Suppressors of *YCK*-encoded yeast casein kinase 1 deficiency define the four subunits of a novel clathrin AP-like complex

Heather R.Panek, J.David Stepp¹,
Holly M.Engle², Kim M.Marks,
Philip K.Tan^{3,4}, Sandra K.Lemmon¹ and
Lucy C.Robinson⁵

LSU Medical Center, Department of Biochemistry and Molecular Biology, 1501 King's Highway, Shreveport, LA 71130, ¹Case Western Reserve University, Department of Molecular Biology and Microbiology, Cleveland, OH, ²Moravian College, Bethlehem, PA and ³UCLA, School of Medicine, Department of Biological Chemistry, Los Angeles, CA, USA

⁴Present address: UCSF, Department of Neurology, San Francisco, CA, USA

⁵Corresponding author

In *Saccharomyces cerevisiae*, the redundant *YCK1* and *YCK2* genes (Yeast Casein Kinase 1) are required for viability. We describe here the molecular analysis of four mutations that eliminate the requirement for Yck activity. These mutations alter proteins that resemble the four subunits of clathrin adaptors (APs), with highest sequence similarity to those of the recently identified AP-3 complex. The four yeast subunits are associated in a high-molecular-weight complex. These proteins have no essential function and are not redundant for function with other yeast AP-related proteins. Combination of suppressor mutations with a clathrin heavy chain mutation (*chc1-ts*) confers no synthetic growth defects. However, a *yck^{ts}* mutation shows a strong synthetic growth defect with *chc1-ts*. Moreover, endocytosis of Ste3p is dramatically decreased in *yck^{ts}* cells and is partially restored by the AP suppressor mutations. These results suggest that vesicle trafficking at the plasma membrane requires the activity of Yck protein kinases, and that the new AP-related complex may participate in this process.
Keywords: casein kinase 1/clathrin adaptors/vesicular trafficking

Introduction

The family of protein kinases collectively called casein kinase 1 (CK1) has been characterized biochemically over the past several decades (Tuazon and Traugh, 1991). These protein kinases, ubiquitous among eukaryotic cell types, comprise a large family of related gene products. The members of the five CK1 groups termed α , β , γ , δ and ϵ each share at least 50% amino acid sequence identity within the protein kinase catalytic domain (Rowles *et al.*, 1991; Graves *et al.*, 1993; Fish *et al.*, 1995; Zhai *et al.*, 1995). While different isoforms show different tissue distributions and subcellular localizations, CK1 protein kinases share several characteristics. All are monomeric with sizes varying from 35 to 65 kDa. All exhibit Ser/Thr-specific protein kinase activity, use ATP exclusively as the phosphate donor, are

generally cofactor-independent, and rely on upstream acidic and/or phosphorylated amino acids for substrate recognition (Roach, 1990; Tuazon and Traugh, 1991).

Relatively little is known regarding specific physiological roles of any particular CK1 isoform. However, genetic and molecular characterization of CK1 isoforms in the budding yeast *Saccharomyces cerevisiae* has demonstrated their physiological significance. One, encoded by the *HRR25* gene, is a nuclear protein kinase that functions in DNA recombination and repair (Hoekstra *et al.*, 1991; DeMaggio *et al.*, 1992). A second yeast CK1 isoform, encoded by the *YCK3* (a yeast casein kinase 1 homologue) gene, appears to have functions distinct from the other isoforms but forms an essential gene pair with *HRR25* (Wang *et al.*, 1996). A third CK1 isoform, essential for viability, is encoded by duplicate genes *YCK1* and *YCK2* (Robinson *et al.*, 1992; Wang *et al.*, 1992). This isoform is likely anchored to the plasma membrane by a carboxy-terminal isoprenyl modification, since both Yck1p and Yck2p contain a conserved geranylgeranyl consensus sequence at the extreme carboxy-terminus, and elimination or alteration of this sequence abolishes membrane association (Robinson *et al.*, 1993; Vancura *et al.*, 1993, 1994).

Deletion of both *YCK1* and *YCK2* results in aberrant cellular morphology and growth arrest. A *yck^{ts}* strain, which contains a temperature-sensitive allele of *YCK2* (*yck2-2^{ts}*) and a deletion of *YCK1*, displays a similar phenotype at 37°C to that of *yck1⁻ yck2⁻* cells. At this restrictive temperature, *yck^{ts}* cells undergo several cell cycles without cell division, resulting in dramatically elongated buds. Since these buds usually define a single cellular compartment continuous with the mother cell, this CK1 form is required for cellular functions that include both morphogenesis and cytokinesis (Robinson *et al.*, 1993).

To identify proteins and pathways regulated by Yck-mediated phosphorylation, we have used the *yck^{ts}* mutant for genetic analysis. We have identified a novel clathrin adaptor (AP)-related complex through suppression of the *yck^{ts}* mutant. Clathrin AP complexes AP-1 and AP-2 interact with the cytoplasmic tails of membrane proteins and form an assembly site for clathrin, facilitating vesicular budding. We also provide evidence that wild-type levels of Yck activity are required for efficient constitutive endocytosis of the pheromone receptor Ste3p. Based on these results, we propose that Yck-mediated phosphorylation is required for some aspect of vesicle trafficking at the plasma membrane, and that the novel adaptor-related complex participates in this process.

Results

Isolation of mutations that suppress loss of Yck activity

To aid in identifying cellular pathways that require Yck-mediated phosphorylation, we isolated spontaneous sup-

pressors of temperature-sensitive *yck1⁻ yck2-2^{ts}* (*yck^{ts}*) strains (Robinson *et al.*, 1993; see Materials and methods). Twelve mutants that contained single mutations allowing growth of *yck^{ts}* cells at 37°C were characterized. Two mutants show wild-type growth rate and morphology at all temperatures. Analysis of meiotic progeny of crosses to LRB343 (*yck2::HIS3*) showed that both suppressors failed to recombine with the *YCK2* locus, and are likely to represent true revertants of the *yck2-2^{ts}* allele. Two other strains contain mutations linked to *YCK2* that do not restore wild-type growth rate and morphology, which could represent second-site suppressor mutations.

The remaining eight strains contained mutations, termed *yks* (*yck^{ts}* suppressor) mutations, that showed no genetic linkage to each other, to *YCK1* or *YCK2*. Seven *yks* mutations were fully recessive and complemented one another. One mutation was recessive to wild-type but displayed complex genetic character in complementation tests with the other seven recessive *yks* mutations. None restores wild-type growth and morphology. To determine whether the suppressor mutations could bypass the essential requirement for Yck activity, we examined meiotic progeny of crosses of each *yks yck^{ts}* strain to a *yck2::HIS3* strain. Strains carrying both *yck1::ura3* and *yck2::HIS3* were recovered from such crosses of four of the fully recessive suppressors and the complex recessive suppressor; thus, these mutations eliminate the essential requirement for Yck activity. However, *yck⁻ yks* strains do not show wild-type growth rate or morphology.

We describe here the characterization of the four fully recessive bypass suppressors, *yks4*, *yks5*, *yks6* and *yks7*. As shown in Figure 1, these mutations restore growth at restrictive temperature to the *yck^{ts}* mutant but only partially alleviate its morphological defect. The growth rate of the *yck^{ts} yks* strains is intermediate between the *yck^{ts}* strain and the *YCK⁺* strain (Figure 1A) as is the extent of elongated morphology (Figure 1B). None of these four mutations results in obvious associated growth or morphogenesis defects in *YCK⁺* strains (data not shown).

The *YKS* genes encode proteins resembling clathrin adaptor subunits

The four wild-type *YKS* genes were cloned from low copy genomic libraries by complementation of the ability of *yks yck^{ts}* strains to grow at 37°C. Each gene encodes a protein that shares limited but significant similarity with one of the four clathrin adaptor complex (AP) subunits, which with clathrin constitute a major group of vesicular transport coats. AP complexes are thought to be responsible for receptor capture and clathrin assembly during clathrin-mediated vesicular budding. The two major types of clathrin AP complexes in mammalian cells, AP-1 and AP-2, are restricted to Golgi and plasma membrane-derived coated pits and vesicles, respectively. AP-1 participates in sorting at the *trans*-Golgi, while AP-2 mediates selective endocytosis of plasma membrane proteins (Pearse and Robinson, 1990). The complexes are identical in architecture, each containing two large subunits (γ and $\beta 1$ in AP-1; α and $\beta 2$ in AP-2), one medium ($\mu 1$ or $\mu 2$) and one small subunit ($\sigma 1$ or $\sigma 2$). In addition, a related novel mammalian adaptor-like complex, AP-3, was recently identified (Newman *et al.*, 1995; Simpson *et al.*, 1996, 1997; Dell'Angelica *et al.*, 1997) that also contains two

Fig. 1. The *yks* mutations partially suppress the growth and morphogenesis defects due to thermosensitive Yck activity. (A) Drop growth tests with strains of the indicated genotype. Cells were grown to 2×10^7 cells per ml in rich medium and 5 μ l drops of undiluted culture (left column) and a 5-fold dilution (right column) were placed onto solid rich medium and incubated at the indicated temperatures for 24 h (30, 35 and 37°C) or 36 h (24°C) before plates were photographed. Strains used were: *yck^{ts}*, LRB756; *YCK⁺*, LRB758; *yck^{ts} yks4*, LRB746; *yck^{ts} yks5*, LRB748; *yck^{ts} yks6*, LRB750; *yck^{ts} yks7*, LRB752. (B) Cells of the indicated genotype from the 37°C plate shown in (A) (24 h incubation) were suspended on slides, viewed and photographed using phase optics. Final magnification, $\sim 600\times$, is identical for all panels.

large chains (δ and $\beta 3A$ or $\beta 3B/\beta$ -NAP), one medium ($\mu 3/p47$) and one small chain ($\sigma 3$). Although the function of this complex is not clear, it does not appear to co-localize with clathrin.

The amino acid sequences of the *YKS* gene products were aligned with multiple AP subunit sequences from the GenBank, Swiss-Protein and EMBL databases using the Clustal V program (Figure 2). The *YKS4* (chromosome XVI; also called YPL195w; accession number Z73551) and *YKS5* (chromosome VII; also called YGR261c; accession number S63450) genes encode products with predicted molecular weights of 107 and 92 kDa, respectively. Yks4p shows 18–19% sequence identity over most of its length to α and γ adaptins. Interestingly, as shown in the large subunit dendrogram (Figure 2A), Yks4p is most related (25% identity) to the recently identified AP-3 δ subunit (Simpson *et al.*, 1997). Most striking are the alignments over the first 182 amino acids of Yks4p, which show 44% identity with δ as compared with 20% and 26% identity with α and γ , respectively. Yks5p shows 21–22% identity to the AP β large chains, but again is most related (26% identity) to both neuronal and non-neuronal β isoforms of AP-3 (Figure 2A; Newman *et al.*, 1995; Simpson *et al.*, 1997).

The *YKS6* gene, on chromosome II (Feldmann *et al.*, 1994), was previously called *APM3* and YBR288c. This gene encodes a 55 kDa protein most similar in primary sequence to AP medium (μ) chains. The sequence similarity is again limited (22–24% identity) but extends over

most of the protein's length. Again, Apm3p shows slight preferential similarity to the corresponding AP-3 subunits (μ 3/p47; Figure 2B).

The 21 kDa YKS7 (chromosome X; also called YJL024c) predicted product is likely translated from a spliced message. The RNA that hybridizes to a DNA probe from the complementing region is ~600 bases long, 100 bases smaller than the expected size for an unspliced message

containing this open reading frame (data not shown). No in-frame ATG exists for the longest open reading frame within the complementing region and all conserved splice site elements are present for a 67 nucleotide predicted intron at the extreme 5' end of the coding sequence.

The Yks7 protein is slightly larger than the mammalian 17 and 19 kDa AP small (σ) chains with which it shares 29–33% amino acid identity. However, Yks7p is similar in size and shares strongest sequence similarity (37% identity) with the AP-3 σ 3A and σ 3B proteins (Figure 2C; Watanabe *et al.*, 1996; Dell'Angelica *et al.*, 1997; Simpson *et al.*, 1997).

Genes encoding AP-related proteins have been identified previously in yeast (Kirchhausen, 1990; Kirchhausen *et al.*, 1991; Nakayama *et al.*, 1991; Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). The *APL* genes (AP large chain) *APL1* and *APL2* encode β -like chains, and *APL3* and *APL4* encode α/γ -like proteins. Three other genes, *APM1*, *APM2* and *APM4*, encode proteins similar to AP medium/ μ chains, and two other genes (*APS1* and *APS2*) encode proteins similar to AP small/ σ chains. Some of these gene products show preferential similarity to AP-1 or AP-2 subunits (Figure 2), but none shows preferential similarity either to the Yks proteins or to the AP-3 subunits. Nevertheless, since each of the *YKS* gene products is related to adaptor subunits, we have adopted the established nomenclature for yeast AP subunit homologues. Thus, the revised gene designations are *YKS4* = *APL5*, *YKS5* = *APL6*, *YKS6* = *APM3* and *YKS7* = *APS3*.

The novel AP subunit-like products are associated in a high-molecular weight complex

Since all four subunits of an AP-like complex were identified in the *yck^{ts}* suppressor screen, it seemed likely that these proteins are associated in an adaptor-like complex. To test this prediction, we examined the elution pattern of the Apm3 subunit in a gel sizing column in wild-type and *aps3::LEU2*, *apl5::HIS3* and *apl6::URA3* disruption strains, which are perfectly viable (see below). We predicted that loss of any of the other subunits would alter the elution pattern of Apm3p if these proteins form a complex.

Cells were lysed in a buffer that strips coats from membranes and a 100 000 g supernatant was fractionated over a Superose-12 column. Apm3p extracted from a wild-type strain eluted in a high-molecular-weight complex

Fig. 2. Dendrograms of AP subunits. (A) AP large chains (adaptins α , β , γ and δ). (B) AP medium (μ) chains. (C) AP small (σ) chains. Trees were constructed and displayed as described in Materials and methods. Accession numbers for sequences displayed: Apl1p (G M64998), Apl2p (S P36000), β 2 bovine (G M34177), β 1 human (G L13939), Apl6p/Yks5p (G U35411), β -NAP human (G U37673), β 3A human (G R02669/T98538), Apl3p (S P38065), α rat (S P18484), Apl4p (E Z49274/Z71255), γ mouse (E X54424), Apl5p/Yks4p (G U36858), δ human (G T30164/R54523), Apm1p (E X60288), μ 1 mouse (G M62419), unc-101 (G L26291), Apm4p (E X91067), μ 2 *Schizosaccharomyces pombe* (E Z50113), μ 2 *Dictyostelium discoideum* (G U44890), μ 2 rat (G M23674), CEAP (G L26290), Apm2p (G U09841), Apm3p (E X76053/Z36157), p47a rat (G L07073), p47b rat (G L07074), p47 electric ray (G 07072), Aps1p (E Z20314), σ 1 mouse (G M62418), Aps3p (G U31448), σ 3a human (G N30960), σ 3b mouse (G W97614), Aps2p (G M37193), σ 2 rat (G M37194). Databases: G, GenBank; S, Swiss-Prot.; E, EMBL. Sequences of delta (δ), β 3A adaptins and σ 3 proteins were provided by M.S.Robinson and J.Bonafacino (personal communications).

Fig. 3. The *YKS* gene products are components of an AP-like complex. The 100 000 g supernatants from whole-cell extracts were applied to a Superose-12 column. Samples from collected fractions were separated on SDS-8% polyacrylamide gels and prepared for immunoblotting with anti-Apm3p serum. Molecular size standards are thyroglobulin (85 Å), ferritin (61 Å), catalase (52 Å) and BSA (35.5 Å). Indicated strains are: wild-type, SL1463; *yks4/apl5*, HPY20; *yks5/apl6*, LRB858; *yks7/aps3*, LRB739.

with an apparent Stokes radius of 71 Å (Figure 3, peak fractions 16–18). This size is comparable with those determined for previously described yeast and mammalian AP-like complexes.

When each of the other *YKS*/AP genes was disrupted, the elution pattern of Apm3p shifted to lower-molecular-weight species. The most dramatic shift was observed when the β -like chain encoded by *APL6/YKS5* was absent. Here, Apm3p eluted almost exclusively in fractions 26–29 (40 Å), which we suspect is the size of the monomeric species. This suggests that *in vivo* the β chain is most important for μ association in the adaptor complex, consistent with previous studies showing preferential interaction of μ with β (Page and Robinson, 1995; Seaman *et al.*, 1996). In addition to the putative monomeric Apm3p species, intermediate-size complexes were observed from mutants with disruptions of the δ -like (*apl5/yks4*) and σ -like (*aps3/yks7*) subunit genes. Although antibodies to the other subunits will be required to identify the components of the intermediate complexes, these biochemical data indicate that Apl5p, Apl6p, Apm3p and Aps3p likely function together in a unique adaptor-like complex.

The novel AP-like proteins are not essential for growth and do not share essential functions with previously identified yeast AP subunits

We generated strains disrupted for each of the four AP subunit-related genes (Materials and methods) and examined the consequences of loss of function. Single and multiple *yks* AP disruptions in *YCK*⁺ strains, including a quadruple mutant, confer no obvious growth defect. In contrast to yeast clathrin mutants, processing of the α -factor mating pheromone, reflecting proper late Golgi retention of Kex2p, and sorting and processing of the soluble vacuolar hydrolase carboxypeptidase Y appear normal in *yks* strains (data not shown). The disruptions were also introduced into *yck*^{ts} strains. In every case, the single obvious phenotype of disruption was suppression of defects resulting from loss of Yck function.

Previous studies of other yeast AP subunit genes also failed to uncover any obvious growth or protein trafficking defects associated with deletion of these genes in otherwise wild-type genetic backgrounds (G.Payne, personal communication; Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp

et al., 1995). This is surprising given the severe growth and sorting phenotypes associated with mutation of clathrin heavy chain. One explanation for the lack of phenotypic effect associated with loss of yeast APs is the presence of a functionally redundant subunit or complex(es). However, the gene products described here do not appear to be wholly redundant with other yeast AP subunits. Single deletions of *APS1*, *APS2*, *APM2*, *APL2* and *APL3* (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995) were generated in a *yck*^{ts} strain. None of these deletions supports growth of *yck*^{ts} strains at 37°C, indicating that the suppression phenotype is unique to loss of function of the *YKS* AP genes.

We also combined single *YKS* deletions with single deletions of *APS1*, *APM2* and *APL3*. All combinations resulted in strains with wild-type growth properties. We conclude that the new AP-like subunits carry out a function(s) distinct from the role(s) of other yeast adaptor-related proteins.

Normal clathrin function is required in a *yck*^{ts} strain

The only phenotypic consequences of loss of function of any yeast AP-like subunit have been observed in strains impaired for clathrin function (G.Payne, personal communication; Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). Mutations in *APS1*, *APM1*, *APL2* and *APL4* display synthetic growth defects with, and exacerbate the late-Golgi retention phenotype of, strains carrying a conditional clathrin heavy chain allele, *chc1-ts* (Seeger and Payne, 1992). We tested whether disruption of the *yks* genes confers similar synthetic growth defects with the *chc1-ts* mutant. No difference in growth rate was observed for single or multiple *yks* AP mutations in the *chc1-ts* background. Thus, the relationship of these AP-like proteins with clathrin is unclear, as it is for several other yeast AP-related proteins (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995).

We next examined the genetic relationship between Yck activity and clathrin function by combining *yck*^{ts} with *chc1-ts*. Unlike either single mutant, the growth of *yck*^{ts} *chc1-ts* strains is extremely slow, even at 24°C, and such mutants are inviable at temperatures above 30°C (Figure 4). The morphology of the *yck*^{ts} *chc1-ts* cells at 24°C is identical to that of *yck*^{ts} cells at restrictive temperature; therefore Yck activity may be required for a pathway that is parallel to or intersects a clathrin-dependent pathway.

We tested for the ability of the *yks* mutations to suppress the severe growth defect of *chc1-ts yck*^{ts} strains. If loss of this AP-like complex affects a clathrin-independent pathway, then these mutations should restore growth of the *chc1-ts yck*^{ts} strains to that of the single *chc1-ts* mutant. Instead, deletion of the novel AP genes had no effect on the growth of *chc1-ts yck*^{ts} mutants. One explanation for these results is that suppression of the *yck*^{ts} growth defects by the AP mutations requires clathrin function.

Endocytosis of Ste3p is normal in *YKS* AP-deleted strains

The *yks* AP mutations suppress the lethality due to loss of plasma membrane-localized Yck activity and the predicted Yks products form an AP-like complex, together suggesting a function in an endocytic pathway. Therefore,

Fig. 4. The *chc1-ts* mutation shows a strong synthetic growth defect with decreased Yck activity. Cells were grown in YEPglucose at 24°C to $\sim 2 \times 10^7$ cells/ml. Drops of undiluted culture (left columns) and a 5-fold dilution (right columns) were placed onto solid rich medium and plates were incubated at the indicated temperatures for 24 h (30, 35 and 37°C) or 36 h (24°C) before plates were photographed. Strains are: *yckts*, LRB756; *YCK+*, LRB758; *chc1-ts*, LRB781; *yckts chc1-ts*, LRB779.

we tested whether deletion of the *YKS* AP genes had any effect on constitutive endocytosis of the *STE3*-encoded α -factor pheromone receptor. Ste3p is internalized at a high constitutive rate and degraded in the vacuole (Davis *et al.*, 1993; Givan and Sprague, 1997); thus, its rate of turnover directly reflects its internalization rate.

Analysis of Ste3p turnover was performed using a *GALI*-driven *STE3* gene (Davis *et al.*, 1993), whose expression is induced by galactose and rapidly shut off upon addition of glucose. Assays of Ste3p stability during a glucose chase at 24°C were carried out by immunoblot analysis. As shown in Figure 5A, the amount of Ste3p in wild-type cells declines rapidly, little protein being detectable after 90 min. The half-life estimated from these and other data is ~ 30 min, slightly longer than that reported for Ste3p at 30°C (Davis *et al.*, 1993). Although the initial level of Ste3p appears higher in the *YCK+* *apl5/yks4* mutant, the rate of degradation is comparable with that in the wild-type strain (Figure 5B). Similar results were obtained for other *YCK+* *yks* mutant strains (data not shown). These data indicate that this novel AP-like complex is not required for constitutive internalization of Ste3p.

***YCK*-encoded CK1 activity is required for internalization of Ste3p and the *yks* AP mutations partially alleviate this requirement**

The identification of AP-related proteins in the *yckts* suppressor screen, the plasma membrane localization of the Yck proteins, and the genetic interaction with clathrin

Fig. 5. Stability of Ste3p is unaffected by *yks* mutations. The stability of the constitutively internalized membrane protein Ste3p was assayed in the wild-type strain KMY28 (A) and the *yks4/apl5* strain KMY25 (B). For pulse-chase assay using a p*GALI*-driven *STE3* construct (see Materials and methods), cells were grown in YEPgalactose, shifted to YEPglucose and sampled at the indicated times after the shift. Western blots were probed with anti-Ste3p (upper panels) and reprobbed with antisera to phosphoglycerate kinase (Pgk) to provide loading controls (lower panels).

activity together suggest that Yck-mediated phosphorylation could regulate vesicle trafficking to or from the plasma membrane. Constitutive endocytosis of Ste3p was therefore examined in *yckts* mutants. Assays of Ste3p stability were carried out with cells grown at 24°C, the permissive temperature for *yckts*. In contrast to the results with the wild-type strain (Figure 6A), Ste3p levels in the *yckts* strain remain approximately the same throughout 120 min of glucose chase (Figure 6B).

To test whether the increase in stability of Ste3p is due to a defect in constitutive internalization or to accumulation in internal compartments, we determined the accessibility of Ste3p to extracellular protease at each time point (Davis *et al.*, 1993). Accessibility to protease is demonstrated by the appearance of a major cleavage product that includes the membrane-spanning and cytoplasmic domains of Ste3p. As shown in Figure 6B, the majority (>70%) of the Ste3p in *yckts* cells remains accessible to protease at all time points, suggesting that these cells are defective in clearance of the receptor from the cell surface rather than in subsequent trafficking of Ste3p to the vacuole.

To determine if the *yks* adaptor mutations suppress the endocytosis defect associated with *yckts*, we assayed the constitutive endocytosis rate of Ste3p in *yks yckts* mutant strains (Figure 6C). Significant internalization of Ste3p in an *apl5/yks4 yckts* strain occurred over the time period assayed, albeit more slowly than in the wild-type strain. The half-life of Ste3p in the *apl5/yks4 yckts* strain estimated from these and other data was 75–80 min. Mutations in the other three *YKS*/AP subunit genes conferred similar

Fig. 6. Constitutive internalization of Ste3p is blocked in *yck^{ts}* strains and is partially restored by loss of the AP-like gene products. The stability of Ste3p and its protease accessibility at the cell surface were assayed as described in Materials and methods. Samples removed at the indicated times after a shift from galactose to glucose medium (minutes chase) were split, incubated without (–) or with (+) Pronase, and prepared for electrophoresis, followed by immunoblotting with anti-Ste3p antiserum. (A) Wild-type strain KMY28. (B) *yck^{ts}* strain KMY29. (C) *yck^{ts} yks4/apl5* strain KMY21. Arrowheads mark the positions of the major Ste3p species; the doublet of intact Ste3p in (A) may represent differentially modified species. Blots were reprobed with anti-Pgk to provide loading controls.

destabilization of Ste3p in *yck^{ts}* strains (data not shown). Therefore, loss of function of the AP complex also partially rescues the internalization defect of the *yck^{ts}* mutant.

Discussion

We describe here the identification of subunits of a novel yeast AP-like complex, encoded by the *APL5* (*YKS4*), *APL6* (*YKS5*), *APM3* (*YKS6*) and *APS3* (*YKS7*) genes. The genes were identified by loss of function suppressor mutations that allow growth of *yck^{ts}* cells at restrictive temperature and partially correct the morphological and endocytic defects associated with loss of Yck protein kinase activity. Genetic and biochemical data suggest that these novel AP-like products associate in a complex. First, mutations in any of the four genes result in the same

phenotype-suppression of loss of Yck activity. Combination of mutations, including the quadruple disruption mutant, has no additive effect on suppression. Finally, Apm3p is detected in a high-molecular-weight complex, and its mobility in gel filtration chromatography is altered in the absence of any of the other Yks AP subunit-like proteins.

Previous characterization of yeast AP-related proteins has been based on the idea that they might operate like mammalian adaptor complexes and act in association with clathrin. Since there is no obvious effect on growth of strains deleted for any yeast AP subunits, the possible function(s) has been investigated by combining deletions of each with a temperature-sensitive allele of clathrin heavy chain (*chc1-ts*) (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). Loss of function of any of four AP-1 subunit-like proteins, Apl2p, Apl4p, Apm1p and Aps1p, exacerbates the growth and α -factor pheromone maturation defects of a *chc1-ts* strain, but does not affect the pheromone receptor endocytosis defect (G. Payne, personal communication; Nakayama *et al.*, 1991; Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). These and other data have led to the conclusion that these four subunits define an AP-1-like complex that functions with clathrin at the Golgi. At this time, no yeast AP subunits have been assigned to an AP-2-like complex, although Apl3p and Apm4p are most related to AP-2 α and μ 2 subunits, respectively (Figure 2). Deletion of other yeast genes encoding AP-like proteins has no synthetic effects with *chc1-ts*, and loss of function of any of these other adaptor-like proteins does not affect any vesicle trafficking process examined, leading to the proposal that these proteins could carry out a clathrin-independent function (Stepp *et al.*, 1995).

Based on their genetic relationship with *yck^{ts}* and the location of Yck protein in the plasma membrane (Vancura *et al.*, 1994), it is possible that the Yks AP-like proteins form a complex associated with the cell surface. However, like the non-AP-1 subunits, the Yks AP-like complex shows no genetic interaction with clathrin, so these new proteins could act differently than classical clathrin adaptor proteins. In this regard, it is interesting that all of the Yks AP-like subunits show closest similarity to subunits of the recently described AP-3 adaptor-like complex, which does not co-localize with clathrin or with AP-1 or AP-2 adaptors and does not co-purify with brain clathrin-coated vesicles (Newman *et al.*, 1995; Simpson *et al.*, 1996, 1997; Dell'Angelica *et al.*, 1997). This mammalian AP-3 complex is proposed to act in a clathrin-independent pathway of trafficking at the TGN and/or an endosomal compartment. The Yks AP complex may function in a similar clathrin-independent manner.

What is the relationship of Yck1 and Yck2 protein kinases with trafficking and with the Yks AP proteins? Our data indicate that the Yck1 and Yck2 protein kinases are required for some trafficking from the cell surface. Whether this role is essential for growth has yet to be determined, since mutants completely blocked for endocytosis show at most a temperature-sensitive growth defect. The major defect may be blockage of temporally specific traffic necessary for membrane remodeling or structural alterations that are necessary at different points during the cell cycle. The location of the Yck2 protein

A Yck phosphorylation of AP promotes further coat assembly and endocytosis **B The Yks AP functions in recycling from an endosomal compartment**

Fig. 7. Models explaining the *yck* internalization defect and its suppression by loss of function of the Yks adaptor complex. **(A)** Yckp phosphorylates the Yks adaptor to promote further coat assembly and endocytosis. In this model the Yks adaptor binds to the cytoplasmic tails of receptors, but phosphorylation of the adaptor is required for assembly of the outer coat that is needed for vesicle budding (left). In the absence of the kinase, receptors would be trapped at the cell surface by association with Yks adaptors (middle). Loss of adaptor function (right) would release receptors, allowing them to be internalized by a less efficient endocytic pathway. **(B)** The Yks adaptor functions in recycling from an endosomal compartment. In this model the kinase phosphorylates receptors to direct trafficking to the vacuole. Both phosphorylated and unphosphorylated receptors are internalized, but unphosphorylated receptors are recycled back to the cell surface from a perivacuolar compartment (PVC) or endosome via the Yks adaptors (left). In the absence of the kinase, all receptors would be in the recycling mode (middle). Additional loss of the adaptor would prevent recycling, allowing slow internalization and transfer of unphosphorylated receptors to the vacuole for degradation (right).

within the plasma membrane changes during the cell cycle, consistent with such a role (L.C.Robinson and C.Bradley, unpublished data).

The simplest model for the suppression of loss of Yck activity by loss of the Yks AP-like complex is that loss of this complex results in misdirection of a related protein kinase to the plasma membrane, where it can substitute for the essential Yck proteins. There are two closely related protein kinases in yeast, Hrr25p and Yck3p (Hoekstra *et al.*, 1991; Wang *et al.*, 1996). Hrr25p is a nuclear protein, but its misdirection to the plasma membrane allows substitution for Yck1/Yck2 function (DeMaggio *et al.*, 1992; Wang *et al.*, 1996). Although Yck3p forms an essential pair with Hrr25p, it carries a consensus sequence for isoprenylation, and a significant fraction is localized to the plasma membrane as well as to internal membranes. Intact *YCK3* acts as a dosage suppressor of *yck^{ts}* (Wang *et al.*, 1996). While overexpression of Yck3p does not support growth of *yck1⁻ yck2⁻*

strains (L.C.Robinson, unpublished data), it is possible that its specific localization or retention in the plasma membrane could account for the bypass suppression.

The relationship of the Yks AP subunit-like proteins with Yck activity could be more direct. A second model to explain the relationship is that the Yks AP-like coat is required for clathrin-independent internalization of a specific subset of plasma membrane proteins, and that Yck phosphorylation of one or more subunits of the complex is required for assembly of an outer coat needed to drive vesicle budding (Figure 7A). In this model, the abnormal stability of Ste3p in the membrane of *yck^{ts}* cells is explained by trapping of the receptors by adaptors at the cell surface. Loss of AP complex function by deletion of one or more subunits would alleviate sequestering of receptors, and allow slow internalization by another mechanism, e.g. a clathrin-dependent mechanism.

This model explains both the synthetic growth defect of *yck^{ts}* with *chc1-ts*, as well as the lack of suppression

of this defect by the *yks*/AP mutations. The loss of both Yck-stimulated and clathrin-dependent endocytic mechanisms, combined with other *chc1-ts* defects, could account for the synthetic growth defect. The suppression of *yck^{ts}* by the *yks* mutations would require a clathrin-dependent pathway, so these adaptor mutations would not be predicted to rescue the synthetic *yck^{ts} chc1-ts* defect.

Another prediction of this model is that loss of the kinase might result in the accumulation of unphosphorylated Yks adaptor complex at the plasma membrane. Further studies are under way to test whether any subunits of the Yks complex are phosphorylated in a Yck1/Yck2-dependent manner and whether this affects association with membranes. Classical mammalian AP subunits are phosphorylated *in vivo* (Bar-Zvi *et al.*, 1988; Pauloin and Thurieau, 1994; Wilde and Brodsky, 1996), although in most cases the significance of the modification is unknown. In one study, phosphorylation of β 2 adaptin blocked association of clathrin with the AP-2 complex (Wilde and Brodsky, 1996). This effect is opposite from that predicted by our model, where phosphorylation by Yck1p/Yck2p would promote outer coat assembly, but different coats are probably involved and phosphorylation by distinct protein kinases could have distinct effects on assembly.

A third possible model is that the kinase phosphorylates cargo proteins directly to regulate their trafficking. Both the Ste3p and Ste2p receptors are phosphorylated, and phosphorylation has been suggested to regulate internalization (Zanolari *et al.*, 1992; Hicke and Riezman, 1996; Roth and Davis, 1996). However, our data do not support a model in which Yck phosphorylation of plasma membrane cargo promotes adaptor binding and internalization. In the *yck^{ts}* mutant, cargo would remain unphosphorylated and unable to bind adaptors, so absence of AP function should not suppress the internalization defect.

Alternatively, the Yks AP complex could act at an endosomal compartment to sort proteins destined for return to the cell surface. Phosphorylation of the cytosolic domains of membrane proteins by the Yck kinases would signal their movement to the vacuole for degradation, and lack of phosphorylation would result in return to the cell surface after recognition by the Yks AP-like complex (Figure 7B). Loss of Yck activity would result in the inability to subject this set of proteins to regulated degradation, such as is required for remodeling at the cell surface during cell growth. This model explains the abnormal stability of Ste3p in *yck^{ts}* cells as resulting from continual removal from and recycling to the surface. Loss of the Yks AP-like complex would allow removal of these proteins to the vacuole by other mechanisms, which would be unregulated and less efficient. The fact that cells lacking both Yck activity and the AP-like complex are not wild-type in growth rate, appearance or Ste3p turnover is consistent with this idea.

A model in which the Yks AP-like complex functions in recycling is appealing, since mammalian AP-3 seems to localize to an endosome-like compartment. As yet there is no direct evidence for a recycling endosome in yeast, although recent studies of yeast chitin synthases 1 and 3 have suggested that an internal compartment, the chitosome, has properties of a recycling endosome (Chuang and Schekman, 1996; Ziman *et al.*, 1996). Other models could explain the genetic interactions and phenotypes

observed in our studies, but further studies, including localization of the adaptor complex, are needed to distinguish between these possibilities.

Overall, our results provide direct evidence that the Yck protein kinases are involved in vesicle transport in yeast. Other studies have suggested such a role for CK1 enzymes. A mammalian CK1 isoform localizes to cytoplasmic vesicular structures and is highly enriched in synaptic vesicles (Brockman *et al.*, 1992; Gross *et al.*, 1995). Also, both *YCK1* and *YCK2* act as dosage suppressors of the *gcs1* mutation, which was reported to confer an endocytic defect (Wang *et al.*, 1996). *Gcs1p* is homologous to a mammalian ADP ribosylation factor (ARF) GTPase activating protein (Cukierman *et al.*, 1995), and genetic interactions of *gcs1* and *arf* mutants have been observed (Poon *et al.*, 1996). ARF small GTPases are thought to be important for the recruitment of certain coat proteins, including coatamer (COP1) and AP-1 adaptors, onto membranes (Stamnes and Rothman, 1993; Traub *et al.*, 1993). Recruitment of the mammalian AP-3 adaptor to membranes may also be regulated by small GTPases (Simpson *et al.*, 1996). It is intriguing to speculate that the function of this new Yks adaptor is affected by a *Gcs1p*-regulated small GTPase. Thus, the suppression of *gcs1* by the YCK-encoded CK1s could reflect modulation of the same pathway(s) that require the novel AP-3-like complex.

Materials and methods

DNA manipulation and analysis

Standard techniques were used for DNA manipulation (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989). Restriction and modification enzymes were used as recommended by the manufacturers (Promega, US Biochemical and New England Biolabs). The low copy YCp50-based libraries of Rose *et al.* (1987) and D.S.Conklin (unpublished) were used for cloning.

Insertion mutagenesis of the *YKS* plasmids was carried out in *Escherichia coli* strain RR1 (Maniatis *et al.*, 1982) using λ phage carrying Tn5 (Sullivan *et al.*, 1985). Plasmids carrying insertions were directly selected by addition of kanamycin to growth media. Positions of insertion were determined by restriction site mapping, and mutants were tested for function by complementation analysis in mutant strains.

DNA sequence was determined for both strands using the Sequenase 2.0 kit as recommended by the manufacturer (US Biochemical). Analysis of DNA sequences was carried out using DNAsis software and Clustal V multiple sequence alignment software. Database searches with predicted protein sequences were carried out either from the National Center of Biological Information or from the Saccharomyces Genome Database, using the Blast algorithm (Altschul *et al.*, 1990). Dendrograms were constructed by aligning sequences using the Clustal V program and generating tree diagram output using the Hypercard stack ClusToTree (programs courtesy of B.Fuller).

Yeast culture and manipulation

Yeast strains (Table I) were grown on standard rich (YEP) or synthetic medium at temperatures as indicated, and standard techniques were used for phenotypic and genetic analyses (Sherman *et al.*, 1986). Yeast genomic DNA for Southern hybridization was prepared by a rapid glass bead lysis method (Hoffman and Winston, 1987).

Yeast transformation was by the LiAc procedure (Ito *et al.*, 1983), modified by buffering all solutions with Tris-EDTA at pH 8.0. All gene disruptions were constructed *in vitro* and introduced into diploid strains by standard gene replacement (Rothstein, 1983). Strains confirmed by genomic Southern analysis to carry a disrupted allele were sporulated and haploid progeny were analyzed.

Isolation of suppressors of *yck^{ts}*

Strains deleted for *YCK1* and carrying the *yck2-2^{ts}* allele (*yck^{ts}*) are temperature-sensitive for growth with a restrictive temperature of 37°C

Table I. Yeast strains

Strain name	Genotype	Source/Reference
LRB264	<i>MATa his3 leu2 ura3-52 yck1-1::URA3</i>	Robinson <i>et al.</i> (1993)
LRB341	<i>MATa his3 leu2 ura3-52</i>	Robinson <i>et al.</i> (1993)
LRB342	<i>MATa his3 leu2 ura3-52</i>	Robinson <i>et al.</i> (1993)
LRB343	<i>MATa his3 leu2 ura3-52 yck2::HIS3</i>	Robinson <i>et al.</i> (1993)
LRB362	<i>MATa his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts}</i>	this study
LRB363	<i>MATα his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts}</i>	this study
LRB758	<i>MATa his3 leu2 ura3-52</i>	this study
LRB759	<i>MATα his3 leu2 ura3-52</i>	this study
LRB756	<i>MATa his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts}</i>	this study
LRB757	<i>MATα his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts}</i>	this study
LRB746	<i>MATa his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts} yks4-1</i>	this study
LRB747	<i>MATα his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts} yks4-1</i>	this study
HPY23	<i>MATα his3 leu2 ura3-52 yck1-2::LEU2 yck2-2^{ts} yks4::HIS3</i>	this study
HPY20	<i>MATα his3 leu2 ura3-52 yks4::HIS3</i>	this study
HPY21	<i>MATα his3 leu2 ura3-52 yks4::HIS3</i>	meiotic segregant of LRB524 transformed with <i>yks4::HIS3</i>
LRB748	<i>MATa his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts} yks5-1</i>	this study
LRB749	<i>MATα his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts} yks5-1</i>	this study
LRB750	<i>MATa his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts} yks6-1</i>	this study
HPY25	<i>MATa his3 leu2 trp1 ura3-52 yck1-2::LEU2 yks6::TRP1</i>	this study
LRB752	<i>MATa his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts} yks7-1</i>	this study
LRB743	<i>MATa his3 leu2 ura3-52 yks7::LEU2 yck1-1::ura3 yck2-2^{ts}</i>	this study
LRB739	<i>MATa his3 leu2 ura3-52 yks7::LEU2</i>	this study
LRB816	<i>MATa his3 leu2 ura3-52 yks4::HIS3 yks5::URA3 yks6::TRP1 yks7::LEU2 yck1-1::ura3 yck2-2^{ts}</i>	this study
LRB781	<i>MATa his3 leu2 ura3-52 chc1-ts:URA3</i>	transformant of LRB758 with Yipchc721 (<i>chc1-ts</i> ; Seeger and Payne, 1992)
LRB779	<i>MATa his3 leu2 ura3-52 chc1-ts:URA3 yck1-1::ura3 yck2-2^{ts}</i>	meiotic segregant from a cross of LRB781 by RB757
LRB556	<i>MATa/MATα his3/his3 leu2/leu2 ura3-52/ura3-52 yck1-1::ura3/yck1-1::ura3 yck2-2^{ts}/yck2-2^{ts}</i>	cross of LRB362 by LRB363
KMY21	<i>MATα his3 leu2 ura3-52 yks4-1 yck1-1::ura3 yck2-2^{ts} GAL1:STE3:LEU2</i>	introduction of <i>GAL1:STE3:LEU2</i> (pSL1683; Givan and Sprague, 1996) into LRB747
KMY22	<i>MATα his3 leu2 ura3-52 yks5-1 yck1-1::ura3 yck2-2^{ts} GAL1:STE3:LEU2</i>	introduction of pSL1683 into LRB749
KMY25	<i>MATα his3 leu2 ura3-52 yks4::HIS3 GAL1:STE3:LEU2</i>	introduction of pSL1683 into HPY21
KMY28	<i>MATα his3 leu2 ura3-52 GAL1:STE3:LEU2</i>	introduction of pSL1683 into LRB759
KMY29	<i>MATα his3 leu2 ura3-52 yck1-1::ura3 yck2-2^{ts} GAL1:STE3:LEU2</i>	introduction of pSL1683 into LRB757
LRB524	<i>MATa/MATα his3/ his3 leu2/leu2 ura3-52/ura3-52</i>	cross of LRB758 by LRB759
SL1463	<i>MATα leu2 ura3-52 trp1 his3-Δ200</i>	this study

(Robinson *et al.*, 1993). To isolate spontaneous mutations that rescue the temperature-sensitive mutant, 10^7 – 10^8 cells per plate of strains LRB362 (*MATa yck^{ts}*) and LRB363 (*MATα yck^{ts}*) were incubated at 37°C. Colonies arose at a frequency higher than 10^{-7} and colonies from each plate were retested for growth at 37°C and were examined for additional growth phenotypes.

Each mutant was backcrossed to the parental *yck^{ts}* strain to determine dominant or recessive character and to determine single gene character of mutations by segregation in meiotic progeny. Mutants were then crossed by one another and by strains with marked *yck1* and *yck2* alleles (LRB264 *MATa yck1::URA3* and LRB343 *MATa yck2::HIS3*) to assign complementation and linkage groups. Additional crosses were carried out to *YCK⁺* strains (LRB341 and LRB342, *MATa* and *MATα*, respectively) to assay for associated growth phenotypes among haploid progeny.

Cloning of *yck^{ts}* suppressor (*YKS*) genes

The *YKS* genes were cloned from YCp50 genomic libraries by complementation of the temperature-independent growth of *yck^{ts} yks* strains. To test for identity of cloned genes with mutant loci, genomic sequences were subcloned into a *URA3* integrating vector (YIp5 or YIp352), linearized within the genomic fragment, and introduced into *yck^{ts}* strains by transformation. Genomic Southern hybridization analysis confirmed integration at the appropriate loci. Genetic linkage of the *Ura⁺* phenotype with the original *yks* mutation was assayed by tetrad analysis of crosses of *yck^{ts} YKS:URA3* transformants by the original *yck^{ts} yks* mutant strains. The complementing region of each genomic clone was delimited by transposon insertion analysis (see above).

The *YKS* genes were disrupted *in vitro* by insertion of selectable markers followed by introduction into yeast. A 2.5 kb *Bam*HI fragment containing *HIS3/Kan^r* was inserted into *YKS4* at a unique *Bam*HI site, interrupting the coding sequence at codon 326. The *YKS5* gene was

disrupted by insertion of the *Hind*III *URA3* fragment at a unique *Hind*III site, corresponding to codon 492. A Tn5 insertion in this region abolished *YKS5* function. The *YKS6/APM3* gene was disrupted by substitution of a 2.8 kb *Bam*HI–*Cla*I fragment containing *TRP1/Kan^r* for a *Bam*HI–*Cla*I fragment containing codons 247–452. The *YKS7* gene was disrupted by insertion of the *LEU2 Sal*I–*Xho*I fragment at a unique *Sal*I site at codon 15 of the spliced message. The disrupted *YKS* genes were introduced into wild-type and *yck^{ts}/yck^{ts}* diploid strains (LRB524 and LRB556, respectively).

Characterization of the AP-like complex

Apm3p antisera were generated in rabbits using a His₆-tagged fusion protein as antigen. The region of *APM3* encoding amino acids 206 to 483 was subcloned into pRSETC (Invitrogen) fused in-frame at the *Nhe*I site following the His₆ leader coding region. The resulting plasmid, pDS22, was transformed into *E. coli* strain BL21 (DE3, pLysS). Expression was induced with IPTG, cells were lysed and denatured fusion protein was purified over Ni–NTA agarose as described by the manufacturer (Qiagen). The fusion protein solution was exchanged into 1× PBS + 0.1% SDS, and sent to Pocono Rabbit Farm and Laboratory for antibody generation.

Antisera detected a major immunoreactive band of ~55 kDa on immunoblots of whole cell extracts at a primary antibody dilution of up to 1/10 000. This band was missing from an extract prepared from an *apm3-Δ* strain, and was amplified in extracts from strains overexpressing *APM3* from a 2 μ plasmid. Immunoblots were developed using goat anti-rabbit IgG secondary antibody (Zymed Laboratories) followed by ECL-enhanced chemiluminescence (Amersham). Extraction of cellular protein and fractionation of the Apm3p-containing complex by Superose-12 (Pharmacia) column chromatography was performed as described previously (Stepp *et al.*, 1995).

Ste3p internalization assays

Assay of Ste3p stability was carried out using a Gal-inducible *STE3* construct (pSL1683; kindly provided by P.Kinsey and G.F.Sprague, Jr). Strains with *pGALI::STE3* integrated at *STE3* were grown in YEPraffinose to mid-log phase and shifted for 90 min into YEPgalactose to induce Ste3p synthesis, then shifted into YEPglucose to shut off Ste3p production. Stability and protease accessibility (membrane association) of Ste3p were assayed essentially as described (Givan and Sprague, 1997). To test stability, cells were removed from the galactose culture prior to glucose shift (time 0) and at 30 min intervals post-shift. To test accessibility to protease, samples were split for Pronase (Calbiochem) treatment. Mock-treated and protease-treated cells were harvested and lysed, samples were denatured by addition of urea-SDS buffer and 3 min incubation at 42°C, and were electrophoresed in SDS-10% polyacrylamide gels.

Immunoblotting was carried out using monoclonal anti-Ste3p antibody (provided by G.F.Sprague, Jr) and goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma), with subsequent detection using the ECL system (Amersham). Blots were reprobbed with anti-Pgkp antisera to provide a control for loading and cell lysis.

Acknowledgements

We thank G.S.Payne for providing unpublished data, reagents, and helpful discussions, M.S.Robinson and J.Bonifacio for providing unpublished data, N.Davis for reagents, G.Sprague and P.Kinsey for reagents, protocols and helpful discussions, and K.Tatchell for helpful discussions and assistance with figure preparation. This work was supported by the LSU Medical Center, Department of Biochemistry and NSF MCB-9601294 to L.C.R., and by NSF MCB-9305287 to S.K.L. H.R.P. is supported by Louisiana Educational Quality Support Fund Doctoral Student Training Program GF11, J.D.S. is supported by NIH pre-doctoral training grant AG00105, and P.K.T. was supported by a UCLA Dissertation Year Fellowship and by NIH 39040 (G.S.Payne).

References

- Altschul,S.F., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410.
- Bar-Zvi,D., Mosley,S.T. and Branton,D. (1988) *In vivo* phosphorylation of clathrin-coated vesicle proteins from rat reticulocytes. *J. Biol. Chem.*, **263**, 4408–4415.
- Brockman,J.L., Gross,S.D., Sussman,M.R. and Anderson,R.A. (1992) Cell cycle-dependent localization of casein kinase I to mitotic spindles. *Proc. Natl Acad. Sci. USA*, **89**, 9454–9458.
- Chuang,J.S. and Schekman,R.W. (1996) Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p. *J. Cell Biol.*, **135**, 597–610.
- Cukierman,E., Huber,I., Rotman,M. and Cassel,D. (1995) The ARF1 GTPase-activating protein: zinc finger motif and Golgi complex localization. *Science*, **270**, 1999–2002.
- Davis,N.G., Horecka,J.L. and Sprague,J.G.F., III (1993) *Cis*- and *trans*-acting functions required for endocytosis of the yeast pheromone receptors. *J. Cell Biol.*, **122**, 53–65.
- Dell'Angelica,E.C., Ohno,H., Ooi,C.E., Rabinovich,E., Roche,K.W. and Bonifacio,J.S. (1997) An adaptor-like complex with ubiquitous expression. *EMBO J.*, **5**, 917–928.
- DeMaggio,A.J., Lindberg,R.A., Hunter,T. and Hoekstra,M.F. (1992) The budding yeast *HRR25* gene product is a casein kinase I isoform. *Proc. Natl Acad. Sci. USA*, **89**, 7008–7012.
- Feldmann,H., Aigle,M. and Aljinovic,G. (1994) Complete DNA sequence of yeast chromosome II. *EMBO J.*, **13**, 5795–5809.
- Fish,K.J., Cegielska,A., Getman,M.E., Landes,G.M. and Virshup,D.M. (1995) Isolation and characterization of human casein kinase I ϵ (CKI), a novel member of the CKI gene family. *J. Biol. Chem.*, **270**, 14875–14883.
- Givan,S.A. and Sprague,G.F., Jr (1997) The ankyrin repeat-containing protein Akrlp is required for the endocytosis of yeast pheromone receptors. *Mol. Biol. Cell*, in press.
- Graves,P.R., Haas,D.W., Hagedorn,C.H., DePaoli-Roach,A.A. and Roach,P.J. (1993) Molecular cloning, expression, and characterization of a 49-kilodalton casein kinase I isoform from rat testis. *J. Biol. Chem.*, **268**, 6394–6401.
- Gross,S.D., Hoffman,D.P., Fiset,P.L., Baas,P. and Anderson,R.A. (1995) A phosphatidylinositol 4,5 bis phosphate-sensitive casein kinase I α associates with synaptic vesicles and phosphorylates a subset of vesicle proteins. *J. Cell Biol.*, **130**, 711–724.
- Hicke,L. and Riezman,H. (1996) Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell*, **84**, 277–287.
- Hoekstra,M.F., Liskay,R.M., Ou,A.C., DeMaggio,A.J., Burbee,D.J. and Heffron,F. (1991) HRR25, a putative protein kinase from budding yeast: association with repair of damaged DNA. *Science*, **253**, 1031–1034.
- Hoffman,C.S. and Winston,F. (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene*, **57**, 267–272.
- Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163–168.
- Kirchhausen,T. (1990) Identification of a putative yeast homolog of the mammalian beta chains of the clathrin-associated protein complexes. *Mol. Cell. Biol.*, **10**, 6089–6090.
- Kirchhausen,T., Davis,A.C., Frucht,S., Greco,B.O., Payne,G.S. and Tubb,B. (1991) AP17 and AP19, the mammalian small chains of the clathrin-associated protein complexes, show homology to Yap17p, their putative homolog in yeast. *J. Biol. Chem.*, **266**, 11153–11157.
- Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Nakayama,Y., Goebel,M., O'Brine,B.G., Lemmon,S., Chow,E.P. and Kirchhausen,T. (1991) The medium chains of the mammalian clathrin-associated proteins have a homolog in yeast. *Eur. J. Biochem.*, **202**, 569–574.
- Newman,L.S., McKeever,M.O., Okano,H.J. and Darnell,R.B. (1995) β -NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein. *Cell*, **82**, 773–783.
- Page,L.J. and Robinson,M.S. (1995) Targeting signals and subunit interactions in coated vesicle adaptor complexes. *J. Cell Biol.*, **131**, 619–630.
- Pauloin,A. and Thureau,C. (1994) The 50 kDa protein subunit of assembly polypeptide (AP) AP-2 adapter from clathrin-coated vesicles is phosphorylated on threonine-156 by AP-1 and a soluble AP50 kinase which co-purifies with the assembly polypeptides. *Biochem. J.*, **296**, 409–415.
- Pearse,B.M.F. and Robinson,M.S. (1990) Clathrin, adaptors, and sorting. *Annu. Rev. Cell Biol.*, **6**, 151–171.
- Phan,H.L., Finlay,J.A., Chu,D.S., Tan,P.K., Kirchhausen,T. and Payne,G.S. (1994) The *Saccharomyces cerevisiae* *AP51* gene encodes a homolog of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex. *EMBO J.*, **13**, 1706–1717.
- Poon,P.P., Wang,X.M., Rotman,M., Huber,I., Cukierman,E., Cassel,D., Singer,R.A. and Johnston,G.C. (1996) *Saccharomyces cerevisiae* Gcs1 is an ADP-ribosylation factor GTPase-activating protein. *Proc. Natl Acad. Sci. USA*, **93**, 10074–10077.
- Rad,M.R., Phan,H.L., Kirchrath,L., Tan,P.K., Kirchhausen,T., Hollenberg,C.P. and Payne,G.S. (1995) *Saccharomyces cerevisiae* Apl2p, a homologue of the mammalian clathrin AP β subunit, plays a role in clathrin-dependent Golgi functions. *J. Cell Sci.*, **108**, 1605–1615.
- Roach,P.J. (1990) Control of glycogen synthase by hierarchical protein phosphorylation. *FASEB J.*, **4**, 2961–2968.
- Robinson,L.C. et al. (1992) Yeast casein kinase I homologues: an essential gene pair. *Proc. Natl Acad. Sci. USA*, **89**, 28–32.
- Robinson,L.C., Menold,M.M., Garrett,S. and Culbertson,M.R. (1993) Casein kinase I-like protein kinases encoded by *YCK1* and *YCK2* are required for yeast morphogenesis. *Mol. Cell. Biol.*, **13**, 2870–2880.
- Rose,M.D., Novick,P., Thomas,J.H., Botstein,D. and Fink,G.R. (1987) A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene*, **60**, 237–243.
- Roth,A.F. and Davis,N.G. (1996) Ubiquitination of the yeast α -factor receptor. *J. Cell Biol.*, **134**, 661–674.
- Rothstein,R.J. (1983) One-step gene disruption in yeast. *Methods Enzymol.*, **101**, 202–210.
- Rowles,J., Slaughter,C., Moomaw,C., Hsu,J. and Cobb,M.H. (1991) Purification of casein kinase I and isolation of cDNAs encoding multiple casein kinase I-like enzymes. *Proc. Natl Acad. Sci. USA*, **88**, 9548–9552.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

- Seaman,M.N.J., Sowerby,P.J. and Robinson,M.S. (1996) Cytosolic and membrane-associated proteins involved in the recruitment of AP-1 adaptors onto the trans-Golgi network. *J. Biol. Chem.*, **271**, 25446–25451.
- Seeger,M. and Payne,G.S. (1992) Selective and immediate effects of clathrin heavy chain mutations on Golgi membrane protein retention in *Saccharomyces cerevisiae*. *J. Cell Biol.*, **118**, 531–540.
- Sherman,F., Fink,G.R. and Hicks,J.B. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Simpson,F., Bright,N.A., West,M.A., Newman,L.S., Darnell,R.B. and Robinson,M.S. (1996) A novel adaptor-related protein complex. *J. Cell Biol.*, **133**, 749–760.
- Simpson,F., Peden,A.A., Christopolou,L. and Robinson,M.S. (1997) Characterization of the adaptor-related protein complex, AP-3. *J. Cell Biol.*, **137**, 835–845.
- Stammes,M.A. and Rothman,J.E. (1993) The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell*, **73**, 999–1005.
- Stepp,J.D., Pellicena-Palle,A., Hamilton,S., Kirchhausen,T. and Lemmon,S.K. (1995) A late Golgi sorting function for *Saccharomyces cerevisiae* Apm1p, but not for Apm2p, a second yeast clathrin AP medium chain-related protein. *Mol. Biol. Cell*, **6**, 41–58.
- Sullivan,M.A., Cannon,J.F., Webb,F.H. and Bock,R.M. (1985) Antisuppressor mutation in *Escherichia coli* defective in biosynthesis of 5-methylaminomethyl-2-thiouridine. *J. Bacteriol.*, **161**, 368–376.
- Traub,L.M., Ostrom,J.A. and Kornfeld,S. (1993) Biochemical dissection of AP-1 recruitment onto Golgi membranes. *J. Cell Biol.*, **123**, 561–573.
- Tuazon,P.T. and Traugh,J.A. (1991) Casein kinase I and II- multipotential serine kinases: structure, function and regulation. *Adv. Second Messenger Phosphoprotein Res.*, **23**, 123–164.
- Vancura,A., O'Connor,A., Patterson,S.D., Mirza,U., Chait,B.T. and Kuret,J. (1993) Isolation and properties of YCK2, a *Saccharomyces cerevisiae* homolog of casein kinase I. *Arch. Biochem. Biophys.*, **305**, 47–53.
- Vancura,A., Sessler,A., Leichus,B. and Kuret,J. (1994) A prenylation motif is required for plasma membrane localization and biochemical function of casein kinase I in budding yeast. *J. Biol. Chem.*, **269**, 19271–19278.
- Wang,P.-C., Vancura,A., Mitcheson,T.G. and Kuret,J. (1992) Two genes in *Saccharomyces cerevisiae* encode a membrane bound form of casein kinase-I. *Mol. Biol. Cell*, **3**, 275–286.
- Wang,X., Hoekstra,M.F., DeMaggio,A.J., Dhillon,N., Vancura,A., Kuret,J., Johnston,G.C. and Singer,R.A. (1996) Prenylated isoforms of yeast casein kinase I, including the novel Yck3p, suppress the *gcsI* blockage of cell proliferation from stationary phase. *Mol. Cell. Biol.*, **16**, 5375–5385.
- Watanabe,T.K., Shimizu,F., Nagata,M., Takaichi,A., Fujiwara,T., Nakamura,Y., Takahashi,E. and Hirai,Y. (1996) Cloning, expression pattern and mapping to 12p13.2→p13.1 of CLAPS3, a gene encoding a novel clathrin-adaptor small chain. *Cytogenet. Cell Genet.*, **73**, 214–217.
- Wilde,A. and Brodsky,F.M. (1996) *In vivo* phosphorylation of adaptors regulates their interaction with clathrin. *J. Cell Biol.*, **135**, 635–645.
- Zanolari,B., Rath,S., Singer-Kruger,B. and Riezman,H. (1992) Yeast pheromone receptor endocytosis and hyperphosphorylation are independent of G protein-mediated signal transduction. *Cell*, **71**, 755–763.
- Zhai,L., Graves,P.R., Robinson,L.C., Italiano,M., Culbertson,M.R., Rowles,J., Cobb,M.H., DePaoli-Roach,A.A. and Roach,P.J. (1995) Casein kinase Iγ subfamily: molecular cloning, expression and characterization of three mammalian isoforms, and complementation of defects in the *Saccharomyces cerevisiae* YCK genes. *J. Biol. Chem.*, **270**, 12717–12724.
- Ziman,M., Chuang,J.S. and Schekman,R.W. (1996) Chs1p and Chs3p, two proteins involved in chitin synthesis, populate a compartment of the *Saccharomyces cerevisiae* endocytic pathway. *Mol. Biol. Cell*, **7**, 1909–1919.

Received on February 27, 1997; revised on April 28, 1997