Novel developmentally regulated phosphoinositide binding proteins from soybean whose expression bypasses the requirement for an essential phosphatidylinositol transfer protein in yeast


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Phosphatidylinositol transfer proteins (PITPs) have been shown to play important roles in regulating a number of signal transduction pathways that couple to vesicle trafficking reactions, phosphoinositide-driven receptor-mediated signaling cascades, and development. While yeast and metazoan PITPs have been analyzed in some detail, plant PITPs remain entirely uncharacterized. We report the identification and characterization of two novel soybean PITPs, Ssh1p and Ssh2p, whose structural genes were recovered on the basis of their abilities to rescue the viability of PITP-deficient Saccharomyces cerevisiae strains. We demonstrate that, while both Ssh1p and Ssh2p share ~25% primary sequence identity with yeast PITP, these proteins exhibit biochemical properties that diverge from those of the known PITPs. Ssh1p and Ssh2p represent high-affinity phosphoinositide binding proteins that are distinguished from each other both on the basis of their phospholipid binding specificities and by their substantially non-overlapping patterns of expression in the soybean plant. Finally, we show that Ssh1p is phosphorylated in response to various environmental stress conditions, including hyperosmotic stress. We suggest that Ssh1p may function as one component of a stress response pathway that serves to protect the adult plant from osmotic insult.

Keywords: phosphatidylinositol transfer proteins/phosphoinositides/signaling/soybean/stress response

Introduction

Phosphatidylinositol/phosphatidylcholine transfer proteins (PITPs) transfer either phosphatidylinositol (PI) or phosphatidylcholine (PC) between membrane bilayers in vitro (Cleves et al., 1991a; Wirtz, 1991), and this class of proteins exhibits two hallmark features of interest. First, PITPs are unique in that while these polypeptides contain one phospholipid (PL) binding site per protein monomer, PITPs have the ability to accommodate binding of two dissimilar PLs (PI and PC) in a mutually exclusive binding reaction. Secondly, PITPs exhibit a high level of primary sequence conservation. These homologies among PITPs are presently bifurcated into two distinct branches. PITPs of mammalian and insect origin share at least 40% primary sequence identity (Dickeson et al., 1989; Vihtelic et al., 1993; Tanaka and Hosaka, 1994; Chang et al., 1997). Fungal PITPs are also highly similar to each other at the primary sequence level (Bankaitis et al., 1989, 1990; Salama et al., 1990; Lopez et al., 1994), but share no primary sequence similarity with metazoan PITPs (Bankaitis et al., 1989; Dickeson et al., 1989; Vihtelic et al., 1993).

The Saccharomyces cerevisiae SEC14 gene product (Sec14p) represents the major PITP of yeast, and plays an essential role in protein exit from the yeast Golgi complex (Bankaitis et al., 1989, 1990). A dissection of how Sec14p translates its PI/PC-exchange activity to biological function has been driven by the characterization of mutations that relieve cells of the normally essential Sec14p requirement for Golgi function and cell viability (Cleves et al., 1989, 1991b; Alb et al., 1996; Fang et al., 1996). Characterization of these ‘bypass Sec14p’ mutations has generated the proposal that Sec14p maintains the integrity of a critical Golgi diacylglycerol (DAG) pool that is required for Golgi secretory function (McGee et al., 1994; Kearns et al., 1997). An important aspect of this function is the ability of the PC-bound form of Sec14p to repress the activity of the CDP-choline pathway for PC biosynthesis (a potent DAG consumer) in yeast Golgi membranes (McGee et al., 1994; Skinner et al., 1995). The PI-bound form of Sec14p may function independently in potentiating PI metabolism in an action which would resupply the Golgi DAG pool. This mode of action is inferred from the demonstration that accelerated PI-turnover represents one in vivo mechanism for effecting a ‘bypass Sec14p’ phenotype (Kagiwada et al., 1996; Kearns et al., 1997), that the PI-transfer activity of mammalian PITP is necessary for rescue of Sec14p defects (Alb et al., 1995), and that both Sec14p and mammalian PITPs stimulate PI-metabolism in phosphoinositide-dependent reactions that have been reconstituted in permeabilized mammalian cells (Hay and Martin, 1993; Thomas et al., 1993; Hay et al., 1995; Cunningham et al., 1996). Indeed, PITP function prevents the onset of specific neurodegenerative diseases in Drosophila and mammals, although the in vivo mechanisms of PITP action remain unclear (Hamilton et al., 1997; Milligan et al., 1997).

While fungal and metazoan PITPs have been analyzed in some detail, plant PITPs remain uncharacterized. Herein, we report the identification and characterization of two novel soybean Sec14p homologs, designated Ssh1p and Ssh2p. These homologs share some 25% sequence identity with Sec14p, and expression of these proteins is developmentally regulated in the plant. The primary sequence
homologies shared by these SSH proteins with Sec14p translate to some level of functional relatedness since high-level expression of either Ssh1p or Ssh2p is sufficient for rescue of the growth and Golgi secretory defects associated with haploid lethal sec14Δ mutations. While Ssh2p exhibits robust PI-transfer activity in vitro, Ssh1p exhibits no such activity. Moreover, neither Ssh1p nor Ssh2p are capable of effecting PC transfer in vitro. In fact, both Ssh1p and Ssh2p are high-affinity phosphoinositide binding proteins that exhibit distinct phospholipid binding specificities. As such, Ssh1p and Ssh2p represent new members of the Sec14p family of proteins that exhibit novel biochemical properties. Finally, we report that Ssh1p is rapidly phosphorylated in response to the exposure of cells to a variety of environmental stresses (e.g. hyper-osmotic stress) and that the phosphorylated form of Ssh1p fails to associate with membranes. The distinct biochemical properties of Ssh1p and Ssh2p, when coupled with their differentially regulated patterns of expression in the plant, suggest that Ssh1p and Ssh2p play distinct physiological roles. Ssh1p, in particular, may play a role in regulating the response of soybean plants to environments of high osmolarity.

Results

Identification of two functional homologs of yeast Sec14p from a higher plant

Functional rescue of yeast Sec14p defects represents a simple method for recovering genes encoding heterologous PITPs (Skinner et al., 1993; Tanaka and Hosaka, 1994). To isolate higher plant PITP genes, we constructed a developing soybean seed cDNA library that was engineered for high-level expression in yeast (see Materials and methods). Fifteen cDNA clones, representing two distinct cDNA species designated SSH1 and SSH2, were recovered by virtue of their ability to rescue the growth defects of a sec14Δ strain at 37°C (Figure 1A). Importantly, plasmid shuffle experiments (see Lopez et al., 1994) demonstrated that SSH1 and SSH2 expression also remedied the unconditional lethality associated with

Fig. 1. (A) Expression of soybean SSH1 and SSH2 genes rescues the growth defects of Δsec14 yeast and sec14Δ strains. Growth properties of a wild-type yeast strain (CTY182), its isogenic sec14Δ strain (CTY182Δ), and various sec14Δ derivative strains carrying either YEp(SSH1) or YEp(SSH2) plasmids are shown. The sec14Δ strains (CTY937 and CTY938) all represent strain CTY1-1A carrying the indicated plasmid, while the Δsec14 strains (CTY897 and CTY898) were generated by plasmid shuffle as described by Lopez et al. (1994). These yeast strains were streaked onto YPD plates and incubated for 36 h at the indicated temperatures. High-level expression of either SSH1 or SSH2 not only restored growth of sec14Δ strains at the normally restrictive temperature at 37°C, but also restored viability at all temperatures to yeast strains carrying the haploid-lethal Δsec14 allele. Thus, when expressed at high levels, Ssh1p and Ssh2p are individually able to fulfill in yeast the essential cellular functions of Sec14p. (B) Efficiency of invertase secretion at 37°C for Δsec14 strains carrying the indicated YEp(SSH) plasmids. The secretion index relates extracellular secreted invertase to total cellular invertase as described (Salama et al., 1990). The secretion indices of wild-type and sec14Δ strains represent measures of normal secretory proficiency and the magnitude of the sec14Δ Golgi secretory block, respectively. The Δsec14 strains CTY897 and CTY898 (see Table I) were analyzed for secretory competence to determine the efficiency with which SSH1 and SSH2 expression rescued Δsec14-associated secretory defects, respectively. These data indicate that Ssh1p and Ssh2p expression in yeast significantly alleviated the Golgi secretory defect associated with complete loss of Sec14p function. (C) Ssh1p and Ssh2p share primary sequence homology with fungal PITPs. An alignment of the entire Sec14p, Ssh1p, and Ssh2p primary sequences is shown, and corresponding residue numbers are designated at the far right of each column. These soybean polypeptides each share ~25% primary sequence identity (and 50% similarity) with Sec14p. Composite identities are indicated by the residues boxed in black, while the Sec14p residues within the white boxes represent amino acid residues conserved in four of the fungal Sec14ps (i.e. Saccharomyces cerevisiae, Kluyveromyces lactis, Yarrowia lipolytica and Schizosaccharomyces pombe) we have characterized to date.
sec14Δ alleles (Figure 1A). Thus, the SSH1 and SSH2 gene products were able to substitute for Sec14p when expressed in yeast. However, this functional substitution required high-level expression of Ssh1p or Ssh2p, as evidenced by our finding that the introduction of centromeric plasmids bearing P<sub>PGK</sub>::SSH1 or P<sub>PGK</sub>::SSH2 cassettes was insufficient to support rescue of either sec14Δ or sec14<sup>Δts</sup> alleles (e.g. strains CTY1013 and CTY1015; see Table I). Moreover, Ssh2p expression consistently yielded a superior improvement in growth of Sec14p-deficient strains relative to Ssh1p expression (Figure 1A).

SSH1 or SSH2 expression alleviated sec14 growth defects and elicited significant relief of the secretory block associated with Sec14p insufficiencies. As shown in Figure 1B, wild-type strains grown at 37°C exhibited a secretion index (90.9 ± 0.4%) that indicated efficient trafficking of invertase through the secretory pathway to the cell surface. In contrast, an isogenic sec14<sup>Δts</sup> strain exhibited a secretion index of only 28.2 ± 2.4%. This value was diagnostic of the accumulation of a major intracellular pool of invertase that is blocked in transit from the yeast Golgi complex (Bankaitis et al., 1989; Franzusoff and Schekman, 1989; Cleves et al., 1991b). However, expression of SSH1 and SSH2 elevated the secretion index of a sec14 null strain grown at 37°C to 55.8 ± 6.2% and 80.2 ± 1.8%, respectively, i.e. values substantially greater than those determined for the sec14<sup>Δts</sup> strain at the restrictive temperature (Figure 1B). Again, in accordance with the growth phenotypes described above, Ssh2p expression elicited a reproducibly more efficient rescue of Sec14p-related Golgi secretory defects than did Ssh1p expression.

**SSH1 and SSH2 are developmentally regulated in soybean**

The high frequency of recovery for SSH2 cDNA clones, relative to SSH1 cDNA clones, in our functional screen suggested the possibility that SSH2 was more highly expressed in developing seeds than was SSH1. Northern blot analyses indicated that such was indeed the case. The steady-state abundance of SSH1 transcripts was greatest in leaf and root tissue, and was of only low abundance in developing seeds (Figure 2). In contrast, SSH2 transcripts were most abundant in developing seeds. Only low levels of SSH2 poly(A)<sup>+</sup>-RNA were detected in leaf tissue and we were unable to detect SSH2 expression in roots (Figure 2). Immunoblotting experiments with the corresponding tissue extracts were entirely consistent with the Ssh1p and Ssh2p expression pattern as deduced from the Northern blot analysis (not shown). Finally, Southern blot analyses, where soybean genomic DNA was individually probed with SSH1 or SSH2 cDNA, revealed a rather simple banding pattern. While the intron/exon organization and pseudogene repertoire for each SSH gene remains undetermined, the banding complexity obtained is consistent with each SSH gene being either unique, or belonging to a small multigene family (Figure 2).

**SSH1p and SSH2p are not typical PITPs**

High-level expression of mammalian PITP in yeast is sufficient to rescue the growth and secretory defects associated with sec14<sup>Δts</sup> defects (Skinner et al., 1993). The rescue of sec14 defects associated with expression of Ssh1p or Ssh2p in yeast, when coupled with the primary sequence relatedness of Ssh1p and Ssh2p to Sec14p (Figure 1C), suggested that Ssh1p and Ssh2p represented soybean PITPs. As a qualitative test of this possibility, we individually expressed Ssh1p and Ssh2p in the <i>cki</i>, sec14Δ yeast strain CTY303 (Table I) and measured the...
ability of cytosol prepared from these strains to effect PI and PC transfer in vitro. CTY303 was employed for these studies because it is devoid of endogenous PI- or PC-transfer activity as a consequence of the sec14Δ lesion. This strain retains full viability, in spite of the haploid- lethal nature of sec14Δ, because of the ‘bypass Sec14p’ property of the cki lesion which inactivates choline kinase, the first enzyme of the CDP–choline pathway for PC biosynthesis (Cleves et al., 1991b; Skinner et al., 1993). In these experiments, cytosol represented a clarified salt- stripped fraction of broken cell lysate (see Materials and methods). Salt-stripping was performed because significant fractions of both Ssh1p and Ssh2p were membrane associated (see below).

Analysis of the phospholipid transfer properties of Ssh1p and Ssh2p cytosol, prepared from CTY303, yielded unanticipated results, and data are shown in Figure 3A and B. As positive control, the phospholipid transfer activities of wild-type yeast (Sec14p) cytosol were individually measured, and robust PI- and PC- transfer activities were recorded. Both transfer activities were linear in the range of 0 to at least 2 mg added cytosol, and 2 mg wild-type yeast cytosol effected ~8 and 16% of total input radiolabeled PI- and PC-substrate, respectively, under the employed experimental conditions. Ssh2p cytosol also exhibited robust PI-transfer activity. Indeed, the PI-transfer activity of Ssh2p cytosol was in excess of that measured for Sec14p cytosol, which resulted from Ssh2p expression being driven by a powerful promoter from an expression cassette carried by a multicopy plasmid. Immunoblotting data were consistent with Ssh2p levels in cytosol preparations markedly exceeding those recorded for Sec14p in wild-type yeast cytosol (not shown). Yet, PC-transfer activity was essentially undetectable in Ssh2p cytosol (Figure 3A and B). The demonstration that Ssh2p cytosol effected efficient PI-transfer indicated that our inability to measure PC-transfer was not the trivial result of inefficient recovery of Ssh2p, nor the result of catastrophic degradation of Ssh2p during cytosol preparation. Characterization of Ssh1p cytosol also provided unanticipated results as it failed to support significant PI- or PC-transfer activity, even at high concentrations (Figure 3A and B). The stability of Ssh1p during the cytosol preparation was not a contributory factor to our inability to record PI- and PC-transfer activity since high levels of full-length Ssh1p were detected by immunoblotting of input cytosol (not shown).

The yeast cytosol data suggested that neither Ssh1p nor Ssh2p represented typical PITPs, i.e. proteins that exhibit both PI- and PC-transfer activity. To effect a more quantitative comparison of the phospholipid transfer activities of these Sec14p homologs, we expressed His6-tagged Ssh1p and Ssh2p in E.coli. The phospholipid transfer properties of the purified proteins were then determined. As illustrated in Figure 3C, the data obtained with Ssh1p and Ssh2p purified from E.coli broadly recapitulated the results generated with the corresponding yeast cytosol preparations. Ssh1p was inactive with respect to PI- or PC-transfer activity, even when 200 µg of purified protein were assayed. Yet, we believe that the relevant functional properties of Ssh1p are retained in the recombinant protein (see below). Recombinant Ssh2p scored as an active PI-transfer protein (Figure 3C), but was substantially weaker than Sec14p in activity. From titration experiments, we estimate His6-Sec14p purified from E.coli exhibited ~10-fold greater specific activity for PI-transfer than did His6-Ssh2p recovered from the same source. As expected, in marked contrast to the robust PC-transfer activity elaborated by His6-Sec14p, purified His6-Ssh2p was inactive for PC transfer (Figure 3C), even at 200 µg Ssh2p per PC-transfer assay (not shown).

The collective data indicate Ssh1p and Ssh2p exhibit biochemical properties that diverge from those associated with all other presently known PITPs that are characterized by their abilities to effect both PI and PC transfer. Ssh1p is devoid of both PI- and PC-transfer activity, whereas Ssh2p is a novel PI-transfer protein that elaborates PI-transfer activity in the absence of accompanying PC-transfer activity.

Ssh1p and Ssh2p are novel phosphoinositide binding proteins

We had previously demonstrated that the PI-transfer activity of mammalian PITP was required for expression of this protein to effect a heterologous rescue of Sec14p growth and secretory defects in yeast (Alb et al., 1995). Since mammalian PITP stimulates phosphatidylinositol-4,5-bisphosphate (PIP2) synthesis in permeabilized cells (Cunningham et al., 1995; Hay et al., 1995), we used a photoaffinity radiolabeling strategy to ascertain whether
Ssh1p and Ssh2p represented phosphoinositide binding proteins. Specifically, we employed \[ ^{3}H \] triester-BZDC-Pi(4,5)P$_2$ \{\(( ^{3}H \)\( \text{p-benzoyldihydrocinnamidyl-amino} \) propyl-phosphatidylinositol-4,5-bisphosphate\} and \[ ^{3}H \] triester-BZDC-InsP$_3$ \{\(( ^{3}H \)\( \text{p-benzoyldihydrocinnamidyl-amino} \) propyl-inositol-1,4,5-trisphosphate\} as photolabile affinity ligands (Dorman and Prestwich, 1994; Prestwich, 1996; Prestwich et al., 1997, 1998). Both ligands had previously been shown to exhibit highly selective IP$_3$ and PIP$_2$-displaceable photocovalent modification of the pleckstrin homology domain of phospholipase C$_{\delta}$ (Tall et al., 1997), and \[ ^{3}H \] triester-BZDC photoprobe was successfully employed to characterize the phosphoinositide binding specificity of the mammalian Golgi coatomer complex (Chaudhary et al., 1998).

Neither His$_6$-Ssh1p nor His$_6$-Ssh2p were efficiently photolabeled by the PIP$_2$ headgroup photoprobe \[ ^{3}H \] BZDC-IP$_3$, even though the control PIP$_2$ binding protein, gelsolin, exhibited intense photolabeling that was competed by excess unlabeled PIP$_2$ (not shown). However, both His$_6$-Ssh1p and His$_6$-Ssh2p were successfully labeled by the \[ ^{3}H \] BZDC-PIP$_2$ ligand, and binding of this phosphotriester photoprobe was competed by challenge with excess unlabeled PIP$_2$ (Figure 4A). As expected, the known PIP$_2$ binding protein gelsolin also exhibited PIP$_2$-displaceable binding of the \[ ^{3}H \] BZDC-PIP$_2$ photoprobe (Figure 4A). We estimated the IC$_{50}$s of PIP$_2$ for \[ ^{3}H \] BZDC-PIP$_2$ binding to Ssh1p and Ssh2p to be on the order of 0.5 and 1.0 \(\mu\)M, respectively (not shown). Thus, the demonstration that His$_6$-Ssh1p and His$_6$-Ssh2p efficiently bound \[ ^{3}H \] BZDC-PIP$_2$, but not \[ ^{3}H \] BZDC-IP$_3$, suggested that the diacylglycerol backbone was critical for...
specificities of Ssh1p and Ssh2p. Recombinant His$_6$-tagged Ssh1p and Ssh2p associated PIP$_2$ transfer activity not only by their inability to catalyze PC transfer, but also Ssh1p and Ssh2p are minimally distinguished from Sec14p probes (Figure 4A) at the concentrations tested. Thus, challenge of the photolabeling reaction with other acidic phospholipids (i.e. PI, PI-4-P, phosphatidylserine and phosphatidic acid) failed to exert a competitive effect on binding of photoprobe to Ssh1p (Figure 4B). Similarly, PC and phosphatidylethanolamine were not effective competitors for photoprobe binding to Ssh1p.

Ssh2p exhibited a phospholipid binding specificity that was distinct from that of Ssh1p. Whereas PI-(4,5)-P$_2$ competed effectively with photoprobe binding to Ssh2p, PI, PI-(3,5)-P$_2$ and PI-4-P failed to exert a competitive effect on Ssh2p–photoprobe interaction (Figure 4C). Yet, challenge with other acidic phospholipids (i.e. phosphatidylserine and phosphatidic acid) strongly diminished the efficiency of Ssh2p photolabeling. In this light, the inability of a 135-fold molar excess of PI to inhibit photolabeling was unexpected, especially given that Ssh2p must bind PI at some level since it is able to effect PI transfer in vitro (see above). Since both Ssh2p and Ssh1p exhibit high binding affinities for PI-(4,5)-P$_2$, it is likely that the affinity of Ssh2p for PI is much lower (a reasonable possibility since Ssh2p does not exhibit a particularly robust specific activity for PI transfer in vitro relative to Sec14p; see Figure 3C). Again, PC and phosphatidylethanolamine were not effective competitors for photoprobe binding to Ssh2p. These data indicate that, while both Ssh1p and Ssh2p are high-affinity phosphoinositide binding proteins, these proteins exhibit differing phospholipid binding specificities. In particular, by the competitive photolabeling assay employed, Ssh1p exhibited a more restricted phospholipid binding spectrum than Ssh2p.

**Ssh1p and Ssh2p associate with membrane fractions**

Sec14p is primarily a cytosolic protein that also exhibits a specific peripheral association with yeast Golgi membranes (Bankaitis et al., 1989; Cleves et al., 1991b). To determine the localization of Ssh1p and Ssh2p, we subjected cell-free lysates prepared from CTY303-derivative strains harboring individual YEp($\text{P}_{\text{ADH1}}$::SSH1 and $\text{P}_{\text{SEC14}}$::SSH2myc) plasmids to a differential centrifugation regime in order to generate a set of defined membrane and cytoplasmic fractions (see Materials and methods). The distribution of Ssh1p or Ssh2p across these fractions was subsequently assessed by immunoblotting.

Approximately 70% of the cellular Ssh1p pool was estimated to localize to the 100 000 g supernatant fraction (S100), while the remainder of the material was sedimented with the 13 000 g pellet fraction (P13) (Figure 5A). Previous studies have established that the P13 is enriched in endoplasmic reticulum (ER) and plasma membrane, while the P100 is relatively enriched for Golgi and endosomal membrane (Cleves et al., 1991b; Bowser et al., 1992; Horazdovsky and Emr, 1993). Ssh1p routinely labeling assay was employed. These experiments were performed in the context of a mixed detergent–phospholipid micelle system to standardize presentation of phospholipid to protein (see Materials and methods). As shown in Figure 4B, of the various phospholipids tested, Ssh1p exhibited the highest affinity for bis-phosphorylated inositol phospholipids. Under the conditions employed, both PI-(4,5)-P$_2$ and PI-(3,5)-P$_2$ effectively competed with photoprobe for binding to Ssh1p. In contrast, challenge of the photolabeling reaction with other acidic phospholipids (i.e. PI, PI-4-P, phosphatidylserine and phosphatidic acid) failed to exert a competitive effect on binding of photoprobe to Ssh1p (Figure 4B). Similarly, PC and phosphatidylethanolamine were not effective competitors for photoprobe binding to Ssh1p.

**Photolabeling reaction**

From these data, we conclude that Ssh1p and Ssh2p represent novel phosphoinositide binding proteins. In that regard, we note that His$_6$-Sec14p failed to bind efficiently either the [$^3$H]BZDC-PIP$_2$ or the [$^3$H]BZDC-PI-PI$_2$ photoprobe in the absence (T) or the presence (C) of a 36-fold excess of unlabeled PIP$_2$. Samples were equilibrated in the dark and exposed to longwave UV radiation to induce formation of covalent adducts of protein–phospholipid complexes. In these experiments, the known high-affinity PIP$_2$ binding protein gelsolin was used as positive control. Protein–photoprobe adducts were separated by SDS–PAGE, the gels were fixed, and resolved adducts were visualized by fluorography. Like the purified gelsolin control, both the 37 kDa Ssh1p and the 29 kDa Ssh2p formed adducts with the PIP$_2$ photoprobe in a manner that was competed by challenge with excess unlabeled PIP$_2$. In contrast, Sec14p failed to bind PIP$_2$ efficiently by this assay. (B) and (C) Phospholipid binding specificities of Ssh1p and Ssh2p. Recombinant His$_6$-tagged Ssh1p and Ssh2p were photolabeled with [$^3$H]BZDC-PIP$_2$ photoprobe either in the absence or the presence of a 135-fold molar excess of unlabeled competitor phospholipid (as indicated). Abbreviations are: PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP(4,5)P$_2$, phosphatidylinositol-4,5-bisphosphate; PIP(3,5)P$_2$, phosphatidylinositol-3,5-bisphosphate; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Binding was conducted in a Triton X-100-phospholipid mixed micelle system such that both photoprobe and competitive phospholipids were minute micellar constituents (<0.1 mol%). Competitive binding was scored as a function of reduced photolabeling efficiency.

**Phospholipid binding specificities of Ssh1p and Ssh2p**

To characterize more fully the phospholipid binding spectrum exhibited by Ssh1p and Ssh2p, a competitive photoprobe binding by either polypeptide (see Prestwich et al., 1998 for discussion of protein:photoprobe binding modes).

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Fig. 5. Ssh1p and Ssh2p are peripheral membrane proteins. (Top panel) Cell-free lysates were prepared from (A) the Δsec14/YEpSSH1 strain (CTY937) and (B) the Δsec14/YEpSSH2 strain (CTY938), using an osmotic lysis procedure that maintains the integrity of subcellular organelles. The lysates were subjected to three rounds of differential centrifugation resulting in the production of whole cell lysate (WC), 13 000 g supernatant and pellet fractions (S13 and P13, respectively), and 100 000 g supernatant and pellet fractions (S100 and P100, respectively). The volumes of the various fractions were adjusted so that equal cell equivalents were resolved by SDS–PAGE. After electrophoresis, the proteins were transferred to nitrocellulose and immunoblotted for Ssh1p (A) and Ssh2p (B) immunoreactive species, and the following markers: the integral endoplasmic reticulum (ER) membrane protein dolichol-phosphomannose synthase (DPM), the 62 kDa cytosolic marker phosphoglucosamutase (PGM), and Kar2p, the 70 kDa soluble lumenal ER protein employed as a marker for organelle integrity during the fractionation procedure. Primary antibodies were used at 1:3000, 1:3000, 1:1500, 1:10 000 and 1:1000 dilutions for Ssh1p, Ssh2p, DPM, Kar2p and PGM, respectively. All antibodies were rabbit polyclonal antibodies. In the case of Ssh1p and Ssh2p, the cognate antisera were raised against His6-tagged proteins purified from E.coli (see Materials and methods). (Bottom panel) Cell-free lysates were prepared from the Δsec14 strain carrying YEpSSH1 (CTY937) (A) and the Δsec14/YEpSSH2myc strain (CTY940) as described above. The lysates were adjusted to a final concentration of 0 mM (Buffer), 100 mM and 500 mM KCl, as indicated at the top, and incubated for 45 min on ice. Lysates were then centrifuged at 100 000 g to generate P100 (P) and S100 (S) fractions. Ssh1p and Ssh2p immunoreactive species (along with the DPM, PGM and Kar2p markers) were resolved and visualized as described for the top panel. (B) The details for both the top and bottom panels are as described in the top panel except that Ssh2p was visualized by immunoblotting with the c-myc epitope-directed 9E10 monoclonal antibody (1:3000 dilution) (Evan et al., 1985).

resolved itself by SDS–PAGE as a doublet of 37 and 38 kDa species, and the form of higher molecular weight (Ssh1p*) represents a phosphorylated form of the polypeptide (see below). Interestingly, while Ssh1p* failed to associate with membranes at all, a significant fraction of Ssh1p was reproducibly membrane associated (Figure 5A). The distinction between which form was membrane associated came from two lines of evidence in addition to the fractionation data of Figure 5A. First, we generated a monoclonal antibody against Ssh1p which immunoreacts with 37 kDa Ssh1p, but not 38 kDa Ssh1p*. Immunoblotting experiments with this monoclonal antibody demonstrated membrane-associated immunoreactivity. Secondly, [32P]orthophosphate radiolabeling experiments demonstrated that 32P-radiolabeled Ssh1p (i.e. the Ssh1p* form, see below) was restricted to cytosolic fractions (not shown).

The association of Ssh1p with membranes was salt-labile, as a 100 mM KCl wash stripped most of the Ssh1p from membranes and 500 mM KCl quantitatively released the membrane-associated Ssh1p. Unlike Ssh1p, the 29 kDa Ssh2p resolved itself as a single species by SDS–PAGE (Figure 5B). Approximately 40–60% of the Ssh2p was routinely recovered in the P13 fraction with the remainder distributing to the S100. Similar to the case of Ssh1p, the association of Ssh2p with the P13 was salt-sensitive since Ssh2p was quantitatively stripped from membranes by a 100 mM KCl wash (Figure 5B).

Control markers exhibited expected behavior in both the fractionation and salt-extraction experiments (Figure
Specific stresses cultured in standard yeast medium predominantly resulted in the 38 kDa Ssh1p* form (Figure 6A). YEp(HO) was present to stimulate conversion of the 37 kDa Ssh1p to the higher molecular weight species (Ssh1p*) represented a post-translationally modified form of Ssh1p. One possibility is that Ssh1p is phosphorylated by a kinase, the activity of which may be up-regulated under specific conditions. Indeed, challenge of yeast cells with conditions of low osmotic stress, and NaCl as indicated on top. After a 30 min incubation of the cultures at 25°C with shaking, cell-free extracts were prepared, proteins were resolved by SDS–PAGE, and Ssh1p-immunoreactive species were visualized by immunoblotting. The positions of the 37 kDa Ssh1p and the 38 kDa Ssh1p* forms are indicated at left. Formation of Ssh1p* was observed when cells were subjected to conditions of high osmotic stress, and NaCl was a particularly effective inducer of Ssh1p* formation. (B) Strain CYT899 was cultured in uracil-free minimal medium at 30°C with shaking and the culture was split into two portions. One portion was incubated as before without addition of NaCl (mock), while the second portion was adjusted to a final concentration of 1 M NaCl as indicated at top. At various times after NaCl addition (indicated in min at bottom), aliquots were removed, cells were lysed in sample buffer, and protein samples were electrophoresed in SDS–polyacrylamide gels. Resolved proteins were transferred to PVDF membranes and immunoblotted with polyclonal anti-Ssh1p serum (1:3000 dilution). The positions of the 37 kDa Ssh1p and 38 kDa Ssh1p* forms are indicated, right. Induction of the Ssh1p* form was induced rapidly in response to NaCl challenge, and Ssh1p* levels persisted throughout. No induction of Ssh1p* formation was recorded in the mock challenge control.

5A and B). The lumenal ER marker, Kar2p, sedimented quantitatively with the P13 fraction in a salt-resistant manner, and these results confirmed that intracellular organelle integrity was maintained during fractionation and salt-extraction procedures. The integral ER membrane protein marker, dolichol-phosphomannose synthase (Dpm), also exhibited a predominant and salt-resistant distribution to membrane fractions (i.e. P13). Under the fractionation conditions employed, the cytosolic protein phosphoglucomutase (PgM) was exclusively recovered from the S100 fraction, regardless of whether or not KCl was present (Figure 5A and B).

Conversion of Ssh1p to Ssh1p* represents a post-translational modification that is induced by specific stresses

The fact that Ssh1p resolved itself by SDS–PAGE as a doublet of 37 and 38 kDa species (Figure 5A) suggested that the higher molecular weight species (Ssh1p*) represented a post-translationally modified form of Ssh1p. One possibility is that Ssh1p is phosphorylated by a kinase, the activity of which may be up-regulated under specific stress conditions. Indeed, challenge of yeast cells with conditions of elevated environmental osmolarity was sufficient to stimulate conversion of the 37 kDa Ssh1p to the 38 kDa Ssh1p* form (Figure 6A). YEp(SSHI) yeast strains cultured in standard yeast medium predominantly exhibited the Ssh1p form. Less than 10% of the total Ssh1p was detected as Ssh1p*. Relatively modest increases in the osmotic strength of the medium (e.g. introduction of sorbitol or NaCl to final concentrations of 1 and 0.3 M, respectively, for 30 min) had little effect on the Ssh1p:Ssh1p* ratio in these strains. However, more substantial osmotic challenges involving elevation of the sorbitol or NaCl concentrations in the medium to 1.5 and 0.9 M for 30 min, respectively, elicited a dramatic shift in the Ssh1p:Ssh1p* ratio (Figure 6A). This change in the Ssh1p:Ssh1p* ratio was observed in the face of the protein synthesis inhibitor cycloheximide, indicating that generation of the Ssh1p* form was a post-translational event (not shown).

The dramatic increase in Ssh1p* levels in response to hyperosmotic challenge occurred rapidly (Figure 6B). Within 5 min of raising the extracellular NaCl concentration from 0 to 1.0 M, the molar fraction of Ssh1p* rose from <10% of the total cellular Ssh1p to ~25%. Significant increases in Ssh1p* were detected within 1 min of NaCl challenge (not shown). After 15 min of salt exposure >60% of the Ssh1p was in the 38 kDa Ssh1p* form, and this distribution was stably maintained for the duration of the 135 min experiment (Figure 6B). Mock-challenged control cultures did not induce conversion of Ssh1p to the Ssh1p* form (Figure 6B).

More detailed analyses indicated that conversion of the 37 kDa Ssh1p to the 38 kDa Ssh1p* form represented a rather specific cellular stress-response in yeast. Challenge of these same YEp(SSHI) strains with other environmental stresses such as heat shock (42°C for 15 min and 1 h), ethanol (5% for 1 h), or amino acid analogs (10 µg/ml canavanine for 1 h) failed to induce generation of the Ssh1p* form (not shown). However, exposure of Ssh1p-expressing yeast strains to glucose-free conditions for 15 min effectively induced a shift to the Ssh1p* form (not shown).

Ssh1p* is a phosphorylated form of Ssh1p

We considered the possibility that Ssh1p* represents a phosphorylated form of Ssh1p, and two lines of evidence demonstrate that this is indeed so. First, incubation of cell-free extracts with alkaline phosphatase in vitro resulted in the collapse of Ssh1p* into the Ssh1p form (Figure 7). Secondly, we were able to radiolabel Ssh1p* with [32P]orthophosphate and to measure increased incorporation of [32P]-radiolabel into Ssh1p in response to environmental cues that induced conversion of Ssh1p to Ssh1p* (Figure 7). Phosphoamino acid analysis indicated that this modification represents phosphorylation of serine and threonine residues, but not tyrosine (not shown).

The induction of Ssh1p* formation by increased growth medium osmolality suggested that Ssh1p could potentially represent a downstream target for the high osmolality/glycerol-activated MAP kinase pathway (the HOG pathway; Maeda et al., 1994) in yeast. To test this possibility, we incorporated a hog1Δ::TRP1 allele (which represents a null mutation in the structural gene for Hog1p, the MAP kinase of the HOG pathway) into Ssh1p-expressing yeast strains and tested whether conversion of Ssh1p to Ssh1p* was affected. Our data demonstrated the hog1Δ::TRP1 allele did not compromise the ability of yeast to phosphorylate Ssh1p in response to osmotic stress (not shown). Thus, Ssh1p* formation was not dependent on Hog1p activity.
Alkaline phosphatase  

Immppt

Fig. 7. Ssh1p* represents a phosphorylated form of Ssh1p. (Left panel) A clarified cell-free extract was prepared from the SSH1 expressing strain CTY987. Extract (0.75 µg) was incubated in 50 mM Tris–HCl pH 7.6, 0.1 mM EDTA (20 µl reaction volume) at 30°C for 15 min in the absence (−) or the presence (+) of 26.5 units of calf intestinal alkaline phosphatase (Gibco-BRL). The reactions were subsequently boiled in the presence of sample buffer, displayed by SDS–PAGE, and Ssh1p species were visualized by immunoblotting. In the absence of alkaline phosphatase, the standard Ssh1p profile was observed where ~15% of the Ssh1p (37 kDa; lower band) was in the Ssh1p* form (38 kDa; upper band). Alkaline phosphatase treatment collapsed the Ssh1p* form into the Ssh1p species, indicating that Ssh1p* is a phosphorylated form of Ssh1p. (Right panel) A 5 ml culture of an His6-Ssh1p-expressing strain (CTY920) was incubated in minimal medium, supplemented with 0.5 mCi of [32P]orthophosphate, for 2 h at 30°C with shaking. Clarified cell-free extracts were prepared and Ssh1p species were purified by sequential rounds of Ni2+-NTA and immunoprecipitation. Immunoprecipitated proteins were resolved by SDS–PAGE and transferred to PVDF membrane. Radiolabeled species were then visualized by autoradiography. Only a single 32P-radiolabeled species was detected, and its recovery was dependent on both Ssh1p expression and inclusion of anti-Ssh1p serum in the immunoprecipitation. After autoradiography, the PVDF membrane was wetted with methanol and subjected to immunoblotting with anti-Ssh1p serum. Comparison of the autoradiogram with the immunoblot confirmed that the 32P-radiolabeled polypeptide corresponded to the topmost band of the two His6-Ssh1p species (i.e. the Ssh1p* form).

Discussion

Although the existence of monospecific phospholipid transfer proteins has been reported in higher plants (Tanaka and Yamada, 1982), the only characterized plant lipid transfer proteins to date have been the low molecular weight, non-specific transfer proteins that facilitate transfer of a broad spectrum of phospholipids and galactolipids (Kader, 1996). No higher plant PITPs have yet been described. As fungal and metazoan PITPs are now under-}

et al., 1990; Gu et al., 1992; Sato et al., 1993; Chinen et al., 1996).

All presently characterized PITPs from fungal, mammalian, and insect sources are capable of effecting the transfer of either PI or PC between membrane bilayers in vitro. Yet, mutant mammalian, insect and yeast PITPs can be generated that exhibit specific defects in one transfer activity (Alb et al., 1995; Milligan et al., 1997; Sha et al., 1998; S.E.Phillips and V.A.Bankaitis, unpublished data). These findings not only demonstrate that there is no obligate biochemical coupling between these activities at the level of the in vitro transfer assay, but also raise the question of why this dual substrate specificity has been evolutionarily conserved. Whereas a physiological rationale for the dual PI- and PC-binding properties of Sec14p has been described (Kears et al., 1997; Fang et al., 1996), it is not yet clear that the same rationale applies to metazoan PITPs (see Milligan et al., 1997). Ssh2p is the first example of a PI-transfer protein that fails to transfer PC in vitro (Figure 3A–C). Ssh1p is also atypical in that it does not exhibit measurable PI- or PC-transfer activity in vitro (Figure 3A–C). Thus, Ssh2p is biochemically more similar to Sec14p. Perhaps this accounts for why Ssh2p expression consistently effected a superior rescue of Sec14p growth and secretory defects than did Ssh1p expression (Figure 1A and B).

Photolabeling experiments demonstrated that both Ssh1p and Ssh2p are high-affinity PI-(4,5)-P2 binding proteins, whereas Sec14p is not (Figure 4A–C). Thus, sec14-1 yeast strains are appropriate hosts not only for heterologous ‘complementation’ experiments designed to recover PITP genes, but such strategies also have the potential for recovering genes that encode phosphoinositide binding proteins. In that regard, there is some specificity as to which phosphoinositide binding protein genes will pass this functional screen. High-level expression of either the mammalian PIP2/PIP binding protein centaurin-α (Hammonds-Odie et al., 1996), or its yeast ARF–GTPase activating protein counterpart Gcs1p (Poon et al., 1996), fails to rescue the growth defects associated with Sec14p deficiency (not shown). Finally, our data indicate that genes encoding proteins sharing primary sequence homology with Sec14p, recovered on the basis of rescue of sec14 mutations, cannot be assumed to represent PITP structural genes. The biochemical properties of the heterologous Sec14p homologs must also be characterized.

Although even the general biological functions executed by Ssh1p and Ssh2p remain to be determined, these proteins do exhibit clear developmental regulation of expression in the plant. Whereas Ssh2p expression is highest in the developing seed and very low in roots and leaves, Ssh1p is most highly expressed in roots and leaves and is only very poorly expressed in developing seed (Figure 2). These data, when coupled with the distinct phospholipid binding/transfer properties of Ssh1p and Ssh2p (Figures 3 and 4), suggest that these proteins fulfill distinct physiological functions within the plant. We speculate that Ssh1p may be involved in the plant response to various environmental stresses, particularly stresses associated with conditions of elevated osmolarity. Two lines of evidence are independently consistent with this proposal. First, Ssh1p is rapidly phosphorylated when yeast are subjected to hyperosmotic stress (Figure 6A and...
plant tissues such as roots and leaves is consistent with these suggested osmoprotective functions. How Shs1p* may harness phosphoinositide binding to biological function also remains unknown, but the possibility that Shs1p* and Shs1p exhibit distinct phosphoinositide binding properties is an attractive one. Characterization of purified Shs1p* will be required for the unambiguous test, but we have as yet been unable to isolate sufficient Shs1p* for this purpose. Finally, our finding that Shs2p is most highly expressed in developing seed, a structure undergoing a developmental program of dehydration, is also intriguing. It raises the possibility that Shs2p too is involved in the proper execution of controlled stress conditions imposed by the plant for specific developmental purposes.

**Materials and methods**

**Strains, media and genetic techniques**

Yeast complex and minimal media have been previously described (Sherman et al., 1983), as have yeast plasmid transformations and gene disruption techniques (Ito et al., 1983; Rothstein, 1983; Sherman et al., 1983). The plasmid shuffle assay performed to determine complementation of Δsec14 was done using an ADE3, SEC14 plasmid as outlined by Lopez et al. (1994). Complete genotypes of yeast strains are listed in Table I. Generally, all SSH cDNAs were placed under transcriptional control of the yeast ADH promoter except where it is indicated that SSH cDNAs were expressed from the yeast PKG promoter (P<sub>PKG::SSH</sub>). The sec14Δ::hisG allele represents a null mutation whose detailed description is found elsewhere (Skinner et al., 1993). YEp195 and pPH plasmids (Gietz and Sugino, 1988; Sikorsky and Hieter, 1989) represented the vectors into which SSH constructs were cloned. Ni<sup>2+</sup>-NTA resin and pQE plasmids were obtained from Qiagen (Chatsworth, CA), while phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) was purchased either from Calbiochem or Ethrecel Research Laboratories (Salt Lake City, UT). Fine chemicals were purchased from Sigma Co. (St Louis, MO) unless specified otherwise. Restriction enzymes were purchased from Promega (Madison, WI). [α-<sup>32</sup>P]dATP was purchased from American Radiolabeled Chemicals, Inc. [α-<sup>32</sup>P]dATP was purchased from American Radiolabeled Chemicals, Inc.

**Recovery of SSH1 and SSH2 clones from soybean**

Size selected cDNA (>500 bp) was synthesized from mRNA isolated from developing soybean (Glycine max cv Dare) seeds 25 days after flowering using moloney murine leukemia virus reverse transcriptase as described by the manufacturer (Gibco-BRL, Grand Island, NY). The cDNAs were subsequently ligated to BoSI adapters, cloned into the yeast expression vector pDB20 and amplified as outlined by Becker et al. (1991). pDB20 is a yeast 2 µ circle plasmid that employs URA3 as a selectable marker, and constitutive expression of cloned cDNAs is driven by the powerful ADH1 promoter. Using 0.1 µg of a cDNA pool, a library containing ~5×10<sup>7</sup> primary transformants was produced.

A Saccharomyces strain (CTY1-1A) carrying the hyperosmotic M<sup>1</sup> allele was transformed with the soybean cDNA library according to the protocol of Gietz et al. (1992). Ura<sup>+</sup> transformants were selected on solid minimal-media lacking uracil with a plating density of ~5000 colonies per 90 mm Petri dish. After overnight growth at 30°C, the plates were transferred to the restrictive temperature of 37°C for 2 days and Ts<sup>+</sup> colonies isolated. Plasmid dependence of the Ts<sup>+</sup> phenotype was determined by two independent methods: (i) curing the strains of plasmid by selection for growth on minimal media containing 5-fluoroorotic acid (Boeke et al., 1984) and screening for re-acquisition of the ts<sup>+</sup> growth phenotype, and (ii) recovery of resident plasmids from Ts<sup>+</sup> transformants and reintroduction of plasmid into strain CTY1-1A with subsequent assessment of reacquisition of a Ts<sup>+</sup> growth phenotype as an unselected trait. Fifteen independent plasmids that passed both of these criteria were recovered, and the corresponding cDNA inserts were subcloned into pBluescript SK<sup>+</sup>. Detailed restriction enzyme analyses identified two distinct classes of cDNA clones. One clone, designated SSH1, exhibited a restriction map that failed to resemble those of the remaining 14 clones. Of those 14 remaining clones, one representative clone was selected and designated SSH2.

**Fig. 8. Speculative model for Shs1p function in specific stress responses.** Challenge of cells with specific environmental stresses (e.g., hyperosmotic stress) induces phosphorylation of Shs1p to the Shs1p<sup>*</sup> form by an as yet unidentified protein serine/threonine kinase (PKase). This modification mobilizes Shs1p<sup>*</sup> from either the plasma membrane (A), or the membrane of an intracellular organelle (B), to a cytosolic location. The ultimate result is facilitation of an osmoprotective response. As only ~60% of the Shs1p is converted to Shs1p<sup>*</sup> and redistributed, we suggest that Shs1p<sup>*</sup> plays an active role in this response (rather than formation of Shs1p<sup>*</sup> representing an inactivation of Shs1p). Mobilization of Shs1p<sup>*</sup> may permit this polypeptide to mediate regulation of some aspect of phosphoinositide metabolism at a distinct subcellular location. Alternatively, modification of Shs1p to the Shs1p<sup>*</sup> form by an as yet unidentified protein serine/threonine kinase (PKase) is mobilized. Dephosphorylation of Shs1p<sup>*</sup> by a protein serine/threonine phosphatase (PPase) would complete the cycle.

and B), and we find this environmental stress-induced modification of Shs1p to be a conserved response. A rapid and dramatic conversion of Shs1p to the Shs1p<sup>*</sup> form is also recorded in young soybean roots and transgenic tobacco leaf disks bathed in high salt solutions (manuscript in preparation). These data suggest that the kinase responsible for Shs1p phosphorylation in response to environmental stress may itself be functionally conserved, and identification of this kinase(s) from soybeans and yeast represents an important direction for further work. In this regard, we find that high osmolarity-induced phosphorylation of Shs1p is a Hog1p-independent reaction. The phosphorylation data highlight yet another difference between Sec14p and Shs1p. Sec14p is not phosphorylated in yeast and imposition of osmotic stresses onto yeast does not induce phosphorylation of Sec14p (not shown). Secondly, Shs1p binds PI-(3,5)-P<sub>2</sub> (Figure 4B), a phosphoinositide found in plant, animal and yeast cells (Dove et al., 1997). In yeast, PI-(3,5)-P<sub>2</sub> is produced in response to osmotic stress (Dove et al., 1997), and therefore this phospholipid represents an excellent candidate for a physiological Shs1p ligand in the osmotically stressed plant.

Phosphorylation of Shs1p has consequences for protein localization since Shs1p associates with yeast membranes and Shs1p<sup>*</sup> does not (Figure 5A). We suggest that Shs1p phosphorylation may regulate the release of this phosphoinositide binding protein from specific membranes so as to initiate, or otherwise sustain, a protective response to osmotic stress (Figure 8). The expression of Shs1p in...
Invertase assays

Strains were grown to mid-logarithmic phase in 2% glucose medium at 25°C with shaking. Cells were pelleted, washed with 2 vols of water, resuspended in 0.5 M of pre-warmed glucose (0.1%)YP medium, and incubated at 37°C. After 2 h, the samples were adjusted to 10 mM NaNO₃, washed twice with ice-cold 10 mM NaNO₃ and resuspended in 0.5 M of the same. Each sample was split into two equal aliquots and the volumes made up to 0.5 ml with 10 mM NaNO₃ or 10 mM NaNO₃, 0.2% Triton X-100 in order to measure external and total invertase activities, respectively (Salama et al., 1990).

Nucleotide sequence analysis

The dyeoxy chain termination method (Sanger et al., 1977) was employed using double-stranded DNA as the template, the Sequenase version 2.0 kit (Amer sham) and [α-35S]dATP as a radiolabel.

Nucleic acid blot

Total genomic DNA from young soybean leaves was isolated as described previously (Murray and Thompson, 1980). Ten micrograms of genomic DNA was digested with restriction enzymes and separated by electrophoresis on a 1% agarose gel. Total cellular RNA was recovered from the leaves, roots and developing seeds (25 days after flowering) as outlined by Grimes et al. (1992). Poly(A)+ RNA was isolated using the mRNA Separator Kit according to the manufacturer’s protocol (Clontech) and separated electrophoretically on a 1.2% formaldehyde gel. Ten micrograms of poly(A)+ RNA were loaded per lane; equal loading of each preparation was confirmed by the equivalency of ethidium bromide staining of the gel prior to transfer. All nucleic acids were transferred to Magnagraph nylon membranes as described by the manufacturer (Micron Separations Inc.) and hybridized to randomly primed 32P-labeled probes according to the method of Feinberg and Vogelstein (1983).

Preparation of cytosol and phospholipid transfer assays

Appropriate strains were grown to mid-logarithmic phase in media lacking uracil, and harvested by centrifugation. Cell pellets were resuspended in spheroplasting buffer (1.1 M sorbitol, 10 mM Tris–HCI, pH 7.5), and 2-ME added to a final concentration of 25 mM. Cells were incubated at room temperature for 10 min, pelleted, and resuspended in spheroplasting buffer. Lyticase (5000 U) (Enzymes, Corvallis, OR) was incubated at 37°C. After 2 h, the samples were adjusted to 10 mM Tris–HCl pH 7.4, 1 mM EDTA, and [3H]BZDC-PIP₂, pH 7.4, 1 mM EDTA, and [3H]BZDC-PIP₂ in a microtiter plate. [3H]BZDC-PIP₂ was used at a concentration of 0.47 µM for the binding studies. Reactions were allowed to equilibrate for 10 min at 0°C before exposure to long range UV light (360 nm) at a distance of 1 cm for 1 h with a lamp power of 30 W. Sample buffer was added to the reactions and proteins were resolved on a 10% SDS–PAGE gel. Gels were fixed and fluorographed with Intensify (Amer sham), dried and exposed to film. Exposures were quantified using a Bio-Rad Model GS-670 Imaging Densitometer. Competitive binding assays were supplemented with PIP₂ (10 µM final concentration). Details of [3H]BZDC-PIP₂ synthesis have been described by Gu and Prestwich (1991).

To assess the specificity of phospholipid binding, a competitive photolabeling procedure was employed in the context of a mixed micelle system. Purified Ssh1p or Ssh2p (0.75–1.0 µg) was incubated in 25 µl 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 2% (v/v) Triton X-100. This concentration of detergent was well in excess of the critical micellar concentration (0.24 mM) for Ssh2p. The reaction mixture was added to the system to a final concentration of 10 µM and the concentration of [3H]BZDC-PIP₂, photoprobe was held at 0.074 µM. The reaction mixture was equilibrated in Dynatech Immulon™ microtiter wells, irradiated and resolved by SDS–PAGE as described above. The protein gel was fixed in 10% acetic acid, treated with Intensify, dried and exposed to film at −80°C for two weeks. Exposures were quantified using a Bio-Rad Model GS-670 Imaging Densitometer. PI, PI-4-phosphate and PI-(4,5)-bisphosphate were purchased from Echelon Laboratories (Salt Lake City, UT). PI-(3,5)-bisphosphate was synthesized by Dr Jing Peng (Peng and Prestwich, 1998).

Epitope-tagged Shs2p

To permit immunodetection of Ssh2p, we generated a c-myc epitope-tagged version of this polypeptide. Briefly, the SSH2 coding region was fused to the transcriptional and translational control elements of a SECI4 gene that harbors a nucleotide sequence inserted between SECI4 codons 3 and 4 which encodes the c-myc epitope (EQKLISEEDL) plus a KL motif. A unique HindIII site encodes the KL residues that follow the c-myc epitope. This basic cassette is designated PSECI4-myc. PCR technology was employed to engineer the appropriate restriction sites flanking SSH2 to facilitate construction. Primers were designed to create 5′-HindIII and 3′-SphI sites in SSH2 to enable an in-frame cloning of Ssh2p with respect to PSECI4-myc.

These expression plasmids were subsequently employed for purification of Ssh1p and Ssh2p.

Large scale protein preparations were started by seeding 1 l of pre-warmed LB + Ampicillin (100 µg/ml) with a 10 ml overnight culture of the desired clone. Cultures were grown at 37°C for an additional 2 h and induced with a final concentration of 1 mM IPTG (Fisher Scientific, Pittsburgh, PA). Cultures were grown an additional 4–5 h post-induction and cells harvested by centrifugation. Cells were washed in ice-cold lysis buffer (50 mM NaPO₄ (pH 7.4), 300 mM NaCl, 2 mM-2-ME, 0.2% Triton X-100 (v/v) (PMSF) and resuspended in 20 ml of lysis buffer. Lysozyme was added and cells were incubated on ice for 10 min. Glass beads (0.1 mm diameter, Biospec) were added to one half the final volume and samples vortexed for a total of 7 min in 1 min bursts with 1 min rests between each burst. Lysate was serially centrifuged for 10 min at 5000 r.p.m., and twice at 12 000 r.p.m. for 20 min in a Beckman centrifuge using a JA14 rotor. Supernatants were centrifuged for 1 h (100 000 g) to generate clarified protein lysates.

His₆-tagged Ssh1p and Ssh2p were purified as follows. Clarified protein lysates were bound in batch to Ni-NTA resin (1 ml; Qiagen) overnight at 4°C. The resin was washed three times with lysis buffer and resuspended in 5 ml of the same. The resin was packed into a column (Bio-Rad) and proteins eluted using a 0–200 mM imidazole gradient. Column fractions were run on SDS–PAGE gels and proteins visualized by Coomassie Blue staining. Enriched fractions were pooled and protein concentrations determined using a BCA (Pierce) protein detection kit.

Photoaffinity radiolabeling of Ssh1p and Ssh2p with [3H]BZDC-PIP₂

His₆-tagged Ssh1p and Ssh2p were over-produced in E.coli and purified as described above, using 10 mM Tris pH 7.4, 1 mM EDTA and 100 mM NaCl as lysis buffer throughout purification. After purification, enriched fractions were pooled and dialyzed extensively against 10 mM Tris–HCl pH 7.4, 1 mM EDTA. Protein concentration was determined using a BCA assay (Pierce). Protein samples (0.04 nmol) were incubated in 50 µl 10 mM Tris–HCl pH 7.4, 1 mM EDTA, and [3H]BZDC-PIP₂, in a microtiter plate. [3H]BZDC-PIP₂ was used at a concentration of 0.47 µM for the binding studies. Reactions were allowed to equilibrate for 10 min at 0°C before exposure to long range UV light (360 nm) at a distance of 1 cm for 1 h with a lamp power of 30 W. Sample buffer was added to the reactions and proteins were resolved on a 10% SDS–PAGE gel. Gels were fixed and fluorographed with Intensify (Amer sham), dried and exposed to film. Exposures were quantified using a Bio-Rad Model GS-670 Imaging Densitometer. Competitive binding assays were supplemented with PIP₂ (10 µM final concentration). Details of [3H]BZDC-PIP₂ synthesis have been described by Gu and Prestwich (1991).

Expression and purification of His₂-tagged Ssh1p and Ssh2p

To allow expression of full length Ssh1p and Ssh2p, tagged in the N-terminus by six sequential histidine residues, the coding regions of the proteins were subcloned into the plasmid pQE30. Oligonucleotide primers for PCR amplification were designed to fuse to Ssh1p with 5′-BamHI and 3′-SphI sites, and to clamp Ssh2 with 5′-BamHI and 3′-SphI sites. Ssh1A-5′ (5′-GGGACATCCGATACGACCA-CCATTGCTGT-3′) were used to amplify the Ssh1 coding region while Ssh2-A (5′-GGGACATCCCGATACGACCA-CCATTGCTGT-3′) and Ssh2-C (5′-GGGACATCCGATACGACCA-CCATTGCTGT-3′) were used to amplify the Ssh2 coding region. The resulting PCR products were digested with the appropriate restriction enzymes ligated into E.coli vector pTZ18U (Mead et al., 1985) and confirmed for accurate amplification by nucleotide sequence analysis. The corresponding PCR products were subcloned into the BamHI and SphI sites of pQE30 and the resultant plasmids transformed into E.coli strain K2186. The Ssh1p and Ssh2p expression plasmids were designated pRE298 and pRES82, respectively.
were used as forward and reverse primers, respectively, to amplify the SSH2 coding region. The relevant HindIII and Sphl restriction site sequences are underlined, and the SSH2 initiator codon is highlighted in bold. The resulting PCR product was digested with the appropriate restriction enzymes and subcloned into the HindIII and Sphl sites of pTZ18U. The identity of the construct and the fidelity of the amplification reaction was subsequently confirmed by nucleotide sequence analysis. The HindIII/Sphl cassette of SSH2 was then subcloned into the corresponding sites of the P3/Emc2 plasmid pE2E51. The resulting gene fusion results in the expression of an Ssh2p that harbors a 15 residue amino acid extension at the N-terminus of which 10 residues comprise the epitope-tag. This N-terminal extension does not compromise Ssh2p function in vivo or in vitro.

**Generation of mouse anti-Ssh1p serum**

Recombinant His-Ssh1p was expressed in E.coli, purified, dialyzed extensively against PBS and employed as immunogen. Mouse polyclonal antibodies were raised against His-Ssh1p at the University of Alabama at Birmingham HybriDOMA Facility, employing standard methods.

**Subcellular fractionation and extraction of Ssh1p and Ssh2p**

Appropriate strains were grown to mid-logarithmic growth phase in media lacking uracil and were harvested by centrifugation. Whole cell lysate was generated and subjected to a centrifugation regimen. The whole cell lysate was centrifuged at 13 000 g for 15 min to yield supernatant (S13) and pellet (P13) fractions. A portion of the S13 was saved for further analysis while the remaining sample was centrifuged at 100 000 g for 1 h to generate supernatant (S100) and pellet (P100) fractions. Fraction volumes between samples were normalized as a function of cell equivalents with lysis buffer.

Sample buffer was added to the individual sub-cellular fractions and the samples were loaded on a 10% SDS–PAGE gel. The gel was transferred to nitrocellulose for Western blot analysis. Membranes were blocked in 5% dry milk, 1% BSA in TTBS (Tween-20/Tris buffered saline) for 1 h at 37°C. After washing in TTBS for 15 min, primary antibody was added in 1% BSA in TTBS, and blots were incubated for 2 h at 25°C. Blots were then washed with TTBS and incubated with either goat anti-mouse or goat anti-rabbit horseradish peroxidase conjugated antibodies (Bio-Rad) for 2 h at 25°C. Immune complexes were detected by ECL reagents (Amersham).

For extraction experiments yeast whole cell lysates were generated as described above. Whole cell lysate was resuspended in equal volumes of the following and incubated on ice for 45 min: osmotic lysis buffer, 200 mM KCl in lysis buffer, and 1 M KCl in lysis buffer. Samples were centrifuged at 100 000 g for 1 h and supernatant and pellet fractions collected. The fraction loads were normalized for gel-loading on the basis of cell equivalents with lysis buffer.

**Osmotic stress experiments**

CTY 899 (Δsec14/YEpSH2) was grown to mid-logarithmic phase in minimal media lacking uracil and harvested by brief centrifugation. Cell pellets were resuspended in a minimal volume of the same growth medium and equal culture aliquots were adjusted with NaCl or sorbitol to yield final concentrations of 0.3 M NaCl, 0.9 M NaCl, 1 M sorbitol and 1.5 M sorbitol. Cultures were subsequently incubated at 25°C for 30 min with shaking, after which 10 mM NaN₃ was added to poison the cells. The cells were then harvested by centrifugation and whole cell lysates generated as described above. The resulting protein lysates were displayed on a 10% SDS–PAGE gel, transferred to nitrocellulose, and Ssh1p was detected by immunoblotting.

**[^32P]orthophosphate radiolabeling and immunoprecipitation**

A 5 ml culture of a His-Ssh1p-expressing strain (CTY920) was incubated in minimal medium supplemented with 0.5 μCi of [^32P]orthophosphate for 2 h at 30°C with shaking. Clarified cell-free extracts were prepared and proteins bound to Ni²⁺-NTA resin by standard methods. Bound proteins were eluted with a 0–200 mM imidazole gradient, and fractions enriched in Ssh1pHis6 were identified by immunoblotting, and pooled. The pooled fractions were dialyzed against PBS and concentrated in dialysis tubing placed on a bed of dry PEG 8000 at 4°C. Concentrated protein was diluted to a final volume of 1 ml in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) and Ssh1p immunoreactive species were precipitated with polyclonal rabbit anti-Ssh1p immunoglobulin and protein G-agarose (Boehringer Mannheim) according to standard procedures (Harlow and Lane, 1988). Immunoprecipitated proteins were resolved by SDS–PAGE and transferred to PVDF membrane. Radiolabeled species were then visualized by autoradiography using Kodak XAR-5 film.

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## References


Novel Sec14p homologs from soybean


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