The Saccharomyces cerevisiae APS1 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

Huan L. Phan1, Judith A. Finlay2, Diana S. Chu1, Philip K. Tan2, Tomas Kirchhausen3 and Gregory S. Payne1,2,4

1Molecular Biology Institute and 2Department of Biological Chemistry, School of Medicine, University of California, Los Angeles, CA 90024 and 3Department of Cellular Biology, Harvard Medical School and Center for Blood Research, Boston, MA 02115, USA
4Corresponding author

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Clathrin-associated protein (AP) complexes have been implicated in the assembly of clathrin coats and the selectivity of clathrin-mediated protein transport processes. We have identified a yeast gene, APS1, encoding a homolog of the small (referred to herein as σ) subunits of the mammalian AP-1 complex. Sequence comparisons have shown that Aps1p is more similar to the σ subunit of the Golgi-localized mammalian AP-1 complex than Aps2p, which is more related to the plasma membrane AP-2 σ subunit. Like their mammalian counterparts, Aps1p and Aps2p are components of distinct, large (>200 kDa) complexes and a significant portion of the AP proteins co-fractionate with clathrin-coated vesicles during gel filtration chromatography. Unexpectedly, even though the evolutionary conservation of AP small subunits is substantial (50% identity between mammalian and yeast proteins), disruptions of APS1 (aps1Δ) and APS2 (aps2Δ), individually or in combination, elicit no detectable mutant phenotypes. These data indicate that the AP proteins are not absolutely required for clathrin-mediated selective protein transport in cells expressing wild type clathrin. However, aps1Δ accentuated the slow growth and α-factor pheromone maturation defect of cells carrying a temperature-sensitive allele of clathrin heavy chain (Chc) (cht1-ts). In contrast, aps2Δ did not influence the effects of cht1-ts on vacuolar protein sorting or receptor-mediated endocytosis. The aps2Δ mutation resulted in a slight effect on cht1-ts cell growth but had no additional effects. The growth defect of cells completely lacking Chc was compounded by aps1Δ but not aps2Δ. These results comprise evidence that Aps1p is involved in a subset of clathrin functions at the Golgi apparatus. The effect of aps1Δ on cells devoid of clathrin function suggests that Aps1p also participates in clathrin-independent processes.

Key words: α-factor maturation/AP complex/clathrin/Golgi complex/yeast

Introduction

Clathrin-coated membranes participate in selective protein transport from the plasma membrane and the Golgi complex. Clathrin-mediated processes include receptor-mediated endocytosis, sorting of soluble lysosomal or vacuolar precursors to lysosomes or vacuoles, and retention of resident Golgi membrane proteins (reviewed in Brodsky, 1988; Pearse and Robinson, 1990; Payne, 1990; Seeger and Payne, 1992a). The principal components of clathrin coats are trimeric complexes of clathrin heavy chain (Chc) and clathrin light chain (Clc) assembled into polyhedral lattices, and heterotetrameric complexes of clathrin-associated proteins (APs) (reviewed in Morris et al., 1989; Keen, 1990; Pearse and Robinson, 1990; Kirchhausen, 1993). AP complexes were initially identified on the basis of their ability to stimulate clathrin assembly into cages (Zaremba and Keen, 1983). In clathrin-coated vesicles, APs are positioned between the outer clathrin lattice and the vesicle membrane where they interact with clathrin and, presumably, with the cytoplasmic tails of transmembrane cargo proteins (reviewed in Robinson, 1992). Two major AP complexes have been identified in mammalian cells (reviewed in Morris et al., 1989; Keen, 1990; Pearse and Robinson, 1990). AP-1 is located at the trans-Golgi network while AP-2 is located at the plasma membrane. Both APs consist of two α, one β and one γ subunit (Virshup and Bennett, 1988; Matsui and Kirchhausen, 1990). The large subunits of AP-1 are α and β', the medium subunit is AP47 (referred to here as μ1) and the small subunit is ρ1 (AP19). AP-2 is made up of α and β large subunits and μ2 (AP50) and o2 (AP17). In addition to being differentially localized and compositionally distinct, AP-1 and AP-2 are functionally different as well. For example, AP-2, but not AP-1, can stimulate in vitro assembly and invagination of coated pits at the plasma membrane (Mahaffey et al., 1990; Lin et al., 1991; Schmid and Smythe, 1991; Peeler et al., 1993). Addition of AP-2, but not AP-1, causes aggregation of liposomes prepared in vitro (Beck et al., 1992). Finally, in vitro, AP-1 and AP-2 appear to bind to different domains on the cytoplasmic tail of the mannose-6-phosphate receptor (Glickman et al., 1989). These findings have led to the model that APs function in the assembly of clathrin coats and provide the selectivity for clathrin-mediated sorting at different intracellular compartments.

cDNAs encoding the subunits of AP-1 and AP-2 have been isolated and sequenced (Thurieau et al., 1988; Kirchhausen et al., 1989; Robinson, 1989, 1990; Ponnambalam et al., 1990; Kirchhausen et al., 1991; Nakayama et al., 1991). Database searches using the predicted amino acid sequences of these proteins as probes have uncovered a number of putative homologs in the budding yeast Saccharomyces cerevisiae. APL1 (formerly YAP80) is predicted to encode an 80 kDa protein with 35% identity to the β subunit of AP-2 (Kirchhausen, 1990). The APM1 (formerly YAP54) product is 56% identical to μ1 (Nakayama et al., 1991) and the APS2 (formerly YAP17) product is 50% identical to o2 (Kirchhausen et al., 1991). The function of the proteins encoded by these genes has not been assessed.
As part of our studies of clathrin coat function in yeast, we have identified a second APS gene, APS1, which appears to encode a cognate of the mammalian σ1 subunit. With genes encoding homologs of both mammalian σ subunits, a genetic and biochemical analysis of ApS function in yeast has been initiated. Our results indicate that the products of the APS genes are components of clathrin-coated vesicles but are not absolutely necessary for clathrin function. However, genetic interactions between mutations in APS1 and CHC1 uncover a role for ApS1p in both clathrin-dependent and clathrin-independent processes.

## Results

### Isolation of a second APS gene, APS1

Two mammalian AP small subunits, σ1 and σ2, and one putative σ2 yeast homolog, APS2, have been identified and cloned (Kirchhausen et al., 1991). Given the precedent of two related σ subunits in mammals, we undertook a search for additional yeast σ subunit genes using PCR. Degenerate oligonucleotide primers were designed against two conserved 5 amino acid (aa) stretches in σ1, σ2, and APS2 (Materials and methods, see the underlined amino acids in Figure 2). In order to avoid amplification of APS2, chromosomal DNA from a yeast strain lacking most of the wild type APS2 sequence was used as the template. The PCR products were separated by PAGE and products of the expected size were cloned. Four clones were chosen for sequencing. Translation of one of the sequenced PCR products revealed multiple stop codons and was not studied further. The remaining three sequences were identical to each other and showed significant amino acid sequence identity to all of the previously identified σ subunits, with the highest degree of identity to mammalian σ1.

One of the cloned PCR fragments was used to probe a yeast genomic library in λEMBL3 (see Materials and methods). Two positive clones were identified and characterized by restriction endonuclease mapping. A 1.3 kb KpmI—SalI fragment encompassing the sequences present in the PCR products was sequenced. The complete nucleotide sequence of the KpmI—SalI fragment (see Materials and methods) is shown in Figure 1. Also shown is the amino acid translation of an open reading frame (ORF) with significant identity to AP small subunits. Starting with the first available methionine, this ORF predicts a protein of 18.1 kDa, without any stretches of unusual amino acid composition or outstanding structural features. This ORF is 52% identical to σ1, 41% identical to σ2 and 39% identical to ApS2p (Figure 2). When the amino acid sequence of σ1 is aligned to maximize identity with σ2 and ApS2p, σ1 extends 16 aa beyond the C-termini of the other two sequences (Figure 2). Similarly, the ORF sequence exhibits a C-terminal extension, although it is unrelated to the σ2 C-terminus (Figure 2). Based on the comparison of amino acid sequences and the C-terminal extension, the ORF is most similar to σ1. We have designated the gene encoding the ORF as APS1 and its product ApS1p.

### Disruption of APS1 accentuates the growth defect of chc1-ts cells

Deleted versions of both APS genes were constructed for use in targeted gene disruption (Figure 3). It was possible that elimination of APS genes would cause severe growth defects since yeast cells lacking Chc grow slowly or not at all (Payne and Schekman, 1985; Lemmon and Jones, 1987; Munn et al., 1991). Consequently, we initially introduced the disrupted versions of the APS genes, individually or together, into diploid strains to generate strains heterozygous for the mutations. When these strains were induced to undergo meiosis and dissected into tetrads, there was no observable effect on cell growth of the aps mutations alone or in combination (data not shown). This finding allowed us to disrupt the genes directly in haploid cells. The aps disruptions were introduced into wild type strains and congenic strains expressing a temperature-sensitive allele of CHC (chc1-ts). The effects of aps mutations on the chc1-ts strains were evaluated to determine whether combinations of mutations resulted in phenotypes significantly more severe than phenotypes caused by each mutation alone. Such ‘synthetic’ genetic effects often reflect interaction of gene products (Huffaker et al., 1987).

The growth rates of congenic strains containing different combinations of chc1-ts, aps1A and aps2Δ were measured in liquid cultures at 24, 30 and 37°C (Table II). Effects of

### Table I. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source</th>
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<td>MATa leu2–3,112 ura3-52 pep4::URA3 prbl1 his4-519 trpl1</td>
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</tr>
<tr>
<td>GPY1100α</td>
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<td>Payne and Schekman (1989)</td>
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<td>Payne et al. (1987)</td>
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<tr>
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<tr>
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<td>GPY1100a aps2::URA3</td>
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<td>GPY418</td>
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<td>his4-519/his4-519 trpl1-289/trpl1-289 can1/can1 gal2/gal2</td>
<td>This work</td>
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Materials and methods (Figure 1). Consequently, we initially introduced the disrupted versions of the APS genes, individually or together, into diploid strains to generate strains heterozygous for the mutations. When these strains were induced to undergo meiosis and dissected into tetrads, there was no observable effect on cell growth of the aps mutations alone or in combination (data not shown). This finding allowed us to disrupt the genes directly in haploid cells. The aps disruptions were introduced into wild type strains and congenic strains expressing a temperature-sensitive allele of CHC (chc1-ts). The effects of aps mutations on the chc1-ts strains were evaluated to determine whether combinations of mutations resulted in phenotypes significantly more severe than phenotypes caused by each mutation alone. Such ‘synthetic’ genetic effects often reflect interaction of gene products (Huffaker et al., 1987).

The growth rates of congenic strains containing different combinations of chc1-ts, aps1A and aps2Δ were measured in liquid cultures at 24, 30 and 37°C (Table II). Effects of
Fig. 1. Nucleotide sequence of the 1.3 kb KpnI-SalI APS1 DNA fragment and the predicted protein product. These sequence data will appear in the EMBL Database under the accession number Z30314.

the *aps* mutations were apparent only in *chcl*-ts cells grown at 37°C. The *chcl*-ts mutation by itself effectively doubles the doubling time at 37°C to 2.9 h compared with the wild type rate (1.6 h). Loss of APS2 in a *chcl*-ts background (Table II, *chcl*-ts *aps2A*) led to a 1 h increase in doubling time over *chcl*-ts alone, while loss of *APS1* in the same background (Table II, *chcl*-ts *aps1A*) led to a 3 h increase in doubling time. The effect of *aps1Δ* and *aps2Δ* on the growth rate of *chcl*-ts cells (Table II, *chcl*-ts *aps1Δ* *aps2Δ*) may be additive (6.9 h), but *chcl*-ts *aps1Δ* *aps2Δ* and *chcl*-ts *aps1A* strains grow so slowly that small differences in their growth rates may not be significant.

Because *Aps1p* and *Aps2p* are nearly 40% identical, it was possible that the two proteins are functionally redundant. We examined this possibility by determining whether elevated levels of *Aps2p* could rescue the synthetic growth defect of the *chcl*-ts *aps1Δ* strain. Overproduction of *Aps2p* by at least 5-fold over endogenous levels (as estimated by immunoblotting, data not shown) did not rescue the synthetic growth defect of the *chcl*-ts *aps1Δ* strain (Table II, *chcl*-ts *aps1Δ* YeEp-APS2).

**Disruption of APS1 accentuates the growth defect of chcl-ts cells**

At 37°C, *chcl*-ts cells grow more rapidly than cells carrying a deletion of the Chc gene (*chclΔ*) (G.S.Payne, unpublished), suggesting that the temperature-sensitive Chc

Fig. 2. Amino acid sequence alignment of mammalian and yeast clathrin AP small subunits (see Materials and methods). Amino acids conserved among the four sequences are shown as a consensus sequence. The degenerate oligonucleotide primers used in PCR were based on the underlined sequences.

Fig. 3. Restriction map of *APS1* and *APS2* and deletion constructs used to generate *aps1Δ* and *aps2Δ* mutants. Arrows represent ORFs.
Table II. Strain doubling times with increasing temperature

<table>
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<td>CHCI aps1Δ aps2Δ</td>
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</tr>
<tr>
<td>chcl-ts</td>
<td>3.2</td>
</tr>
<tr>
<td>chcl-ts aps2Δ</td>
<td>3.3</td>
</tr>
<tr>
<td>chcl-ts aps1Δ</td>
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</tr>
<tr>
<td>chcl-ts aps1Δ aps2Δ</td>
<td>3.1</td>
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<tr>
<td>chcl-ts aps1Δ YEp-APS2</td>
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Cells were grown on SD CAA or SD CAA-trp (cells with YEp-APS2) at 24°C. An aliquot of each strain was diluted into fresh medium and shifted to 24, 30 or 37°C and growth at each temperature was monitored for at least 14 h by light scattering at 500 nm.

**Fig. 4.** aps1Δ aggravates the growth defect of chclΔ. (A) Tetrad from a sporeulation of strain GPY746 (chclΔΔ/ps aps2ΔΔ) or (B) from strain GPY747 (chclΔΔ/ps aps1ΔΔ).

retains a small degree of activity at the non-permissive temperature. It was therefore possible that aps1Δ accentuated the 37°C growth defect caused by chcl-ts by abolishing residual Chc activity. If so, aps1Δ should not exaggerate the growth defect caused by chclΔ since no Chc is produced from this allele. This prediction was tested by tetrad analysis of the meiotic progeny of a diploid strain heterozygous for disruptions of APS1 and CHCI. For comparison, the same analysis was performed on a diploid strain heterozygous for APS2 and CHCI disruptions. Figure 4 shows representative dissections of the two strains. Similar to diploids heterozygous for just chclΔ (Munn et al., 1991), the chclΔ aps2Δ double heterozygote yielded tetrads which gave rise to two small colonies and two wild type-sized colonies (Figure 4A). In 18 tetrads from the chclΔ aps2Δ double heterozygote, colony size segregated 2:2 in 16 tetrads and in every case (32/32) the large colonies were CHCI and the small colonies (32/32) were chclΔ. Two tetrads (one of which is at the right-hand end of Figure 4A) consisted of only three colonies. In these tetrads, colony size and chclΔ also co-segregated. The genotype at the APS2 locus did not visibly affect the growth rate of either CHCI or chclΔ segregants. In contrast, colonies of three sizes were apparent in the tetrads derived from the chclΔ aps1Δ double heterozygote (Figure 4B). Spores that gave rise to large colonies were always CHCI (66/66), regardless of whether they were wild type or mutant at the APS1 locus. Spores carrying chclΔ produced small colonies like the chclΔ colonies in Figure 4A, or distinctly smaller colonies. In every case (35/35), the genotype of the small colonies was chclΔ APS1. Only the chclΔ aps1Δ spores (31/31) gave rise to very small colonies. These cells grew so slowly that it was difficult to score the genetic markers and often their genotypes were inferred from markers in their sibling segregants. Therefore aps1Δ, but not aps2Δ, accentuates the growth phenotype of chclΔ, indicating that Aps1p plays a role in cell growth in the complete absence of clathrin function.

**aps1Δ accentuates the α-factor processing defect caused by the chcl-ts allele**

The synthetic growth defects caused by aps1Δ and aps2Δ in the chcl backgrounds encouraged an investigation of specific transport pathways. In particular, secretion of the mating pheromone α-factor was examined. Mutations in CHCI result in defective maturation of the α-factor mating pheromone precursor (Payne and Schekman, 1989; Lemmon et al., 1991; Seeger and Payne, 1992a). In wild type cells, the glycosylated α-factor precursor is proteolytically processed during passage through the Golgi apparatus to yield the 13 aa α-factor peptide (Fuller et al., 1988). Strains expressing the chclΔ allele and chcl-ts strains at the non-permissive temperature secrete the glycosylated precursor (Payne and Schekman, 1989; Seeger and Payne, 1992a). The maturation defect is due to inefficient retention of Kex2p, the endoprotease which initiates maturation, in the Golgi apparatus. Instead, Kex2p is mislocalized to the plasma membrane of the chclΔ cells (Seeger and Payne, 1992a). We monitored the effect of aps mutations on α-factor precursor maturation by assessing the form of α-factor secreted into the medium of chcl-ts cells. The chclΔ allele provides a sensitive background to measure the effects of the aps mutations since chcl-ts cells display α-factor maturation defects at 30°C, a temperature where no growth defect is apparent (Table II). Congenic strains were grown overnight at 24°C then divided into three equal portions which were incubated at 24, 30 or 37°C for 2 h. After the 2 h incubation, each sample was labelled at the incubation temperature with [35S]methionine for 45 min and then α-factor was immunoprecipitated from the culture medium. Precipitated pheromone was analyzed by SDS–PAGE and autoradiography. An example of such an experiment is shown in Figure 5A. Four separate experiments were quantified by determining the relative levels of radioactivity in precursor and mature species (Figure 5B). At 24°C, CHCI aps1Δ aps2Δ cells, chcl-ts cells and chcl-ts aps2Δ cells secreted only mature α-factor (Figure 5A, lane 1–3). Less than 4% of the α-factor secreted by these strains was in the highly glycosylated form (Figure 5B, open bars). However, chcl-ts strains which also harbored aps1Δ secreted between 10 and 12% of the α-factor as the highly glycosylated precursor (Figure 5A, lanes 4–6; Figure 5B, open bars). Overproduction of Aps2p from YEp-APS2 did not rescue the synthetic α-factor maturation defect of aps1Δ chcl-ts strains (Figure 5A, lane 6; Figure 5B, open bar).

The aggravation of the α-factor processing defect in chcl-ts strains by aps1Δ was also observed at 30°C. chcl-ts aps1Δ strains (Figure 5A, lanes 10–12; Figure 5B, stippled bars) secreted ~55% precursor α-factor, compared with 21% for chcl-ts strains and 29% for chcl-ts aps2Δ strains (Figure 5A, lanes 7 and 9; Figure 5B). aps2Δ increased the amount of precursor α-factor secreted by chcl-ts strains at 30°C (Figure 5A, lane 9). This effect was much smaller than the effect of aps1Δ.

At 37°C, no effect of aps1Δ or aps2Δ on chcl-ts strains was detected (Figure 5A, lanes 13–18). All chcl-ts strains secreted 62–65% precursor α-factor (Figure 5B, solid bars).
Fig. 5. Increased α-factor maturation defect in aps1Δ chcl-ts cells. Cells were grown at 24°C and shifted to 24, 30 or 37°C for 2 h. After the temperature shift, cells were metabolically labelled with [35S]cysteine and [35S]methionine for 45 min, and the α-factor was immunoprecipitated from the culture supernatant. (A) Immunoprecipitated proteins were subjected to SDS–PAGE and autoradiography. + and − represent presence or deletion of a gene (CHCI, APS1 or APS2) or plasmid (YEp-APS2) in a strain. (B) Quantitation of precursor α-factor secreted from cells mutant for CHCI and/or APS1 and APS2. Bars represent the average amount of precursor α-factor secreted by various strains from four independent experiments, performed as described in panel A. The error bars represent the standard error.

compared with 100% precursor secreted by kex2Δ cells (Payne and Schekman, 1989). The residual levels of Chc activity in the chcl-ts cells at 37°C do not contribute to α-factor precursor maturation; in contrast to growth rates, the α-factor maturation defect in chcl-ts cells at 37°C is comparable with that in chclΔ cells (Seeger and Payne, 1992a).

Based on these results, we conclude that neither aps1Δ nor aps2Δ are necessary for α-factor maturation when fully functional Chc is expressed. However, aps1Δ, but not aps2Δ, has a significant effect on the α-factor processing in strains expressing the altered Chc encoded by the chcl-ts allele. This synthetic defect is evident when there are substantial levels of Chc function (at 24 and 30°C), but is not apparent in cells with slight levels of Chc function (at 37°C), suggesting that the absence of Aps1p reduces clathrin function. In addition, Aps1p and Aps2p do not appear to be functionally redundant in the process of α-factor maturation since overexpression of Aps2p did not reverse the additional α-factor processing defect imposed by aps1Δ in a chcl-ts background at any temperature (Figure 5A, lanes 6, 12 and 18; Figure 5B).

In cells expressing chcl alleles, the Kex2 endopeptidase is not retained in the Golgi apparatus and proceeds to the cell surface (Payne and Schekman, 1989; Seeger and Payne, 1992a). The failure to retain Kex2p in the Golgi apparatus is the probable cause of the α-factor maturation defect. Cell surface iodinations of cells incubated at each of the three temperatures did not reveal differences in the levels of Kex2p at the surface of chcl-ts and aps1Δ chcl-ts cells (data not shown). It is likely that the changes in the levels of Kex2p mislocalization that account for the observed differences in α-factor maturation in aps1Δ chcl-ts cells compared with chcl-ts cells are not large enough to be detected by the iodination procedure.

Other clathrin-mediated transport processes are not affected by aps mutations

We examined the role of Aps proteins in another clathrin-mediated transport process which occurs in the Golgi apparatus, sorting of the soluble vacuolar protease carboxypeptidase Y (CPY). In wild type cells, CPY enters the secretory pathway as a precursor and, upon reaching the Golgi apparatus, is sorted to the vacuole (Stevens et al., 1982). Along the way, CPY undergoes a series of characteristic modifications. Removal of the signal sequence and core glycosylation in the ER produces the p1 precursor form. Passage through the Golgi apparatus is accompanied by additional glycosylation producing the larger p2 form. CPY is then diverted from the secretory pathway and sorted to the vacuole where it is proteolytically processed to mature CPY (the ‘m’ form). In chcl-ts cells shifted to 37°C for short periods of time, CPY is not sorted from the secretory pathway and consequently is secreted as the p2 form (Seeger and Payne, 1992b). This result suggests that clathrin is necessary for the sorting of CPY from the Golgi apparatus to the vacuole.

The sorting of CPY can be monitored in a pulse–chase experiment by analyzing the forms of cell-associated and secreted CPY. chcl-ts cells carrying both aps1Δ and aps2Δ were subjected to this type of analysis at both 24 (Figure 6A) and 37°C (Figure 6B). A congeneric chcl-ts strain with wild type APS genes was included as a control. At 24°C, after a 10 min pulse labelling, all of the CPY was found inside the chcl-ts cells as either the p1 or p2 form (Figure 6A, lanes 1 and 2). After an additional 40 min, the labelled CPY was efficiently sorted to the vacuole where it was proteolytically matured (m form, Figure 6A, lanes 3 and 4). Elimination of the two APS genes had no effect on CPY sorting and maturation in the chcl-ts cells (Figure 6B, lanes 5–8). The slight amount of CPY in the extracellular fraction
Fig. 6. Deletions of ASP1 and ASP2 do not affect the CPY processing phenotypes of chcl-ts. (A) chcl-ts APS1 APS2 (GPY418) and chcl-ts apsΔ aps2Δ (GPY720) cells were grown at 24°C and labelled with [35S]cysteine and [35S]methionine for 10 min. Excess unlabelled amino acids were added and an aliquot was harvested from each sample (0' chase). The remaining cells were incubated an additional 40 min (40' chase). Intracellular (I) and extracellular fractions were prepared and CPY was immunoprecipitated from each fraction and analyzed by SDS–PAGE and autoradiography. (B) Cells were first shifted from growth at 24–37°C for 5 min, followed by the labelling regimen described for panel A. The ER and early Golgi form of CPY is labelled as P1. The late Golgi form is labelled as P2 and the mature form is labelled m.

of the chcl-ts apsΔ aps2Δ cells at the 40 min chase point (Figure 6A, lane 7) was probably not due to missorting since it was not the p2 form. The extracellular mCPY was most likely due to a small degree of cell lysis during removal of the cell wall.

When chcl-ts cells were preshifted to 37°C for 5 min, followed by a 10 min pulse labelling and a subsequent 40 min chase incubation at 37°C, a CPY sorting defect was observed. In the pulse-labelled sample, p1 and p2 CPY were found inside the cells, but in addition, a significant amount of p2 CPY was secreted (Figure 6B, lanes 1 and 2). After the 40 min chase period, most of CPY was not processed to the mature form, but was secreted as the p2 form (Figure 6B, lanes 3 and 4). The sorting defect in chcl-ts apsΔ aps2Δ strain mimicked that of the chcl-ts cells (Figure 6B, lanes 5–8). Also, no difference in CPY processing and sorting was detected in these two strains at 30°C, although at this temperature both strains efficiently sort CPY (data not shown). When chcl-ts strains are subjected to long incubations at 37°C (2 h or more) the CPY sorting defect diminishes by an unknown mechanism (Seeger and Payne, 1992b). The ability of the chcl-ts strains to recover CPY sorting after prolonged exposure to 37°C was unaffected by apsΔ and aps2Δ (data not shown). Taken together, these experiments show that apsΔ and aps2Δ do not impinge in any detectable way on clathrin-mediated or clathrin-independent sorting of CPY in the Golgi apparatus.

Clathrin is also necessary for efficient endocytosis of the yeast mating pheromone receptors (Tan et al., 1993). In chcl-ts cells, α-factor uptake occurs at normal rates at 24 and 30°C but is reduced by 2–3-fold at 37°C when compared with wild type cells. The effect of apsΔ and aps2Δ on endocytosis was determined by comparing uptake of radiolabelled α-factor into chcl-ts apsΔ aps2Δ cells with uptake into chcl-ts cells at 24, 30 and 37°C. There was no difference in α-factor internalization in the two strains. Thus, the aps mutations do not influence the rate of α-factor internalization in the absence or presence of functional clathrin.

Subcellular distribution of Aps proteins
As a means to characterize Aps1p and Aps2p, specific antibodies were generated (see Materials and methods). The subcellular distribution of Aps proteins was evaluated by analysis of fractions obtained by differential centrifugation of a yeast cell lysate. We applied the centrifugation protocol conventionally used to prepare yeast clathrin-coated vesicles (Mueller and Branton, 1984; Payne and Schekman, 1985). This involves centrifugation of an RNase-treated lysate (L) at 20 000 g for 30 min to yield pellet (P1) and supernatant (S1) fractions, then centrifugation of the S1 fraction at 100 000 g for 60 min to yield P2 and S2 fractions. In addition to the Aps proteins, we also monitored the fractionation of Chc, cytosolic glucose-6-phosphatase dehydrogenase (G6PD) and vacuolar membrane alkaline phosphatase (ALP). The distribution of the cytosolic and membrane markers is presented in Figure 7A and B. As expected, G6PD was present almost exclusively in the soluble fractions (Figure 7A, lanes 2 and 4) while ALP sedimented in the pellet fractions (Figure 7B, lanes 3 and 5). Chc (Figure 7C) was found in both soluble and membrane fractions. When normalized for the total amount of protein in each fraction,
**Aps proteins are associated with clathrin-coated vesicles**

Although Chc and Aps proteins exhibited distinct distribution patterns during differential centrifugation, all three proteins were present in the P2 fraction. In order to determine whether the Aps proteins in P2 were associated with clathrin-coated vesicles, we subjected the fraction to gel filtration chromatography through Sephacryl S-1000. This procedure yields a highly enriched preparation of clathrin-coated vesicles (Mueller and Branton, 1984; Payne and Schekman, 1985). As shown in Figure 8A, the protein profile of fractions from the Sephacryl S-1000 column indicates that clathrin-coated vesicles, as revealed by the presence of Chc and Clc eluted in fractions 41–53. Immunoblotting of these same fractions shows that Aps1p co-fractionated with the clathrin-coated vesicles (Aps1p, Figure 8A). Although Aps2p was spread through all the column fractions (Aps2p, Figure 8A) there was a slight but reproducible increase in the coated vesicle fractions (fractions 47–51). Three other proteins did not co-fractionate with clathrin-coated vesicles during Sephacryl S-1000 chromatography (data not shown): Sec62p, a marker for the ER (Deshaies and Schekman, 1989); ALP, a marker for vacuoles (Wiemken et al., 1979); and the plasma membrane ATPase (Willsky, 1979). In a separate experiment, we also monitored the fractionation of the Golgi membrane protein Kex2p. Since Kex2p is mislocalized in chclΔ mutants, it has been proposed to interact with clathrin coats (Payne and Schekman, 1989; Seeger and Payne, 1992a). Although Kex2p was apparent in fractions which eluted before the coated vesicles, there was a distinct peak of Kex2p which coincided with the peak fractions of clathrin-coated vesicles (Kex2p, Figure 8B).

If the co-elution of Aps proteins and clathrin truly reflects association with clathrin-coated vesicles, then the fractionation properties of the Aps proteins should be altered in cells lacking clathrin-coated vesicles due to a disruption of the CHC gene (*chclΔ*). Using the same protocol that was applied to the wild type cells, P2 membranes were prepared from congenic *chclΔ* cells and subjected to gel filtration on Sephacryl S-1000. The overall protein elution profile (Figure 8C) is very similar to that of the wild type strain, except for the absence of Chc and Clc. [Clc was not present because it does not associate with vesicles in the absence of Chc (Payne and Schekman, 1985)]. In contrast, the fractionation of Aps1p from *chclΔ* was dramatically altered (Aps1p, Figure 8C). Aps1p no longer peaked in fractions 41–53 but instead was distributed throughout the fractions. This result suggests that Aps1p is not incorporated into vesicles of discrete size in cells lacking clathrin-coated vesicles. The
heterogeneity of these Aps1p-containing vesicles may be generated by the disruption of larger membrane structures during the lysis procedure, or could signify a role for clathrin in regulating vesicle size. In either case, the altered fractionation of Aps1p in chclΔ cells provides further evidence that Aps1p is a component of clathrin-coated vesicles in wild type cells.

We were unable to draw conclusions from the elution patterns of Aps2p and Kex2p from chclΔ cells. The broad elution profile of Aps2p from wild type cells precluded detection of any increase in heterogeneity of Aps2p from the mutant cells and the instability of Kex2p in chclΔ cells (Payne and Schekman, 1989) made detection of this protein difficult (data not shown). However, the elution pattern of these proteins from wild type cells suggests that a portion of both Aps2p and Kex2p are associated with clathrin-coated vesicles.

**Aps proteins are components of high molecular weight complexes**

The AP complexes of mammalian cells consist of four proteins totalling nearly 300 kDa (Pearse and Robinson, 1984; Keen, 1987; Manfredi and Bazari, 1987; Ahle et al., 1988; Virshup and Bennett, 1988; Matsu and Kirchhausen, 1990). Gel filtration chromatography was used to determine whether the yeast Aps proteins were components of similarly sized complexes. To obtain a sample for gel filtration, a P1 fraction was prepared directly from a cell lysate without RNase treatment and then was incubated in 0.5 M Tris–HCl buffer. Like mammalian AP complexes (Keen et al., 1979), Aps1p and Aps2p are peripherally associated with membranes and can be released from membrane fractions by high concentrations of Tris–HCl (data not shown). The Tris–HCl extract was cleared of membranes by centrifugation and chromatographed through Sepharose CL4B. The bulk of Aps2p eluted between the 669 and 200 kDa markers (Figure 9). A second peak of Aps2p, representing a small fraction of the total, eluted in later fractions (Figure 9, fraction 52). This minor fraction of Aps2p may be a monomeric species. A similar elution profile was obtained for Aps1p extracted from microsomes (data not shown). Cytosolic Aps proteins also eluted between 669 and 200 kDa (data not shown). These results suggest that under native conditions, both Aps proteins are components of high molecular weight complexes.

**Aps proteins are components of biochemically distinct complexes**

To determine whether Aps1p and Aps2p are part of distinct complexes, we used hydroxyapatite chromatography. This is an established procedure for separating mammalian AP complexes (Pearse and Robinson, 1984). A Tris–HCl extract of P1 membranes was chromatographed through Sepharose CL4B to obtain a fraction enriched for the Aps complexes. The gel filtration fractions containing Aps proteins were then applied to hydroxyapatite and eluted with sequential steps of increasing sodium phosphate concentration. The Aps proteins bound to the hydroxyapatite and were not eluted by extensive washes with the imidazole binding buffer. As shown in Figure 10, Aps2p was eluted with 0.1 and 0.2 M sodium phosphate whereas Aps1p required 0.5 M sodium phosphate for elution. Both eluted proteins were still part of high molecular weight complexes as assessed by subsequent gel filtration chromatography (data not shown). The differential fractionation of Aps1p and Aps2p with hydroxyapatite provides evidence that the two proteins are components of distinct complexes.

**Discussion**

We have isolated a yeast gene, APs1, which encodes a putative homolog of the mammalian clathrin APσ subunits. Based on sequence homologies and size comparisons, the APs1 product is most similar to the σ1 subunit of mammalian Golgi-localized AP-1. By the same criteria, the previously identified APs2 (formerly YAPI7) is more similar to the plasma membrane AP-2 σ2 subunit. Additional biochemical evidence strengthens the analogy with mammalian AP subunits. The two APs gene products are associated with clathrin-coated vesicles and are components of distinct high molecular weight complexes which are peripherally associated with membranes. Taken together, these results provide evidence that the yeast Aps proteins are components of complexes analogous to the mammalian heterotetrameric APs. However, definitive proof awaits purification and characterization of the complexes.
Molecular clones of the APS genes have allowed us to initiate a genetic study of Aps function. In an otherwise wild type background, deletion of both APS genes had no detectable effect on cell growth, α-factor maturation, sorting of CPY to the vacuole and receptor-mediated endocytosis of α-factor. The absence of detectable phenotypes is particularly striking in view of the significant sequence conservation (~50% identity) between small AP subunits in yeast and mammals. In contrast, each of these processes is perturbed by mutations in Chc which also displays 50% amino acid identity with its mammalian counterpart (Lemmon and Jones, 1987; Payne et al., 1988; Payne and Schekman, 1989; Lemmon et al., 1991; Seegar and Payne, 1992a,b). These findings could be accounted for if yeast expresses additional proteins with functions that are redundant with the Aps proteins. Two observations make this possibility less likely but do not completely rule out the existence of related Aps genes. First, overproduction of Aps2p, which is 39% identical to Aps1p, did not reverse the synthetic defects caused by aps1Δ in chcl-ts cells. Second, PCR amplification of genomic DNA from an aps1Δ aps2Δ strain with the same primers and conditions used to isolate APS1 did not uncover any additional APS genes (data not shown). More likely, the AP complexes may be stable and functional in the absence of the Aps proteins. Support for this hypothesis derives from in vitro studies of mammalian APs which demonstrated that complexes reconstituted from large subunits alone, thereby devoid of μ and σ subunits, retain the ability to assemble clathrin trimers into cages (Prasad and Keen, 1991). Thus, the Aps proteins may serve to enhance the efficiency of AP complexes, perhaps by conferring some aspect of regulation. In the presence of wild type clathrin, the decrease in AP efficiency or regulation caused by elimination of the APS genes may not be detectable by our assays. Identification of other components of the yeast AP complexes will be necessary to test this model.

A role for Aps1p in a clathrin-mediated process was uncovered in cells expressing a temperature-sensitive Chc. The loss of APS1 aggravated the α-factor maturation defect caused by the chcl-ts allele. It seems likely that the chcl-ts mutation by itself leaves Chc with borderline function at 24°C since a relatively mild temperature increase to 30°C causes an α-factor maturation defect. Thus, the effect of the loss of Aps1p on AP complex function becomes apparent when Chc function is sub-optimal. In the presence of the partly debilitated Chc, the elimination of APS1 resulted in detectable levels of a-α-factor precursor in the medium even at 24°C, a temperature where only mature α-factor is secreted by APS1 chcl-ts cells. Furthermore, the maturation defect in aps1Δ chcl-ts strains is maximal at 30°C compared with 37°C in chcl-ts strains. If aps1Δ perturbs α-factor maturation by a clathrin-independent process then the loss of Aps1p in cells with a non-functional Chc would be expected to exaggerate the maturation defect beyond that caused by the non-functional Chc by itself. However, the most extensive maturation deficiency in aps1Δ chcl-ts cells at elevated temperatures (60–65% secreted precursor) did not exceed that in APS1 chcl-ts cells at 37°C or APS1 chcl-ts cells, suggesting that the effect of aps1Δ was exerted through the mutant clathrin. The effect of the aps1Δ mutation on clathrin-mediated processes was specific to α-factor maturation since endocytosis and vacuolar protein sorting defects were indistinguishable in chcl-ts and aps1Δ chcl-ts strains.

The aps1Δ mutation also perturbs the growth rate of chcl-ts cells, retarding the doubling rate at 37°C from 2.9 (chcl-ts cells) to 6 h (aps1Δ chcl-ts cells). In addition, aps1Δ also reduces the growth rate of cells carrying a chclΔ allele. Many complementation groups have been identified which display similar synthetic growth defects when combined with chclΔ (Lemon and Jones, 1987; Munn et al., 1991). Synthetic effects (mutation combinations which produce significantly more severe defects than either mutation alone) can be taken as genetic evidence for interaction with CHCl (Huffaker et al., 1987). Alternatively, the synthetic effects may be due to non-specific additional stress imposed by a second mutation on an already growth-compromised clathrin-deficient cell (Munn et al., 1991). The ability to distinguish between these two possibilities relies on additional evidence for interaction. Alleles of two complementation groups (SCD1 and CTD1) aggravate the growth and α-factor processing defects caused by chcl-ts (Munn et al., 1991; H.L. Phan, unpublished). Because the identity of these two genes remains obscure, we are not able to determine whether the products of either of these genes directly interact with clathrin. In contrast, there is additional evidence supporting the model that Aps1p, or more likely an Aps1p-containing AP complex, interacts with Chc. First, the predicted sequence of Aps1p is 52% identical to a mammalian protein (a1) which has been shown to be in a complex found in clathrin-coated vesicles and localized to the Golgi apparatus. Second, Aps1p is found in a high molecular weight complex like its mammalian counterpart. Third, Aps1p, along with Kex2p, co-fractionates with clathrin-coated vesicles. Taken together these data provide evidence for the interaction of Aps1p-containing AP complexes with Chc. Furthermore, the results are consistent with the idea that Aps1p is involved in only a subset of clathrin functions at the Golgi apparatus—those necessary for Kex2p retention.

Since Aps2p is more similar to the a2 subunit of the plasma membrane AP-2 complex in mammalian cells (Kirchhausen et al., 1991) and AP-2 has been shown to have a role in endocytosis (Chin et al., 1989), it was an attractive possibility that aps2Δ would affect endocytosis of α-factor in chcl-ts cells. However, we have not observed any specific effects of aps2Δ on clathrin-dependent processes in chcl-ts cells, including endocytosis. Therefore, our results provide no new information on the function or location of Aps2p.

The effect of aps1Δ on the growth of chclΔ cells indicates that, in the complete absence of clathrin, Aps1p still provides some function in cell growth. This result supports proposals that AP complexes may carry out functions which are independent of clathrin (Ahle et al., 1988; Beck et al., 1992; Hansen et al., 1993). In light of this possibility it is worth noting that fractionation of cell extracts revealed a population of Aps proteins on P1 membranes that lack clathrin. Although further studies will be needed to establish the significance of this observation, there is precedent from studies of mammalian cells for AP complexes on membranes without clathrin (Heuser and Keen, 1988; Guagliardi et al., 1990). Several roles have been proposed for membrane-associated AP complexes in the absence of clathrin. These include roles in vesicle recognition of target organelles (Ahle et al., 1988), aggregation of endocytic vesicles prior to fusion (Beck et al., 1992) and clustering of specific cargo
proteins before assembly of the clathrin lattice to form a clathrin-coated pit (Hansen et al., 1993). The Aps proteins could act in any or all of these capacities, or they could provide other, as yet unidentified, functions.

In summary, our studies represent the first genetic investigations of Aps function in vivo. The results suggest that AP function is not strictly dependent on the small subunits. The effect of an APS1 gene disruption on α-factor maturation in cells expressing a temperature-sensitive Chc suggests that Aps1p-containing AP complexes interact with Chc to carry out a specific function at the Golgi apparatus.

Finally, the effects of APS mutations in cells devoid of Chc reveal a role for Aps proteins in clathrin-independent processes.

Materials and methods

Materials

Unless noted, all reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Plasmids and nucleic acid techniques

Plasmid constructions were carried out using standard molecular biology techniques (Sambrock et al., 1989). pAPS2-D5 contains a 1.1 kb HindIII URA3 fragment (Rose et al., 1984) replacing APS2 sequences between BamHI and EcoRV sites. This construct results in the replacement of sequences between amino acids 27 and 82 in the 147 aa APS2 sequence. pAPS1-A1 was created by replacing the SpI-EcoRV fragment of APS1 with a HpaI fragment containing LEU2 (Rose and Brouch, 1991). The disrupted APS1 gene lacks sequences between amino acid 5 and 116 in the 157 aa APS1 sequence.

YPE24T is an altered version of the multicopy YEP24 plasmid (Botstein et al., 1979) in which the Cln1-BamHI URA3 fragment has been replaced by the SpI-Sul fragment of TRPI from YEp17 (Tschumper and Carbon, 1980). YEP24TAPS2 contains a 2.4 kb APS2 SulI fragment inserted into YEP24T. YEP24TAPS1 contains a 1.2 kb APS1 KpnI-BamHI fragment inserted into YEP24T.

pChcl-Δ12 contains the 1.1 kb HindIII fragment of URA3 inserted between the BglII site located 205 bp 5' of the initiating ATG codon and the BamHI site located 306 bp 5' of the terminating TAA codon of CHCl1 (Payne et al., 1987). The URA3 HindIII sites are eliminated in the construct.

Strains, genetic methods and media

Yeast strains used in this study are listed in Table I. Yeast mating, sporulation and tetrad analyses were conducted as described by Sherman et al. (1974). DNA transformations were performed by the lithium acetate procedure (Ito et al., 1983). The aps1-A1, aps2-Δ5 and chcl-Δ12 mutations were made by single-step gene replacement (Rothstein, 1991) using plasmids pAPS1-Δ1 cleaved with PsvII and SstI and pAPS2-Δ5 cleaved with EcoRI and HindIII. pChcl-Δ12 was cleaved with HindIII. All gene replacements were verified by Southern blotting or immunoblots that to the genes were disrupted.

Diploid GPY746 was generated by mating GPY1100a with GPY715 (aps2-Δ5::URA3) then introducing chcl1-A10::LEU2 (Payne et al., 1987) by single step gene replacement (Rothstein, 1991). Diploid GPY747 was generated by mating GPY1100a with GPY716 (aps1-Δ1::LEU2) then introducing chcl1-A12::URA3 by single step gene replacement (Rothstein, 1991).

SD medium is 0.67% yeast nitrogen base (Difco Laboratories Inc., Detroit, MI) and 2% dextrose. Supplemented SD is SD with 20 μg/ml histidine, uracil and tryptophan, and 30 μg/ml leucine, adenine and lysine. SD CAA medium is SD with 5 mg/ml vitamin assay casamino acid mix (Difco Laboratories Inc.) with 15 μg/ml adenine and 10 μg/ml methionine, histidine, uracil and tryptophan. SD CAA-trp is SD CAA without tryptophan. SDYE is SD with 0.2% yeast extract. Cell densities in liquid culture were measured in a 1 cm plastic cuvette using DU62 Beckman spectrophotometer. One A590 unit is equivalent to 2.3 × 10^6 cells/ml.

Isolation and cloning of APS1

Four degenerate oligonucleotide primers were synthesized (ABI 391 DNA Synthesizer): A: 5'-GGGCGGATCTACTG(A/C)(A/G)(A/C)(A/G)NTAGTCG-3' B: 5'-GGGCGGATCTACTG(A/C)(A/G)(A/C)(A/G)NTAGGC3' C: 5'-CCCGGATCT(A/G)(A/T)(C/T)CA(A/G)(A/T)(C/T)AC3-3' D: 5'-CCCGGATCTC(G/A)(G/T)(C/T)CA(A/G)(A/T)(C/T)AC3-3' A and B codes for the amino acid sequence YR/KRYA (as 59–63 in 1, see Figure 2) and differ only at nucleotide position 22. C and D are primers complementary to the amino acid sequence VCAELD (aa 110–112 in 1, see Figure 2) and differ only at nucleotide position 25. Bold nucleotides are recognized sequences for EcoRI and BamHI. All possible productive combinations of these primers were used in 100 μl PCR, which included 10 mM Tris–HCl pH 8.3, 50 mM KC1, 1.5 mM MgCl2, 0.001% (w/v) gelatin, 200 μM each dNTP, 1 mM of each primer, = 0.5 μg yeast genomic DNA (Rose et al., 1990) deleted for APS2 sequences from strain GPY 297 and 2.5 U AmpliTaq DNA polymerase (Perkin Elmer–Cetus, Norwalk, CT). Each reaction was overlaid with 100 μl mineral oil, heated for 5 min at 94°C, subjected to 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min 72°C, followed by a final extension for 5 min at 72°C. The products were separated on a 10% polyacrylamide gel and a band of the expected size (= 150 bp) was present in the reaction employing primers A and D. This band was excised, purified, digested with BamHI and EcoRI, and cloned into pBKCS(+) (Strategene, La Jolla, CA). Plasmid DNA was prepared from four independent bacterial transformants and sequenced using the Sequence DNA Sequencing Kit (USB, Cleveland, OH).

A yeast ÆEMBL3 genomic library (Schmidt et al., 1989) was screened by hybridization using one of the cloned PCR fragments. Two positive λ clones were identified and a 1.2 kb Aps28–SulI fragment from one of these clones was subcloned into pBluescriptII KS(+) for sequencing (pAPS1-4) by the dideoxynucleotide procedure of Sanger (1981). Complete sequence of both strands of this fragment revealed an ORF with significant homology to the small subunits of APs. There appeared to be a frame shift near nucleotide position 540 in this ORF. Three independent PCR amplifications of this ORF from yeast genomic DNA and subsequent sequencing indicated that the amplified copies of the genomic ORF contained one less cytosine at nucleotide position 538. The absence of this cytosine eliminated the frameshift. This mutation is most likely an artifact that was introduced during construction or passage of the ÆEMBL3 library. A wild type clone of APS1 (pAPS1-wt) was created by replacing a SpI–HindII fragment of pAPS1-4 (containing the frameshift) with the corresponding fragment from the product of one of the genomic PCR amplifications. The sequence is presented without the frameshift in Figure 1. Sequence alignments were carried out using the University of Wisconsin programs ‘pileup’ and ‘pretty’.

Radiolabelling and immunoprecipitations

For metabolic labelling of a-α-factor, cells were grown to mid-logarithmic phase in SD CAA or SD CAA-trp (strain with plasmid) at 24°C. Cultures were shifted to 24, 30 and 37°C for 2 h. Labelling and immunoprecipitation was performed as described in Seeger and Payne (1992a) except that labelling was for 45 min instead of 10 min. Quantitation of the various forms of α-factor was carried out using an AMBIUS phosphoimager. For metabolic labelling of CPY, cells were grown to mid-logarithmic phase in SDYE at 24°C. Cultures were shifted to 30 and 37°C for 5 min or 2 h. Labelling and immunoprecipitation was conducted as described in Seeger and Payne (1992b). Cell surface iodinations were carried out according to Seeger and Payne (1992a).

Endocytosis assay

Endocytosis assays were carried out as described in Dulic et al. (1991) and Tan et al. (1993).

Antibodies and immunoblotting

Rabbit anti-yeast G6PD antibodies were purchased from Sigma Chemical Co. Monoclonal antibodies to yeast Clc were a gift from Sandra Lemmon (Case Western Reserve University). Monoclonal antibodies to Sec52p were a gift from David Meyer (University of California, Los Angeles). Antiserum to yeast ALP was prepared as described by Seeger and Payne (1992a). Kex2p antibodies were prepared from a β-galactosidase–Kex2p fusion protein expressed from plasmid pLZ-KXX as described by Fuller et al. (1989).

To prepare antibodies against yeast Clc, a β-galactosidase–Clc fusion protein was constructed. A 400 bp HindIII–Xhol fragment from Clc1 carboxy pLS10 (Silverman et al., 1990) was inserted into YEp138 gelatin and DeRobertis, 1987). The β-galactosidase–Clc fusion protein was expressed and purified according to Burglin and DeRobertis (1987).

To generate Aps1p antibody, the coding region of APS1 was amplified by the PCR from yeast genomic DNA. The 5' PCR primer contained an NdeI site overlapping the initiating methionine codon. The 3' PCR primer was designed to introduce a total of 50 nucleotides onto the C-terminus of Aps1p, followed by a stop codon (TAG) and a BamHI site. The PCR product was cloned into the NdeI–BamHI sites of pET3c (Studier
et al., 1990) to produce pApS1-His. pApS1-His was transformed into BL21 (DE3) (Studier et al., 1990). Expression of the recombinant ApS1–His protein was accomplished by growing the BL21 (DE3) strain containing pApS1-His in Luria broth with 50 mg/ml ampicillin to an A660 of 0.8. Isopropyl thio-galactoside was added to 0.4 mM and the cells were incubated for an additional 2 h. Cells were sedimented at 4000 × g for 10 min and the cell pellets were stored at −20°C prior to subsequent purification. Purification of the ApS1–His fusion protein by Nickel ion–NTA affinity chromatography (Qiagen Inc., Chatsworth, CA) was carried out as described in Bush et al. (1991) with the following modification: the fusion protein was eluted from the resin with buffer E (8 M urea, 0.1 M NaH2PO4, 0.01 M Tris–HCl pH 4.5) and buffer F (6 M guanidine–HCl, 0.2 M acetic acid) instead of an imidazole wash. Fractions containing the ApS1–His protein were dialyzed against PBS and used as antigen.

In vitro-coupled rRNA templates were generated using the terminal peptide acetyl-C-MLLERLSILDRLD coupled via the cysteine to BSA. The peptide was synthesized by the UCLA peptide synthesis facility and was coupled to BSA using the method of Green et al. (1982).

Antibodies were generated in rabbits according to standard procedures (Harlow and Lane, 1988) and affinity-purified using Sephacrose- or Affi-Gel 10-coupled (Bio-Rad, Hercules, CA) antigen. Prior to affinity purification, anti-Aps2p serum was passed through a column of BSA coupled to Sepharose and anti-Cle serum was passed through a column of β-galactosidase coupled to Sepharose. Affinity-purified antibodies were used at 1–5 µg/ml.

Immunoblotting was carried out essentially according to Burnette (1981) with secondary antibodies coupled to ALP (Bio-Rad Laboratories, Richmond, CA) or coupled to horseradish peroxidase (HRP, Bio-Rad Laboratories). Antibodies were visualized using color development for ALP (Bio-Rad Laboratories) or chemiluminescence (ECL) for HRP (Amersham, UK). For analysis of the Aps antibodies, cell extracts were prepared directly by glass bead lysis in SDS (Payne et al., 1987).

Aps antibody detects a protein of ~18 kDa in cells expressing the single chromosomal copy of Aps1 (GPY418) which is absent in the aps1Δ strains (GPY719) and is present at higher levels in cells carrying APS1 on a multicopy vector (GPY733). Similarly, the Aps2 antibody detects a 17 kDa protein species (GPY418), absent in the aps2Δ strains (GPY718) and overexpressed in the strain carrying the gene on a multicopy plasmid (GPY732). In both cases, cross-reaction with the homologous Aps protein was not observed, even when overexpressed, demonstrating the specificity of the antibodies (data not shown).

Fractionation procedures

Clathrin-coated vesicles were prepared as described in Payne and Schekman (1985) with the following modifications. 4–6 l cells were grown to 8×10^6 cells/ml (25–65 g wet weight) in YC. Buffer A (100 mM MES–NaOH pH 6.5, 0.5 mM MgCl2, 1 mM EGTA, 0.2 mM DTT and 1 mM sodium azide) contained 1 mM PMSF, 1 mM benzamidine, 0.01 mM leupeptin (Boehringer Mannheim Biochemicals, Indianapolis, IN), 2 ng/ml pepstatin (Boehringer Mannheim Biochemicals), 1 µg/ml chymostatin (Boehringer Mannheim Biochemicals), 1 µg/ml aprotinin and 1 mM sodium azide. The lysate was added to a 0.1 M imidazole pH 7.0, 1 mM EDTA. Fractions with the greatest absorbance at 280 nm were pooled from each step, dialyzed against imidazole buffer, precipitated with acetone and resuspended in LSB.

Following incubation overnight at 4°C, the suspension was centrifuged at 138 000 g for 1 h. Five millilitres of the resulting supernatant were applied to a 2.5×95 cm Sepharose CL4B column equilibrated in 0.25 M imidazole pH 7.0, 1 mM EDTA, 0.2 mM DTT, 0.6 mM PMSF and 0.02% sodium azide and 6 ml fractions were collected. For hydroxyapatite chromatography, microsomal membranes were prepared and extracted as described above. The membranes were pelleted by centrifugation at 100 000 g for 48 min and the resulting supernatant was brought to 30% saturation of ammonium sulfate. The precipitate (which contained all of the Aps proteins) was collected by centrifugation, resuspended in 0.1 M imidazole pH 7.0, 1 mM EDTA and passed through Sepharose CL4B. Aps-containing fractions were pooled and applied to a 1 ml hydroxyapatite (Calbiochem, San Diego, CA) column at room temperature. Bound proteins were eluted with sequential steps of 0.1, 0.2 and 0.5 M sodium phosphate, followed by 0.6 M fractions. Fractions with the greatest absorbance at 280 nm were pooled from each step, dialyzed against imidazole buffer, precipitated with acetone and resuspended in LSB.

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References


Our recent results show that the sequence of APS1 has been reported by Nakai et al. [(1993) Biochem. Biophys. Res. Commun., 1714, 282–284]. The sequence is identical to that shown here in Figure 1.