Mutant rat phosphatidylinositol/phosphatidylcholine transfer proteins specifically defective in phosphatidylinositol transfer: Implications for the regulation of phospholipid transfer activity

JAMES G. ALB, JR., ALMA GEDVILAITE, ROBERT T. CARTEE, HENRY B. SKINNER, AND VYTAS A. BANKAITIS[†]

Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294-0005

Communicated by Randy Schekman, University of California, Berkeley, CA, June 6, 1995 (received for review April 14, 1995)

ABSTRACT The mammalian phosphatidylinositol/phosphatidylcholine transfer proteins (PI-TPs) catalyze exchange of phosphatidylinositol (PI) or phosphatidylcholine (PC) between membrane bilayers *in vitro*. We find that Ser-25, Thr-59, Pro-78, and Glu-248 make up a set of rat (r) PI-TP residues, substitution of which effected a dramatic reduction in the relative specific activity for PI transfer activity without significant effect on PC transfer activity. Thr-59 was of particular interest as it is a conserved residue in a highly conserved consensus protein kinase C phosphorylation motif in metazoan PI-TPs. Replacement of Thr-59 with Ser, Gln, Val, Ile, Asn, Asp, or Glu effectively abolished PI transfer capability but was essentially silent with respect to PC transfer activity. These findings identify rPI-TP residues that likely cooperate to form a PI head-group binding/recognition site or that lie adjacent to such a site. Finally, the selective sensitivity of the PI transfer activity of rPI-TP to alteration of Thr-59 suggests a mechanism for *in vivo* regulation of rPI-TP activity.

All eukaryotic cells harbor cytosolic phospholipid transfer proteins (PL-TPs) that can transport lipid monomers between membrane bilayers *in vitro* (1–3). The phosphatidylinositol/phosphatidylcholine transfer proteins (PI-TPs) represent an interesting class of PL-TPs in that (i) PI-TPs can utilize either phosphatidylinositol (PI) or phosphatidylcholine (PC) as transfer substrates and (ii) PI-TPs define two highly conserved protein families. The mammalian PI-TPs are 35-kDa proteins that share a very high degree of primary sequence identity (4–6). Yet another PI-TP homolog is represented by the *Drosophila* rdgB protein (rdgBp); an integral membrane protein with an N-terminal rat (r) PI-TP-like domain that catalyzes PI transfer *in vitro* when expressed as a soluble polypeptide (7). The fungal SEC14 proteins (SEC14ps) are also some 35 kDa in molecular mass and are highly homologous to each other (8–11) but do not resemble metazoan PI-TPs (5, 8). While the evidence suggests that SEC14p functions in *Saccharomyces cerevisiae* as a PL sensor that controls the PC content of yeast Golgi membranes by regulating the activity of the CDP-choline pathway for PC biosynthesis (12–14), the *in vivo* function of mammalian PI-TPs is unresolved. It is widely assumed that the PI and PC binding/transfer activities of rPI-TP are somehow relevant to *in vivo* function. However, as there exists no understanding of how rPI-TP executes or regulates its PL binding/transfer activities, the necessity for a functional analysis of the PL transfer activities of rPI-TP is emphasized.

Herein, we describe the uncoupling of the PI and PC transfer activities of rPI-TP and report genetic and biochemical data that identify at least one consensus protein kinase C (PKC) phosphorylation site in rPI-TP (Thr-59) as a structural element required for efficient PI transfer. Finally, the data suggest

a means by which PI-TP activity could be regulated in mammalian cells.

MATERIALS AND METHODS

Yeast Strains, Plasmids, Media, and Transformation. *S. cerevisiae* CTY182 (Mata *ura3-52 Δhis3-200 lys2-801^{am}*); CTY1-1A (Mata *ura3-52 Δhis3-200 lys2-801^{am} sec14-1^{ts}*); CTY303 (Mata *ura3-52 Δhis3-200 cki sec14ΔP::hisG*), and the YE_p (URA3, *SrPI-1*) plasmid, pCTY161, and basic yeast methods have been described (8, 15–17).

Random Mutagenesis of the *SrPI-1* Expression Construct. Random chemical mutagenesis employed hydroxylamine (18). Aliquots of mutagenized DNA were transformed into *Escherichia coli* MC1066 [F^- , $\Delta(lac)X74$ *hsr⁻*, *hsm⁺*, *rpsL*, *galU*, *galK*, *trpC9830*, *leuB600*, *pyrF::Tn5*]. Ampicillin-resistant transformants were selected and replica plated onto M9 medium lacking uracil to assess the frequency of noncomplementation of the *pyrF::Tn5* uracil auxotrophy. Base M9 minimal medium has been described (19). Suitably mutagenized DNA aliquots (3–5% Ura⁻ plasmids) were used to transform yeast strain CTY1-1A to Ura⁺ at 25°C, and transformants that grew at 25°C but failed to grow at 37°C were rescreened for the temperature-sensitive phenotype. Those temperature-sensitive mutants whose growth defect at 37°C was not uracil-remedial were kept for analysis.

Phospholipid Transfer Assays. Yeast cytosol was prepared, and PI and PC transfer assays were performed as described (15, 20, 21). Quantitative ELISAs were performed by using a direct sandwich assay with polyclonal rabbit anti-rPI-TP serum (4 μg/ml) directed against the C-terminal 128 residues of rPI-TP (12, 15). Secondary mouse anti-rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad) were used at 400 ng/ml for development of signal in the presence of *o*-phenylenediamine. After quenching, A_{450} was measured on an EL311sx automated microplate plate reader (Bio-Tek, Winooski, VT).

Nucleotide Sequence Analysis. Sequencing of the *SrPI-1* mutants was performed by the chain-termination method (22) by using double-stranded plasmid DNA as template and the Sequenase version 2.0 sequencing kit (Amersham).

Site-Directed Mutagenesis. To mutagenize codon 59 of rPI-TP, pCTY161 was digested with *Hpa* I and *Bam*HI to liberate the 0.8-kb rat PI-TP coding sequence. This fragment was cloned into the SK Bluescript plasmid (Stratagene) to yield pRE547. Single-stranded DNA was prepared and mutagenized as described (23) by using the synthetic primer 5'-GTAGATCTTGTGTGCGTACTGGCCTTTCTCG-3' to effect the T59A substitution (mutagenized codon is under-

Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; PKC, protein kinase C; PI-TP, PI/PC transfer protein; rPI-TP, rat PI/PC transfer protein; PL, phospholipid; PPase, protein phosphatase.
[†]To whom reprint requests should be addressed at: Department of Cell Biology, 6th Floor Basic Health Services Building, University of Alabama at Birmingham, Birmingham, AL 35294-0005.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

lined). The remaining substitutions were constructed utilizing the same mutagenic primer except that rPI-TP codon 59 was altered to TTC, GTC, TGA, CTG, TAC, or ATT to introduce the T59E, T59D, T59S, T59Q, T59V, or T59N substitutions, respectively. Mutants were confirmed by nucleotide sequence analysis and the mutagenized *Hpa* I–*Bam*HI cassettes were cloned into *Hpa* I/*Bam*HI-digested pCTY161.

RESULTS

Isolation and Characterization of Mutant rPI-TPs That Fail to Rescue *sec14-1^{ts}*. Expression of rPI-TP in yeast effects a phenotypic rescue of *sec14-1^{ts}* mutants at 37°C, and the rPI-TP engineered for such expression is referred to as the SrPI-1 protein (15). From 6000 mutagenized SrPI-1 expression plasmids, we identified 31 that genuinely failed to rescue growth of *sec14-1^{ts}* yeast at 37°C. Nucleotide sequence analysis of these 31 mutant plasmids indicated that 21 of the corresponding mutations mapped within the SrPI-1 structural gene (Fig. 1A).

Mutant <i>rpi</i> allele		<i>rpi</i> Mutation	Relative protein level
<i>rpi-11</i>		R146 (CGA) → Nonsense (TGA)	<10%
<i>rpi-31</i>	<i>rpi-35</i>	R74 (CGA) → Nonsense (TGA)	
<i>rpi-36</i>	<i>rpi-54</i>	E6 (GAA) → K (AAA)	
<i>rpi-43</i>	<i>rpi-48</i>	E66 (CAG) → Nonsense (TAG)	
<i>rpi-53</i>		E185 (CAG) → Nonsense (TAG)	
<i>rpi-59</i>		P12 (CCT) → S (TCT)	
<i>rpi-65</i>		C192 (TGT) → Y (TAT)	
<i>rpi-17</i>		E113 (GAA) → K (AAA)	10%–40%
<i>rpi-57</i>		W204 (TGG) → Nonsense (TAG)	
<i>rpi-58</i>		H85 (CAT) → Y (TAT)	
<i>rpi-13</i>		P78 (CCA) → L (CTA)	>40%
* <i>rpi-15</i>		T198 (ACA) → I (ATA)	
* <i>rpi-22</i>	* <i>rpi-34</i>	T59 (ACA) → I (ATA)	
* <i>rpi-38</i>	* <i>rpi-66</i>	H60 (CAC) → Q (CAG)	
* <i>rpi-46</i>		E248 (GAA) → K (AAA)	
<i>rpi-52</i>		S25 (TCT) → F (TTT)

FIG. 1. Primary sequence of SrPI-1 mutants that fail to rescue yeast *sec14-1^{ts}* growth defects at 37°C. (A) The 21 mutations that render SrPI-1 incapable of rescuing yeast *sec14-1^{ts}* growth defects at 37°C identify 16 unique *rpi* (rat PI-TP) alleles. Each *rpi* mutation is identified by the corresponding base change involved, by the codon affected, and by the corresponding amino acid substitution or termination mutation introduced at the affected codon. The *rpi* mutations are also compiled as a function of the relative steady-state levels of their respective products compared to the steady-state level of the wild-type SrPI-1. These values were estimated by immunoblot analysis with anti-rPI-TP serum and by quantitative ELISA. (B) Immunoblot analysis of the relative steady-state levels of representative *rpi* gene products. Clarified extracts were prepared from yeast strain CTY303 carrying the appropriate YEp (*rpi*, *URA3*) plasmids that was cultured in uracil-deficient medium to midlogarithmic growth phase at 25°C. Lysates (10 µg protein) were individually resolved by SDS/PAGE. Immunoblots were developed by using the enhanced chemiluminescence (ECL) system (Amersham). SrPI-1 is indicated at left, and the corresponding mutational alterations are indicated at bottom. Am designates an amber mutation.

Immunoblot analyses identified three mutant categories: (i) seven mutants that either failed to express detectable levels of SrPI-1 antigen or expressed greatly reduced levels of SrPI-1 antigen, (ii) three mutants that expressed reduced levels of full-length SrPI-1 (10–40% of wild-type levels), and (iii) six mutants that expressed substantially wild-type (>40%) levels of full-length SrPI-1 (Fig. 1). In this collection, we noted five missense mutations that exerted either wholesale (E6K, P12S, or C192Y) or considerable (E113K or H85Y) destabilizing effects on SrPI-1. These five mutations likely identify residues that either play important roles in the rPI-TP folding pathway or are required for the maintenance of stable rPI-TP tertiary structure. The *rpi57* allele represented a peculiar case of significant translational read-through across a termination codon. Finally, the six remaining dysfunctional SrPI-1 (i.e., S25F, T59I, H60Q, P78L, T198L, and E248K) were expressed at steady-state levels approaching those of SrPI-1 and represented the most interesting category of mutants, henceforth referred to as SrPI-1*.

SrPI-1 Mutants Exhibit Defects in PL Transfer Activity. Analysis of the PL transfer properties of the six SrPI-1* identified two mutant classes. Class I SrPI-1* (H60Q and T198I) exhibited relative specific activities for PI and PC transfer at 25°C and 37°C that were at least 70% of the parental SrPI-1-specific activities measured at those temperatures (Fig. 2A). It is not obvious why these mutants failed to complement *sec14-1^{ts}* defects. While both mutations reduced the expression of bulk PI and PC transfer activities in yeast relative to those sustained by SrPI-1 (i.e., the H60Q and T198I PI-TP* cytosols exhibited $63 \pm 3.8\%$ and $56.4 \pm 4.1\%$ of the bulk PI transfer activity measured for SrPI-1 cytosol at 37°C and $65.2 \pm 3.1\%$ and $63.3 \pm 6.1\%$ of the bulk PC transfer activity measured for SrPI-1 cytosol at 37°C), the bulk PI and PC transfer activities measured for H60Q and T198I cytosols nevertheless exceeded those measured for SrPI-1myc cytosol (PI and PC transfer activities of $31.3 \pm 4.1\%$ and $29.1 \pm 4.1\%$ of those measured for SrPI-1 cytosol at 37°C, respectively). Yet, SrPI-1myc expression phenotypically rescues *sec14-1^{ts}* (15). These results suggest that rescue of *sec14-1^{ts}* growth and secretory defects by SrPI-1 expression may not solely rely on the ability of SrPI-1 to effect PI or PC transfer in yeast.

The class II mutants (S25F, T59I, P78L, and E248K) exhibited no detectable PI transfer activity at either 25°C or 37°C (<0.3% SrPI-1-specific activity; Fig. 2B). In contrast, the relative specific PC transfer activities measured for these SrPI-1* at 25°C and 37°C always exceeded 70% of that measured for SrPI-1 (Fig. 2B). That the PC transfer activities measured in these mutant cytosols were derived from the SrPI-1* was indicated by the demonstration that isogenic yeast cytosol devoid of SrPI-1* failed to exhibit PC transfer activity in excess of background or of that measured for *E. coli* cytosol (Fig. 2C). The magnitude of the PI transfer defects recorded for the class II SrPI-1*, in the face of substantially wild-type PC transfer capability, demonstrated that (i) class II mutations did not result in global SrPI-1 folding defects and (ii) that the PI transfer defect was not merely attributable to enhanced proteolysis of SrPI-1* relative to SrPI-1 in cell-free lysates. Finally, the remarkable defects in the specific activity of PI transfer measured for the class II SrPI-1* were not the result of modest reductions in steady-state SrPI-1* levels diminishing SrPI-1*-associated PI transfer activity to levels below the sensitivity of the assay. Titration experiments demonstrated that (i) PI transfer activity was linear between 100 µg and 2.5 mg of wild-type SrPI-1 cytosol per assay (i.e., the PI transfer assay recorded activities that were at least 10-fold reduced relative to standard assays) and (ii) addition of 3 mg of class II SrPI-1* cytosol to the PI transfer assay still failed to generate detectable activity (data not shown). Finally, we noted that two of the four class II SrPI-1* mutations (T59I and E248K) involved

FIG. 2. SrPI-1* mutations define two biochemical classes of mutants that are distinguished by their effects on the PL transfer properties of rPI-TP. (A) Cytosolic fractions prepared from derivatives of strain CTY303, a strain lacking endogenous PI/PC transfer activity (ref. 16 and C), carrying isogenic YEp plasmids driving individual expression of the wild-type SrPI-1, SrPI-1myc, and the indicated SrPI-1*. The corresponding cytosols (1 mg of cytosolic protein) were assayed for PI (solid bars) and PC (stippled bars) transfer at 25°C and 37°C. The specific activities for PI and PC transfer were determined for each SrPI-1 species by dividing the percent radiolabeled PL transferred during the experiment by the amount of SrPI-1 antigen detected in the cytosol preparations by specific ELISA (12). The percent radiolabeled PL transferred was corrected by subtraction of background (determined by mock transfer reactions using buffer as input sample). Cytosol (1 mg) from strain CTY303 exhibited PI and PC transfer efficiencies that were indistinguishable from mock transfer reactions. Relative SrPI-1 specific activities were calculated by comparing the specific activities of SrPI-1myc and SrPI-1* cytosols to that measured for SrPI-1 cytosol (set to 100%). Efficiencies of PL transfer typically ranged from 4.0% to 7.3% and 6.3% to 11.5% for PI transfer at 25°C and 37°C, respectively, and from 1.5% to 2.4% and 2.2% to 3.9% for PC transfer at 25°C and 37°C, respectively. Transfer activity was linear with respect to protein and up to levels of 25% and 15% transfer of PI and PC, respectively. Determinations represent the average obtained from at least three experiments. WT, wild type. (B) Relative PI (solid bars) and PC (stippled bars) transfer activities of the indicated SrPI-1* were determined as described in A. Determinations represent the average obtained from at least three experiments. (C) The indicated amounts of cytosol prepared from *E. coli* (solid squares), yeast strain CTY303 (open circles), and a T59I SrPI-1*-expressing derivative of CTY303 (solid circles) were assayed for PC transfer at 37°C. The transfer values were corrected for background as determined by mock transfer reactions using buffer as input sample. Only the T59I strain exhibited measurable PC transfer activity.

residues positioned within or adjacent to consensus PKC phosphorylation sites in SrPI-1 (Fig. 1A).

PI Transfer Activity of rPI-TP Is Exquisitely Sensitive to the Replacement of Thr-59. The specific PI transfer defect associated with T59I indicated a sensitivity of the PI transfer activity to the nature of the amino acid side chain at position 59 of rPI-TP. Interestingly, Thr-59 is an invariant residue among both the mammalian PI-TPs and *Drosophila* rdgBp, and it resides in a consensus PKC motif that is itself highly conserved among these PI-TPs (4–7). Thus, we surveyed PI transfer activity as a function of a range of missense mutations at residue 59. The T59V, T59S, T59D, T59E, T59Q, and T59N mutants uniformly behaved as class II SrPI-1* with regard to (i) their inability to phenotypically rescue *sec14-1^{ts}* growth defects at 37°C (Fig. 3A), (ii) the similarities of their steady-state protein levels to that of SrPI-1 as determined by immunoblot analysis (not shown), and (iii) their specific PI transfer defects in the face of near wild-type PC transfer capability at both 25°C and 37°C (Fig. 3B). The class II SrPI-1* phenotype of the T59S mutant emphasized the sensitivity of SrPI-1-

catalyzed PI transfer to the identity of the amino acid at position 59 as T59S represents a conservative substitution. Moreover, our finding that the T59D and T59E mutations selectively inactivated PI transfer activity suggests that phosphorylation of Thr-59 will also specifically inactivate the PI transfer activity of rPI-TP.

One exceptional case was encountered. The T59A SrPI-1* supported phenotypic rescue of *sec14-1^{ts}* (Fig. 3A), and the T59A SrPI-1* cytosol exhibited PI and PC transfer activity *in vitro* (Fig. 3B). The specific PI transfer activity of this SrPI-1* was only some $50 \pm 4\%$ of that recorded for SrPI-1, however, while the specific activity for PC transfer was considerably less affected (Fig. 3B). Thus, while Thr-59 was not obligatorily required for SrPI-1 to effect significant PI transfer, the T59A mutation nevertheless effected a specific attenuation of PI transfer activity.

DISCUSSION

Recent studies have implicated rPI-TP as a cofactor in the priming of regulated exocytosis in semiintact PC12 cells and in

FIG. 3. PI transfer activity of SrPI-1 is selectively attenuated by mutations involving Thr-59 of SrPI-1. (A) Biological activity of SrPI-1 in yeast is sensitive to alterations at Thr-59. The wild-type strain CTY182 (WT), the isogenic *sec14-1^{ts}* strain CTY1-1A, and CTY-1A derivative strains expressing the indicated Thr-59 SrPI-1* were streaked for isolation on YPD solid medium and incubated at 37°C for 48 h. (B) Relative specific activities for PI and PC transfer associated with SrPI-1, SrPI-1myc, and the indicated Thr-59 SrPI-1* were determined (see Fig. 2A). The relative specific activities measured at the indicated temperatures are identified. Determinations represent the averages from at least three experiments.

the stimulation of PI-specific phospholipase C activity in permeabilized HL60 cells (24, 25). These findings have largely been interpreted in the context of rPI-TP effecting vectorial transfer of PI to signaling membranes. We shall restrict the discussion of our results to (i) rPI-TP residues that are required for PI transfer and (ii) what the data imply for regulation of rPI-TP activity *per se*. While we interpret our results in the context of a PL transfer function for rPI-TP, the concepts described also apply to regulation of differential PL binding by rPI-TP (14).

Two corollaries are implied by a vectorial PI transfer reaction. First, it predicts a critical role for vectorial PI transfer in rPI-TP function *in vivo* and predicts that vectorial PI transfer must be sustained in the direction of the signaling membrane. If vectorial PI transfer is balanced by countertransfer of PC (26), there should exist a mechanism to uncouple the PI and PC transfer activities of rPI-TP at the signaling membrane. Our findings that the S25F, T59I, P78L, and E248K mutations manifested specific PI transfer defects *in vitro*, in the face of unadulterated PC transfer capabilities, demonstrate that the PI and PC transfer activities can be uncoupled in the context of rPI-TP itself (Fig. 2B). The specificity of these mutations further suggests that (i) Ser-25, Pro-78, Thr-59, and Glu-248 cooperate to form a PI head-group recognition/binding site in rPI-TP or (ii) these residues lie adjacent to such a site.

Second, as efficient rPI-TP-mediated PL transfer *in vitro* is executed via PL exchange reactions that exhibit neither membrane specificity nor vectoriality (3), models invoking rPI-TP-

driven vectorial PI transfer to specific signaling membranes in mammalian cells suggest the *in vivo* superimposition of some regulatory action upon rPI-TP. While such regulation can potentially be achieved by a coupling of PL transfer to modification of the transferred PI monomer (27), our finding that the class II T59I and E248K SrPI-1* involve consensus PKC phosphorylation motifs in rPI-TP suggests the additional possibility that at least one consensus PKC phosphorylation site serves as a regulatory site on rPI-TP. In particular, the genetic data demonstrating the sensitivity of PI transfer activity to mutations involving Thr-59 identify this residue as a potential site on rPI-TP through which the PL transfer activities of rPI-TP can be selectively regulated (Fig. 3).

The intimacy between the residue present at position 59 and PI transfer function is underscored by two striking allele-specific effects on PL transfer activity. (i) We noted that H60Q elicited only modest effects on the specific activities of PI and PC transfer. In contrast, all Thr-59 mutations tested, including T59Q, selectively attenuated PI transfer activity (Figs. 2A and B and 3B). (ii) We found that, while both T59I and T198I mutations involve consensus PKC phosphorylation sites, only the former elicited selective PI transfer defects (Fig. 2A and B).

The sensitivity of PI transfer to Thr-59 predicts that phosphorylation of this residue by mammalian PKC will effectively and selectively downregulate the PI transfer activity of PI-TP. A model for how a PKC/protein phosphatase (PPase) cycle might operate in the context of vectorial PI transfer by rPI-TP *in vivo* is depicted in Fig. 4. The demonstration that phosphor-

FIG. 4. PKC/PPase cycle for vectorial transfer of PI from a donor to an acceptor membrane in mammals. Upon discharge of PI (I) or PC (C) into the acceptor membrane (upper legs of cycle), a resident PKC phosphorylates rPI-TP on Thr-59 and renders it unable to reload with PI. rPI-TP then disengages from the acceptor membrane either as a PL-free protein or as a PC-bound species (lower legs of cycle). Upon reengagement of PI-TP with the PL-donating membrane, a resident PPase dephosphorylates PI-TP and permits reloading with PI for the next round of transfer.

ylation of rodent PI-TP is stimulated by PKC agonists *in vivo* (28), when coupled with our finding that the T59D and T59E mutants were specifically defective in PI transfer (Fig. 3), supports the basic tenets of the proposed PKC/PPase cycle. This model is also appealing from a physiological perspective. As PKC is recruited to membranes engaged in inositol PL-driven signaling events and activated there (29), the kinase is properly poised to effect downregulation of the PI transfer activity of rPI-TP at membranes expected to function as efficient PI acceptors, but inefficient PI donors, in vectorial PI transfer reactions. Finally, this cycle identifies a strategy for reconstitution of an efficient vectorial PI transfer reaction with purified components (Fig. 4).

This work was supported by grants from the U.S. Public Health Service (GM-44530) and the American Tobacco Council (3937) to V.A.B. J.G.A. and R.T.C. were partially supported by a Basic Mechanisms of Lung Disease predoctoral training grant from the National Institutes of Health (5T32HL07553) and a predoctoral fellowship from the Helen Keller Eye Research Foundation, respectively.

1. Wirtz, K. W. A. (1991) *Annu. Rev. Biochem.* **60**, 73–99.
2. Rueckert, D. G. & Schmidt, K. (1990) *Chem. Phys. Lipids* **56**, 1–20.

3. Cleves, A., McGee, T. & Bankaitis, V. (1991) *Trends Cell Biol.* **1**, 30–34.
4. Geijtenbeek, T. B. H., de Groot, E., van Baal, J., Brunink, F., Westerman, J., Snoek, G. T. & Wirtz, K. W. A. (1994) *Biochim. Biophys. Acta* **1213**, 309–318.
5. Dickeson, S. K., Lim, C. N., Schuyler, G. T., Dalton, T. P., Helmkamp, G. M., Jr., & Yarbrough, L. R. (1989) *J. Biol. Chem.* **264**, 16557–16564.
6. Tanaka, S. & Hosaka, K. (1994) *J. Biochem.* **115**, 981–984.
7. Vihtelic, T. S., Goebel, M., Milligan, S., O'Tousa, J. E. & Hyde, D. R. (1993) *J. Cell Biol.* **122**, 1013–1022.
8. Bankaitis, V. A., Malehorn, D. E., Emr, S. D. & Greene, R. (1989) *J. Cell Biol.* **108**, 1271–1281.
9. Bankaitis, V. A., Aitken, J. R., Cleves, A. E. & Dowhan, W. (1990) *Nature (London)* **347**, 561–562.
10. Salama, S. R., Cleves, A. E., Malehorn, D. E., Whitters, E. A. & Bankaitis, V. A. (1990) *J. Bacteriol.* **172**, 4510–4521.
11. Lopez, M. C., Nicaud, J.-M., Skinner, H. B., Vergnolle, C., Kader, J. C., Bankaitis, V. A. & Gaillardin, C. (1994) *J. Cell Biol.* **124**, 113–127.
12. Cleves, A. E., McGee, T. P., Whitters, E. A., Champion, K. M., Aitken, J. R., Dowhan, W., Goebel, M. & Bankaitis, V. A. (1991) *Cell* **64**, 789–800.
13. McGee, T. P., Skinner, H. B., Whitters, E. A., Henry, S. A. & Bankaitis, V. A. (1994) *J. Cell Biol.* **124**, 273–287.
14. Skinner, H. B., McGee, T. P., McMaster, C., Fry, M. R., Bell, R. M. & Bankaitis, V. A. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 112–116.
15. Skinner, H. B., Alb, J. G., Whitters, E. A., Helmkamp, G. M., Jr., & Bankaitis, V. A. (1993) *EMBO J.* **12**, 4775–4784.
16. Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168.
17. Sherman, F., Fink, G. R. & Hicks, J. B. (1983) *Methods in Yeast Genetics: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
18. Busby, S., Irani, M. & De Crombughe, B. (1982) *J. Mol. Biol.* **154**, 197–215.
19. Miller, J. H. (1972) *Experiments in Molecular Genetics: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
20. Aitken, J. F., van Heusden, G. P. H., Temkin, M. & Dowhan, W. (1990) *J. Biol. Chem.* **265**, 4711–4717.
21. Paulus, H. & Kennedy, E. P. (1956) *J. Biol. Chem.* **235**, 1303–1311.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
23. Kunkel, T. A., Roberts, J. D. & Zahour, R. A. (1987) *Methods Enzymol.* **154**, 367–382.
24. Hay, J. C. & Martin, T. F. J. (1993) *Nature (London)* **366**, 572–575.
25. Thomas, G. M. H., Cunningham, E., Fensome, A., Ball, A., Totty, N. F., Truong, O., Hsuan, J. & Cockcroft, S. (1993) *Cell* **74**, 919–928.
26. Van Paridon, P. A., Gadella, T. W. J., Somerharju, P. J. & Wirtz, K. W. A. (1987) *Biochim. Biophys. Acta* **903**, 68–77.
27. Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukami, K., Takenawa, T., Anderson, R. A. & Martin, T. F. J. (1995) *Nature (London)* **374**, 173–177.
28. Snoek, G. T., Westerman, J., Wouters, F. & Wirtz, K. W. A. (1993) *Biochem. J.* **291**, 649–656.
29. Nishizuka, Y. (1992) *Science* **258**, 607–614.