Analysis of oxysterol binding protein homologue Kes1p function in regulation of Sec14p-dependent protein transport from the yeast Golgi complex

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Oxysterol binding proteins (OSBPs) comprise a large conserved family of proteins in eukaryotes. Their ubiquity notwithstanding, the functional activities of these proteins remain unknown. Kes1p, one of seven members of the yeast OSBP family, negatively regulates Golgi complex secretory functions that are dependent on the action of the major yeast phosphatidylinositol/phosphatidylcholine Sec14p. We now demonstrate that Kes1p is a peripheral membrane protein of the yeast Golgi complex, that localization to the Golgi complex is required for Kes1p function in vivo, and that targeting of Kes1p to the Golgi complex requires binding to a phosphoinositide pool generated via the action of the Pik1p, but not the Stt4p, PtdIns 4-kinase. Localization of Kes1p to yeast Golgi region also requires function of a conserved motif found in all members of the OSBP family. Finally, we present evidence to suggest that Kes1p may regulate adenosine diphosphate-ribosylation factor (ARF) function in yeast, and that it may be through altered regulation of ARF that Kes1p interfaces with Sec14p in controlling Golgi region secretory function.

Introduction

Oxysterol binding proteins (OSBPs)* define a large protein family whose members are found in mammals (Dawson et al., 1989), Drosophila (Alphey et al., 1998), C. elegans (Caenorhabditis elegans Sequencing Consortium, 1998), and yeast (Jiang et al., 1994; Fang et al., 1996; Beh et al., 2001). Initial studies suggested that OSBPs mediate the potent downregulation of sterol biosynthesis by oxysterols (Taylor et al., 1984; Dawson et al., 1989). Although evidence consistent with this hypothesis was culled from genetic experiments in yeast and mammalian cells (Jiang et al., 1994; Lagace et al., 1997; Beh et al., 2001), other evidence indicates that OSBPs are not direct mediators of oxysterol signaling (Brown and Goldstein, 1999). Rather, these proteins have other functions that interface with membrane trafficking from the Golgi complex (Fang et al., 1996), progression of cells through the cell cycle (Alphey et al., 1998), and tumor metastasis (Fournier et al., 1999).

Yeast express seven OSBPs. Three (Osh1p, Osh2p, and Osh3p) are classified as long OSBPs, typified by the mammalian OXYB, where a large extension is found N-terminally to the 430 amino acid oxysterol binding homology domain (Fig. 1 A). The remaining four are homologous to the oxysterol binding domain of OXYB and are classified as short OSBPs (Fig. 1 A). None of the yeast OSBP genes individually plays an essential function, but deletion of all seven OSBP genes results in inviability (Jiang et al., 1994; Fang et al., 1996; Levine and Munro, 1998; Beh et al., 2001).

Sec14p is the major yeast PI/PC-transfer protein (PITP) (Bankaitis et al., 1990; Kearns et al., 1998a; Li et al., 2000b). Analyses of mutations that allow yeast to grow in the absence of exogenously provided sterols indicate that Sec14p functions as a phosphatidylinositol-phosphatidylcholine allomixotrophic phosphatidylcholine phosphatidylinositol transfer protein (PITP) (Lagace et al., 1997; Beh et al., 2001). These PITP functions are not independent and Sec14p is also required for the function of the major yeast PI/PC-transfer protein (PITP) (Bankaitis et al., 1990; Kearns et al., 1998a; Li et al., 2000b). Analyses of mutations that allow yeast to grow in the absence

Key words: Kes1p; phosphoinositides; yeast Golgi; Sec14p; ARF

*Abbreviations used in paper: ARF, adenosine diphosphate-ribosylation factor; ARFGAP, ARF guanosine triphosphatase activating protein; BZDC, (β-benzoyldihydrocinnamidyl)-amino)propyl; GFP, green fluorescent protein; IP$_3$, inositol-1,4,5-trisphosphate; MALDI-TOF, matrix-assisted laser ionization coupled with time-of-flight mass analysis; OSBP, oxysterol binding protein; PA, phosphatidic acid; PC, phosphatidylcholine; PH, Pleckstrin homology; PI, phosphatidylinositol; PIP, phosphoinositide; PLD, phospholipase D.

Key words: Kes1p; phosphoinositides; yeast Golgi; Sec14p; ARF

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Results

Kes1p functional domains

We independently recovered 51 kes1 loss-of-function mutations in a “bypass Sec14p” mutant screen (Cleves et al., 1991b; Fang et al., 1996). Of these, nine sustain wild-type levels of Kes1p. Gap repair experiments, coupled with DNA sequence determinations, revealed these nine mutations define six alleles (Fig. 2 A). Four represent a GAA → AA mutation that substitutes lysine for glutamate at Kes1p residue 312 (E312K). One represents a double mutation that converts codons 201 and 202 from TTAGCAT to TTCAAT, and substitutes phenylalanine-asparagine for leucine-histidine (LH201/202FN).

The sequences surrounding P30T, S43Y, and LH201/202FN revealed no informative homologies. However, clues were gleaned from regions surrounding the E312K, E107K, and K109A substitutions. E312K lies within a domain that exhibits features of Pleckstrin homology (PH) domains. Although this region is not scored as a PH domain by various search programs, it includes the signature invariant tryptophan (W317) that is positioned downstream of a GIL motif (Kes1p residues 205–207). PH domains are often bounded by a GXL motif at the NH2 terminus, and the signature tryptophan is positioned ~110 residues downstream of that motif (Lemmon, 1999). Alignment of the putative Kes1p PH domain (residues 180–330) with known PH domains is shown (Fig. 2 B). Although proof that this domain conforms to the PH domain fold awaits structural analyses, for purposes of convenience we will refer to this domain as the Kes1p PH domain.

BLAST analyses also identify a motif (Kes1p residues 108–149) that is present only in OSBPs and is highly conserved among them (Fig. 2 C). This OSBP domain consists of two 11-residue motifs separated by a linker of ~20 divergent residues. Two of the kes1 alleles alter residues adjacent to (E107K), or within (H143Y), this domain.

Kes1p is a PIP binding protein

As PI(4,5)P2 and other PIPs are common ligands for PH domains, we assessed binding of purified Kes1p to
PI(4,5)P₂ or its soluble headgroup inositol-1,4,5-trisphosphate (IP₃). To this end, [³H]-BZDC-PI(4,5)P₂ and [³H]-BZDC-IP₃ were used as photo-affinity ligands (Dorman and Prestwich, 1994; Prestwich, 1996; Prestwich et al., 1997; Chaudhary et al., 1998a,b; Feng et al., 2001). The [³H]-BZDC-PI(4,5)P₂ binding experiments were performed in a mixed micelle system where the photo-affinity ligand was a trace component. Thus, the photolabeling assay requires Kes1p to interact with photo-probe monomers in the face of large detergent excess. Although Sec14p fails to bind either probe (unpublished data), Kes1p binds both [³H]-BZDC-PI(4,5)P₂ and [³H]-BZDC-IP₃ avidly, as indicated by recovery of appropriate covalent adducts (Fig. 3 A). The intensities of Kes1p photolabeling were similar to those scored for the PIP binding protein gelsolin.

Kes1p-[³H]-BZDC-IP₃ solution binding experiments also impose rigorous constraints on binding. Both photo-probes are displaced from Kes1p by challenge with excess unlabeled PI(4,5)P₂ or IP₃ competitor. However, an 83-fold molar excess of unlabeled PI(4,5)P₂ displaces both photo-probes, whereas a 10³-fold molar excess of IP₃ is required for displacement of [³H]-BZDC-PI(4,5)P₂ (Fig. 3 A). These data suggest that Kes1p has a 10³-fold higher affinity for PI(4,5)P₂ than it does for IP₃.

To quantify Kes1p affinity for PI(4,5)P₂, we determined the concentration of competitor required for 50% displacement of [³H]-BZDC-PI(4,5)P₂ from Kes1p (IC₅₀). Although photoaffinity labeling is a nonequilibrium process (Dorman and Prestwich, 1994) and cannot directly give equilibrium dissociation constants, displacement by competing ligands yields a rank order of relative affinities. A dose-dependent reduction in photolabeling efficiency was observed when [³H]-BZDC-IP₃ was employed as competitor (Fig. 3 B). The IC₅₀ for PI(4,5)P₂ is 1.9 μM with an estimated Kᵣ = 2.5 μM for Kes1p-[³H]-BZDC-PI(4,5)P₂ binding. These values resemble those measured for PI(4,5)P₂ and [³H]-BZDC-PI(4,5)P₂ binding by PLC-δ₁ (Tall et al., 1997; Lemmon, 1999).

**Kes1p PH domain is sufficient for PIP binding**

We tested whether the Kes1p PH domain represents the PI(4,5)P₂ and IP₃ binding module. Indeed, a 144 amino acid Kes1p fragment that consists largely of the PH domain (residues 171–314) is sufficient for PI(4,5)P₂ and IP₃ binding (Fig. 3 C). A 110 amino acid region of Kes1p (residues 205–314) that defines the putative Kes1p PH domain (Fig. 2 B) also binds IP₃ photo-probe (unpublished data). Neither a smaller region of Kes1p (residues 205–309) that fully overlaps this domain, nor a Kes1p domain (residues 220–330) that incompletely overlaps this domain, nor a Kes1p domain (residues 205–330) that incompletely overlaps this domain, exhibits any inositide binding activity (unpublished data). Interestingly, the minimum Kes1p inositol binding module does not include W317, the signature residue of PH domains.

**Specificity of PIP binding**

To characterize specificity of Kes1p binding to PIPs, we employed a competitive displacement strategy using a variety of binding substrates in a Kes1p-[³H]-BZDC-PI(4,5)P₂ photolabeling assay. At 400-fold molar excess, all PIPs tested and other acidic phospholipids (phosphatidylserine, phosphatidic acid, and cardiolipin) displaced [³H]-BZDC-PI(4,5)P₂ (Fig. 3 D). Other lipids (phosphatidylethanolamine, phosphatidylcholine, ceramide, and diacylglycerol) were ineffective. Soluble inositol-polypolyphosphates (IP₃, IP₄, IP₅) also failed to displace [³H]-BZDC-PI(4,5)P₂ from Kes1p (unpublished data). When unlabelled competitor lipids were reduced to a 10-fold molar excess relative to [³H]-BZDC-PI(4,5)P₂, only PI(4,5)P₂ displaced photo-probe. PI, PI(4)P, phosphatidic acid (PA), and other acidic phospholipids were ineffective (unpublished data). A 10-fold molar excess of PI(3,4,5)P₃, PI(3,4)P₂, or PI(3,5)P₂ also failed to displace [³H]-BZDC-
Thus, PI(4,5)P\(_2\) (Fig. 3 D). Thus, PI(4,5)P\(_2\) is the preferred Kes1p ligand in vitro.

**Mutations in the Kes1p PH domain influence PIP binding**

Since the Kes1p PH domain binds PI(4,5)P\(_2\), we investigated whether integrity of this domain is required for PIP binding. To this end, we characterized the binding properties of Kes1p\(_{E312K}\) and Kes1p\(_{W317A}\) (Kes1p\(_{W317A}\)) using the photolabeling assay described above. In neither case could we detect binding of full-length mutant protein to \([^3H]BZDC-PI(4,5)P_2\) (Fig. 4 A) or to \([^3H]BZDC-IP_3\) (unpublished data). In vivo complementation experiments reveal that Kes1p\(_{E312K}\) and Kes1p\(_{W317A}\), while stable proteins in yeast, are nonfunctional (unpublished data).

**Suppression of PIP binding defects by COOH-terminal truncation of Kes1p**

During the course of the photolabeling experiments, we noted that Kes1p\(_{W317A}\) purifies as two species. Both species bind polyclonal anti-Kes1p immunoglobulin, suggesting that the smaller species represents a Kes1p fragment. Surprisingly, this truncated Kes1p (Kes1p\(_{W317A}\)) binds PI(4,5)P\(_2\), whereas full-length Kes1p\(_{W317A}\) does not (Fig. 4 A). Thus, removal of COOH-terminal Kes1p sequences rescues PIP binding by Kes1p\(_{W317A}\). Although Kes1p\(_{W317A}\) was most prominent in exhibiting this degradation product, preparations of wild-type Kes1p also contain detectable amounts of such a degradation product. This fragment also retains PI(4,5)P\(_2\) binding capability (unpublished data).
Eleven cycles of Edman degradation indicate a sequence of MRGSH6M for the Kes1pW317A* NH2 terminus, and this sequence corresponds to the NH2-terminal sequence of the His6-tagged Kes1p produced in *Escherichia coli* (see Materials and methods). Consequently, the proteolytic event that generates Kes1pW317A* must remove a COOH-terminal Kes1p domain.

The site of Kes1p proteolysis that generates Kes1pW317A* was determined. Matrix-assisted laser ionization coupled with time-of-flight mass analysis (MALDI-TOF) analyses yield a mass of 50.663 kD for Kes1p and 36.838 kD for Kes1pW317A* (Fig. 4 B). These data assigned the Kes1pW317A* cleavage site to the amide bond of a dibasic motif comprised of Kes1p residues R314 and K315, indicating that Kes1pW317A* is devoid of the COOH-terminal 120 residues (Fig. 4 B). Interestingly, this cleavage site lies upstream of the W317A substitution. Thus, Kes1pW317A* represents a proteolytic fragment consisting solely of wild-type Kes1p primary sequence.

To confirm this result, we purified GST-tagged versions of full-length Kes1p and of a protein fragment generated by engineering a Kes1p truncation immediately following residue R314. This truncation product is designated Kes1p(1–314). Consistent with the Kes1pW317A* data, Kes1p(1–314)
binds [3H]BZDC-IP3 with an affinity that is similar to that exhibited by Kes1p itself (Fig. 4 C). We also tested whether the COOH-terminal truncation restores PIP binding to the mutant Kes1pE312K. This experiment was of interest for two reasons. First, it tests the allele specificity of the suppression effect elicited by the COOH-terminal truncation mutation. Second, because E312K is retained in the truncated product (unlike the W317A case where the fragment consists of wild-type Kes1p sequence), this experiment tests whether E312K imposes an intrinsic PIP binding defect on Kes1p.

Photolabeling data demonstrated that Kes1pE312K binds PIPs, whereas full-length Kes1pE312K cannot (Fig. 4 C), indicating deletion of the COOH-terminal 120 Kes1p residues rescues both Kes1pE312K and Kes1pW317A PIP binding defects. Thus, neither E312 nor W317 are intrinsically essential for PIP binding. Rather, COOH-terminal Kes1p sequences inhibit PIP binding by the Kes1p PH domain.

**Kes1p mutants intrinsically defective in PIP binding are nonfunctional**

The data indicate that Kes1pE312K and Kes1pW317A exhibit PIP binding defects of a regulatory nature. This complicates conclusions that can be drawn regarding the significance of PIP binding for Kes1p activity from functional analyses of Kes1pE312K and Kes1pW317A alone. To more directly assess the functional significance of PIP binding by Kes1p, we generated Kes1p derivatives with intrinsic defects in PIP binding. Our criteria for such mutants was that these will fail to bind inositide photo-probe, and that these binding failures will not be rescued by COOH-terminal Kes1p truncation.

To generate mutant Kes1p intrinsically defective in PIP binding, we were guided by structural analyses indicating that basic residues in the PH domain variable loop 1 and 2 regions engage inositide headgroups (Lemmon, 1999). We generated a triple mutant Kes1p (Kes1p3E) where residues R236, K242, and K243 in the presumed loop 2 region of the Kes1p PH domain were replaced with glutamate (see Fig. 2 B). Kes1p3E fails to bind photo-probe in vitro, and truncation of Kes1p3E distal to residue K314 fails to restore PIP binding (Fig. 4 D). Thus, Kes1p3E is intrinsically defective in PIP binding.

The biochemical PIP binding defect translates to loss of Kes1p activity in yeast. Complementation analyses demonstrate that Kes1p3E is nonfunctional in vivo (Fig. 4 D). This defect is manifested even though Kes1p3E is a stable cellular protein that accumulates to steady-state levels that are several-fold greater than those of wild-type Kes1p. Thus, PIP binding is required for Kes1p function in vivo.

**Kes1p OSBP domain is nonessential for PIP binding**

To investigate the OSBP motif in detail, we performed alanine-scanning mutagenesis throughout the OSBP domain (Fig. 5 A). Complementation experiments show that Kes1pH143/144AA, Kes1pE109A, and Kes1pE117A are nonfunctional in vivo (Fig. 5 B), as are Kes1pH143/144AA and Kes1pE117A. All of these proteins are expressed as stable polypeptides in cells (unpublished data). Thus, the OSBP domain is critical for Kes1p function in yeast. Photolabeling experiments demonstrate that OSBP domain mutants avidly bind photo-probe in a manner that is subject to competitive displacement by unlabeled PI(4,5)P2 competitor (Fig. 5 A). These data show that the OSBP domain is not required for PI(4,5)P2 binding as scored by the photolabeling assay.

Other mutant forms of Kes1p (S43Y, E107K, LH201/202FN) harbor unaltered PH and OSBP domains, yet are nonfunctional in vivo (Fig. 2 A). We find that Kes1pH143/144AA, Kes1pE109A, and Kes1pE117A are all proficient in [3H]BZDC-PI(4,5)P2 binding. Moreover, bound photo-probe is efficiently displaced by the incorporation of excess unlabeled PI(4,5)P2 into the photolabeling assay (Fig. 5 C). Competitive displacement assays using purified Kes1pH143/144AA revealed an IC50 of photo-probe displacement of 6.1 µM when PI(4,5)P2 was employed as competitor (unpublished data), a value that resembles the IC50 of 1.9 µM re-
corded for wild-type Kes1p (Fig. 3 B). Thus, these mutant Kes1ps bind PI(4,5)P₂ with affinities similar to that measured for Kes1p (Kₑᵤ = 8.0 µM vs. 2.5 µM, respectively). We conclude that PIP binding is necessary, but insufficient, for Kes1p function in vivo.

**Kes1p localizes to Golgi membranes**

Fractionation studies revealed that Kes1p resides in both soluble and membrane-bound pools (Fang et al., 1996). To localize Kes1p more precisely, a YCp(KES1-YFP) plasmid was constructed that drives physiological expression levels of a chimera consisting of Kes1p fused via its COOH terminus to the green fluorescent protein (GFP) NH₂ terminus. This chimera is both stable and functional (unpublished data). To assess Kes1p-GFP distribution, appropriate yeast strains were cultured to midlogarithmic growth phase in minimal medium at 26°C and cells were imaged. As control, we also monitored localization of GFP expressed from an isogenic YCP plasmid carrying a KES1 promoter cassette. Whereas GFP is distributed diffusely throughout the cytosol, Kes1p-GFP adopts both a diffuse cytosolic location and localization to 3–10 punctate structures dispersed throughout the cytoplasm (Fig. 6).

The punctate component of Kes1p-GFP staining primarily corresponds to a Golgi membrane–associated Kes1p-GFP pool. We observed that ~85% of the Kes1-GFP–positive structures coexist with the yeast Golgi complex marker Kes2p-RFP (Fig. 6). The remaining 15% of the Kes1-GFP–positive structures coexist with the fluorescent dye FM4-64 under conditions that trap this lipophilic molecule in early endosomes.

**Localization of Kes1p to Golgi membranes requires PIP binding**

Previous reports had documented that PIP binding is necessary and sufficient for localization of long OSBPs to target membranes (Levine and Munro, 1998). To determine whether PIP binding plays the same role in targeting of short OSBPs, representative mutations in the Kes1p PH domain were introduced into a YCp(KES1-GFP) expression cassette. Localization of mutant Kes1p-GFP proteins was then monitored in living cells by fluorescence microscopy. The excitatory filters employed for visualization of GFP/YFP and RFP fluorescence were the quinacrine mustard filter (excitation wavelength 495 nm) and rhodamine filter (excitation wavelength 545 nm), respectively. Images were collected by IP Lab 2.0 software and processed with Adobe Photoshop 5.0. Arrows identify some examples of colocalization.

**Defects in PI-4-P synthesis compromise Kes1p localization to Golgi membranes**

To test whether defects in PIP synthesis release of Kes1p from Golgi membranes, we expressed Kes1p-GFP in strains carrying ts alleles of each of the two known yeast phosphatidylinositol (PI) 4-kinases (Pik1p and Str4p). As described above, Kes1p-GFP adopts a characteristic punctate localization pattern in wild-type cells incubated at 26°C (Fig. 7 B). By contrast, Kes1p-GFP redistributes from Golgi membranes to the cytosol in the pik1-101ts mutant incubated at either 26°C or 37°C (Fig. 7 B). Thus, Pik1p, which localizes to Golgi membranes (Walch-Solimena and Novick, 1999), catalyzes synthesis of a PIP pool, PI(4)P and/or PI(4,5)P₂, that is required for localization of Kes1p to yeast Golgi membranes. Kes1p mislocalization in the pik1-101ts strain at 26°C indicates that this pik1ts allele is quite defective even at permissive temperatures, a result in accord with the potent synthetic lethality exhibited by pik1ts in combination with a number of other mutations that compromise secretory pathway function (Walch-Solimena and Novick, 1999). By contrast, inactivation of Str4p at 37°C results in some mislocalization of Kes1p to cytosol at 26°C (Fig. 7 B). Relative to pik1-101ts, these effects are modest. Thus, Str4p–derived PIPs play more minor roles in Kes1p targeting to Golgi.
membranes than do Pik1p-derived PIPs. To distinguish whether PI(4)P or PI(4,5)P2 is a Golgi region ligand for Kes1p, we assessed Kes1p-GFP localization in mutants inactivated for Mss4p, the yeast PI(4)P 5-kinase. Even long shift of mss4-2ts mutants to restrictive temperatures (37°C) fails to compromise Kes1p-GFP association with Golgi membranes (Fig. 7 B).

We also tested whether inactivation of the Golgi complex-associated Sec14p affects Kes1p localization. Sec14p stimulates PIP synthesis in a variety of in vitro systems (Hay and Martin, 1993; Cunningham et al., 1996; Jones et al., 1998). Moreover, Sec14p stimulates PI(4)P synthesis in yeast (Hama et al., 1999; Phillips et al., 1999). Kes1p localization is not compromised in sec14-1ts strains at 26°C. Shift of the sec14-1ts strain to 37°C for 30 min reduces, but does not abolish, Kes1p association with Golgi membranes (Fig. 7 B).

**Localization of Kes1p to Golgi membranes requires a functional OSBP domain**

Analyses of mutants compromised for OSBP domain function demonstrate that an intact OSBP domain is also essential for proper localization of Kes1p-GFP in living cells. Imaging experiments show that the Kes1pK109A-GFP chimera adopts a diffuse cytosolic distribution with no visible concentration in punctate structures (Fig. 7 C). Kes1p-GFP chimeras with mutant OSBP domains (i.e., Kes1pK109AAA-A-GFP, Kes1pK1115A/G1116AA-A-GFP, and Kes1pV141/142AA-A-GFP) also exhibit exclusively cytosolic staining profiles (unpublished data). Of interest is the allele specificity that underlies these localization defects. E107K represents a missense substitution at a position only two residues upstream of K109A. Yet, Kes1pE107K does not mistarget to the cytosol (unpublished data), whereas Kes1pK109A does. Finally, mutations that release Kes1p-GFP to the cytosol are not limited to the OSBP domain. For example, LH201/202FN also has this effect (unpublished data). We conclude the region bounded by Kes1p residues 109–202 is critical for the Kes1p localization to membranes, and that the OSBP domain is an essential component of this membrane targeting information.

Because Kes1ps with defective OSBP domains bind PIPs, we tested whether this domain contributes to Golgi region targeting by binding another lipid. To this end, we determined whether the phospholipase D (PLD)-driven accumulation of PA that accompanies Sec14p inactivation is re-

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**Figure 7. Kes1p targeting to yeast Golgi membranes.** (A) Kes1p localization domains. YCp(GFP), YCp(KES1-GFP) and mutant YCp(kes1E-GFP), YCp(kes1E312K-GFP), YCp(kes1W317A-GFP), and YCp(kes1K109A-GFP) plasmids were introduced into a kes1 yeast strain. Fluorescence images were captured at 26°C. The kes1E-GFP and chimeras exhibit a diffuse cytosolic distribution that mimics that of GFP alone. In these three cases, signal is excluded from vacuoles (the dark circular structures). The wild-type (WT) control Kes1p-GFP and the mutant Kes1pE312K-GFP and Kes1pW317A-GFP chimeras exhibit typical punctate staining patterns. (B) PIP synthesis and Kes1p localization. A YCp(KES1::GFP) plasmid was introduced into wild-type, pik1-101ts, stt4ts, sec14-1ts, and mss4-2ts strains as indicated at right. Fluorescence images were captured at 26°C and after shift to 37°C for 2 h (indicated at top) at an excitation wavelength of 495nm. Pik1p defects exert major defects in Kes1p localization to Golgi membranes, whereas inactivation of Stt4p and Sec14p has more modest effects.
required for Kes1p localization to the yeast Golgi region. This effort was motivated by the weak binding of Kes1p to PA monomers (see above). PLD deficiency markedly reduces Golgi region/endosomal PA levels in Sec14p-deficient yeast (Li et al., 2000a), but has no effect on Kes1p localization in Sec14p-deficient strains (unpublished data). In accord with our previous findings that the involvement of Kes1p in yeast Golgi region function is independent of metabolic flux through the yeast sterol biosynthetic pathway (Fang et al., 1996), we also find that mutations compromising sterol biosynthesis (e.g., er6) do not result in obvious mislocalization of Kes1p from Golgi membranes (unpublished data).

Relationship between magnitude of Kes1p overexpression required for compromise of “bypass Sec14p” and PI(4)P levels

Since genetic data indicate Kes1p antagonizes activity of the Sec14p pathway for Golgi region secretory function, we overexpressed Kes1p in “bypass Sec14p” mutant strains. In agreement with previous demonstrations (Fang et al., 1996), all mechanisms for “bypass Sec14p” are sensitive to increased Kes1p gene dosage (Fig. 8 A). Kes1p is unique in this respect. Increased dosage of structural genes for enzymes of the CDP-choline pathway (i.e., CKI1, PCT1, and CPT1) or the Sac1p PIP phosphatase (SAC1) have no effect on nonallelic mechanisms for “bypass Sec14p” (unpublished data). Although modest overproduction of Kes1p compromises all other mechanisms of “bypass Sec14p”, robust overexpression of Kes1p is required for this effect in sac1 mutants (Fig. 8 A).

Because sac1 mutants are unique among “bypass Sec14p” mutants in their massive overproduction of PI(4)P (Guo et al., 1999; Rivas et al., 1999; Stock et al., 1999), there is a correlation between PI(4)P levels and the magnitude of Kes1p overproduction required to abolish “bypass Sec14p”.

To investigate whether the Kes1p levels required to abolish “bypass Sec14p” in sac1 mutants are proportional to PI(4)P levels, we investigated the relationship between magnitude of Kes1p overexpression required for compromise of “bypass Sec14p” and PI(4)P levels. (A) Distinct sensitivities of different mechanisms for “bypass Sec14p” to increased Kes1p dosages. Kes1p was modestly overexpressed by introduction of YCp (KES1), or highly overexpressed by introduction of YEplac119::KES1, which drives Kes1p expression from a powerful PGM promoter (see Materials and methods). All mechanisms of “bypass Sec14p” except that mediated by sac1 are sensitive to modest increases (two- to threefold) in Kes1p levels. A high degree (~20-fold) of Kes1p is required for compromise of “bypass Sec14p” in sac1 mutants. (B) Structure of Sac1pΔTM. The first 462 codons of Sac1p, which include the catalytic domain but not the transmembrane domain, were fused to a protein A tag. This construct is designated Sac1pΔTM and its expression is under control of the native SAC1 promoter. Details of the construction are available from the authors by request. (C) PI(4)P levels as a function of Sac1pΔTM expression. A wild-type SAC1 strain, a sac1 mutant strain, and isogenic sac1 derivatives transformed either with YCp(sac1ΔTM) or YEplac119::sac1ΔTM were pulse radiolabeled for 20 min at 26°C with [32P]-orthophosphate (10 μCi/ml), phospholipids were extracted under acidic conditions, lipids were resolved by two-dimensional paper chromatography, and PI(4)P was identified and quantified by phosphorimaging (Rivas et al., 1999). PI(4)P is expressed as a percentage of total extractable phospholipid. Relevant strain genotypes are indicated. The various strains incorporated similar amounts of [32P]-radiolabel into total lipid. Data are expressed as the mean ± standard deviation from at least three independent experiments. (D) Sac1pΔTM expression sensitizes sac1-mediated “bypass Sec14p” to Kes1p. Isogenic sac1 and sac1/YEp(sac1ΔTM) yeast strains were transformed with YCp(KES1). Transformants were subsequently isolated and streaked for isolated colonies on selective medium at 37°C. Growth was scored after 48 h of incubation. Relevant genotypes are given.
levels, we expressed in *sac1* mutants a truncated Sac1p. This truncated Sac1pΔTM contains the PIP phosphatase catalytic domain but lacks the COOH-terminal transmembrane domain (Fig. 8 B). Sac1pΔTM is a partially functional protein as it partially alleviates the derangement of PIP metabolism that characterizes *sac1* mutants (Fig. 8 C). As shown previously, PI(4)P constitutes 17.8 ± 0.8% and 3.6 ± 0.6% of extractable phospholipid in *sac1* mutants and wild-type yeast, respectively (Guo et al., 1999; Rivas et al., 1999; Stock et al., 1999). By comparison, PI(4)P levels are markedly reduced to 8.1 ± 1.2% in the *sac1* mutant that expresses Sac1pΔTM from YEp(*sac1*ΔTM) (Fig. 8 C). Expression of Sac1pΔTM from a YCp(*sac1*ΔTM) vector effects only minor reductions in PI(4)P levels in a *sac1* genetic background (15.3 ± 0.5%).

The reduction in PI(4)P recorded in the YEp(*sac1*ΔTM) strain corrects the inositol auxotrophy and es growth phenotypes of *sac1* mutants, but fails to ablate *sac1*-mediated “bypass Sec14p” (unpublished data). However, Sac1pΔTM-mediated reduction in PI(4)P levels strongly sensitizes the “bypass Sec14p” phenotype of *sac1* mutants to elevated Kes1p. Whereas large increases in KES1 dosage are required for compromise of “bypass Sec14p” in *sac1* mutants, modest increases in KES1 dosage ablate “bypass Sec14p” in *sac1* YEp(*sac1*ΔTM) strains (Fig. 8 C). Thus, the magnitude of Kes1p overproduction required for compromise of “bypass Sec14p” is proportional to cellular PI(4)P levels.

**Kes1p is mislocalized in *sac1* mutants**

Based on analyses of PI(4)P levels in *sac1* mutants bearing pik1<sup>es</sup> and stt4<sup>es</sup> mutations, it is clear that the accumulated PI(4)P is predominantly synthesized via the Straitp PI 4-kinase and not the Pik1p kinase (Nemoto et al., 2000; Foti et al., 2001). Because Pik1p is the Golgi region–localized PI 4-kinase (Walch-Solimena and Novick, 1999) and PI(4)P is likely the physiological ligand for Kes1p, we assessed the localization of Kes1p-GFP in *sac1* mutants. As shown in Fig. 9 A, the intracellular profile of Kes1p-GFP distribution in *sac1* mutants is dramatically different from its normal punctate distribution in wild-type strains. Rather, Kes1p-GFP localizes to a few large patches in *sac1* cells that are frequently located in a juxtanuclear position. This altered Kes1p localization pattern does not reflect abnormal *sac1* Golgi region morphology. Distribution of the Golgi complex marker Kex2p is not altered in *sac1* mutants (Fig. 9 B). Rather, consistent with the significant (if not exclusive) localization of a Sac1p to ER compartments in yeast and mammalian cells (Whitters et al., 1993; Nemoto et al., 2000; Foti et al., 2001), we conclude that Kes1p is mistargeted to what are likely ER membranes in *sac1* mutants. In support of this idea, the Kes1p PH domain is required for this mislocalization as Kes1p<sup>3E-GFP</sup> retains its cytosolic localization in *sac1* mutants, and fusion of the Sac1p PIP phosphatase catalytic domain to the ER-resident protein Sec61p yields a fully functional Sac1p whose expression complements all
sac1Δ phenotypes (including the “bypass Sec14p” phenotype [unpublished data]).

Genetic interactions of kes1Δ with arf1Δ, gcs1Δ, and pik1-101ts
To further understand how Kes1p functions in yeast, we screened for instructive genetic interactions. The phenotypes associated with Kes1p overproduction in many ways mimic those associated with reduced PLD activity (Xie et al., 1998), suggesting that Kes1p is an inhibitor of PLD in yeast. However, our in vivo experiments indicate PLD activity is not sensitive to high levels of Kes1p (unpublished data). Rather, we observed a linkage between Kes1p function and the yeast ARF cycle. Both arf1Δ and gcs1Δ alleles abrogate kes1Δ-mediated “bypass Sec14p”. Although an sec14-1ts kes1Δ mutant cannot grow at 37°C, the sec14-1ts kes1Δ derivative grows at wild-type rates. However, the isogenic sec14-1ts kes1Δ arf1Δ and sec14-1ts kes1Δ gcs1Δ triple mutants are no longer viable at 37°C (Fig. 10 A). This is not the result of more complex genetic interactions between kes1Δ and arf1Δ or gcs1Δ as introduction of SEC14 into sec14-1ts kes1Δ arf1Δ and sec14-1ts kes1Δ gcs1Δ mutants restores viability at 37°C (unpublished data). Gcs1p is an ARFGAP, and it is this biochemical activity that is required for kes1-mediated “bypass Sec14p” (Yanagisawa, L., and V.A. Bankaitus, unpublished data).

Second, we recorded a genetic interaction between kes1Δ and a mutation that compromises activity of the Pik1p PI 4-kinase. Yeast strains carrying pik1-101ts grow at 26°C, but not at 35°C or 37°C (Walch-Solimena and Novick, 1999; Fig. 10 B). A kes1Δ allele clearly improves the growth of pik1-101ts strains at 35°C. In a serial dilution array incubated at 35°C, the pik1-101ts mutant forms colonies only in the undiluted culture inoculum. By contrast, the wild-type control exhibits growth in undiluted and low dilution inocula; i.e., single colonies are scored in regions spotted with aliquots of 1,000-fold dilutions of culture sample. Thus, relative to wild-type, there is an ~1,000-fold reduction in the viability of pik1-101ts mutants cultured at 35°C. The kes1Δ pik1-101ts mutant exhibits a growth profile intermediate between those of wild-type and pik1-101ts strains (Fig. 10 B). Growth was recorded in the inoculum of a 10-fold culture dilution. Thus, kes1Δ increases the viability of pik1-101ts mutants some 10-fold at 35°C. This effect is not observed at 37°C, indicating kes1Δ exerts only partial suppression of pik1-101ts defects (unpublished data). Nonetheless, this effect is specific by two criteria. First, kes1Δ allele has no beneficial effect on the growth of yeast strains defective in Str4p (unpublished data). Second, no other “bypass Sec14p” alleles (including sac1 alleles) suppress pik1-101ts-associated growth or secretory defects at 35°C (unpublished data).

Genetic interactions of kes1Δ with ARF and ARFGAP deficiencies
Kes1p deficiency elicits a “bypass Sec14p” that, like all “bypass Sec14p” phenotypes, requires Gcs1p ARFGAP activity (Yanagisawa, L., and V.A. Bankaitus, unpublished data). Our finding that Kes1p overproduction mimics Gcs1p defects in “bypass Sec14p” mutants further supports the possibility that Kes1p influences regulation of the ARF cycle. Therefore, we tested whether kes1Δ suppresses phenotypes associated with Gcs1p or ARF dysfunction. Sodium fluoride (NaF) sensitivity is associated with defects in the yeast ARF cycle (Zhang et al., 1998). Gcs1p-deficient mutants are sensitive to NaF, as are arf2Δ strains carrying the arf1-3Δ mutation (Zhang et al., 1998; Table I). Based on the results described above, we predicted that Kes1p defects might suppress NaF sensitivity in gcs1Δ and arf1-3 arf2Δ strains. Although both gcs1Δ and arf1-3 arf2Δ mutants fail to grow in the presence of 30 and 50 mM NaF, isogenic wild-type strains are NaF resistant (Table I). However, kes1Δ suppresses Gcs1p and ARF1p deficiency in this assay as isogenic kes1Δ gcs1Δ and kes1Δ arf1-3 arf2Δ mutants grow when challenged with either 30 or 50 mM NaF (Table I). Serial dilution experiments indicate that the viability of gcs1Δ strains is increased ~1,000-fold relative by kes1Δ.

This is not a trivial result of Kes1p defects reducing either the permeability of cells to NaF or the capacity of yeast to accumulate NaF as kes1Δ does not increase the threshold of yeast tolerance to NaF. Moreover, kes1Δ only modestly influences other phenotypes associated with Gcs1p defects (e.g., sensitivity to hyperosmotic stress). The viability of gcs1Δ mutants is reduced ~10-fold relative to that of isogenic gcs1Δ kes1Δ mutants (unpublished data). Zhang et al. (1998) reported that overproduction of any one of several ARFGAPs, including Gcs1p, suppresses NaF sensitivity in arf1-3 arf2Δ yeast strains. Thus, kes1Δ phenocopies the elevation of ARFGAP levels in arf1-3 arf2Δ yeast mutants. Finally, we note that the pik1-101ts mutant resembles gcs1Δ and arf1-3 strains in its NaF sensitive growth, and that NaF sensitivity is relieved by kes1Δ (Table I).

Discussion
Herein, we show Kes1p binds PIPs and this PIP-binding property, in conjunction with its conserved OSBP domain, is essential for Kes1p localization to Golgi membranes. The PIP pool required for Kes1p targeting to Golgi membranes is driven by activity of the Pik1p PI 4-kinase, and Golgi region targeting of Kes1p is required for inhibition of Sec14p-dependent Golgi region function in vivo. We further demonstrate that inactivation of Kes1p not only affects “bypass Sec14p”, but that it also elicits a suppression of some phenotypes associated with ARF and ARFGAP dysfunction. We propose that it is through its effects on ARF that Kes1p regulates Sec14p-dependent Golgi membrane secretory function in yeast.

The PIP binding activity resides in what appears to be a Kes1p PH domain. Although the existence of PH domains has been documented for long OSBPs (e.g., OXYB yeast Os1; Levine and Munro, 1998), our findings represent the first demonstration that short OSBPs also binds PIPs. Moreover, our data demonstrate that PIP binding, and its role in targeting an OSBP to Golgi membranes, is a functionally important activity in cells. With regard to the binding of Kes1p to other lipids, we failed to detect significant binding of Kes1p to 25-hydroxycholesterol (unpublished data).

One unanticipated result obtained from analysis of mutations in the Kes1p PH domain is our finding that the E313K

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Figure 10. Genetic interactions between kes1Δ, arf1Δ, gcs1Δ, and pik1-101Δ alleles. (A) “Bypass Sec14p” in arf1Δ strains. Isogenic sets of wild-type (WT), sec14-1ts, sec14-1ts kes1Δ, sec14-1ts arf1Δ, and sec14-1ts kes1Δ gcs1Δ strains were incubated on YPD agar at 26°C or 37°C (a restrictive temperature for sec14-1ts strains) as indicated for 72 h. WT and sec14-1ts strains represent positive and negative growth controls, respectively. The kes1Δ allele rescues sec14-1ts lethality at 37°C, and this rescue is abolished by arf1Δ and gcs1Δ. The arf1Δ and gcs1Δ alleles do not compromise growth of otherwise WT strains at 37°C. (B) Kes1p deficiency partially suppresses growth defects of the pik1-101Δ strain. Each strain was serially diluted from original culture (OD600 = 0.6) by 10−, 100-, and 1,000-fold. 3 μL of each dilution was inoculated onto YPD plates, and plates were incubated for 3 d at 26°C and 35°C, respectively.

Table 1. Interactions of kes1 mutations with perturbations in Arf function

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NaF (0 mM)</th>
<th>NaF (30 mM)</th>
<th>NaF (50 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>sec14-1Δ</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>arf1-3 arf2Δ</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>arf1-3 arf2Δ kes1Δ</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>sec14-1Δ gcs1Δ</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>sec14-1Δ gcs1Δ kes1Δ</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pik1-101Δ</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pik1-101Δ kes1Δ</td>
<td>+++</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Genetic interactions between Kes1p and ARF cycle components. KES1 was deleted in gcs1Δ, arf1-3ts arf2D, and in pik1ts mutants, and strains were grown on YPD agar with indicated NaF concentrations. Wild-type colony size is indicated by +++, smaller colonies by ++, and − indicates no growth at all after 3 d at 26°C. In arf1, gcs1, and pik1 mutants challenged with 50 mM NaF, kes1Δ affects 103-fold increases in cell viability.

and W317A mutations abolish PIP binding in the context of full-length Kes1p, but not when the COOH-terminal 120 Kes1p residues are removed. Thus, E312K and W317A do not affect PIP binding directly. Rather, these substitutions likely affect Kes1p conformation in such a way that the PH do-
in eukaryotic cells remain unresolved. We suggest that Kes1p function somehow interfaces with activity of the yeast ARF cycle. We find that arf1Δ and ges1Δ both abrogate kes1Δ-mediated “bypass Sec14p”, and several other lines of genetic evidence indicate that Kes1p dysfunction mimics elevated ARFGAP function, whereas elevated Kes1p phenocopies reduced ARFGAP function. Thus, Kes1p may act as: (a) an inhibitor of the Gcs1p ARFGAP activity that is required for yeast Sec14p-dependent Golgi complex function (Yanagisawa, L., and V.A. Bankaitis, unpublished data), (b) a novel ARF nucleotide exchange factor, or (c) an activator of an ARF nucleotide exchange factor. In our hands, Kes1p fails to inhibit the ARFGAP activity of Gcs1p in vitro and we have not detected intrinsic ARF nucleotide exchange activity associated with Kes1p (unpublished data).

Our data suggest that Kes1p may regulate ARF function through its effects on PIP synthesis via the Golgi complex–associated Pik1p as evidenced by the demonstration Kes1p defects partially suppress growth defects associated with Pik1p dysfunction. Kes1p may function, directly or indirectly, as a Pik1p inhibitor in vivo. Alternatively, effects of Kes1p on the ARF cycle may influence Pik1p activity. Linkage of Kes1p localization and function to Pik1p-mediated PI(4)P synthesis suggests that PI(4)P is the physiological Kes1p ligand. Although PI(4,5)P2 is bound by Kes1p with a 10-fold higher affinity, PI(4)P mass is fourfold greater than that of PI(4,5)P2 in yeast. Moreover, defects in PI(4,5)P2 synthesis do not compromise Kes1p localization to Golgi membranes, and massive accumulation of PI(4)P in a non-Golgi compartment entices Kes1p from Golgi membranes. The latter result suggests that a component of the mechanism by which sec14Δ-mediated PI(4)P accumulation contributes to “bypass Sec14p” is an indirect one that involves Kes1p mistargeting to what is likely the ER. Finally, the data identify Kes1p as a reporter for Pik1p-driven PIP synthesis on yeast Golgi membranes. Thus, Pik1p facilitates the paradoxical recruitment to Golgi membranes of a protein whose dysfunction permits Sec14p-independent secretory function of Golgi membranes.

### Materials and methods

#### Strains, media, and reagents

Genotypes for yeast strains are listed in Table II. Media are described elsewhere (Sherman et al., 1983), as are E. coli and yeast plasmid transformation techniques (Ito et al., 1983). Vector pQE30 was from QIAGEN. Hi-Trap Ni+ chelating columns and glutathione-Sepharose 4B beads were from Amersham Pharmacia Biotech. PI(4,5)P2, PI(4)P, and IP3 were from CalBiochem. PI(3,4)P2 and PI(3,5)P2 were from Echelon Research Laboratories. Other lipids were from Avanti Polar Lipids, Inc. Fine chemicals were from Sigma-Aldrich unless specified. Restriction enzymes were purchased from Promega. [α-35S]dATP was obtained from Amersham Pharmacia Biotech.

#### Recovery of genomic kes1 alleles

An integrative Kes1p plasmid (pCTY204) was restricted at a unique site within the Kes1p promoter with AflII. Linearized plasmid was transformed into kes1 strains and recombinants at the Kes1 locus were identified. Genomic DNA was prepared from transformants, restricted with Clal, and ligated at 15°C. Following transformation into E. coli DH5α, plasmid DNA was purified and used as template for nucleotide sequencing.

#### Site-directed mutagenesis

Mutageneses employed QuickChange™ (Stratagene) and confirmed by DNA sequencing analysis (Sanger et al., 1977) using the Sequenase version 2.0 kit (Amersham Pharmacia Biotech).

#### Plasmid construction

Details of the various plasmid constructions are included in supplemental materials or are available from the authors by request.

#### Expression and purification of His6-tagged and GST-tagged proteins from E. coli

One liter of Superbroth (12 g tryptone, 24 g yeast extract, 4 g glycerol, 0.17 M KH2PO4, 0.72 M K2HPO4, 50 μg/ml ampicillin) was inoculated with 10 ml of an overnight culture of E. coli strain KK2186 harboring plasmids expressing His6– or GST-tagged proteins. Protein production was induced with isopropyl β-D-thiogalactopyranoside (0.5 mM) and, after 3–5 h, cells were harvested by centrifugation. Cells were disrupted by sonication, and Triton X-100 was added (final concentration 0.1% vol/vol). After addition of DNase I (10 μg/ml) and MgCl2 (10 mM), lysate was clarified and filtered.

His6-tagged proteins were purified by HiTrap Ni-chelating column chromatography (Amersham Pharmacia Biotech) as per the manufacturer’s instructions.

#### Peptide sequencing

Purified proteins were transferred to Immuno-Blot PVDF membranes (Bio-Rad Laboratories) using the semidy Transblot apparatus (Bio-Rad Laboratories). Blotted PVDF membrane were washed with distilled water and...
stained with Ponceau S. Kes1p species were excised from membranes and subjected to automated NH2-terminal Edman degradation.

**Mass spectrometry**
MALDI-TOF mass spectrometry was performed using positive mode on a Voyager Elite unit with delayed extraction technology (PerSeptive Biosystems). Samples were diluted 1:10 with matrix, and 1 μl of the resulting mix was deposited onto a smooth plate. Acceleration voltage was set at 25 kV and 10–50 laser shots were summed. Sinapinic acid (D13, 460-0; Sigma-Aldrich) dissolved in acetonitrile: 0.1% TFA (1:1) was used as matrix. The mass spectrometer was calibrated with bovine serum albumin.

**Online supplemental materials**
PPIP photolabeling assay. Photolabeling and displacement assays were performed as described previously (Kearns et al., 1998b). Details are available at http://www.jcb.org/cgi/content/full/jcb.200201037/DC1 or from the authors by request.

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Bankaitis, V.A., J.R. Aitken, A.E. Cleves, and W. Dowhan. 1990. An essential role of the resulting mix was deposited onto a smooth plate. Acceleration voltage was set at 25 kV and 10–50 laser shots were summed. Sinapinic acid (D13, 460-0; Sigma-Aldrich) dissolved in acetonitrile: 0.1% TFA (1:1) was used as matrix. The mass spectrometer was calibrated with bovine serum albumin.

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