions described represent an incomplete description of this transition. This incompleteness derives in part from the lack of provision in the MD simulations for how a phospholipid ligand may influence Sec14p conformational transitions. How these motions relate to those involved in ejection of bound phospholipid from the Sec14p hydrophobic cavity (i.e., are these perfectly symmetrical processes?) also remains unclear. These unresolved issues notwithstanding, we posit the Sec14p structural elements described herein are relevant to the execution of the major Sec14p conformational transitions that occur throughout a cycle of phospholipid exchange. Finally, these studies raise the question of how is the activity of the Sec14G-module regulated so that the dynamics of the helical gate are properly coordinated with the phospholipid exchange cycle?

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