The EMBO Journal vol.15 no.23 pp.6447–6459, 1996

Kes1p shares homology with human oxysterol binding protein and participates in a novel regulatory pathway for yeast Golgi-derived transport vesicle biogenesis

Min Fang, Brian G.Kearns, Alma Gedvilaite, Satoshi Kagiwada, Melissa Kearns, Mark K.Y.Fung and Vytas A.Bankaitis[1]

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

[1]Corresponding author

The yeast phosphatidylinositol transfer protein (Sec14p) is required for biogenesis of Golgi-derived transport vesicles and cell viability, and this essential Sec14p requirement is abrogated by inactivation of the CDP-choline pathway for phosphatidylcholine biosynthesis. These findings indicate that Sec14p functions to alleviate a CDP-choline pathway-mediated toxicity to yeast Golgi secretory function. We now report that this toxicity is manifested through the action of yeast Kes1p, a polypeptide that shares homology with the ligand-binding domain of human oxysterol binding protein (OSBP). Identification of Kes1p as a negative effector for Golgi function provides the first direct insight into the biological role of any member of the OSBP family, and describes a novel pathway for the regulation of Golgi-derived transport vesicle biogenesis.

Keywords: Golgi/oxysterol binding protein homolog/phosphatidylinositol transfer protein/secretion/Sec14p

Introduction

The *Saccharomyces cerevisiae* Sec14p belongs to an enigmatic class of proteins, the phospholipid transfer proteins (PLTPs), that have classically been identified by their ability to catalyze the *in vitro* transport of phospholipid monomers across an aqueous milieu from one membrane bilayer to another (Cleves *et al.*, 1991a; Wirtz, 1991). These proteins have posed a long-standing enigma with regard to: (i) what the genuine *in vivo* functions for PLTPs are, and (ii) how PLTPs harness their associated phospholipid transfer activities to the execution of their various *in vivo* functions. Based upon the activity by which these proteins are operationally defined, there has been considerable speculation with regard to the involvement of such proteins as diffusible lipid carriers in intracellular lipid trafficking and lipid sorting events *in vivo* (Wirtz, 1991).

Sec14p is a phosphatidylinositol (PI)/phosphatidylcholine (PC) transfer protein that is required for secretory protein transport from a late Golgi compartment: a role that is essential for yeast cell viability (Bankaitis *et al.*, 1989, 1990; Franzusoff and Schekman, 1989; Cleves *et al.*, 1991b). The available evidence suggests that Sec14p functions to stimulate the biogenesis of Golgi-derived transport vesicles (Novick *et al.*, 1980; Bankaitis *et al.*, 1989; Franzusoff and Schekman, 1989; Cleves *et al.*, 1991b), and the involvement of Sec14p in this process is considered to be a direct one as a pool of Sec14p exists in a specific association with yeast Golgi membranes *in vivo* (Cleves *et al.*, 1991b). Current evidence suggests that at least some aspects of PI/PC-transfer protein (TP) function may be conserved from yeast to mammals as rat PI/PC-TP has recently been implicated in the biogenesis of both dense core granules and constitutive secretory vesicles at the *trans*-Golgi membrane surface, and in the priming of dense core granules for regulated fusion to the plasma membrane, in neuroendocrine cells (Hay and Martin, 1993; Hay *et al.*, 1995; Ohashi *et al.*, 1995). Moreover, expression of rat PI/PC-TP in yeast is able to effect a partial correction of the growth and secretory defects associated with Sec14p dysfunction (Skinner *et al.*, 1993).

Mutations in any one of at least seven genes (*CKI*, *CCT*, *CPT*, *BSR3*, *SAC1*, *BSD1* and *BSD2*) effect an efficient bypass of the normally essential Sec14p requirement, and analysis of these 'bypass Sec14p' mutants is driving a detailed dissection Sec14p function *in vivo* (Cleves *et al.*, 1989, 1991b; McGee *et al.*, 1994). Indeed, the very existence of 'bypass Sec14p' mutations indicates that Sec14p serves a regulatory function in the biogenesis of Golgi-derived transport vesicles. Penetrating insight into mechanism of 'bypass Sec14p' was initially gained from the demonstration that three of the seven known classes of 'bypass Sec14p' mutations (i.e. *cki*, *cct* and *cpt*) define loss-of-function mutations in the three structural genes for enzymes of the CDP-choline pathway for PC biosynthesis (illustrated in Figure 7), thereby establishing that specific inactivation of PC biosynthesis via the CDP-choline pathway represents one mechanism by which 'bypass Sec14p' can be realized (Cleves *et al.*, 1991b; McGee *et al.*, 1994). This discovery not only provided an integration of secretory pathway function with phospholipid metabolism, but also demonstrated that CDP-choline pathway activity is specifically toxic to Golgi secretory function.

Biochemical and genetic evidence now suggest that Sec14p functions to down-regulate the activity of the rate-determining enzyme of the CDP-choline pathway on Golgi membranes, thereby coupling the activity of the CDP-choline pathway to Golgi PC levels (McGee *et al.*, 1994; Skinner *et al.*, 1995). Thus, Sec14p appears to employ its phospholipid binding/transfer properties in the context of a molecular switch that controls the CDP-choline pathway-directed negative effector function of Sec14p (Skinner *et al.*, 1995). The precise basis for why CDP-choline pathway activity might be toxic to the function of yeast Golgi membranes, however, remains obscure.

In this report we demonstrate that Kes1p, a yeast member of the human oxysterol binding protein (OSBP)

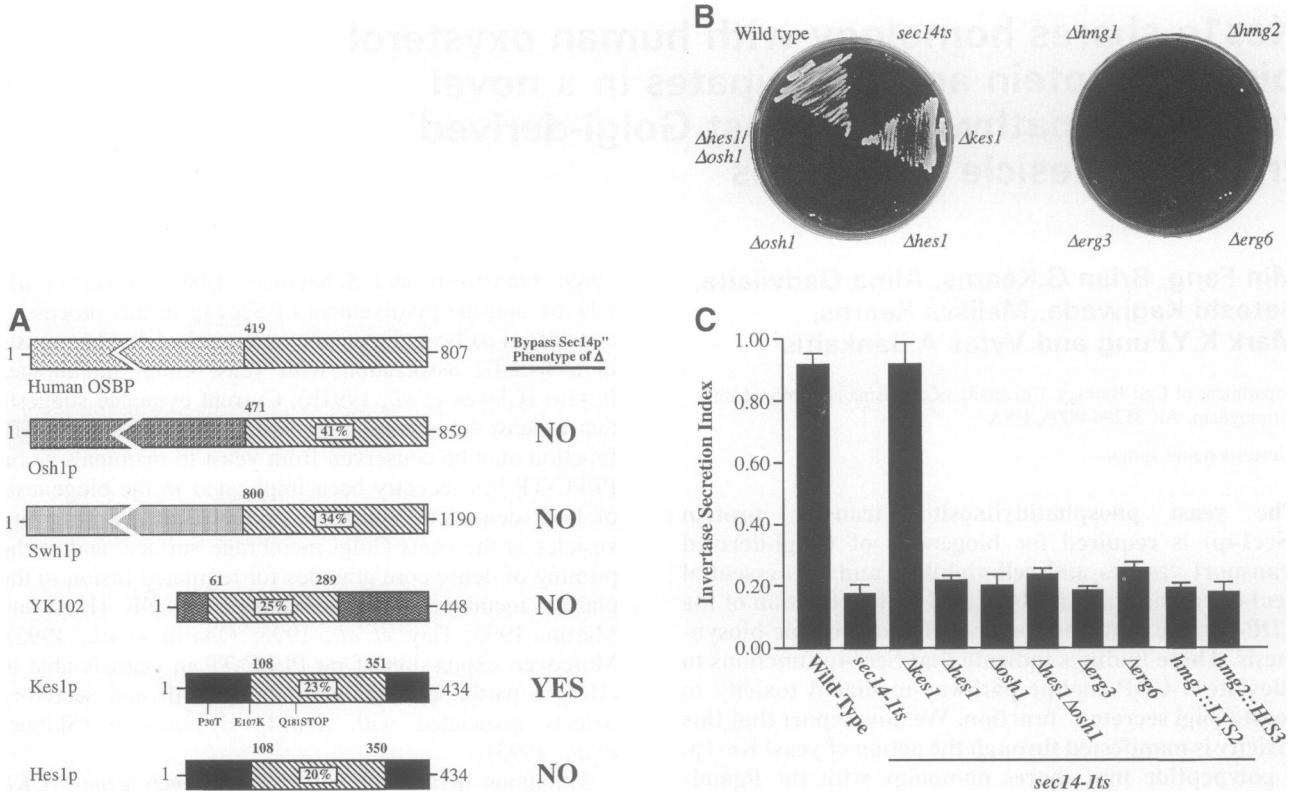

Fig. 1. (**A**) Alignment of non-essential yeast OSBP homologs. Homologous regions (striped; boundary residues numbered at top) are restricted to the oxysterol binding domain of OSBP (Ridgway *et al.*, 1992; Jiang *et al.*, 1994). Primary sequence identities shared with OSBP therein are given, and the 'bypass Sec14p' phenotype of each corresponding deletion (Δ) is indicated. The 'bypass Sec14p' phenotypes were assessed by plasmid loss assays that gauged the ability of the indicated deletion mutations to alleviate the dependence of Δ*sec14* strains on a YEp(*SEC14*) plasmid for viability; as visualized by mitotic instability of the plasmid in cells grown on YPD (Lopez *et al.*, 1994; see Materials and methods). (**B**) The wild-type yeast strain CTY182 (WT) and a representative series of *sec14-1*^{ts} derivative strains (relevant genotypes indicated) were streaked on YPD medium at 37°C and incubated for 48 h. Only the *sec14-1*^{ts}, *kes1Δ1::URA3* derivative grew at 37°C, indicating phenotypic suppression of *sec14-1*^{ts} by *kes1Δ1::URA3*. The other indicated mutations failed to relieve *sec14-1*^{ts}-associated growth defects. (**C**) Efficiency of invertase secretion in wild-type, *sec14-1*^{ts} and *sec14-1*^{ts} derivative strains at 37°C. Secretion indices of wild-type and *sec14-1*^{ts} parental strains were calculated as (extracellular invertase/total cellular invertase×100%) as previously described (Salama *et al.*, 1990), and these values provided indicators of secretory efficiency under Sec14p-proficient and deficient conditions, respectively. Only the *sec14-1*^{ts}, *kes1Δ1::URA3* strain exhibited a secretion index resembling that of wild-type, and *kes1Δ1::URA3* similarly restored wild-type secretory capacity to Δ*sec14* strains (not shown). The other indicated mutations did not significantly relieve the *sec14-1*^{ts} secretory block.

family, is a cytosolic factor that functions in a specific and previously unanticipated role as a negative effector of Golgi-derived transport vesicle biogenesis. Moreover, we provide evidence to indicate that the Golgi-directed toxicity of the CDP-choline pathway is likely mediated through potentiation of Kes1p function. These data not only provide functional insight into the biological role of a member of the OSBP family, but also describe the basic form of an altogether novel pathway (which we refer to as the Sec14p pathway) for the regulation of Golgi-derived transport vesicle biogenesis.

Results

BSR3 is allelic to KES1

The yeast *BSR3* gene is defined by three recessive 'bypass Sec14p' alleles (Cleves *et al.*, 1991b). A candidate *BSR3* gene was recovered from a yeast genomic library on the basis of its ability to confer a *ts* phenotype to *sec14-1*^{ts}, *bsr3-1* yeast strains at 37°C, and nucleotide sequence analysis revealed its identity to *KES1*, a previously recognized gene of 434 codons that encodes a non-essential OSBP homolog (see Materials and methods; Jiang *et al.*, 1994). That *KES1* and *BSR3* are the same gene was unambiguously established by three independent methods.

First, we tested whether a naive *kes1* mutation exhibited an unselected 'bypass Sec14p' phenotype. We constructed a *kes1Δ1::URA3* null allele, and recombined it into a *sec14-1*^{ts} strain. The growth and secretory properties of the resulting double mutant were subsequently assessed. The phenotypic data clearly indicated that *kes1Δ1::URA3* was able to effect an efficient suppression of the growth defects associated with the *sec14-1*^{ts} mutation and with haploid lethal Δ*sec14* mutations (Figure 1A and B). Moreover, *kes1Δ1::URA3* restored wild-type secretory capability to *sec14-1*^{ts} strains at 37°C, a normally restrictive temperature, as evidenced by the measurement of a wild-type invertase secretion index (0.93 ± 0.7) for *kes1Δ1::URA3*, *sec14-1*^{ts} double mutant strains (Figure 1C). This was in contrast to the very poor efficiency of invertase secretion exhibited by isogenic *sec14-1*^{ts} strains at 37°C (secretion index = 0.18 ± 0.02). Similarly, *kes1Δ1::URA3*, Δ*sec14* double mutant strains were also able to secrete invertase to the cell surface with wild-type efficiencies (not shown). These data indicated that an inherent property of *kes1* mutations was a 'bypass Sec14p' phenotype.

Second, genetic mapping experiments demonstrated a strong linkage between the *BSR3* and *KES1* loci. A *kes1Δ1::URA3*, *sec14-1^{ts}* strain was mated to a *bsr3-1*, *sec14-1^{ts}* strain, the resulting diploid was subsequently induced to sporulate, and the haploid progeny recovered from that meiosis were analyzed for the segregation of *kes1Δ1::URA3* and *bsr3-1*. In this cross, both the *kes1Δ1::URA3* and *bsr3-1* loci were followed by their individual abilities to render *sec14-1^{ts}* strains Ts$^+$ (i.e. able to grow at 37°C). From a total of 60 four-spore tetrads analyzed, only parental ditype asci (i.e. yielding four Ts$^+$: 0 *ts* meiotic progeny each) were recovered. Our failure to resolve the *bsr3-1* and *kes1Δ1::URA3* loci in this meiotic segregation analysis suggested that these two mutations were essentially superimposed upon each other in the yeast genome (i.e. <100 bp apart).

Finally, we tested whether the three original *bsr3* alleles represented authentic *kes1* mutations. The *KES1* gene was recovered from each of the *bsr3-1*, *bsr3-2* and *bsr3-3* mutant strains by PCR amplification, and the entire nucleotide sequence was determined in each case from three independent amplifications. The nucleotide sequence data were uniformly consistent for each allele and clearly showed that *bsr3-2* and *bsr3-3* represented *kes1* missense mutations (Figure 2A). The *bsr3-2* allele involved a GAG to AAG transition event that resulted in a non-conservative E107K substitution, and *bsr3-3* represented a CCT to ACT transversion resulting in a non-conservative P30T substitution. Thus, the *bsr3-2* mutation lay at the amino-terminal boundary of the Kes1p domain that exhibits homology to the oxysterol binding domain of human OSBP, while the *bsr3-3* allele did not reside within that region of homology. By contrast, *bsr3-1* involved a CAA to TAA transition that introduced an ochre mutation at codon 181 of the *KES1* gene (Figure 2A).

Kes1p immunoblotting experiments generated protein profiles that were entirely consistent with the nucleotide sequence data. A specific Kes1p-immunoreactive species with the expected molecular mass of ~51 kDa was detected in lysates prepared from wild-type, *bsr3-2* and *bsr3-3* strains, while no such Kes1p species was observed in *kes1Δ1::URA3* and *bsr3-1* lysates (Figure 2B). The *bsr3-2* and *bsr3-3* alleles did not remarkably diminish steady-state levels of Kes1p as estimated by the immunoblotting regimen employed (Figure 2B). The demonstration that Kes1p dysfunction rendered yeast independent of their normally essential Sec14p requirement identified Kes1p as a negative effector in the Sec14p pathway for Golgi secretory function.

Interaction of Kes1p with the Sec14p pathway exhibits functional specificity

The *SWH1*, *OSH1*, *YK102* and *HES1* genes encode additional yeast OSBP homologs that, like Kes1p, share varying degrees of primary sequence homology with the oxysterol-binding domain of OSBP (Figure 1A) (Ridgway *et al.*, 1992; Jiang *et al.*, 1994). We therefore investigated whether deletion of any of the structural genes for these other OSBP homologs could also generate a 'bypass Sec14p' phenotype. With the exception of cases involving *kes1Δ* alleles, neither individual nor combinatorial disruption of these genes resulted in restoration of growth of *sec14* null strains (as determined by plasmid loss assays; see Materials and methods) or of *sec14-1^{ts}* strains at 37°C (Figure 1A and B). Moreover, disruption of the corresponding structural genes for these OSBP homologs failed detectably to alleviate the *sec14-1^{ts}* secretory block as measured by efficiency of invertase secretion to the cell surface (Figure 1C). The derivative *sec14-1^{ts}* strains carrying disruptions in yeast OSBP homolog structural genes exhibited invertase secretion indices at 37°C that ranged from 0.20 ± 0.02 for the *Δosh1*, *sec14-1^{ts}* strain to 0.23 ± 0.02 for the *Δhes1*, *Δosh1*, *sec14-1^{ts}* triple mutant. These values were indistinguishable from the secretion index measured for the *sec14-1^{ts}* strain under these conditions (0.18 ± 0.02). Of particular note was the lack of functional redundancy between Kes1p and Hes1p. These polypeptides share a 70% primary sequence identity (Jiang *et al.*, 1994). Yet, these two homologs were functionally distinguished not only by the inability of *hes1* mutations to manifest themselves as 'bypass Sec14p' alleles (Figure 1A–C), but also by the failure of increased *HES1* gene dosage to compromise *kes1*-mediated suppression of *sec14-1^{ts}* defects [as indicated by introduction of a YEp(*HES1*) plasmid into *kes1Δ1::URA3*, *sec14-1^{ts}* strains and demonstration that these strains remained phenotypically Ts$^+$; data not shown].

The collective data indicated a lack of functional relatedness among the OSBP homologs of yeast with regard to genetic interaction with the Sec14p pathway, and revealed a specificity for the involvement of Kes1p in the negative regulation of Golgi-derived transport vesicle biogenesis.

Kes1p localizes predominantly to the yeast cytosol

The *KES1* nucleotide sequence predicts a largely hydrophilic polypeptide with no obvious candidate regions for transmembrane domains (Jiang *et al.*, 1994). To determine the localization of Kes1p, we employed a differential centrifugation regimen to resolve cell-free lysates into a set of defined membrane and cytoplasmic fractions (see Materials and methods). The distribution of Kes1p across these fractions was subsequently assessed by immunoblotting. Single-copy *KES1* and YEp(*KES1*) strains exhibited very similar fractionation profiles for Kes1p (Figure 3). Typically, we estimated that >70% of the cellular Kes1p pool was localized to the 100 000 *g* supernatant fraction (S100), while the remainder of the material distributed rather equally between large membranes that pelleted at 12 000 *g* (P12) and microsomal fractions that pelleted only at 100 000 *g* (P100). The P12 fraction is enriched in endoplasmic reticulum (ER) and plasma membrane, while the P100 is enriched for Golgi and endosomal membranes (Cleves *et al.*, 1991b; Bowser *et al.*, 1992; Horazdovsky and Emr, 1993). Kes1p was not tightly associated with the P12 and P100 fractions, as evidenced by our finding that a 150 mM KCl wash had the effect of efficiently stripping Kes1p from these membranes (data not shown).

Control markers behaved as expected. The integral ER membrane protein marker dolichol–phosphomannose synthase distributed predominantly to membrane fractions, and very predominantly to the P12 fraction while, under the fractionation conditions employed, the cytosolic protein phosphoglucomutase (PGM) was essentially exclusively recovered from the S100 fraction (Figure 3). Finally, a lumenal ER marker (Kar2p) sedimented largely with the

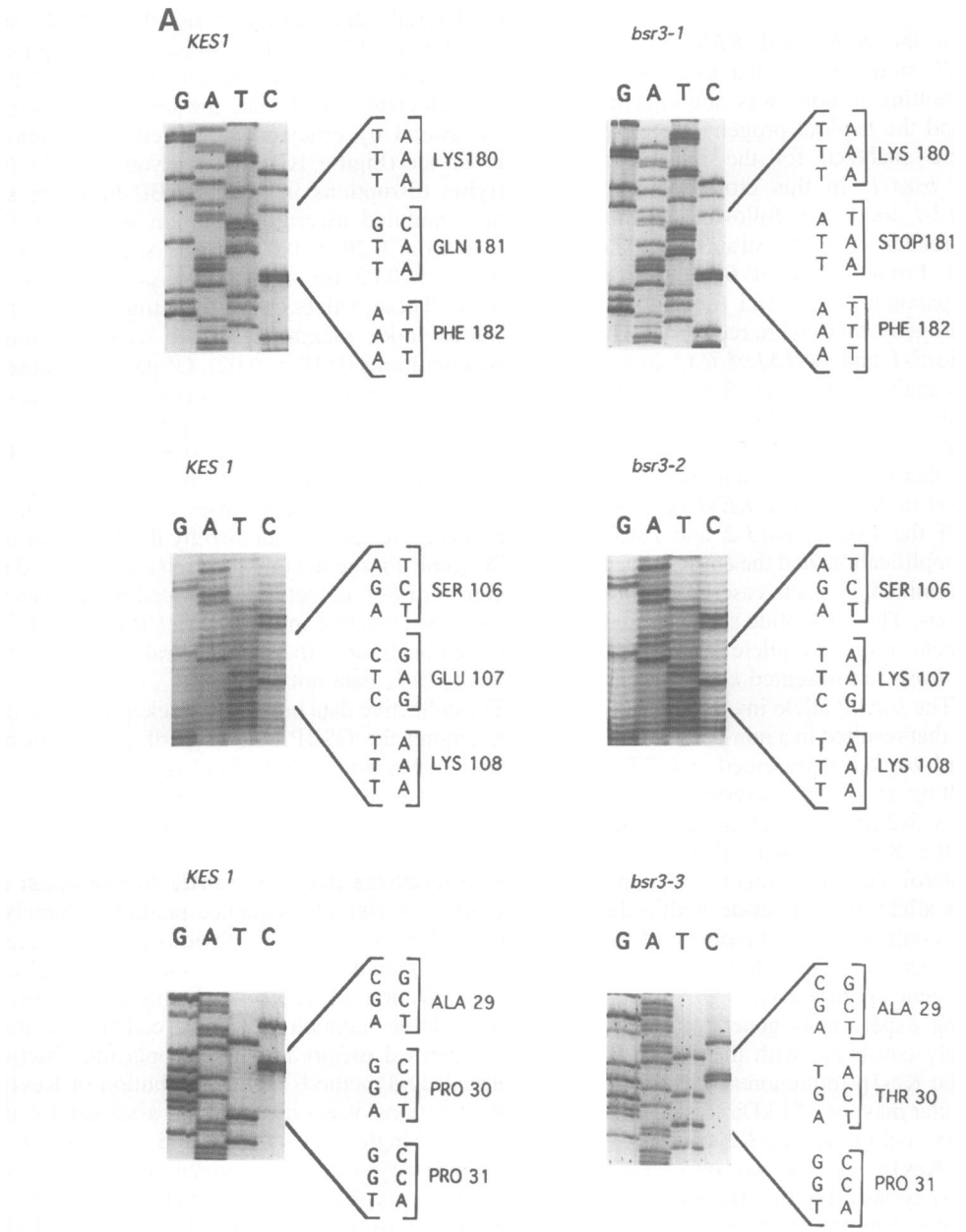

Fig. 2. (**A**) Nucleotide sequence determination that *bsr3* alleles represent *kes1* mutations. The *KES1* gene was recovered three independent times from each of the corresponding *bsr3* strains by PCR amplification, the PCR products subcloned, and the corresponding nucleotide sequences determined (see Materials and methods). The primary data are shown. Relevant nucleotide and primary sequence data are indicated in the *KES1* regions of interest for both wild-type and the indicated *bsr* alleles. (**B**) Kes1p immunoblots from *KES1* and *kes1Δ1::URA3* strains, and mutants harboring the three original *bsr3* alleles. The corresponding Kes1p amino acid substitutions and *bsr3* allelic designations are indicated. Sample loading consistency (22 μg per lane) was normalized by monitoring vacuolar carboxypeptidase Y (CPY) antigen. Immunoblots were developed using the ECL™ system (Amersham). Primary antibodies were used at 1:1000 and 1:4000 dilutions for Kes1p and CPY, respectively.

P12 pellet, indicating that the integrity of intracellular organelles was maintained by the lysis procedure employed (Figure 3). The similarities in the fractionation profiles for PGM and Kes1p suggest that Kes1p was predominantly localized to the yeast cytosol.

The routine detection of Kes1p in pellet fractions further suggested that a pool of Kes1p might exist in specific association with intracellular membranes (e.g. the yeast Golgi complex). To this point, however, we have failed to identify a subcellular compartment with which Kes1p

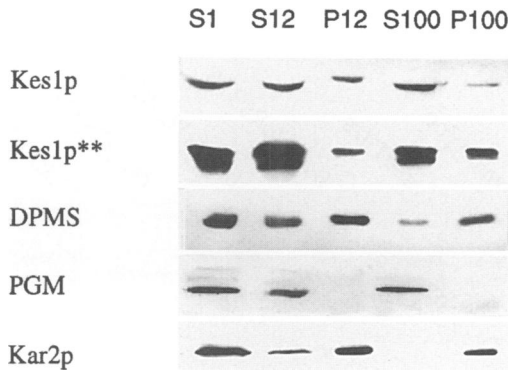

Fig. 3. Kes1p fractionates as a cytoplasmic polypeptide. Strains were grown in minimal medium, converted to spheroplasts and lysed osmotically. The resultant lysate was subjected to three rounds of differential centrifugation to yield whole-cell (S1), 12 000 *g* pellet and supernatant fractions (P12 and S12, respectively) and 100 000 *g* pellet and supernatant fractions (P100 and S100, respectively) (see Materials and methods). These various fractions were subsequently immunoblotted for the integral ER membrane protein dolichol–phosphomannose synthase (DPMS), the marker for the lumen of the ER (Kar2p), the cytosolic protein PGM and Kes1p. Samples were then resolved by SDS–PAGE, and specific markers identified by immunoblotting. The various fractions represent equal cell equivalents of sample with the exception of the S1 fractions, which were underloaded relative to the other fractions by a factor of 2. Immunoblots were developed using the ECL™ system (Amersham). Primary antibodies were used at 1:1000, 1:1500, 1:10000 and 1:1000 dilutions for Kes1p, DPMS, Kar2p and PGM, respectively. Recoveries of each protein analyzed are estimated as >70%. Kes1p** designates the Kes1p fractionation profile obtained from a YEp(*KES1*) strain that overproduces Kes1p.

might associate specifically. We have attempted a number of fixation and epitope-tagging strategies to localize Kes1p by immunofluorescence in both wild-type and Kes1p-overproducing strains without success, as we have been unable to detect a specific Kes1p signal relative to the *kes1Δ1::URA3*, *hes1Δ::LEU2* negative control strain. Moreover, the results obtained from more refined biochemical fractionation experiments also have not permitted the formulation of strong conclusions as Kes1p is rather broadly distributed throughout the equilibrium density gradients we have employed to resolve specific membrane fractions (not shown). Thus, we have as yet been unable to establish the existence of a yeast Golgi-localized pool of Kes1p.

Increase in Kes1p levels specifically eliminates the 'bypass Sec14p' phenotype associated with CDP-choline pathway dysfunction and dominant bsd1 mutations

During the process of screening for candidate *BSR3/KES1* clones, we tested the expectation that authentic clones should complement only *bsr3* mutations and not other recessive 'bypass Sec14p' mutations, e.g. such as those that involve structural genes for CDP-choline pathway enzymes. To our surprise, however, all authentic *KES1* clones obtained exhibited a pleiotropic phenotypic suppression of 'bypass Sec14p' mutations. As the representative data depicted in Figure 4A show, even an estimated 2- to 4-fold elevation in *KES1* gene dosage [elicited by introduction of a YCp(*KES1*) plasmid into such strains] was sufficient to neutralize completely the 'bypass Sec14p' growth phenotypes associated with loss-of-function mutations in the structural genes for enzymes of the CDP-choline pathway for PC biosynthesis. This pleiotropic effect was not limited to recessive 'bypass Sec14p' mutations as it also was observed with dominant *bsd1* mutations. Interestingly, *bsd1* mutations represent the most frequently encountered class of 'bypass Sec14p' alleles initially obtained as 80 *bsd1* mutants were recognized in the first 107 'bypass Sec14p' alleles isolated (Cleves *et al.*, 1991b). The trivial possibility that *bsd1* and *kes1* are allelic was excluded by linkage analyses that demonstrated these to represent distinct genetic loci (not shown). The additional possibility that increased *KES1* gene dosage was causing some non-specific *ts* phenotype was discounted by the following observations: (i) that introduction of YEp(*KES1*) into *sec14-1^{ts}* strains carrying either recessive *sac1* or dominant *bsd2-1* mutations was without consequence to the respective 'bypass Sec14p' phenotypes associated with these alleles; and (ii) that YEp(*KES1*) had no discernible effect on the growth properties of otherwise wild-type yeast strains (not shown).

To determine whether the Kes1p-mediated reversal of these 'bypass Sec14p' phenotypes was associated with a restoration of the *sec14-1^{ts}* secretory block, we employed two independent experimental strategies. First, we employed a pulse-radiolabeling strategy to determine the efficiency at which the vacuolar proteinase carboxypeptidase Y (CPY) was delivered to the yeast vacuole. As shown in Figure 4B, wild-type yeast strains accumulated the 61 kDa mature form of radiolabeled CPY that is diagnostic of CPY that has completed transit through the secretory pathway and reached the vacuole. Only trace amounts of either the core-glycosylated ER form of proCPY (p1) or the fully glycosylated Golgi form of proCPY (p2) were detected. In accord with previous results, these data indicated rapid delivery of this proteinase through the secretory pathway to the yeast vacuole (Stevens *et al.*, 1982; Bankaitis *et al.*, 1986; Rothman and Stevens, 1986). The radiolabeled CPY profile for *sec14-1^{ts}* mutants challenged with the restrictive temperature of 37°C revealed a dramatic accumulation of the p2 proCPY form, however, and this effect was reflective of the *sec14-1^{ts}* Golgi-secretory block. The *sec14-1^{ts}* defect in CPY trafficking was fully corrected in yeast strains carrying the dominant 'bypass Sec14p' allele *bsd1-128* and the recessive 'bypass Sec14p' allele *cki* that genetically inactivates choline kinase, the first enzyme of the CDP-choline pathway for PC biosynthesis. Introduction of a low-copy *KES1* plasmid YCp(*KES1*) into these *bsd1-128* and *cki* strains eliminated the 'bypass Sec14p' effects of these alleles, however, and fully reimposed the *sec14-1^{ts}* secretory block; as evidenced by the restored accumulation of radiolabeled p2 CPY in these strains (Figure 4B). Confirmatory results were also obtained from experiments where the efficiency of invertase secretion was measured in these same strains (not shown).

These collective data demonstrated that even subtle overproduction of Kes1p eliminated the 'bypass Sec14p' character associated with CDP-choline pathway dysfunction and *bsd1* alleles. With regard to the genetic interaction of *KES1* with the CDP-choline pathway, we considered the rather trivial possibility that such a phenotypic reversal of the 'bypass Sec14p' character of *cki* and *cct* mutants could reflect a resuscitation of CDP-choline pathway

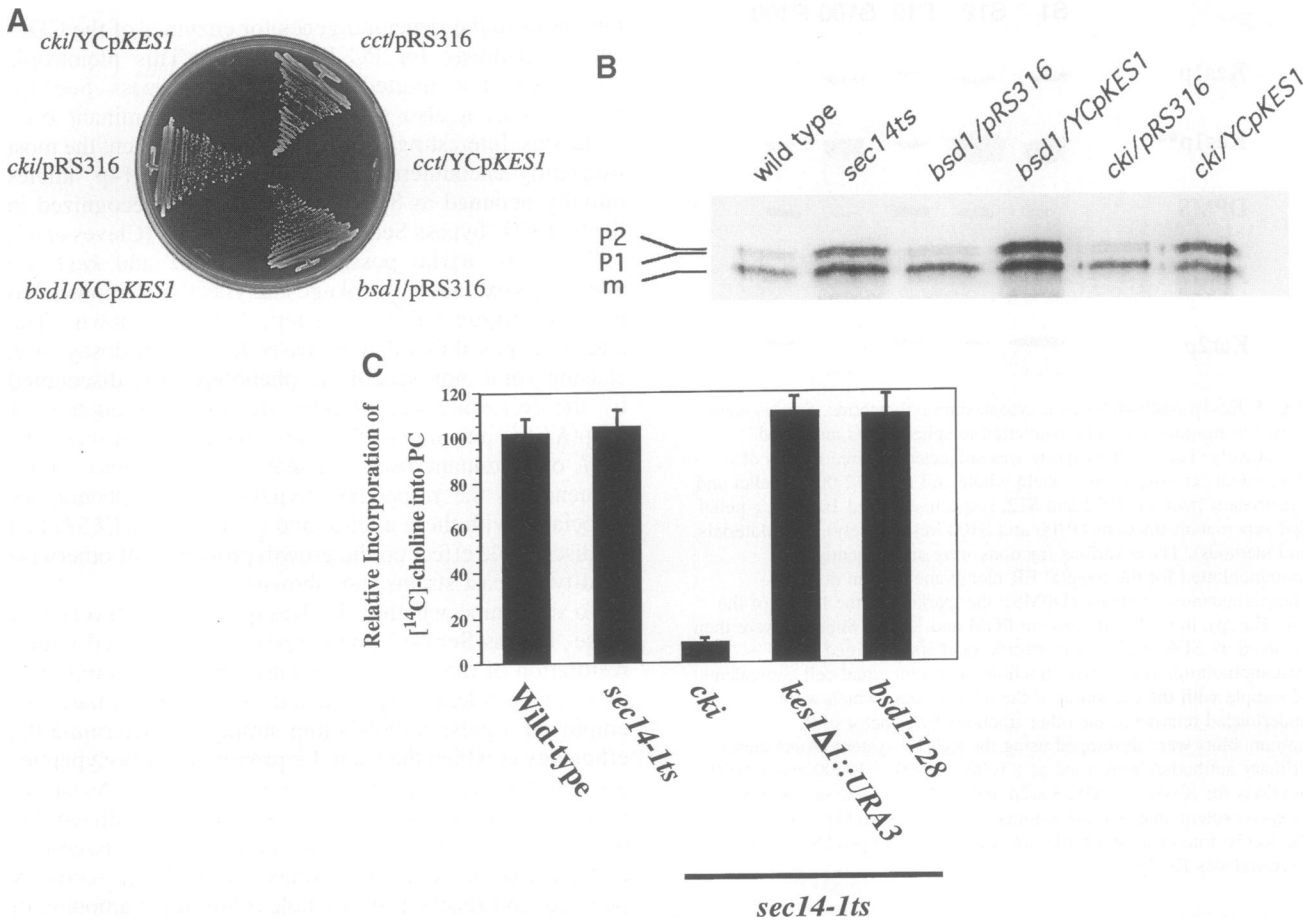

Fig. 4. (**A**) Kes1p-overproduction abolishes phenotypic suppression of *sec14-1*^{ts} growth defects afforded by disruption of structural genes for enzymes of the CDP-choline pathway enzymes (choline kinase, *cki*; choline-phosphate cytidylyltransferase, *cct*), and by dominant *bsd1* mutations. Isogenic *sec14-1*^{ts} strains carrying *cki*, *cct* or *bsd1-128* mutations, and either a control yeast centromeric plasmid (pRS316; Sikorski and Hieter, 1989) or an isogenic *KES1* plasmid (YCp*KES1*), were streaked for isolation on uracil-free medium and incubated at 37°C. The indicated Kes1p-overproducing strains could no longer grow at 37°C, and these growth defects were *sec14-1*^{ts}-dependent. The *sec14-1*^{ts} strains employed included: CTY128 (*bsd1-128*)/YCp(*KES1*), CTY128 (*bsd1-128*)/pRS316, CTY392 (*cki-284::HIS3*)/YCp(*KES1*), CTY392 (*cki-284::HIS3*)/pRS316, CTY102 (*cct*), cry102 (*cct*)/YCp (*KES1*). Complete genotypes are given in Table I. (**B**) Trafficking of the vacuolar proteinase carboxypeptidase Y (CPY) to the yeast vacuole. Radiolabeled CPY species immunoprecipitated from clarified extracts (appropriate strains radiolabeled for 30 min at 37°C; relevant genotypes at bottom) were analyzed by SDS–PAGE and autoradiography. The p1 (ER), p2 (Golgi) and mature vacuolar (m) forms of CPY are indicated (Stevens *et al.*, 1982). The *sec14-1*^{ts} strains carrying the designated 'bypass Sec14p' mutations and pRS316, the control YCp(*URA3*) vector, exhibited wild-type efficiencies for CPY trafficking. Introduction of YCp*KES1* into these strains resulted in p2 accumulation to the extent observed for the *sec14-1*^{ts} control strain. The strains employed were isogenic and included: CTY182 (wild-type), CTY1-1A (*sec14-1*^{ts}) and the following *sec14-1*^{ts} strains: CTY128 (*bsd1-128*)/YCp(*KES1*), CTY128 (*bsd1-128*)/pRS316, CTY392 (*cki-284::HIS3*)/YCp(*KES1*), and CTY392 (*cki-284::HIS3*)/pRS316. Complete genotypes are given in Table I. (**C**) Flux through the CDP-choline pathway for PC biosynthesis was determined by [^{14}C]choline pulse-radiolabeling (30 min at 26°C). Relevant genotypes are indicated. Samples were normalized on the basis of incorporation of radiolabel into cells as previously described (Skinner *et al.*, 1995). The *kes1Δ1::URA3* and *bsd1-128* mutations effect 'bypass Sec14p' without reducing flux through the CDP-choline pathway. A control *cki* strain (genetically inactivated for this pathway; see Figure 7 for an illustration of the CDP-choline pathway) is unable efficiently to incorporate [^{14}C]choline into PC. The strains employed were isogenic and included: CTY182 (Wild-type), CTY1-1A (*sec14-1*^{ts}), CTY862 (*kes1Δ1::URA3*), CTY160 (*cki*) and CTY128 (*bsd1-128*). Complete genotypes are given in Table I.

activity in these yeast strains. [^{14}C]Choline pulse-radiolabeling experiments indicated, however, that *cki* and *cct* strains were similarly defective in CDP-choline pathway activity regardless of whether Kes1p levels were elevated, or not (unpublished data).

Kes1p and Bsd1p act downstream of the CDP-choline pathway execution point in the Sec14p pathway*

The phenotypic and biochemical data demonstrating that Kes1p overproduction counteracted the 'bypass Sec14p' properties of CDP-choline pathway mutants and *bsd1* mutants identified *KES1* as a multicopy suppressor of both CDP-choline pathway defects and *bsd1* mutations when viewed in the context of their 'bypass Sec14p' phenotypes. Overexpression of downstream components in a regulatory pathway often suppresses defects in upstream components in that same pathway (Rine, 1991; Brennwald *et al.*, 1994). Thus, we predicted that the *kes1Δ1::URA3* and *bsd1* mutations would exhibit execution points in the Sec14p pathway that lay downstream of the CDP-choline pathway execution point, and that *bsd1* and *kes1* mutations would have no effect on metabolic flux through the CDP-choline pathway for PC biosynthesis.

To test this prediction, we employed a [^{14}C]choline pulse-radiolabeling strategy to monitor specifically the activity of the CDP-choline pathway as a function of Kes1p deficiency and *bsd1* mutations. As illustrated in

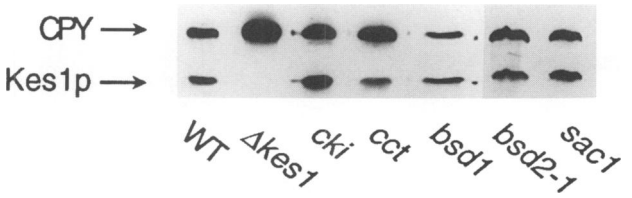

Fig. 5. Representative Kes1p immunoblots of cell-free lysates prepared from *KES1*, *kes1Δ1::URA3* and 'bypass Sec14p' mutants (relevant genotypes at bottom). All 'bypass Sec14p' mutants accumulated essentially normal steady-state levels of Kes1p, indicating that the mechanism of Sec14p bypass was not the trivial result of reducing intracellular Kes1p levels. Sample loading consistency was normalized by co-monitoring CPY antigen. Immunoblots were developed using the ECL™ system (Amersham). Primary antibodies were used at 1:1000 and 1:4000 dilutions for Kes1p and CPY, respectively. The strains employed were isogenic in all cases but for the *bsd2-1* strain, and included: CTY182 (wild-type), CTY862 (*kes1Δ1::URA3*), CTY160 (*cki*), CTY128 (*bsd1-128*), CTY31 (*bsd2-1*) and CTY162 (*sac1-17^{cs}*). Complete genotypes are given in Table I.

Figure 4C, the data demonstrated that *kes1Δ1::URA3* and *bsd1-128* did not significantly attenuate metabolic flux through the CDP-choline pathway. Whereas the control *cki* strain that is genetically inactivated for CDP-choline pathway activity incorporated only ~10% of the [^{14}C]-choline label into PC that the isogenic wild-type and *sec14-1^{ts}* strains were able to incorporate, the *kes1Δ1::URA3* and *bsd1-128* strains exhibited efficiencies of incorporation of radiolabeled choline into PC that were 112 ± 7% and 110 ± 9% those of wild-type. We had previously reported that *bsr3-2* strains exhibit defects in CDP-choline pathway activity as indicated by [^{14}C]choline and [^{32}P]orthophosphate pulse-radiolabeling experiments (McGee *et al.*, 1994). Subsequent analysis has demonstrated, however, that this effect is both peculiar to the *bsr3-2* allele and is strain-dependent (not shown).

These collective data established that *kes1* and *bsd1* mutations exerted their respective 'bypass Sec14p' effects in the face of robust CDP-choline pathway activity. Consequently, the Kes1p and *bsd1* gene product (Bsd1p*) execution points were placed downstream of the CDP-choline pathway execution point in the Sec14p pathway for Golgi-derived transport vesicle biogenesis. In this regard, the finding that Kes1p is a negative effector in the Sec14p pathway suggested the possibility that either preclusion of Kes1p expression or of Kes1p accumulation represented a simple 'bypass Sec14p' mechanism commonly shared by the seven known classes of 'bypass Sec14p' mutations. Immunoblotting experiments demonstrated that steady-state levels of the 51 kDa Kes1p were not generally depressed in strains harboring 'bypass Sec14p' alleles (Figure 5).

kes1 mutations do not effect the 'bypass Sec14p' phenotype through modulation of the yeast sterol/mevalonate pathway

While no biological function has yet been confidently assigned to any member of the OSBP family, these proteins have been suggested to function in the regulation of sterol/mevalonate biosynthetic pathway activity (Ridgway *et al.*, 1992; Jiang *et al.*, 1994; Ridgway and Lagace, 1995). We therefore employed several independent methods to test whether modulation of sterol/mevalonate pathway activity influenced the cellular requirement for Sec14p, and whether the basis for the 'bypass Sec14p' effects of *kes1* mutations was exerted through modulation of the mevalonate/sterol pathway (see Figure 6A).

First, we assessed whether alterations in bulk membrane sterol composition resulted in a 'bypass Sec14p' phenotype. These experiments involved introduction of *erg3Δ* and *erg6Δ* alleles, which strongly alter bulk yeast membrane properties by respectively precluding synthesis of C5=6 sterols and methylated sterols (Gaber *et al.*, 1989; Smith and Parks, 1993), into *sec14-1^{ts}* strains. The data indicated that neither of these *ergΔ* mutations exerted measurable suppression of *sec14-1^{ts}* growth or secretory defects at 37°C; the latter parameter indicated by the quantitative similarity of the secretion indices of the *sec14-1^{ts}* (0.18 ± 0.02), *sec14-1^{ts}*, *Δerg3* (0.19 ± 0.01) and *sec14-1^{ts}*, *Δerg6* strains (0.25 ± 0.02) (Figure 1B and C).

Second, we employed both genetic and pharmacological strategies to determine whether reduction of metabolic flux through the yeast mevalonate/sterol pathway could influence the cellular requirement for Sec14p. We found that neither *hmg1::LYS2* nor *hmg2::HIS3* mutations, which respectively inactivate the major and minor isoforms of yeast 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) (Basson *et al.*, 1986), suppressed *sec14-1^{ts}* growth and secretory defects (Figure 1B and C). [^{14}C]-Acetate pulse-radiolabeling experiments demonstrated that the *hmg1::LYS2* mutation indeed evoked a dramatic reduction (on the order of 100-fold) in metabolic flux through the mevalonate pathway as judged by its strong inhibition of incorporation of ^{14}C-radiolabel into squalene (Figure 6B). In confirmation of those data, pharmacological inhibition of mevalonate/sterol pathway activity with lovastatin or zaragozic acids [i.e. inhibitors of HMG-CoA reductase and squalene synthase, respectively (Alberts *et al.*, 1980; Bergstrom *et al.*, 1993; see Figure 6A)], to the extent that mevalonate/sterol pathway activity actually became growth limiting, also failed to relieve *sec14-1^{ts}* growth and secretory defects (not shown).

Finally, the *kes1Δ1::URA3* mutation itself had no significant effect on metabolic flux through the mevalonate pathway relative to wild-type yeast (Figure 6B). Moreover, neither bulk membrane sterol content nor composition was altered in *kes1Δ1::URA3* strains (not shown). Indeed, no obvious relationship was discerned between the effects of Kes1p, Hes1p or Osh1p dysfunction on flux through the mevalonate pathway and on the cellular Sec14p requirement (Figure 6B). Both *kes1Δ1::URA3* and *osh1Δ::URA3* strains exhibited essentially normal values for metabolic flux through the mevalonate pathway as these strains incorporated 5.2 ± 0.8% and 4.7 ± 0.1% of the lipid-incorporated [^{14}C]acetate label into squalene (relative to 4.9 ± 0.2% and 4.5 ± 0.5% for wild-type and *sec14-1^{ts}* strains, respectively). Yet, *kes1Δ1::URA3* effected 'bypass Sec14p' while *osh1Δ::URA3* did not. Moreover, as also shown in Figure 6B, *hes1Δ::LEU2* strains exhibited a reduced flux through the mevalonate pathway (2.2 ± 0.3% of the lipid-incorporated [^{14}C]acetate label was incorporated into squalene; a reduction of ~55% relative to wild-type), and *hes1Δ::LEU2* failed to effect a 'bypass Sec14p' phenotype. These results clearly demonstrated that neither the cellular requirement for Sec14p

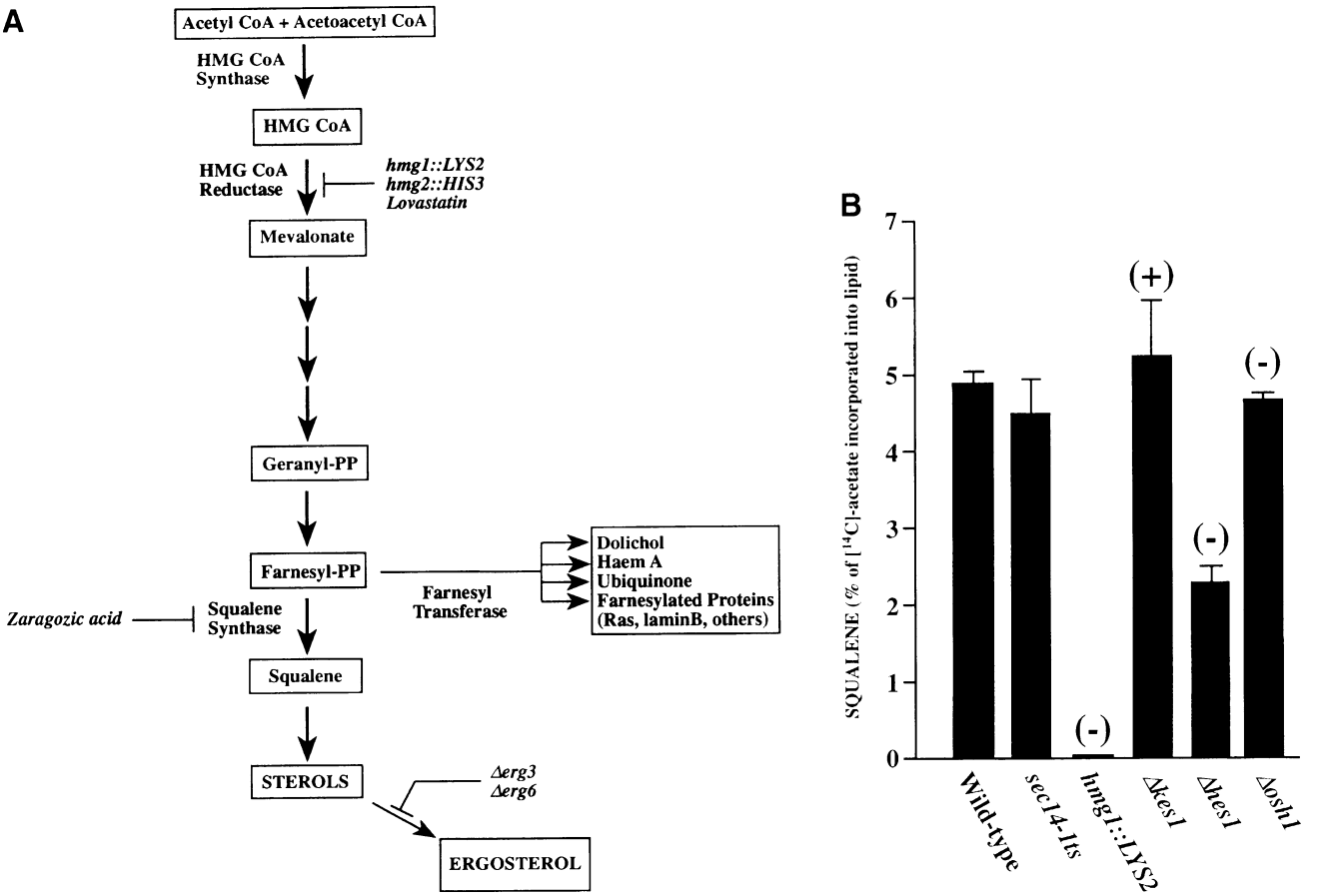

Fig. 6. (**A**) A diagram of the yeast mevalonate/sterol biosynthetic pathway is shown, and hallmark enzymes and pathway intermediates are indicated (adapted from Goldstein and Brown, 1990). Pyrophosphate is abbreviated to PP. The execution points of mutations or inhibitory drugs are also indicated. (**B**) Flux through the early stages of the mevalonate/sterol biosynthetic pathway was determined by [2-^{14}C]acetate pulse-radiolabeling and incorporation of label into squalene as a function of total incorporation of radiolabel into lipid (between 4 and 6×10^4 d.p.m./OD_{600} cells for each strain employed). Quantitation of squalene for each strain is indicated in the bar graph. The 'bypass Sec14p' phenotype of each strain is identified as (+) or (–). The strains employed were isogenic and included: CTY182 (Wild-type), CTY1-1A (*sec14-1^{ts}*), CTY868 (*sec14-1^{ts}, hmg1::LYS2*), CTY862 (*sec14-1^{ts}, kes1Δ1::URA3*), CTY844 (*sec14-1^{ts}, hes1Δ1::URA3*) and CTY869 (*sec14-1^{ts}, oshΔ1::URA3*). Complete genotypes are given in Table I.

nor the *kes1*-mediated mechanism for 'bypass Sec14p' was responsive to bulk membrane sterol composition or to metabolic flux through the sterol/mevalonate pathway.

Discussion

The Sec14p pathway

The Sec14p pathway is unique in a number of physiological respects, two of which are indicated here. First, it exhibits a singular requirement for phosphatidylinositol transfer protein function as evidenced by the demonstration that the first execution point for Sec14p in the yeast secretory pathway is at the level of secretory glycoprotein exit from a late Golgi compartment (Franzusoff and Schekman, 1989; Cleves *et al.*, 1991b). This distinguishes Sec14p from other protein factors known to be required for transport vesicle biogenesis (e.g. COP I and COP II vesicle coat components, GTPases of the ARF family, etc.; see Bednarek *et al.*, 1995; Mallabiabarrena and Malhotra, 1995) that exhibit early and, in some cases, multiple execution points. Second, there exists an intimate physiological linkage between Sec14p function and PC synthesis via the CDP-choline pathway in cells. Sec14p functions to protect Golgi membranes from CDP-choline pathway-mediated inactivation by insuring that this PC biosynthetic pathway is appropriately down-regulated in yeast Golgi membranes (Cleves *et al.*, 1991a,b; McGee *et al.*, 1994; Skinner *et al.*, 1995). Elucidation of the molecular basis for this relationship serves not only to reveal fundamental information regarding how the Sec14p pathway operates, but also to provide novel insight into how cells integrate phospholipid metabolism with membrane dynamics and protein transport.

In this study, we report two unanticipated findings concerning the function and regulation of the Sec14p pathway for Golgi-derived transport vesicle biogenesis in yeast. First, we provide evidence to indicate that a potent negative effector of this pathway is Kes1p, a yeast homolog of human OSBP. Second, we identify Kes1p as the agent through which the exquisite toxicity of CDP-choline pathway-driven PC biosynthesis to Golgi secretory function is likely mediated. The data presented herein describe the general form of an altogether novel regulatory pathway for Golgi-derived transport vesicle biogenesis that employs the opposing actions of a PI-TP and a member of the oxysterol binding protein family.

Kes1p is a negative effector of the Sec14p pathway for Golgi secretory function in yeast

Kes1p is a member of a large family of proteins that share primary sequence homology to the human OSBP (Ridgway *et al.*, 1992; Jiang *et al.*, 1994). Initially, human OSBP was proposed to function as the factor through which oxysterols effect down-regulation of HMG-CoA reductase in mammalian cells (Dawson *et al.*, 1989; Goldstein and Brown, 1990). However, the translocation of OSBP from cytosol to mammalian Golgi upon challenge of cells with 25-hydroxycholesterol is not particularly consistent with its proposed role in down-regulating the ER-localized HMG-CoA reductase (Ridgway *et al.*, 1992). The weight of the *in vivo* data suggests that OSBP executes some as yet undefined regulatory function on mammalian Golgi membranes, and that oxysterols serve to unmask a Golgi-targeting signal in the human OSBP (Ridgway *et al.*, 1992). Thus, the OSBP family of proteins currently represents a biological enigma. These proteins are widely, if not ubiquitously, distributed throughout the eukaryotic kingdom, yet no clear physiological function has been assigned to any of them.

Our demonstration that Kes1p is a negative effector in the Sec14p pathway for yeast Golgi-derived transport vesicle biogenesis provides insight into the physiological function of a member of the OSBP family. First, molecular cloning and meiotic segregation analyses cooperated to demonstrate that *KES1* is allelic to *BSR3*, a gene initially identified by three recessive 'bypass Sec14p' alleles (Cleves *et al.*, 1991b). Second, naive *kes1Δ1::URA3* null alleles themselves exhibited a 'bypass Sec14p' phenotype (Figure 1A), and such *kes1* null alleles were also able to restore both wild-type growth and secretory properties to *sec14-1*^{ts} strains at the normally restrictive temperature of 37°C (Figure 1B and C). Third, nucleotide sequence analyses revealed that the three original *bsr3* alleles represented either non-conservative *kes1* missense mutations (i.e. *bsr3-2* and *bsr3-3*), or a *kes1* nonsense mutation (i.e. *bsr3-1*; Figure 2A and B).

Specificity of Kes1p function in the Sec14p pathway

An outstanding feature of the involvement of Kes1p in the Sec14p pathway was its functional specificity with regard to the other known yeast OSBP homologs. Only *kes1* mutations represented 'bypass Sec14p' alleles (Figure 1A–C). This specificity was most strikingly evident in the case of Hes1p which shares a 70% primary sequence identity with Kes1p (Jiang *et al.*, 1994). Not only were Kes1p and Hes1p functionally distinguished by the inability of *hes1* mutations to manifest themselves as 'bypass Sec14p' alleles (Figure 1A–C), but also by the failure of increased *HES1* gene dosage to compromise *kes1*-mediated suppression of *sec14-1*^{ts} growth and secretory defects. This latter result suggested that Hes1p was not a minor isoform of Kes1p. Finally, genetic, pharmacological and biochemical data demonstrated that: (i) Kes1p, its homology to OSBP notwithstanding, did not effect a detectable regulation of metabolic flux through the mevalonate/sterol biosynthetic pathway; and (ii) that mevalonate/sterol pathway activity did not influence the Sec14p requirement for Golgi secretory function (Figure 1B and C and Figure 6C).

How does Kes1p regulate yeast Golgi secretory function?

The role of Kes1p as a negative effector in the Sec14p pathway for Golgi secretory function poses two additional questions: (i) how is the activity of Kes1p itself regulated? and (ii) what downstream components of the Sec14p pathway does Kes1p regulate? With regard to the former question, we believe the demonstration that a subtle increase in intracellular Kes1p levels eliminated the 'bypass Sec14p' phenotypes associated with CDP-choline pathway dysfunction provides a powerful argument that Kes1p activity is stimulated by PC biosynthesis via the CDP-choline pathway. Thus, the Golgi-directed toxicity of the CDP-choline pathway is at least in part mediated through potentiation of Kes1p function (Figure 4A and B). Additional support for this concept is provided by our findings that: (i) Kes1p dysfunction failed to reduce metabolic flux through the CDP-choline pathway (Figure 4C), thereby indicating that Kes1p acts downstream of the CDP-choline pathway in the Sec14p regulatory circuit; and (ii) Kes1p overproduction did not resuscitate CDP-choline pathway activity in mutants genetically inactivated for that PC biosynthetic pathway (not shown).

The Kes1p overproduction data suggest a simple means for unifying the action of the CDP-choline pathway for PC biosynthesis and Kes1p in a Sec14p-dependent Golgi regulatory pathway, and this general model is illustrated in Figure 7. The homology of Kes1p to a lipid-binding protein (OSBP) is consistent with Kes1p representing a key lipid-regulated component whose activity is ultimately responsive to Sec14p function. The model proposed in Figure 7 offers two general possibilities for how Kes1p function might be regulated. One scenario is that the interaction of Kes1p with PC activates the negative effector function of Kes1p. Thus, increased levels of Golgi PC resulting from CDP-choline pathway biosynthetic activity are posited to result in Kes1p hyperactivity, and drive an inappropriate (and ultimately lethal) down-regulation of Golgi secretory function. Alternatively, Golgi membranes might exhibit an essential pool of diacylglycerol (DAG) that either directly or indirectly effects a down-regulation of Kes1p (McGee *et al.*, 1994; Kagiwada *et al.*, 1996; see Figure 7). In this case, unrestricted CDP-choline pathway activity is proposed to consume that essential lipid precursor pool and thereby inappropriately activate Kes1p, again resulting in a lethal Golgi secretory block. As accelerated turnover of PI appears to define one mechanism by which a bypass of the normally essential Sec14p requirement can be effected (Kagiwada *et al.*, 1996), and we have collected evidence consistent with elevated Golgi DAG bypassing the Sec14p requirement for Golgi secretory function (B.G.Kearns, T.P.McGee and V.A.Bankaitis, manuscript submitted), we presently favor this latter 'consumption of DAG' hypothesis.

Functional interaction of Bsd1p and Kes1p

What components of the Sec14p pathway might interact with Kes1p? While we note that the cytosolic localization of Kes1p is not immediately consistent with a Golgi-directed function for Kes1p (Figure 3), it does not preclude it either. The possibility remains that the association of Kes1p with Golgi membranes is only transient and therefore not readily detectable by the methods employed.

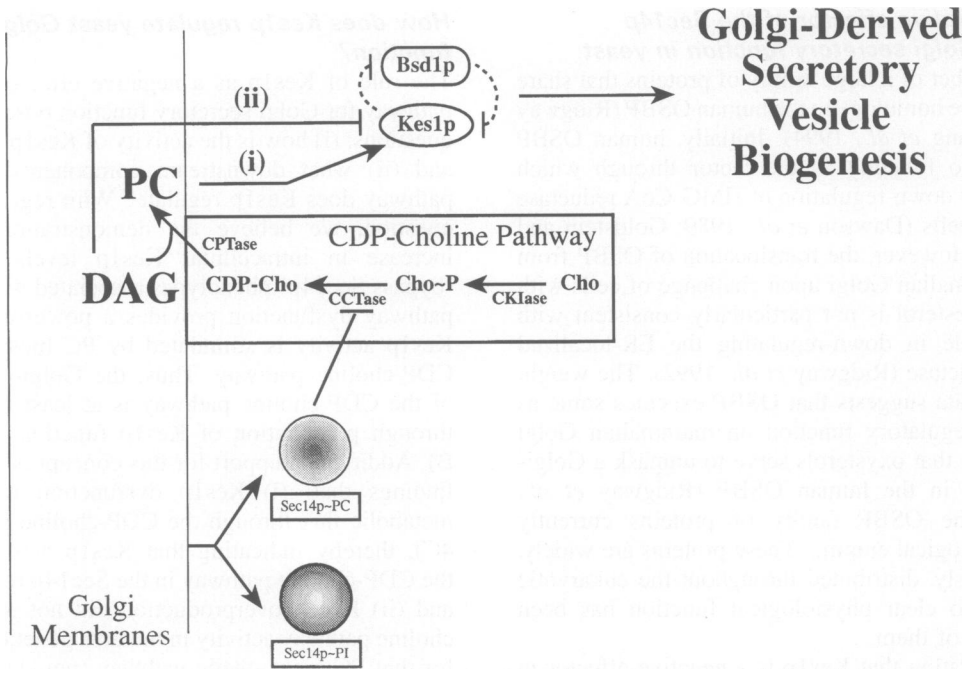

Fig. 7. A negative regulatory role for Kes1p in yeast Golgi secretory function. Sec14p senses the PC content of yeast Golgi membranes, as a function of PI levels, and the PC-bound form of Sec14p (Sec14p~PC) effects a negative feedback regulation on CDP-choline pathway activity (McGee *et al.*, 1994; Skinner *et al.*, 1995). Without Sec14p-mediated attenuation of CDP-choline pathway activity, the negative regulatory effects of Kes1p action could be potentiated either by: (i) synthesis of toxic levels of Golgi PC by the CDP-choline pathway; or by (ii) inappropriate consumption of a critical Golgi pool of lipid precursor (e.g. DAG) by that PC biosynthetic pathway. Kes1p function is opposed to the net positive action of the *BSD1* gene product (Bsd1p) either as a target of inhibition by Bsd1p or as a Bsd1p inhibitor (dashed lines). Abbreviations: Cho, choline; Cho~P, phosphorylcholine; CKIase, choline kinase; CCTase, cholinephosphate cytidylyltransferase; CPTase, cholinephosphotransferase.

Alternatively, Kes1p might function to sequester a Golgi-active component in the cytosol, thereby preventing execution of function by such a component. With these caveats, both scenarios for regulation of Kes1p are consistent with the available biochemical and genetic data, and the concepts proposed here make experimentally testable biochemical predictions.

Our data identify the *BSD1* gene product (Bsd1p) as an especially attractive candidate for a component that functionally interacts with Kes1p. The *BSD1* gene was originally recognized on the basis of dominant 'bypass Sec14p' mutations (Cleves *et al.*, 1991b), thereby implicating Bsd1p as a positive effector of the Sec14p pathway. That a subtle increase in intracellular Kes1p uniformly eliminated the 'bypass Sec14p' phenotypes associated with *bsd1* mutations suggests an intimate, and antagonistic, relationship between Bsd1p and Kes1p (Figure 4A and B).

At this point, we cannot distinguish whether Kes1p acts to regulate Bsd1p, or vice versa (Figure 7). One possibility is that Kes1p negatively regulates Bsd1p, and that dominant *bsd1* alleles represent mutations that reduce the affinity of Bsd1p for Kes1p. We find this hypothesis attractive because it accounts for: (i) the finding that Kes1p overproduction overcame the 'bypass Sec14p' condition associated with *bsd1* mutations (Figure 4A and B); and (ii) the high frequency with which dominant *bsd1* alleles were independently isolated in the initial search for suppressors of Sec14p defects (Cleves *et al.*, 1991b). One generally expects dominant alleles to represent statistically rare gain-of-function mutations. A more precise interpretation of how Bsd1p and Kes1p interact at a functional level will ultimately follow from determination of whether *kes1* null mutations are epistatic to *bsd1* null mutations, or not, and binding studies with purified Kes1p and Bsd1p. These endeavors require the cloning of *BSD1* and detailed characterization of its product.

Materials and methods

Strains, media, genetic techniques and reagents

Genotypes for the yeast strains employed in this study are compiled in Table I. Yeast complex medium supplemented to a final glucose concentration of 2% (YPD), and synthetic complete media have been described (Sherman *et al.*, 1983). Basic yeast genetic techniques, transformation methods and gene disruption technologies have been described (Ito *et al.*, 1983; Rothstein, 1983; Sherman *et al.*, 1983). Plasmid shuffle assays to ascertain the ability of a given disruption allele to effect 'bypass Sec14p' utilized the Δ*sec14* strain CTY558 (Table I) and were performed essentially as described by Lopez *et al.* (1994). Invertase secretion indices were determined exactly as previously described (Salama *et al.*, 1990).

[^{14}C]Choline chloride (40–60 mCi/mmol) and [2-^{14}C]acetate (56 mCi/mmol) were purchased from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL), respectively. Thin-layer silica gel chromatography plates (250 μm) were from Analtech (Newark, DE).

Cloning and characterization of the BSR3 gene

The recovery of *BSR3* genomic clones was predicated on the recessive 'bypass Sec14p' phenotype of *bsr3* strains. Yeast strain CTY156 (MATa, *ura3-52*, *sec14-1^{ts}*, *bsr3-2*) was transformed to Ura$^+$ at 30°C with a yeast genomic library propagated in the centromeric *URA3* shuttle plasmid YCp50 (Rose *et al.*, 1987). This *sec14-1^{ts}* strain is phenotypically Ts$^+$ due to the presence of the suppressing allele *bsr3-2*. The Ura$^+$ transformants were individually patched onto selective medium and subsequently replica plated onto duplicate plates incubated at 30°C and 37°C, respectively. Of the some 20 000 transformants analyzed, only one exhibited an unselected *ts* phenotype (i.e. inability to grow at 37°C) that was both plasmid-linked and remediated by *SEC14*. The genomic insert responsible for reversion of the *bsr3* phenotype was subcloned to

Table I. Yeast strains

Strain	Genotype	Origin
CTY182	MATa, *ura3-52, Δhis3-200, lys2-801*	Bankaitis *et al.* (1989)
CTY1-1A	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}*	Bankaitis *et al.* (1989)
CTY11	MATa, *ade2-201, sec14-1^{ts}, bsr3-1*	this study
CTY14	MATa, *ade2-201, sec14-1^{ts}, bsr3-2*	this study
CTY15	MATa, *ade2-201, sec14-1^{ts}, bsr3-3*	this study
CTY31	MATa, *ade2-201, sec14-1^{ts}, bsd2-1*	McGee *et al.* (1994)
CTY102	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, cct-2*	this study
CTY128	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, bsd1-128*	this study
CTY148	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, bsd1-148*	this study
CTY160	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, cki-1*	Cleves *et al.* (1991b)
CTY162	MATa, *ura3-52, lys2-801, sec14-1^{ts}, sac1-17^{cs}*	Kagiwada *et al.* (1996)
CTY392	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, cki-284::HIS3*	McGee *et al.* (1994)
CTY480	MATa, *ade2-101, ade3, leu2, Δhis3-200, ura3-52, sec14-1^{ts}*	this study
CTY558	MATa, *ade2-101, ade3, leu2, Δhis3-200, ura3-52, sec14Δ::HIS3, pCTY11*	Lopez *et al.* (1994)
CTY835	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, bsd1-128/YCp(KES1)*	this study
CTY836	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, ckiΔ::HIS3/YCp(KES1)*	this study
CTY844	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, hes1Δ::URA3*	this study
CTY846	MATa, *ade2-101, ade3, leu2, Δhis3-200, ura3-52, sec14Δ:HIS3, kes1Δ::URA3*	this study
CTY852	MATa, *ura3-52, leu2-3, sec14-1^{ts}, erg6Δ::LEU2*	this study
CTY861	CTY480, *hes1Δ::LEU2, kes1Δ::URA*	this study
CTY862	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, kes1Δ::URA3*	this study
CTY863	CTY480, *hes1Δ::LEU2, osh1Δ::URA*	this study
CTY868	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, hmg1Δ:LYS2*	this study
CTY869	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, osh1Δ::URA3*	this study
CTY870	MATa, *ura3-52, Δhis3-200, lys2-801, sec14-1^{ts}, hmg2Δ::HIS3*	this study
CTY873	MATa, *ade2-101, ade3, leu2, Δhis3-200, ura3-52, sec14-1^{ts}, erg3Δ::LEU2*	this study
CTY922	MATa, *ura3-52, Δhis3-200, lys2-801, YEp(KES1)*	this study

a 2.4 kb *Cla*I–*Sst*I restriction fragment that was subjected to nucleotide sequence analysis. The proof that the authentic *BSR3* gene was recovered is described in the Results.

Cloning and characterization of the three bsr3 alleles

Yeast genomic DNA samples (0.5 μg) isolated from the isogenic strains CTY11 (*bsr3-1*), CTY14 (*bsr3-2*) and CTY15 (*bsr3-3*) (see Table I) were individually used as templates in at least three independent PCR amplifications that employed the following oligonucleotides as forward and reverse primers, respectively: 5′-ATGGTACCCGTATGTACCTCA-TCTTG-3′ and 5′-ATGGGAGCTCCATCGATTATGTGGTTCTAC-3′. The resulting PCR products amplified *KES1* sequences from nucleotide −582 to +1803 (first nucleotide of *KES1* initiator codon designated as +1). These products were individually purified from agarose gels, restricted with endonucleases *Sst*I and *Kpn*I, and subcloned into the corresponding half-sites of pTZ19U (Mead *et al.*, 1986). The entire amplified sequence was, in each case, subjected to DNA sequence analysis via the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase version 2.0 kit of Amersham (Arlington Heights, IL) and double-stranded plasmid DNA as template. [α-^{32}P]dATP (800 Ci/mmol) radiolabel for the sequencing reactions was also purchased from Amersham.

Construction of kes1Δ::URA3 and hes1Δ::URA3 alleles

To generate the *kes1Δ1::URA3* allele, oligonucleotides KES-A (5′-ATGGGATCCCGTATGTACCTCATCTTG-3′), KES-B (5′-ATGAAGC-TTGGTGATTTCGTTGAAAGCGTG-3′), KES-C (5′-ATGAAGCTTT-CAGGCCAATGGTCTGGC-3′) and KES-D (5′-ATGGGAATTCAT-CGATTATGTGGTTCTAC-3′) were designed to amplify respectively two gene fragments by PCR. The former *KES1* PCR product represented nucleotide sequence that was bounded by *KES1* nucleotides −577 and −87 and lies upstream of the *KES1* initiator AUG codon (representing nucleotides +1 to +3). The latter PCR product was bounded by *KES1* nucleotides +802 to +1803, and this region encodes the C-terminal 171 amino acid residues of the Kes1p. These two PCR products were ligated at their unique *Hin*dIII half-sites and subsequently subcloned into the *Eco*RI–*Bam*HI half-sites of vector pUC18 that had been deleted for its endogenous *Hin*dIII site. The yeast *URA3* gene was then inserted into the unique *Hin*dIII site of that construct to yield plasmid pRE352. This plasmid carries the *kes1Δ1::URA3* allele which represents a deletion of the *KES1* sequences that encode the N-terminal 262 amino acids of Kes1p, and their replacement with *URA3*. The *kes1Δ1::URA3* allele was excised from pRE352 by an *Eco*RI–*Bam*HI restriction digestion for transformation into yeast.

To construct the *hes1Δ1::URA3* allele, oligonucleotides HES-A (5′-TCAGGATCCAGTTGGGTCTTCTTTTCAACC-3′) and HES-B (5′-TCAGGATCCTGACCATTGGCCAGAG-3′) were employed as primers in a PCR that amplified a 924 bp fragment bounded by *HES1* nucleotides −106 and +818. This fragment of *HES1*, which encodes residues 1–272 of Hes1p, was subcloned into the *Bam*HI site of pTZ18R (Mead *et al.*, 1986) to yield pRE353. This plasmid was restricted with restriction endonuclease *Bgl*II to liberate a 219 bp *HES1* fragment (encoding Hes1p residues 23–94), and a *URA3*-containing *Bam*HI fragment was inserted in its stead. The resulting *hes1Δ1::URA3* plasmid was designated pRE354, and this disruption allele was excised from pRE354 for transformation into yeast by *Bam*HI digestion.

Generation of anti-Kes1p serum

The *KES1* codons encoding residues 50–269 of Kes1p were fused in frame to the glutathione *S*-transferase (GST) structural gene in the Gene Fusion vector pGEX-5X-1 (Pharmacia). The resulting GST–Kes1p fusion protein was overproduced in *Escherichia coli* strain MC1066 (Silhavy *et al.*, 1982). Protein expression and purification were carried out according to the manufacturer's instructions. Rabbit polyclonal antibodies against the purified GST–Kes1p fusion protein were generated by Southern Biotechnology Associates, Inc. (Birmingham, AL), and these antibodies were both affinity-purified by binding to GST–Kes1p fusion protein and preabsorbed against GST prior to use.

Fractionation of Kes1p

Osmotic lysis of yeast cells was performed to obtain crude yeast lysate that preserves the integrity of intracellular organelles. Briefly, cells were grown to mid-logarithmic phase and converted to spheroplasts by incubation at 30°C for 1 h in 50 mM potassium phosphate pH 7.5, 1.2 M sorbitol, 50 mM β-mercaptoethanol, 10 mM NaN_3, with addition of lyticase to 0.5 mg/ml. Spheroplasts were pelleted by gentle centrifugation (500 *g*), and resuspended in osmotic lysis buffer (0.3 M sorbitol, 10 mM triethanolamine pH 7.2, 0.1 mM EDTA, 10 mM NaN_3) supplemented with protease inhibitors as previously described (Cleves *et al.*, 1991b; Whitters *et al.*, 1994). Lysis of spheroplasts was performed by incubation on ice for 20 min with frequent agitation. Unbroken cells were pelleted by a brief centrifugation at 1000 *g*, and the resultant clarified lysate was defined as the whole-cell lysate (S1). The S1 fraction was then further subjected to two rounds of differential centrifugation.

First, the S1 was centrifuged at 12 000 *g* for 15 min to yield the pellet (P12) and the supernatant (S12) fractions, and an aliquot of the S12 fraction was saved for subsequent analysis. The remainder of the S12 fraction was then subjected to a 100 000 *g* spin for 1 h to yield the pellet (P100) and the supernatant (S100) fractions. All of the pellet fractions were resuspended in lysis buffer so as to yield the same cell equivalents/unit volume as those represented in the corresponding supernatant fractions.

Pulse-radiolabeling and immunoprecipitation

The appropriate yeast strains were grown in minimal medium at 25°C, shifted to 37°C for 2 h, and pulse-radiolabeled for 30 min with ^{35}S-labeled amino acids (Tran ^{35}S-Label). Detailed methods for pulse-radiolabeling, preparation of clarified extracts, immunoprecipitation of carboxypeptidase Y (CPY) antigen, and resolution of the various CPY forms by SDS–PAGE and autoradiography have been described (Bankaitis *et al.*, 1989; Cleves *et al.*, 1991b).

[^{14}C]Choline pulse-radiolabeling

Methods for pulse-radiolabeling of yeast cells with [^{14}C]choline, quantitation and normalization of [^{14}C]choline incorporation, phospholipid extraction, and thin-layer chromatography have been described (McGee *et al.*, 1994; Skinner *et al.*, 1995). [^{14}C]Choline-radiolabeled phosphatidylcholine was visualized by autoradiography, excised from the thin-layer chromatogram, and quantified by scintillation counting (McGee *et al.*, 1994; Skinner *et al.*, 1995).

Resolution and quantitation of [2-^{14}C]acetate-labeled squalene

The appropriate yeast strains were grown to mid-logarithmic growth phase (OD_{600} = 0.6–1.0) in minimal medium and radiolabeled with [2-^{14}C]acetate (1 μCi/ml) for 30 min at 26°C with shaking. Lipids were extracted as described by McGee *et al.* (1994) and neutral lipids were resolved in one dimension by thin-layer chromatography on silica gel plates (250 μm; Analtech) using a petroleum ether:diethyl ether:acetic acid solvent system (85:15:1, v/v) according to the method of Buttke and Pyle (1982). Squalene was identified by co-chromatography with standards and by reported R_f value (Buttke and Pyle, 1982). ^{14}C-radiolabeled lipids were quantified by phosphorimaging.

Acknowledgements

We thank Randy Hampton, Bo Jiang, Howard Bussey, Todd Graham, Steven Smith and Leo Parks for plasmids, strains and discussion. Jan Tkacz and William Henckler (Merck Corp.) generously donated lovastatin and zaragozic acids, and Martin Bard is acknowledged for measuring bulk sterol content and composition in Δ*kes1* strains. Finally, we thank John Schutzbach, David Bedwell and Randy Schekman for dolichol–phosphomannose synthase, phosphoglucomutase and Kar2p antibodies, respectively. This work was supported by grant from the NIH (GM44530) to V.A.B. S.K., B.G.K. and A.G. were supported by a postdoctoral fellowship from the Human Frontier Science Program (LT-396-93), an NSF predoctoral traineeship (NSF-BIR-9256853) and a grant from the Council for Tobacco Research (3937) to V.A.B., respectively.

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Received on July 3, 1996; revised on August 12, 1996