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A Late Golgi Sorting Function for *Saccharomyces cerevisiae* Apm1p, but not for Apm2p, a Second Yeast Clathrin AP Medium Chain-related Protein

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Mammalian clathrin-associated protein (AP) complexes, AP-1 (trans-Golgi network) and AP-2 (plasma membrane), are composed of two large subunits of 91–107 kDa, one medium chain (μ) of 47–50 kDa and one small chain (σ) of 17–19 kDa. Two yeast genes, *APM1* and *APM2*, have been identified that encode proteins related to AP μ chains. *APM1*, whose sequence was reported previously, codes for a protein of 54 kDa that has greatest similarity to the mammalian 47-kDa μ1 chain of AP-1. *APM2* encodes an AP medium chain-related protein of 605 amino acids (predicted molecular weight of 70 kDa) that is only 30–33% identical to the other family members. In yeast containing a normal clathrin heavy chain gene (*CHC1*), disruptions of the *APM* genes, singly or in combination, had no detectable phenotypic consequences. However, deletion of *APM1* greatly enhanced the temperature-sensitive growth phenotype and the α-factor processing defect displayed by cells carrying a temperature-sensitive allele of the clathrin heavy chain gene. In contrast, deletion of *APM2* caused no synthetic phenotypes with clathrin mutants. Biochemical analysis indicated that Apm1p and Apm2p are components of distinct high molecular weight complexes. Apm1p, Apm2p, and clathrin cofractionated in a discrete vesicle population, and the association of Apm1p with the vesicles was disrupted in *CHC1* deletion strains. These results suggest that Apm1p is a component of an AP-1-like complex that participates with clathrin in sorting at the trans-Golgi in yeast. We propose that Apm2p represents a new class of AP-medium chain-related proteins that may be involved in a nonclathrin-mediated vesicular transport process in eukaryotic cells.

INTRODUCTION

Transfer of proteins between compartments of the secretory and endocytic pathways is initiated by the binding of coat proteins to the cytosolic surface of membranes, which facilitates the capture of cargo molecules and vesicular budding. Clathrin and its associated proteins (APs), also referred to as assembly proteins or adaptors, form a major class of vesicular transport coats (for reviews, see Keen [1990], Pearse and Robinson [1990], Schmid [1992], and Kirchhausen [1993]). Together, they mediate selective endocytosis of cell surface membrane proteins and their ligands. They are also involved in protein sorting or retention at the trans-Golgi, participating in lysosomal hydrolase targeting and regulated secretory granule formation. In mammalian cells the clathrin triskelion, composed of heavy and light chains, is found at both the plasma membrane and Golgi. In contrast, there are two major AP complexes, AP-1 and AP-2, which are restricted to Golgi-associated or to plasma membrane coated pits and coated vesicles, respectively (Ahle *et*

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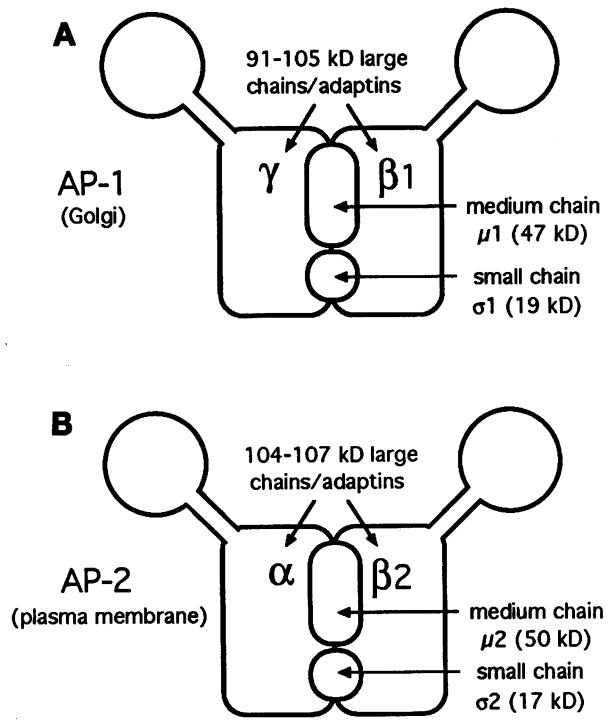

Figure 1. Model of clathrin-associated protein complexes.

al., 1988; Robinson, 1987). The APs are heterotetramers (Figure 1) composed of two large subunits of 90–110 kDa (γ and $\beta1$ in AP-1; α and $\beta2$ in AP-2; also referred to as adaptins), one medium (μ) chain of ~50 kDa ($\mu1$, 47 kDa, in AP-1; $\mu2$, 50 kDa, in AP-2), and one small chain of ~20 kDa ($\sigma1$, 19 kDa, in AP-1; $\sigma2$, 17 kDa, in AP-2) (Ahle *et al.*, 1988; Matsui and Kirchhausen, 1990; Virshup and Bennett, 1988). These complexes are situated between the membrane and the clathrin coat, which forms the striking polyhedral surface lattice found on clathrin-coated membranes (Heuser and Kirchhausen, 1985; Vigers *et al.*, 1986).

Three major functions for the AP complexes have been proposed based on in vivo and in vitro experiments. First, APs seem to play a role in the capture of transmembrane proteins into coated pits, at least in part, by interacting with sorting signals in the cytoplasmic domains of cargo proteins (Beltzer and Spiess, 1991; Chang *et al.*, 1993; Glickman *et al.*, 1989; LeBorgne *et al.*, 1993; Pearse, 1988; Sorkin and Carpenter, 1993; Sosa *et al.*, 1993). APs also bind to docking receptors on their target membranes (Chang *et al.*, 1993; Mahaffey *et al.*, 1990; Peeler *et al.*, 1993; Seaman *et al.*, 1993; Stamnes and Rothman, 1993; Traub *et al.*, 1993; Wang *et al.*, 1993). These docking proteins have been proposed to act in concert with APs to capture membrane proteins or to regulate the recruitment of APs to their specific target membranes at the cell surface or Golgi. Finally, APs promote the assembly of clathrin coats (Zaremba and Keen, 1983) and are required for the binding of triskelions to membranes (Mahaffey *et al.*, 1990; Peeler *et al.*, 1993; Smythe *et al.*, 1992; Unanue *et al.*, 1981).

A number of studies have begun to investigate the function of the individual AP subunits, but the findings are limited thus far. Recent in vitro experiments indicate that the highly related $\beta1$ and $\beta2$ chains (Kirchhausen *et al.*, 1989) are important for the interaction of APs with clathrin and can drive assembly of clathrin coats (Ahle and Ungewickell, 1989; Gallusser and Kirchhausen, 1993; Schroder and Ungewickell, 1991). It has been proposed that the α and γ subunits play a role in AP targeting to membranes or in capture of selected proteins into coated pits (Chang *et al.*, 1993; Robinson, 1993), but there are still little data supporting this. There is also no information on the role of the AP medium ($\mu1$ and $\mu2$) or small chains ($\sigma1$ and $\sigma2$).

In the last several years we have sought homologs of the AP subunits in yeast, where it would be feasible to apply genetic analysis to AP function in an organism with a well-characterized secretory pathway. Several homologs have been identified, including a β chain-related protein encoded by *APL1* (formerly *YAP80*) (Kirchhausen, 1990), $\sigma1$ and $\sigma2$-like proteins encoded by *APS1* and *APS2*, respectively (Kirchhausen *et al.*, 1991; Nakai *et al.*, 1993; Phan *et al.*, 1994), and a $\mu1$ homolog encoded by *APM1* (Nakayama *et al.*, 1991). These findings indicate that the APs, as well as the previously characterized clathrin heavy and light chains (Lemmon and Jones, 1987; Payne and Schekman, 1985; Silveira *et al.*, 1990), have been conserved among all eukaryotes. In this article, we present our further characterization of AP medium chain-related proteins from *S. cerevisiae*. We describe our analysis of *APM1* deletion mutants and provide the first functional evidence that a $\mu1$ chain is important for sorting at the late Golgi. In addition, we have identified a unique AP medium chain-related protein, encoded by the *APM2* gene. We suggest that the medium chains may be members of a larger gene family, and the Apm2p may be a component of a nonclathrin-coat protein complex of another type of transport vesicle.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

Strains used in this study are listed in Table 1 and were derived from S288C background (Mortimer and Johnston, 1986). YEP-dextrose (YEPD), YEP-glycerol (YEPG), YEP-gal, minimal synthetic medium (MV), and dropout media were prepared as described in Nelson and Lemmon (1993). Yeast mating, sporulation, and tetrad analyses were carried out essentially as described in Guthrie and Fink (1991). *Escherichia coli* DH5α was used for plasmid propagation; RR1 (Koerner *et al.*, 1991) was used for trpE-Apmp expression.

Cloning of APM Genes and Plasmid Construction

APM1 was referred to as *YAP54* in a previous report (Nakayama *et al.*, 1991). Since the three letter designation, *YAP*, has already been

Table 1. Yeast strains used in this study

Strain	Genotype	Reference or Source
BJ2164	*MATα CHC1 APM1 APM2 kex2-1 his7*	E. W. Jones
BJ3247	*MATα chc1-Δ::LEU2 trp1 his1 ade6 scd1-v*	Lemmon *et. al.* (1991)
BJ3250	*MATa chc1-Δ::LEU2 ura3-52 scd1-v*	Lemmon *et. al.* (1991)
BJ3475	*MATa/MATα CHC1/CHC1 ura3-52/ura3-52 leu2/leu2 trp1/TRP1 HIS1/his1 ADE6/ade6 gal2/gal2 scd1-i/scd1-i*	Lemmon and Jones (1987)
GPY418.1	*MATα chc1-521^{ts} leu2-3,112 ura3-52 trp1-289 his4-519 can1 gal2*	Seeger and Payne (1992b)
SL214	*MATα GAL1:CHC1 leu2 ura3-52 trp1 his1 GAL2 scd1-i*	Nelson and Lemmon (1993)
SL350	*MATa GAL1:CHC1 leu2 ura3-52 trp1 ade6 GAL2 scd1-v*	Nelson and Lemmon (1993)
SL491	*MATa/MATα CHC1/CHC1 leu2/leu2 ura3-52/ura3-52 trp1/TRP1 ade6/ADE6 gal2/GAL2 scd1-v/scd1-v*	This study
SL638	*MATa/MATα CHC1/CHC1 APM1/apm1-Δ::LEU2 APM2/apm2-Δ::URA3 leu2/leu2 ura3-52/ura3-52 trp1/TRP1 his1/HIS1 ade6/ADE6 gal2/gal2 scd1-i/scd1-i*	This study
SL683	*MATa/MATα CHC1/CHC1 APM1/apm1-Δ::LEU2 APM2/APM2 leu2/leu2 ura3-52/ura3-52 trp1/TRP1 ade6/ADE6 gal2/GAL2 scd1-v/scd1-v*	This study
SL684	*MATa/MATα CHC1/CHC1 APM1/APM1 APM2/apm2-Δ::URA3 leu2/leu2 ura3-52/ura3-52 trp1/TRP1 ade6/ADE6 GAL2/gal2 scd1-v/scd1-v*	This study
SL698	*MATa CHC1 APM1 APM2 leu2 ura3-52 ade6 his1 gal2 scd1-i*	This study
SL701	*MATα CHC1 apm1-Δ::LEU2 apm2-Δ::URA3 leu2 ura3-52 trp1 gal2 scd1-i*	This study
SL703	*MATα CHC1 apm1-Δ::LEU2 APM2 leu2 ura3-52 trp1 his1 gal2 scd1-i*	This study
SL704	*MATα CHC1 apm1-Δ::LEU2 apm2-Δ::URA3 leu2 ura3-52 his1 gal2 scd1-i*	This study
SL705	*MATα CHC1 APM1 APM2 leu2 ura3-52 trp1 his1 gal2 scd1-i*	This study
SL706	*MATα CHC1 APM1 apm2-Δ::URA3 leu2 ura3-52 his1 gal2 scd1-i*	This study
SL760	*MATa/MATα apm1-Δ::LEU2/APM1 apm2-Δ::URA3/APM2 leu2/leu2 ura3-52/ura3-52 trp1/TRP1 ade6/ADE6 gal2/gal2 scd1-v/scd1-v*	This study
SL766	*MATa CHC1 apm1-Δ::LEU2 apm2-Δ::URA3 leu2 ura3-52 trp1 gal2 scd1-v*	This study
SL1003	*MATα CHC1 apm1-Δ::LEU2 APM2 leu2 ura3-52 trp1 his4 can1 gal2 scd1-v*	This study
SL1005	*MATα chc1-521^{ts} apm1-Δ::LEU2 APM2 leu2 ura3-52 trp1 his4 gal2 scd1-v*	This study
SL1007	*MATα chc1-521^{ts} APM1 APM2 leu2 ura3-52 trp1 his4 can1 gal2 scd1-v*	This study
SL1008	*MATα chc1-521^{ts} APM1 APM2 leu2 ura3-52 trp1 gal2 scd1-v*	This study
SL1010	*MATα chc1-521^{ts} APM1 APM2 leu2 ura3-52 gal2 scd1-v*	This study
SL1034	*MATα chc1-521^{ts} APM1 apm2-Δ::URA3 leu2 ura3-52 trp1 his4 gal2 scd1-v*	This study
SL1035	*MATα chc1-521^{ts} apm1-Δ::LEU2 apm2-Δ::URA3 leu2 ura3-52 trp1 his4 gal2 scd1-v*	This study
SL1036	*MATα CHC1 APM1 apm2-Δ::URA3 leu2 ura3-52 trp1 gal2 scd1-v*	This study
SL1037	*MATα CHC1 APM1 APM2 leu2 ura3-52 trp1 his4 gal2 scd1-v*	This study
SL1065	*MATa chc1-521^{ts} apm1-Δ::LEU2 apm2-Δ::URA3 leu2 ura3-52 trp1 his4 gal2 scd1-v*	This study
SL1066	*MATa CHC1 APM1 APM2 leu2 ura3-52 trp1 gal2 scd1-v*	This study
SL1067	*MATα chc1-521^{ts} APM1 apm2-Δ::URA3 leu2 ura3-52 trp1 his4 gal2 scd1-v*	This study
SL1068	*MATα CHC1 apm1-Δ::LEU2 APM2 leu2 ura3-52 trp1 gal2 scd1-v*	This study
SL1069	*MATa CHC1 APM1 apm2-Δ::URA3 leu2 ura3-52 trp1 his4 gal2 scd1-v*	This study
SL1070	*MATα CHC1 apm1-Δ::LEU2 apm2-Δ::URA3 leu2 ura3-52 trp1 gal2 scd1-v*	This study
SL1071	*MATα chc1-521^{ts} apm1-Δ::LEU2 APM2 leu2 ura3-52 trp1 gal2 scd1-v*	This study
SL1072	*MATa chc1-521^{ts} APM1 APM2 leu2 ura3-52 trp1 gal2 scd1-v*	This study
SL1426	*MATa GAL1:CHC1 apm1-Δ::LEU2 APM2 leu2 ura3-52 trp1 ade6 GAL2 scd1-v*	This study
SL1540	*MATa/MATα GAL1:CHC1/GAL1:CHC1 apm1-Δ::LEU2/APM1 leu2/leu2 ura3-52/ura3-52 trp1/trp1 GAL2/GAL2 scd1-v/scd1-v*	This study
SL1541	*MATa/MATα GAL1:CHC1/GAL1:CHC1 apm2-Δ::URA3/APM2 leu2/leu2 ura3-52/ura3-52 trp1/trp1 GAL2/GAL2 scd1-v/scd1-v*	This study
X2180-1B	*MATα gal2 SUC2*	Mortimer and Johnston (1986)

used for another yeast gene (Mortimer *et al.*, 1992), we have renamed the gene *APM1*, for *AP*-medium chain-related protein. *APM1* was cloned by polymerase chain reaction (PCR) using oligonucleotides corresponding to regions flanking the *APM1* open reading frame (ORF), whose sequence was originally reported by Daignan-Fornier and Bolotin-Fukuhara (1989) and corrected by Nakayama *et al.*, (1991). The 5′-oligonucleotide (31 bases; 5′-ggggatccAAAGATGAAGATATTTCAATGG-3′) corresponds to a sequence beginning 288 bases upstream of the translation initiation ATG and contains a leader sequence (lower case letters) with a *Bam*HI restriction site. The 3′ oligonucleotide (25 bases; 5′-ctcgtcgACTCAAACGACAGCGAGC-3′) corresponds to a sequence ending 329 bases downstream of the translation stop codon and has a leader generating a *Sal*I restriction site. Reactions (100 μl) contained 10 ng yeast genomic DNA from strain X2180–1B prepared by the method in Cryer *et al.* (1975), 20 pmol each oligomer, 20 mM Tris-HCl, pH 8.3, 25 mM KCl, 0.05% Tween 20, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, and 200 μM dNTPs. Amplification was carried out for 40 cycles of 1.5 min at 94°C, 1.5 min at 48°C, and 2.5 min at 72°C. The 2.0-kb PCR product was cut with *Bam*HI and *Sal*I and cloned into the polylinker of pBluescript SK^- (Stratagene) generating pSH1. The 2.0-kB insert of pSH1 was used as a probe to screen at YCp50 yeast genomic DNA library (Rose *et al.*, 1987) by colony hybridization (Gergen *et al.*, 1979). A single *APM1* genomic clone (pDS2) was identified from 30,000 colonies screened. Restriction maps of the PCR clone and the insert in pDS2 are shown in Figure

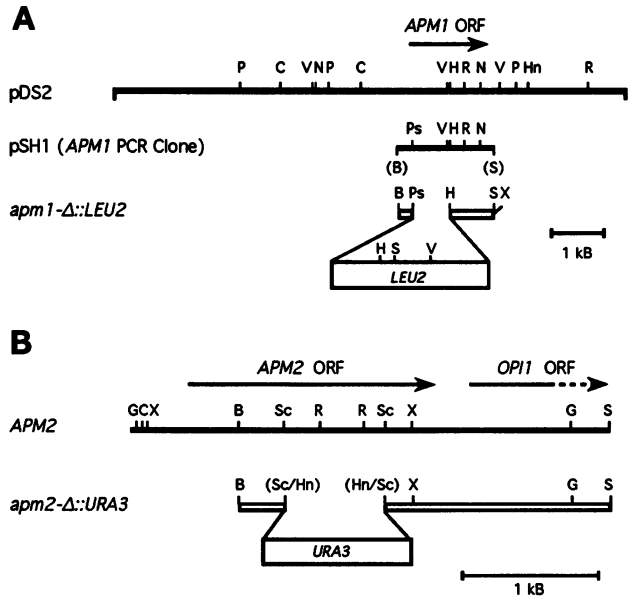

Figure 2. Restriction maps of *APM1* and *APM2* clones and gene disruption constructs. (A) *APM1*. pDS2 was isolated from a YCp50 genomic library using the *APM1* PCR-cloned insert from pSH1 as a probe. *APM1* was disrupted using the *apm1-Δ::LEU2* construct. The *Xho*1 site used to release the disruption construct is in the polylinker of the vector. (B) *APM2*. The region of *APM2* shown contains the entire *APM2* gene as well as the 5′ end of the *OPI1* ORF. *APM2* was disrupted using *apm2-Δ::URA3*. See MATERIALS AND METHODS for details of plasmid isolation and construction. Restriction enzymes: B, *Bam*HI; C, *Cla*1; G, *Bgl*II; H, *Hpa*I; Hn, *Hin*dIII; K, *Kpn*I; N, *Nco*I; P, *Pvu*II; Ps, *Pst*I; R, *Eco*RI; S, *Sal*I; Sc, *Sac*I; V, *Eco*RV; X, *Xho*I). The restriction sites in parentheses for pSH1 were engineered for PCR cloning and are not contained in the genomic DNA.

2. pDS4 is an *APM1* plasmid containing the 4.5-kb *Pvu2* fragment from pDS2 cloned into the *Sma*I site of YEp352, which is a high copy 2μ, *URA3* vector (Hill *et al.*, 1986). pDS6 contains the *APM1* 4.5-kb *Pvu2* fragment, cloned into the *CEN*, *TRP1* vector, pRS314 (Sikorski and Hieter, 1989).

APM2 is located 5′ of the *OPI1* gene, which encodes a negative regulator of phospholipid biosynthesis and is linked to *SPO11* on chromosome VIII (White *et al.*, 1991; Atcheson *et al.*, 1987). Plasmids p(SPO11)1 and pJK613 were obtained from C. Atcheson and S. Esposito. p(SPO11)1 has a 9.4-kb insert containing *APM2*, *OPI1*, and *SPO11*, and has been described previously (Atcheson *et al.*, 1987). pJK613, a YCp50 plasmid, contains the 5.1-kb *Sal*I fragment from p(SPO11)1, which includes *APM2* and all but the last 53 codons of the *OPI1* ORF. pJH344, an *OPI1* clone from M. White and S. Henry (White *et al.*, 1991), contains the 2.8-kb *Bam*HI–*Sal*I fragment in YEp351 (Hill *et al.*, 1986). This clone lacks the first 127 codons of *APM2* and the last 53 codons of the *OPI1* ORF. pDS10 was generated by cloning the *APM2* 3.3-kb *Pst*I–*Bgl*II fragment into pRS314 (*CEN*, *TRP1*) digested with *Pst*I and *Bam*HI. pAP43 contains the *APM2* 3.17-kb *Cla*I–*Bgl*II fragment cloned into pRS426, a 2μ, *URA3* vector (Christianson *et al.*, 1992), digested with *Cla*I and *Bam*HI. pJT2 contains the 3.3-kb *Cla*I–*Sal*I *APM2* fragment cloned into pRS424, a 2μ *TRP1* vector (Christianson *et al.*, 1992).

pAP45, pAP46, and pAP47 are *trpE-APM* fusion constructs made using the pATH expression system (Koerner *et al.*, 1991). pAP45 contains the 0.78-kb *APM1* *Eco*RI-*Sal*I fragment from pSH1 cloned into pATH3 digested with *Eco*RI and *Sal*I. This generates a fusion construct linking the last 149 codons of *APM1* to *trpE*. pAP46 contains the 1.43-kb *APM2* *Eco*RI–*Bgl*II fragment cloned into pATH1 cut with *Eco*RI and *Bam*HI. This fusion construct has the last 173 codons of *APM2* linked to *trpE*. pAP47 contains the 2.8-kb *APM2* *Bam*HI–*Sal*I fragment cloned into pATH10 cut with *Bam*HI and *Sal*I. This generates a fusion construct linking codons 128–605 of *APM2* to *trpE*.

Gene Disruptions

The *APM1* deletion construct (pSH4) was made by substituting the 0.6-kb *Pst*I–*Hpa*I fragment of *APM1* in pSH1 with the 3.18-kb *Pst*I–*Hpa*I *LEU2* fragment from YEp13 (Rose and Broach, 1991). This deletes the *APM1* coding region for amino acids 5–207 (see Figures 2 and 4). pSH4 was digested with *Bam*HI and *Xho*I to release the *apm1-Δ::LEU2* disruption fragment and transformed into diploid SL491 to generate *apm1-Δ::LEU2/APM1* heterozygote SL683. For disruption of *APM2*, the 2.8-kb *Bam*HI–*Sal*I fragment of pJH344 was cloned into pBR322 digested with *Bam*HI and *Sal*I to generate pAP25. pAP25 was digested with *Sac*I to remove 0.75 kb of the *APM2* ORF, and the vector ends were blunted. The 1.16-kb *Hin*dIII *URA3* fragment (ends blunted) from YEp24 (Rose and Broach, 1991) was then cloned in generating pAP40. This deleted the region of *APM2* coding for amino acids 237–485. pAP40 was cut with *Bam*HI and *Sal*I to release the *apm2-Δ::URA3* disruption fragment and transformed into diploid SL491 to generate SL684. The *apm-Δ* double disruption strains were obtained by sequential transformation of BJ3475 with *apm1-Δ::LEU2* and then *apm2-Δ::URA3* to generate SL638 or by crosses of spore progeny from singly disrupted diploids to generate SL760, followed by sporulation and dissection of tetrads.

DNA Sequencing and Computer Analysis

APM2 was identified by performing a computer search (University of Wisconsin Computer Group Sequence Analysis Software Package) on the theoretical translation of the six reading frames of the yeast DNA database (GenBank) using the algorithm TFASTA (Pearson and Lipman, 1988) and the deduced protein sequence of *APM1* as a query. The search identified a matching sequence of 52 amino acids encoded by the 5′ terminal end of the published sequence of the *OPI1* gene (accession no. M57383) (White *et al.*, 1991). These 52 residues map to amino acids 426–475 at the carboxy-terminal end of Apm1p. DNA sequencing of *APM2* was performed by the dideoxy chain termination procedure (Sanger *et al.*, 1977) using the Sequenase sequencing kit from U.S. Biochemical Corp. (Cleveland, OH) and [α-^{35}S]dATP from New England Nuclear (Boston, MA). Complete sequence on both strands was obtained using *APM2* subclones and oligonucleotide primers to bridge gaps. The nucleotide sequence was compiled using the MacVector sequence analysis program (IBI) and submitted to GenBank (accession no. U09841). The multiple protein alignment was generated with the GAP and Pretty programs from the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package, with minor adjustments made visually.

Antibodies

CPY and α-factor antisera were the generous gifts of E.W. Jones and D. Meyer, respectively. Clathrin HC (Chc1p) was detected using a pool of eight anti-yeast clathrin HC monoclonal antibodies (Lemmon *et al.*, 1988). Antibodies to Apm proteins were generated in rabbits using *trpE* fusion proteins as antigens. Fusion proteins were expressed in bacterial strain RR1 carrying pAP45, pAP46, or pAP47 and extracted from the insoluble fraction as described (Koerner *et al.*, 1991). Fusion proteins were further purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), bands were excised, and the proteins were electroeluted. Samples were precipitated with 10 volumes of acetone, dried, and resuspended in phosphate-buffered saline. Rabbits were immunized subcutaneously in Freund's complete adjuvant for primary injec-

tions followed by four to eight boosts in Freund's incomplete adjuvant at 3-wk intervals. For Apm2 antigen, rabbits were immunized with a mixture of the pAP46 and pAP47 trpE-Apm2 fusion proteins. Immunoblots were developed using alkaline phosphatase goat anti-mouse (Chc1p) or goat anti-rabbit (Apm1p and Apm2p) secondary antibodies (Zymed Laboratories) followed by BCIP/NBT detection (Bio-Rad, Richmond, CA).

Apm1p antiserum detected a polypeptide band of 56 kDa on immunoblots of whole yeast extracts. The major 56-kDa band was amplified 5–10-fold in a strain carrying *APM1* on a multicopy plasmid (SL1066 + pDS4). In *apm1*-Δ strains (e.g., SL1068) the 56-kDa band signal decreased ~2-fold; but there was still a residual cross-reacting 56-kDa signal on immunoblots (our unpublished data). We cannot rule out that this cross-reacting band shares antigenic determinants with Apm1p, because it was still detectable in *apm1*-Δ extracts even with the use of partially purified antiserum. However, unlike Apm1p, which elutes in a high molecular weight complex (63–65 Å) on Superose-12, the cross-reacting 56-kDa protein elutes with a native molecular weight of 55–60 kDa (34 Å). Furthermore, the cross-reacting 56-kDa protein was not present in clathrin-coated vesicle fractions (see RESULTS).

Apm2p antibodies detected a protein of 82–84 kDa on immunoblots of whole cell extracts. This size is significantly larger than the 70 kDa predicted for Apm2p; however, specificity of the Apm2p signal was verified by its amplification >10-fold in an *APM2* overexpression strain (SL 705 + pJT2) and its complete absence in extracts of an *apm2*-Δ strain (SL701) (our unpublished results). The basis for the abnormal protein migration in SDS gels is unknown, but we suspect it is an inherent property of the protein, rather than post-translational modification, because the trpE-Apm2p fusions expressed in bacteria also migrated with a higher molecular weight than expected. Neither Apm1p nor Apm2p antisera cross-reacted with Apm2 or Apm1 proteins, respectively.

Radiolabeling and Immunoprecipitation

Cells were grown to log phase overnight at indicated temperatures in MV plus requirements for nutritional markers. For most experiments 1×10^7 cells were harvested by centrifugation, washed once, and resuspended in 0.5 ml fresh growth medium containing 1 mg/ml bovine serum albumin (BSA). Cells were labeled for indicated times and at indicated temperatures with 200 μCi/ml Trans^{35}S (ICN, Costa Mesa, CA). Labeling was stopped by addition of NaN_3 to 0.04% and samples were placed on ice. For α-factor secretion, cells were sedimented and the medium (secreted) fraction was reserved. In one experiment, protocol 1 (Fig. 4), the medium was adjusted to 5% trichloroacetic acid (TCA) and prepared for immunoprecipitation as described previously (Lemmon *et al.*, 1991). In subsequent experiments, protocol 2 (Figures 7 and 8), cells were labeled in 250 μl, and after centrifugation, 200 μl of the supernatant was removed to a fresh microcentrifuge tube to prepare for immunoprecipitation. SDS was added to a final concentration of 0.05%, and samples were boiled for 4 min. Samples were brought to 1.0 ml with immunoprecipitation buffer (IP buffer, 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Tween 20, 0.1 mM EDTA) containing 1 mg/ml BSA. Fifty microliters of protein A-Sepharose (Sigma, St. Louis, MO) (1:5 suspension in 10 mM Tris, pH 7.5, 1 mg/ml BSA, 1 mM NaN_3) was added. After mixing gently for 10 min at room temperature, samples were spun and the supernatant was removed to a fresh tube for immunoprecipitation. For both labeling protocols, 1 μl of α-factor antiserum was then added, samples were incubated for 3 h at 4°C which was followed by the addition of 50 μl of the protein A-Sepharose suspension for 1 h at 4°C. Beads were sedimented and washed twice with IP buffer, two times with 100 mM Tris, pH 7.5, 200 mM NaCl, 2 M urea, 0.5% Tween 20, once with 1% β-mercaptoethanol, and once with 0.1% SDS. The final pellet was resuspended in 50 μl of SDS sample buffer, boiled for 4 min, and spun. The eluted samples were run on 12 or 12.5% SDS gels. Gels were fixed in 15% methanol/10% acetic acid for 30–45 min, washed with H_2O for 30–45 min, enhanced with 1 M salicylic acid for 45 min, dried, and exposed to X-ray film at −70°C. Densitometry was done on autoradiograms using a Sci Scan 5000 (U.S. Biochemical). Where appropriate, lighter exposures of films were used for quantitative scanning.

For CPY experiments, cells were pulse labeled in 0.5 ml for 10 min and chased in the presence of 3 mM $(NH_4)_2SO_4$, 0.01% methionine and cysteine. 250-μl samples were taken at 0 and 40 min after the chase was initiated. Reactions were stopped by addition of NaN_3 as indicated above. For external CPY, medium and periplasmic fractions were prepared and combined as follows. Cells were sedimented, and 200 μl of the supernatant was removed to a fresh microcentrifuge tube, adjusted to 0.5% with SDS, and boiled. IP buffer (800 μl) was added, and the sample was stored on ice. The cell pellet was washed once in spheroplast buffer (1.4 M sorbitol, 50 mM Tris-HCl, pH 7.4) and then resuspended in 100 μl spheroplast buffer containing 0.4% β-mercaptoethanol, 10 mM NaN_3, and 45 U Oxalyticase (Enzymogenetics, Corvallis OR). After 30 min at 30°C, spheroplasts were sedimented in a microfuge for 20 s. Five microliters 2% SDS was added to the supernatant (periplasm), which was then boiled and combined with the medium generating the external CPY fraction. The pellet of spheroplasted cells (internal CPY) was lysed in 100 μl 2% SDS, boiled, and diluted to 1.0 ml with IP buffer and 1 mg/ml BSA. External and internal fractions were subject to immunoprecipitation as described in protocol 2 for α-factor, except samples were incubated overnight with 3 μl CPY antiserum.

Biochemical Procedures

Whole cell yeast extracts were prepared by glass bead homogenization. Typically, 5×10^8 cells from log phase cultures, grown in YEPD or appropriate dropout medium for plasmid selection, were harvested. Cells were washed in dH_2O, resuspended in 1.25 ml 2% SDS, transferred to sample vials (10 mm × 3.5 cm) containing 0.5 volume acid-washed glass beads (0.45 μm), and homogenized for 3 min in a Braun homogenizer with a custom made eight-well sample adapter. Samples were boiled for 10 min and spun 10 min in a microcentrifuge. Soluble extracts (100 μg protein) were fractionated on 7.5% SDS gels and transferred to nitrocellulose for immunoblotting.

Yeast clathrin-coated vesicles were purified by Sephacryl S-1000 (Sigma) chromatography (2.5 cm × 95 cm) as described previously (Lemmon *et al.*, 1988; Phan *et al.*, 1994). Both protocols gave comparable results. Eight-milliliter fractions were collected, and 80-μl aliquots were analyzed by SDS-PAGE and immunoblotting.

For size determination of the Apm1p and Apm2p complexes, 3×10^9 yeast cells, grown at 30°C in YEPD to a density of 2–3 × 10^7 cells/ml, were harvested, washed once in dH_2O and once in clathrin extraction buffer, which contains a 1:1 volume ratio of 1.0 M Tris-HCl, pH 7.0:buffer A (0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.5, 0.5 mM $MgCl_2$, 1.0 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.2 mM dithiothreitol (DTT), and 0.02% NaN_3). The final washed pellet was resuspended in 1.0 ml of clathrin extraction buffer containing a protease inhibitor cocktail consisting of 100 μM L-1-tosylamide-2-phenylethylcholormethyl (TPCK), 1 mM benzamidine-HCl, and 5 mM E64 (from Sigma) plus 25 μM pepstatin A and 4 μM leupeptin (from Boehringer Mannheim, Indianapolis, IN). Samples were homogenized in the Braun glass bead homogenizer for 3 min as described above and centrifuged for 25 min at 27,000×*g*. The supernatant was collected and centrifuged for 1 h at 100,000×*g*. Two hundred microliters of the resulting soluble protein fraction were loaded to a 1 cm × 30-cm Superose-12 FPLC column (Pharmacia, Piscataway, NJ) pre-equilibrated in clathrin extraction buffer and eluted at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected, and 80 μl of each sample were loaded to an 8% SDS gel for immunoblot analysis. The Superose-12 column was calibrated using bovine thyroid thyroglobulin (669 kDa, 85 Å), horse spleen ferritin (440 kDa, 61 Å), bovine liver catalase (232 kDa, 52.2 Å), rabbit muscle aldolase (158 kDa, 48.1 Å), bovine serum albumin (67 kDa, 35.5 Å), and cytochrome *c* (12.4 kDa, 16.3 Å).

Other Methods

Routine cloning and DNA manipulations were performed as described in Sambrook *et al.* (1989). DNA probes were made using the Stratagene random primer labeling kit and [α-^{32}P]dCTP (Dupont-NEN). Yeast strains were transformed by the lithium acetate method (Ito *et al.*, 1983).

RESULTS

Identification and Sequence of APM1 and APM2

APM1 encodes a yeast protein with extensive homology to the medium chain (μ) subunits of the mammalian clathrin AP complexes. *APM1* was described in an earlier report, where it was referred to as *YAP54* (Nakayama *et al.*, 1991). The protein encoded by *APM1* is 475 amino acids long with a predicted molecular weight of 54 kDa. It is 39% identical (59% similar) to rat μ2 and 56% identical (75% similar) to mouse μ1 (Nakayama *et al.*, 1991). Apm1p has similar preferential homology to *C. elegans* μ1 as compared to the μ2 counterpart (Lee *et al.*, 1994). Since Apm1p is more homologous to the μ1 chains than mammalian or *C. elegans* μ1 and μ2 are related to each other, this suggests that Apm1p may be a subunit of an AP-1-like complex involved in clathrin function at the late Golgi.

A second AP-medium chain-related protein was identified by searching GenBank for yeast sequences that encode proteins or portions of proteins with homology to Apm1p using the TFASTA algorithm (Pearson and Lipman, 1988). The search uncovered 52 C-terminal amino acids encoded by a previously unidentified gene in the 5′ region of the reported sequence of the *OPI1* gene, which is upstream of *SPO11* on chromosome VIII (Atcheson *et al.*, 1987; White *et al.*, 1991). Clones containing the remainder of the gene, which we designated *APM2*, were obtained from laboratories working on this region of the genome (see Figure 2 for restriction map) and the sequence was completed (GenBank accession no. U09841).

APM2 encodes a novel AP-medium chain-related protein. Unlike Apm1p, which is similar in size and sequence to the mammalian and *C. elegans* AP medium chains, Apm2p is much larger (605 amino acids with a predicted molecular weight of 70 kDa) and less conserved than the other AP-medium chain subunits. Comparison of Apm2p to Apm1p, mouse μ1 and rat μ2 shows Apm2p has regions of homology throughout its length and there are several segments of high conservation shared among all four proteins, clearly demonstrating their relatedness (Figure 3). However, if one considers strictly the regions that correspond to the 50-kDa medium chains, Apm2p is only 30-33% identical to the other family members and shows no preferential homology to the μ1 or μ2 classes. In addition, Apm2p contains a significant number of insertions causing gaps in the optimized alignment, the largest of which corresponds to an ~100 amino acid segment following residue 136. This large insertion is in a different position than the major insert found after amino acid 227 of Apm1p, which has been proposed

```
Apm2p .MSSSLFILD ENLEPLVSKN IRALPNLSSV ......LSSF KQCYHDG.SP  42
Apm1p .MASAVYFCD HNGKPLLSRR YRDDIPLSAI DKFPILLSDL EE..QSNLIP  47
μ1    MSASAVYVLD LKGKVLICRN YRGDVDMSEV EHFMPILMEK EE..EGMLS.  47
μ2    .MIGGLFIYN HKGEVLISRV YRDDIGRNAV DAFRVNVIHA RQ...QVRSP  46
       * *    *   *  * **  ** *   * *    *    *          **

Apm2p PILSQNDWFF IHLKRDFLHF VSVIHTTDKP NIDLMTILAF LEQFYHLLQK  92
Apm1p PCLNHNGLEY LFIQHNDLYV VAIVTSLSAN AAA...IFTF LQKLVEVLSD  94
μ1    PILAHGGVRF MWIKHNNLYL VA....TSKK NACVSLVFSF LYKVVQVFSE  93
μ2    VTNIARTSFF .HVKRSNIWL AAV....TKQ NVNAAMVFEF LYKMCDVMAA  91
       * *    *    *   *  **      *  *     * * * *   *

Apm2p YFEIEVLTKN VILDNILLVL ELIDECIDFG IVQVTDPSII KDYIRVKVNV 142
Apm1p YL..KTVEEE SIRDNFVIIY ELLDEVMDYG IPQITETKML KQYI..... 136
μ1    YF..KELEEE SIRDNFVIIY ELLDELMDFG YPQTTDSKIL QEYI..... 135
μ2    YF..GKISEE NIKNNFVLIY ELLDEILDFG YPQNSETGAL KTFI..... 133
       **      **  * **** ** *****  ***  ** *    * * **

Apm2p PRVTVDNEEW SPGEESSSSS GSDSDSEYSN TNKRKDKKKK RKKKKGTKGK 192
Apm1p .......... .......... .......... TQKSFKLVKS AKKKRN.... 152
μ1    .......... .......... .......... TQEGHKLETG APRPPA.... 151
μ2    .......... .......... .......... TQQGIKSQHQ TKEE..... 147
                                        **   *      *

Apm2p SVGKSKLKSI MVNNKENRGI NVVETVKETL RNKNDTGKEA ANDELPNDGN 242
Apm1p .......... .......... .......... .......... .......... 152
μ1    .......... .......... .......... .......... .......... 151
μ2    .......... .......... .......... .......... .......... 147

Apm2p DLYINGDIAK TIIMPISWRT KGIHYAKNEF FLDVIERVQY LMDFEKGVIR 292
Apm1p ....ATRPPV ALTNSVSWRP EGITHKKNEA FLDIVESINM LMTQKGQVLR 198
μ1    .......... TVTNAVSWRS EGIKYRKNEV FLDVIEAVNL LVSANGNVLR 191
μ2    ....QSQITS QVTGQIGWRR EGIKYRRNEL FLDVLESVNL LMSPQGQVLS 193
                  *   ***  *** * ***  **** * **  **   * ***

Apm2p KNLIHGEIVC RCYLSGMPKL KISINK..IL .......... .........N 321
Apm1p SEII.GDVKV NSKLSGIPHL KLGIKDKGIF SKYLDDDTNI PSASATTSDN 247
μ1    SEIV.GSIKM RVFLSGMPEL RLGLNDKVLF .......... .........D 221
μ2    AH.VSGRVVM KSYLSGMPEC KFGMNDKIVI .......... .........E 223
         *         ***** * * * ***

Apm2p RDPQFMSNS. .......... ........SF HQCVSLDSIN TIEKDEEKNS 352
Apm1p NTETDKKPSI TSSSATNKKK VNIELEDLKF HQCVRLSKFE .......... 287
μ1    NTGRGKSKS. .......... ..VELEDVKF HQCVRLSRFE .......... 248
μ2    KQGKG..... TADETSKSGK QSIAIDDCTF HQCVRLSKFD S......... 259
            *                    *  * ******* *

Apm2p DDDAGLQAAT DAREIEFIPP DGEFVLCQYE LKRHVKDAPM VRLKDFEIKP 402
Apm1p .......... NEKIITFIPP DGKFDLMNYR LSTTIK..PL IWCDVN..VQ 323
μ1    .......... NDRTISFIPP DGEFELMSYR LNTHVK..PL IWIE..SVIE 284
μ2    .......... .ERSISFIPP DGEFELMRYR TTKDI....I LPFRVIPLVR 294
                    * * **** **** ** ** *    *   *

Apm2p KLKKFKIQIV TKIQTNFKPT NSTSKLNVRI PLTKVFQEYK IDLSKQIRFK 452
Apm1p VHSNSRIEIH CKAKAQIKRK STATNVEILI PVP....... .DDADTPTFK 365
μ1    KHSHSRIEYM VKAKSQFKRR STANNVEIHI PVP....... .NDADSPKFK 326
μ2    EVGRTKLEVK VVIKSNFKPS LLAQKIEVRI PTP....... LN.TSGVQVI 336
            **    * *  **     *   * * *                    **

Apm2p ANIGKVVFNL SDDFLLWEIQ TMKGHREHST NKSSQYNSDE DDPNTCASMV 502
Apm1p YSHGSLKYVP EKSAILWKIR SFPGGKEYS. .......... ........MS 396
μ1    TTVGSVKWVP ENSEIVWSVK SFPGGKEY.. .......... .......LMR 357
μ2    CMKGKAKYKA SENAIVWKIK RMAGMKE... .......... ......SQIS 367
        *  *        * * *     * **                          *

Apm2p AEFPLFNQEE YDRLQEEMKT SMNPPPLRTG PRLEELYRQV HDQQTSHVTP 552
Apm1p AELGL..... ..PSISNNED GNRT..... .......... ..MPKSNAEI 421
μ1    AHFGL..... ..PSVEAEDK EGKPP..... .......... .......... 375
μ2    AEIELLP... .....TNDKK KWARPP.... .......... .......... 385
       **  *                 *

Apm2p RDKLVNIDFE IPYCTCSGLK VEYLKVEEPQ LQY...QSFP WVRYKTVSDE 599
Apm1p SKGPVQIKFQ IPYFTTSGIQ VRYLKINEPK LQY...KSYP WVRYITQSGD 468
μ1    ....ISVKFE IPYFTTSGIQ VRYLKIIE.K SGY...QALP WVRYITQNG. 416
μ2    ....ISMNFE VPFAP.SGLK VRYLKVFEPK LNYSDHDVIK WVRYIGRSG. 429
            ** *** * **   *****  *** * *       * ****** **

Apm2p EYAYIV. 605
Apm1p DYTIRLT 475
μ1    DYQLRTQ 423
μ2    IYETRC. 435
       * *
```

Figure 3. Alignment of the yeast AP-medium chain sequences (Apm2p and Apm1p) with mouse μ1 and rat μ2 (formerly AP47 and AP50) of trans-Golgi network AP-1 and plasma membrane AP-2, respectively. Gaps are indicated with periods. Asterisks indicate positions where there are three or four identities among the four proteins. Note the large insertion after amino acid 136 of Apm2p and the homology at the C-terminal end, which was the region originally identified in the TFASTA search for proteins with homology to Apm1p.

to correspond to a central linker segment that splits the AP medium chains into two functional domains (Matsui and Kirchhausen, 1990; Nakayama *et al.*, 1991). The central linker region is broken up in Apm2p when aligned to the other medium chains due to the unusual placement of the conserved sequence F H Q C V (S/R) L (see Apm2p amino acids 320-363). It is unlikely that any of the nucleotide sequences encoding the insertion regions of Apm2p are removed by splicing, since there are no yeast consensus splice sites in the *APM2* gene. In addition, immunoblot analysis has shown that the Apm2 protein migrates as an 82-84-kDa protein by SDS-PAGE, significantly larger, rather than smaller, than the predicted molecular weight (our unpublished observations).

APM1 and APM2 Disruption Strains Are Phenotypically Normal

If the *APM* gene products are involved in clathrin function, haploid cells carrying deletions of one or both *APM* genes might be expected to display phenotypes characteristic of clathrin-deficient yeast. These include slow growth, temperature sensitivity, genetic instability, and abnormal morphology, mating and sporulation (Lemmon and Jones, 1987; Lemmon *et al.*, 1990; Payne and Schekman, 1985; Payne and Schekman, 1989; Payne *et al.*, 1988). To examine the phenotype of Apm-deficient yeast, gene disruptions of *APM1* and *APM2* were generated by replacing the regions coding for amino acids 5-207 of *APM1* and amino acids 237-485 of *APM2* with the *LEU2* and *URA3* genes, respectively (Figure 2). To provide for the possibility that the genes might be essential for vegetative growth (which has been shown for the clathrin heavy chain gene, *CHC1*, in some yeast strains [Lemmon and Jones, 1987; Munn *et al.*, 1991]), knockouts were first generated in diploids and then tetrad analysis was performed on the *apm*-Δ/*APM* heterozygotes. Surprisingly, null mutations in *APM1* and *APM2*, singly or in combination, resulted in no obvious phenotypic consequences to yeast cells. Virtually all tetrads had four viable spores, and *apm1*-Δ *apm2*-Δ spore clones grew as well as singly disrupted or nonmutant cells (also see Figure 5). Disruptions were verified by DNA blot analysis of diploid transformants and spore clones from tetrads. In all cases there was 2:2 segregation of Leu or Ura phenotypes with the appropriate genomic DNA pattern. Northern blot analysis confirmed the absence of *APM* transcripts in the disruption strains. A larger *TRP1* disruption of *APM2* deleting codons 128–432 gave identical results.

To further confirm that *apm* disruption strains were normal, we examined them for defects in processing of the mating pheromone α-factor. Yeast carrying mutations in *CHC1*, mislocalize Kex2 protease and dipeptidyl amino peptidase A (DPAP-A) from a late Golgi compartment to the plasma membrane (Payne and Schekman, 1989; Seeger and Payne, 1992b). In *MATα* cells, this defect in Golgi retention results in secretion of an inactive highly glycosylated precursor of α-factor (>100 kDa), whose normal maturation requires these enzymes in the late Golgi. To examine the form of α-factor secreted from *apm*-Δ strains, cells were labeled with [^{35}S]Met/[^{35}S]Cys and growth medium was subjected to immunoprecipitation with anti-α-factor antibodies (Figure 4). *chc1*-Δ (lane 6) or *kex2* (lane 1) strains secreted the high molecular weight precursor. In contrast *apm1*-Δ (Δ1) and *apm2*-Δ (Δ2), single and double mutants, showed no defect in α-factor processing and only mature pheromone (~3.5 kDa) appeared in the medium (lanes 2–5). These results indicate that α-factor processing is normal in *apm*-Δ mutants when normal clathrin is present.

Previously we identified a genetic locus (*SCD1*, suppressor of clathrin deficiency) that influences the ability of Chc$^-$ yeast to survive; *chc1*-Δ *scd1-i* strains are inviable, while *chc1*-Δ *scd1-v* strains display the slow growth and other characteristics of viable clathrin-deficient yeast (Lemmon and Jones, 1987). Phenotypic analysis was performed on spores derived from *apm1*-Δ and/or *apm2*-Δ disruption heterozygotes carrying either the *scd1-i* (SL638) or *scd1-v* (SL683, SL684, SL760) allele. None of the resultant spores showed any

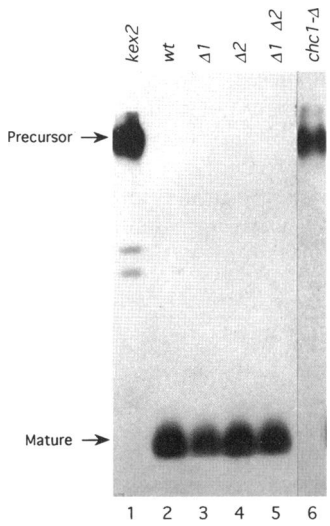

Figure 4. Processing of α-factor is not affected by disruption of the *APM* genes in *CHC1* strains. Cells were grown at 30°C to mid-log phase and labeled with Tran^{35}S at 30°C for 30 min as described in MATERIALS AND METHODS. The medium was immunoprecipitated with α-factor antisera and samples were analyzed on 12% SDS-gels. Lane 1, BJ2164 (*kex2*); lane 2, SL705 (*CHC1 APM1 APM2*); lane 3, SL703 (*CHC1 apm1*-Δ::*LEU2 APM2*); lane 4, SL706 (*CHC1 APM1 apm2*-Δ::*URA3*); lane 5, SL704 (*CHC1 apm1*-Δ::*LEU2 apm2*-Δ::*URA3*); lane 6, BJ3247 (*chc1*-Δ::*LEU2 APM1 APM2*). "Precursor" and "Mature" indicate the highly glycosylated precursor and fully processed α-factor, respectively.

phenotypic defects, indicating that the *SCD1* locus has no obvious effect on *apm*-Δ mutants.

Synthetic Phenotype of apm1-Δ in Combination with a chc1 Temperature Sensitive Mutation

chc1–521^{ts} is a temperature sensitive allele of the clathrin heavy chain gene (Seeger and Payne, 1992b). Yeast cells harboring this mutation (referred to as *chc1-ts*) secrete mature α-factor at 25°C, but after a shift to 30 or 37°C a substantial amount of unprocessed α-factor precursor is secreted due to missorting of DPAP-A and Kex2p from the Golgi to the cell surface (Seeger and Payne, 1992b). In addition, cell growth is slowed at 37°C. We therefore tested whether the combination of *apm1*-Δ and/or *apm2*-Δ with the *chc1-ts* mutation would have any synthetic phenotypes. A triple heterozygote was generated by crossing GPY418.1 (*MATα chc1-ts*) to SL766 (*MAT***a** *apm1-Δ::LEU2 apm2-Δ::URA3*) and tetrad analysis was performed. Scoring of 21 tetrads for growth at 22 and 37°C indicated that *apm1*-Δ, but not *apm2*-Δ, enhanced the temperature sensitive growth defect displayed by *chc1-ts* cells (Table 2). Figure 5 shows an example of the growth phenotypes observed for spore clones representing all eight possible genotype combinations. At 22°C all spore types showed comparable growth. Similar results were obtained at 30°C (see Figure 6). At 37°C, *CHC1* strains grew well regardless of the *APM* genotype, while cells with the *chc1-ts* mutation alone or *chc1-ts apm2*-Δ grew somewhat more slowly than *CHC1* strains (Figure 5). In contrast, the *chc1-ts apm1*-Δ or *chc1-ts apm1*-Δ *apm2*-Δ strains grew very slowly or were completely inviable at 37°C. Since the growth of *chc1-ts apm2*-Δ cells was similar to *chc1-ts* strains carrying wild type *APM* genes, we conclude that the lethality of *chc1-ts* in combination with *apm1*-Δ *apm2*-Δ is due to the lack of the *APM1* gene product alone.

We also observed some variability in growth of *chc1-ts* strains, such that some spore clones grew more slowly at 37°C than others (note range of growth in Table 2). This variation could not be accounted for by differences in the *SCD1* genotype, because back crosses of GPY418.1 confirmed that it is of *scd1-v* genotype, as is SL766. These types of growth differences have been noted previously for *chc1*-Δ *scd1-v* strains (Lemmon *et al.*, 1990; Lemmon and Jones, 1987). Therefore, to confirm that the enhanced growth defect of the *chc1-ts apm1*-Δ strains was due to the *apm1*-Δ mutation, we generated an isogenic set of strains by transforming a *chc1-ts apm1*-Δ mutant (SL1005) with plasmids carrying *APM1*, *APM2*, or no insert (Figure 6). At 37°C, SL1005 was inviable. Growth was rescued to a rate comparable to a *chc1-ts APM1* strain when SL1005 was transformed with *APM1* on a *CEN* vector (p*APM1*, *CEN*). There was no rescue of SL1005 transformed with the parent vector (p*CEN*) or *APM2* on a high copy vector (p*APM2*, 2μ) (Figure 6). Similar results were seen for *APM2* on a centromere vector. This indicates that *APM2*, even when overexpressed, cannot substitute for *APM1*.

α-Factor Processing Is Defective in chc1-ts apm1-Δ at Permissive Temperatures

The enhanced growth defect of *chc1-ts apm1*-Δ at 37°C suggested that vesicular transport might be affected as well. Since *chc1-ts* strains with wild-type *APM* genes exhibit an α-factor processing defect after shift to temperatures as low as 30°C (Seeger and Payne, 1992b), we examined secretion of α-factor in *chc1-ts apm*-Δ mutants at lower temperatures. Our first observation was that some *chc1-ts APM1 APM2* cells secreted significant amounts of α-factor precursor even at 25°C, a permissive growth temperature (Figure 7, lanes 1–3). The amount secreted varied from <1 to 20%, and correlated with the growth phenotype at 37°C; i.e.,

Table 2. Growth of *chc1-ts* spores of different *APM* genotypes

Growth at 37°C	No. of Spores with Genotype[a,b]				
	CHC1[c]	*chc1-ts*			
		APM1 APM2	*apm1*-Δ *APM2*	*APM1 apm2*-Δ	*apm1*-Δ *apm2*-Δ
++	39	0	0	0	0
+	0	4	0	8	0
+/−	0	1	0	5	0
−/+	0	2	7	0	11
−	0	0	3	0	1

[a] GPY418.1 (*MATα chc1-ts*) was crossed to SL766 (*MAT***a** *apm1-Δ:LEU2 apm2-Δ::URA3*) and the diploid was sporulated. Results are from dissection of 21 tetrads. Master plates were grown on YEPD at 22°C, replica plated to YEPD, and grown at 37°C for 2 days.

[b] Three spores did not germinate. Inferred genotypes were as follows: two *CHC1 APM1 APM2* spores, one *CHC1 apm1*-Δ *apm2*-Δ spore.

[c] The number of spores of *CHC1* genotype represent the sum of the data for all four *APM* genotype combinations. Each genotype was represented in approximately equal numbers.

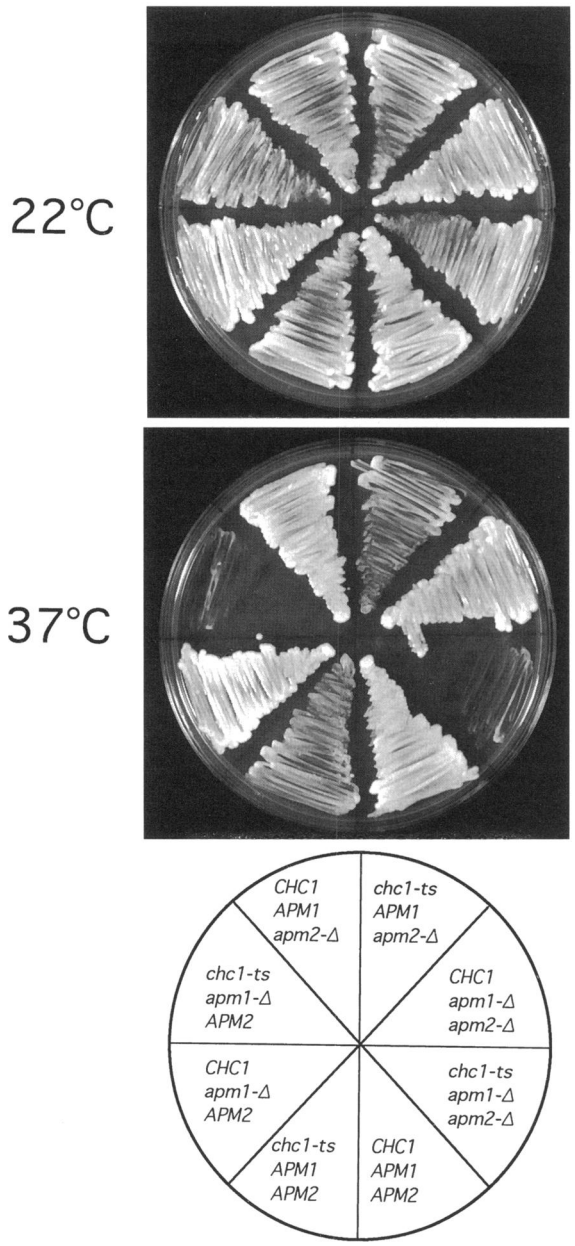

Figure 5. The temperature sensitive growth phenotype of *chc1-ts* mutant cells is enhanced by deletion of *APM1*, but not *APM2*. Strains were streaked onto YEPD plates and grown at 22 or 37°C for 3 days. Starting from the lower left sector and proceeding clockwise are strains: SL1072 (*chc1-ts APM1 APM2*); SL1068 (*CHC1 apm1-Δ APM2*); SL1071 (*chc1-ts apm1-Δ APM2*); SL1069 (*CHC1 APM1 apm2-Δ*); SL1067 (*chc1-ts APM1 apm2-Δ*); SL1070 (*CHC1 apm1-Δ apm2-Δ*); SL1065 (*chc1-ts apm1-Δ apm2-Δ*); SL1066 (*CHC1 APM1 APM2*). These eight spores are segregants from two tetrads that gave all possible genotype combinations.

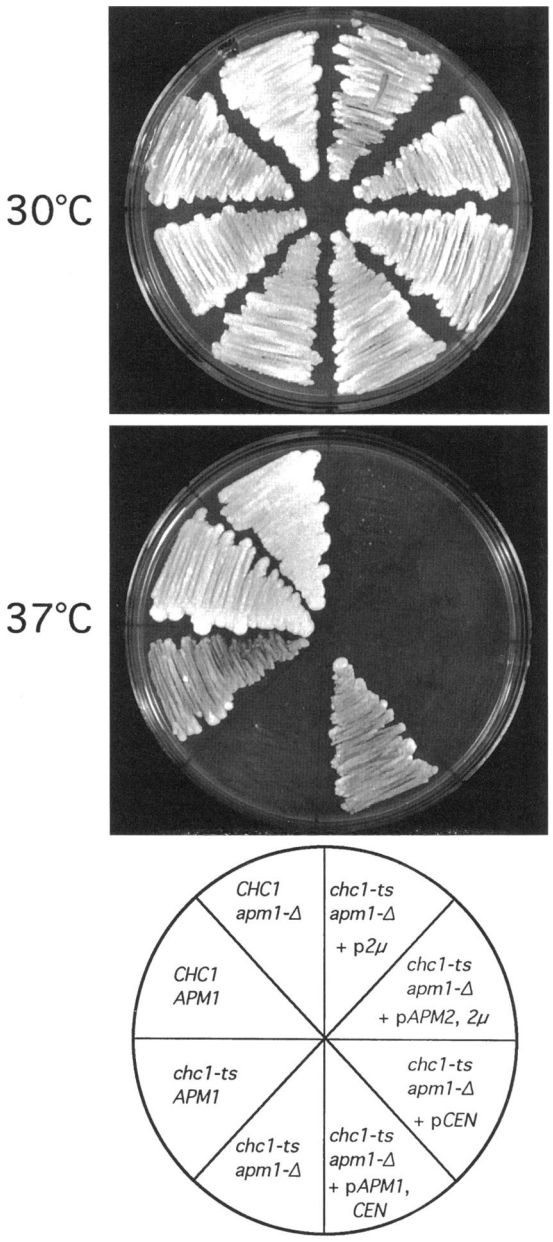

Figure 6. Rescue of the enhanced growth defect of *chc1-ts apm1-Δ* strains by a plasmid bearing *APM1*, but not *APM2*. An isogenic series of strains was generated by transforming a *chc1-ts apm1-Δ* strain (SL1005) with the plasmids indicated. Strains were then streaked onto YEPD plates and grown at 30 or 37°C for 3 days. Starting from the lower left sector and proceeding counter-clockwise are strains: SL1005 (*chc1-ts apm1-Δ*); SL1005 + pDS2 (*chc1-ts apm1-Δ* + p*APM1, CEN*); SL1005 + YCp50 (*chc1-ts apm1-Δ* + p*CEN*); SL1005 + pAP43 (*chc1-ts apm1-Δ* + p*APM2, 2μ*); SL1005 + pRS426 (*chc1-ts apm1-Δ* + p2μ). Control strains shown for comparison: SL1003 (*CHC1 apm1-Δ*); SL1037 (*CHC1 APM1*); SL1072 (*chc1-ts APM1*).

chc1-ts strains that secreted more pro-α-factor at 25°C, showed poorer growth at 37°C.

Due to the variability of *chc1-ts* strains, we examined α-factor processing at 25°C in isogenic sets of *chc1-ts apm1-Δ* strains by transforming a given parental strain with *APM* plasmids or control vectors. Results for

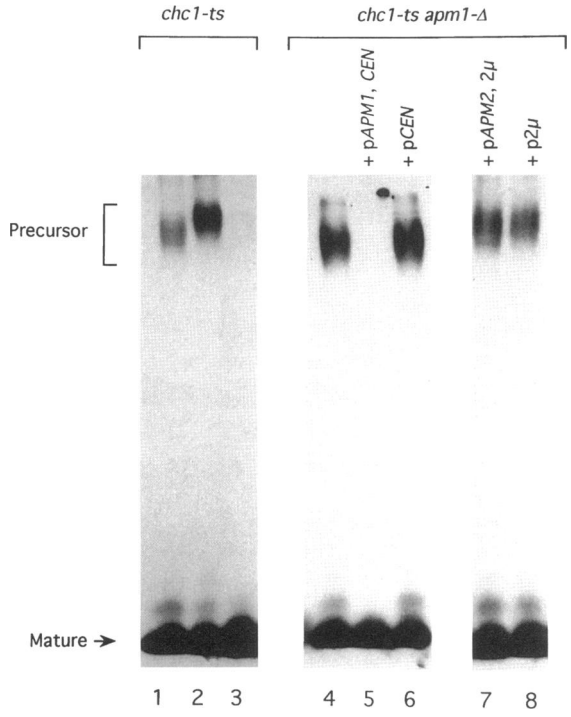

Figure 7. α-factor precursor accumulation is greatly enhanced in *chc1-ts* strains carrying *apm1*-Δ. Cells were grown at room temperature to mid-log phase and labeled with Tran^{35}S at 25°C for 30 min, as described in MATERIALS AND METHODS. Media fractions were immunoprecipitated with α-factor antisera, and samples were analyzed on 12% SDS-gels. Lanes 1–3 (SL1007, SL1008, and SL1010, respectively) show the variability of the α-factor processing defect in *chc1-ts APM1 APM2* strains. Lanes 4–8 show the enhancement of the α-factor processing defect by *chc1-ts* cells in combination with *apm1*-Δ and rescue of the processing defect by *APM1* on a plasmid, but not *APM2*. SL1005, *chc1-ts apm1*-Δ, transformed with: no plasmid (lane 4); an *APM1, CEN* plasmid pDS2 (lane 5); the parent *CEN* vector YCp50 (lane 6). In a separate experiment SL1005 was transformed with an *APM2*, 2μ vector pAP43 (lane 7); or the parental 2μ vector pRS426 (lane 8). "Precursor" and "Mature" indicate the highly glycosylated precursor and fully processed α-factor, respectively.

SL1005 are shown in Figure 7, lanes 4–8. In the untransformed strain 30–40% of the secreted α-factor was in the high molecular weight precursor form, and incompletely processed lower molecular weight peptides were detectable (Figure 7, lane 4). Transformation of SL1005 with the *APM1* containing plasmid (p*APM1, CEN*) completely rescued the processing defect (lane 5), while the vector alone (p*CEN*, lane 6) or the *APM2* high copy plasmid (p*APM2*, 2μ, lane 7) had no effect. Similar results were obtained with a second *chc1-ts apm1*-Δ strain (SL1071). In this case, the parental strain showed a more severe defect in α-factor processing (>40% of secreted α-factor was precursor). Upon transformation with the *APM1* plasmid, processing was substantially improved, but not complete, with 5–7% of the secreted α-factor still in a precursor form due to the underlying variability of *chc1-ts* strains.

α-Factor processing was also examined in *chc1-ts apm2*-Δ strains and found to be comparable to *chc1-ts* strains with wild-type *APM* genes. For example, the *chc1-ts apm2*-Δ strain shown in Figure 8, lane 1 secreted only mature α-factor. This result is consistent with the growth assays, indicating that there is no synthetic interaction between the *chc1-ts* mutation and *apm2*-Δ alone. To determine whether there was an added effect of the combined *apm*-Δ mutations, α-factor secretion was monitored at 25°C in *chc1-ts apm1*-Δ *apm2*-Δ strain SL1035 (Figure 8, lane 3), and compared to its *APM*-transformed counterparts (Figure 8, lanes 4–6). While the *APM1* plasmid significantly improved α-factor processing (lane 4), there was no effect of transformation with an *APM2* plasmid or the parent vector (lanes 5 and 6).

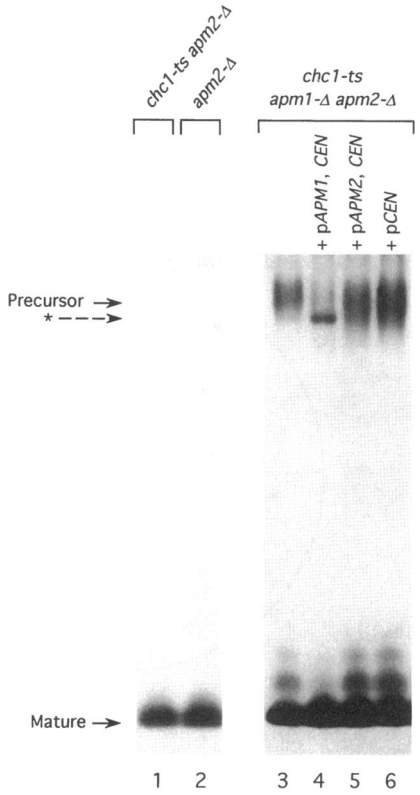

Figure 8. *apm2*-Δ does not affect α-factor processing in *chc1-ts* strains. Cells were grown overnight at room temperature (~22°C) and labeled with Tran^{35}S at 25°C for 30 min. Samples were immunoprecipitated with α-factor antisera and processed as described in Figure 7. Left panel, lane 1, SL1034 (*chc1-ts apm2*-Δ); lane 2, SL1036 (*CHC1 apm2*-Δ). The right panel shows a *chc1-ts apm1*-Δ *apm2*-Δ strain (SL1035) transformed with no plasmid (lane 3); an *APM1, CEN* plasmid pDS6 (lane 4); an *APM2, CEN* plasmid pDS10 (lane 5); or the parent vector pRS314 (lane 6). "Precursor" and "Mature" indicate the highly glycosylated precursor and the fully processed α-factor, respectively. The asterisk denotes a non-specific band that is occasionally found in anti-α-factor immunoprecipitates.

Overall, these results further support the conclusion that *apm1*-Δ, but not *apm2*-Δ, enhances the growth and Golgi sorting defects associated with the *chc1-ts* mutation. Moreover, *APM2* cannot substitute for *APM1* in this late Golgi function.

Processing and Sorting of the Soluble Vacuolar Hydrolase CPY Is Normal in chc1-ts apm1-Δ Strains

Since α-factor processing was affected in *chc1-ts apm1*-Δ strains, we examined sorting to the vacuole, the other major trafficking pathway from the late Golgi. Previous studies have shown that the *chc1-ts* mutation causes a transient defect in processing and sorting of the soluble vacuolar enzyme carboxypeptidase Y (CPY) (Seeger and Payne, 1992a). At early times (5–15 min) after a shift to the non-permissive temperature the Golgi precursor form of CPY (p2 CPY) is secreted from the cell, rather than being matured and sorted to the vacuole. After extended times (3 h) at 37°C cells regain the ability to sort CPY to the vacuole. The basis for this recovery is still not understood.

To examine CPY processing and sorting, pulse/chase experiments were performed on *CHC1 apm*-Δ strains as well as *chc1-ts* strains with various combinations of *apm1*-Δ and *apm2*-Δ. In all cases there was no obvious defect in sorting and processing of CPY at permissive growth temperatures, e.g., 25 and 30°C for *chc1-ts* strains. A typical experiment is shown for *chc1-ts apm1*-Δ (SL1005) and *chc1-ts apm2*-Δ (SL1034) strains shifted to 30°C prior to pulse labeling for 10 min (Figure 9). After the pulse (0 min), CPY was intracellular (I), mostly in p1 (ER, 67 kDa) or p2 (Golgi, 69 kDa) precursor forms with a small amount of mature (m, 61 kDa) CPY present. After the chase (40 min), all CPY was internal and virtually all was mature. In identical experiments shifting to 37°C nearly all CPY was secreted as unprocessed p2 in a *chc1-ts* strain (Seeger and Payne, 1992a). Overall these results indicate that loss of *APM1* and/or *APM2* has no obvious effect on soluble vacuolar hydrolase sorting.

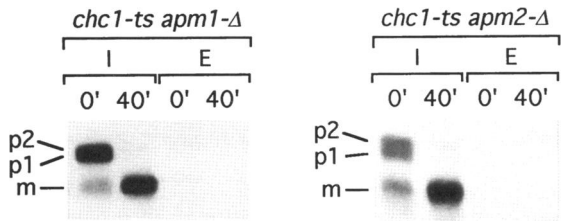

Figure 9. CPY sorting is normal at 30°C in *chc1-ts apm1*-Δ (SL1005) and *chc1-ts apm2*-Δ (SL1034) strains. Cells were grown overnight at room temperature (~22°C), shifted to 30°C for 5 min, pulse labeled with Tran^{35}S for 10 min, and then chased for 40 min. Samples were taken at 0 min and 40 min after initiation of the chase, separated into internal (I) and external (E) fractions and then immunoprecipitated with anti-CPY antibodies as described in MATERIALS AND METHODS. Immunoprecipitates were analyzed on 8% SDS-gels. Note that SL1005 also carried the *CEN* plasmid YCp50.

Disruption of APM1 Enhances the Growth Defect of Cells in the Absence of the Clathrin Heavy Chain

chc1-ts APM strains grow slowly at 37°C, while *chc1-ts apm1*-Δ strains (Table 2, Figure 6) or *chc1*-Δ strains that are viable at 30°C (Lemmon *et al.*, 1991) are dead or nearly dead at 37°. This suggested that the temperature sensitive heavy chain might have some residual activity at the nonpermissive temperature. By this reasoning we would expect that deletion of *APM1* would have no additional effect on growth of cells depleted of clathrin heavy chains. To examine this, tetrads from *apm1*-Δ/*APM1 chc1*-Δ/*CHC1 scd1-v/scd-v* or *apm2*-Δ/*APM2 chc1*-Δ/*CHC1 scd1-v/scd-v* diploids were first analyzed. Normally, *chc1*-Δ/*CHC1 scd1-v/scd1-v* strains with normal *APM* genes give two wild-type Chc$^+$ and two slow growing Chc$^-$ spore clones. In strains with the *APM* disruptions, there was no apparent effect of *apm2*-Δ, but there were an unexpected large number of inviable or very slow growing *chc1*-Δ spore clones in the *apm1*-Δ cross. However, the genotype of many *chc1*-Δ spores from the *apm1*-Δ heterozygote could not be confirmed because both *apm1*-Δ and *chc1*-Δ had *LEU2* as a disruption marker. To circumvent this problem, we made use of strains carrying a repressible *CHC1* gene in which the *GAL1* promoter replaced the normal *CHC1* promoter at the chromosomal heavy chain locus (Nelson and Lemmon, 1993). On galactose medium, *GAL1:CHC1* cells express *CHC1* and grow well. On glucose medium *CHC1* expression is turned off; within 15–20 h Chc1p is depleted and cells acquire the characteristic Chc$^-$ phenotype (Nelson and Lemmon, 1993).

A *GAL1:CHC1/GAL1:CHC1 scd1-v/scd1-v* strain heterozygous for *apm1*-Δ was constructed (SL1540) and segregation analysis was performed, dissecting tetrads on galactose medium to maintain *CHC1* expression. After scoring markers, spore clones were shifted from galactose to glucose medium for 15 h and then streaked on glucose plates. Each tetrad (12 total) had two spore clones that grew slowly and two that showed very slow growth or were inviable after shift to glucose. The poorer growth phenotype cosegregated with *apm1*-Δ. Similar segregational analysis for *apm2*-Δ in a *GAL1:CHC1* diploid (SL1541) showed no accentuated growth defect on glucose in the absence of Apm2p.

The enhanced growth defect of a *GAL1:CHC1 apm1*-Δ strain on glucose and confirmation that the phenotype is due to *apm1*-Δ is shown in Figure 10.

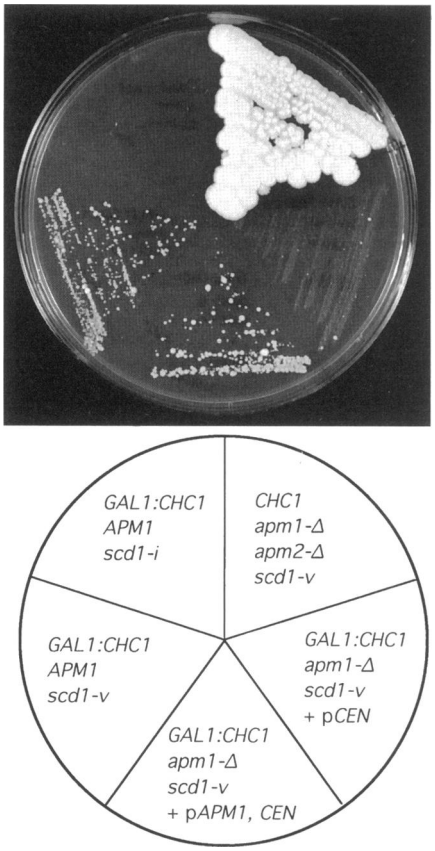

Figure 10. *apm1*-Δ accentuates the slow growth phenotype of cells expressing no clathrin heavy chain. Strains were inoculated from galactose-containing plates into 5 ml glucose containing medium (selective medium for plasmid retention where appropriate) and grown for 15 hours at 30°C to shut down *CHC1* expression and deplete intracellular clathrin. Then cells were streaked onto YEPD (glucose) plates and grown for 6 days at 30°C. Starting at the bottom sector and moving counterclockwise are the following strains: *GAL1:CHC1 apm1*-Δ *scd1-v* + p*APM1, CEN* (SL1426 + pDS6); *GAL1:CHC1 apm1*-Δ *scd1-v* + p*CEN* (SL1426 + pRS314); *CHC1 apm1*-Δ *apm2*-Δ *scd1-v* (SL704); *GAL1:CHC1 APM1 scd1-i* (SL214); *GAL1:CHC1 APM1 scd1-v* (SL350). Note all strains showed comparable growth when restreaked to YEP-galactose after the 15 h in liquid glucose medium.

GAL1:CHC1 apm1-Δ *scd1-v* strain SL1426 was transformed with the *APM1* plasmid pDS6 (p*APM1, CEN*) or the parent plasmid pRS314 (p*CEN*). Cells were then shifted to glucose medium for 15 h and streaked onto glucose plates. SL1426 transformed with the vector alone (p*CEN*) was virtually dead, but showed some residual growth compared to a *GAL1:CHC1 scd1-i* strain (SL214), which is completely inviable on glucose medium. Transformation of SL1426 with the *APM1* plasmid restored growth to a level comparable to that of a *GAL1:CHC1 APM1 scd1-v* strain (SL350), which displays a typical Chc$^-$ growth phenotype on glucose medium. We conclude that *apm1*-Δ enhances the growth defect of cells lacking Chc1p, indicating Apm1p has some function even in the complete absence of clathrin.

Apm1p Fractionates with Clathrin-coated Vesicles

If Apm1p or Apm2p are components of AP-like complexes that mediate clathrin binding to membranes, we would expect that they would be associated with clathrin-coated vesicles. To examine this, coated vesicles were isolated using standard procedures developed for yeast, which involves chromatography of a 100,000×*g* microsomal fraction on Sephacryl S-1000 (Lemmon *et al.*, 1988; Mueller and Branton, 1984; Payne and Schekman, 1985). Column fractions were then analyzed by immunoblotting using antibodies against Chc1p, Apm1p and Apm2p. Figure 11 shows the results for a strain expressing wild type copies of *CHC1*, *APM1*, and *APM2* (SL705). Both Apm1p (56 kDa) and Apm2p (82–84 kDa) coeluted with Chc1p (190 kDa), peaking between fractions 40 and 46; however, our ability to detect Apm2p was inconsistent due to low abundance of the protein in the 100,000×*g* pellet. Control experiments were performed to confirm that the 56 kDa protein in coated vesicle fractions was Apm1p, and not the cross-reacting protein detected by Apm1p antibodies (see MATERIALS AND METHODS). When the 100,000×*g* microsomal fraction was analyzed on the Sephacryl S-1000 column from an *apm1*-Δ strain (SL703), no immunoreactive 56-kDa protein was observed in any column fractions; however, Chc1p eluted in the same region (peak fractions 40–46) as in *CHC1 APM1 APM2* strains. Similar elution of Chc1p was found for an *apm1*-Δ *apm2*-Δ strain (SL704) with no significant decrease in the amount of Chc1p in the coated vesicle peak (our unpublished results). This suggests that other yeast proteins, perhaps additional AP-like complexes, can mediate for-

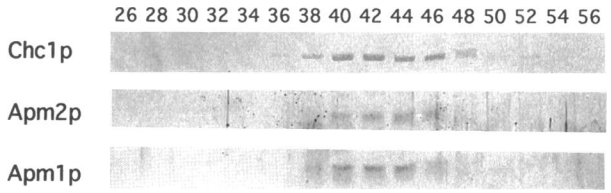

Figure 11. Apm1p and Apm2p cofractionate with the clathrin heavy chain in coated vesicle preparations. Six liters of cells (*APM1 APM2 CHC1* strain SL705) grown to 5 × 10^7 cells/ml in YEP-sucrose were harvested, lysed, and centrifuged as described previously in MATERIALS AND METHODS. The 100,000×*g* pellet was resuspended in 10 ml buffer A, spun 5 min at 12,000×*g* and the supernatant was applied to a 2.5 × 95 cm Sephacryl S-1000 column. Eight-milliliter fractions were collected and 80 μl of fractions indicated were subjected to SDS-PAGE. Gels were transferred to nitrocellulose, after which, the filter was cut into strips and probed with antibodies to Chc1p, Apm2p, or Apm1p. The column void volume was at fraction 23; the total included volume was at approximately fraction 60.

mation of clathrin-coated membrane vesicles. Alternatively, there could be a fairly large pool of clathrin lattices that assemble from the soluble clathrin pool during vesicle purification. We have shown previously that purified yeast clathrin assembles efficiently into clathrin lattices in buffers similar to those used for coated vesicle purification (Lemmon *et al.*, 1988).

If the coelution pattern of clathrin and Apm1p represents coassembly of Apm1p and clathrin followed by budding into a discrete vesicle population, we might expect that the Apm1p elution pattern would be altered in strains lacking the clathrin HC gene. When the coated vesicle purification procedure was performed on a *chc1*-Δ strain (BJ3250), in addition to loss of the Chc1p signal, Apm1p and Apm2p were no longer detectable in immunoblots of column fractions, including those from lower molecular weight regions. This result provides further evidence that Apm1p is packaged into clathrin-coated vesicles in wild type cells. Similar conclusions could not be made for Apm2p, since, even in *CHC1* strains, Apm2p was not reproducibly detectable in coated vesicle fractions.

Apm1p and Apm2p Are Components of Distinct High Molecular Weight Complexes

Mammalian APs are comprised of four subunits that form a noncovalently linked asymmetric heterotetramer with a molecular weight of 250–300 kDa (Ahle *et al.*, 1988; Heuser and Keen, 1988; Keen, 1987; Manfredi and Bazari, 1987; Pearse and Robinson, 1984; Virshup and Bennett, 1988). To determine whether Apm1p or Apm2p are components of high molecular weight complexes, we generated cell extracts for gel filtration chromatography analysis. Wild type cells (SL705) were lysed in a buffer containing 0.5 M Tris-HCl, pH 7.0, which releases clathrin triskelions and AP complexes from membranes (Keen *et al.*, 1979). The soluble fraction (100,000×*g* supernatant) was loaded onto a Superose-12 column and fractions were monitored for absorption at 280 nm (Figure 12A) and for the presence of Apm1p, Apm2p and Chc1p by immunoblot analysis (Figure 12B). Chc1p eluted just after the void volume and before thyroglobulin (669 kDa, 85 Å). This is typical of triskelions which have an extended arm structure. Apm1p antiserum detected 56-kDa proteins in two regions of the elution profile. One 56-kDa protein eluted just before ferritin (440 kDa, 61 Å) with a Stokes radius of 63–65 Å (peak fractions 18–20). This size is very similar to that previously reported for mammalian AP-2 (Pearse and Robinson, 1984; Virshup and Bennett, 1988). The second 56-kDa protein eluted after bovine albumin (67 kDa) with a Stokes radius more similar to a globular protein of 56–60 kDa (34 Å) (peak fractions 28–30). When a cell lysate from an *apm1*-Δ strain

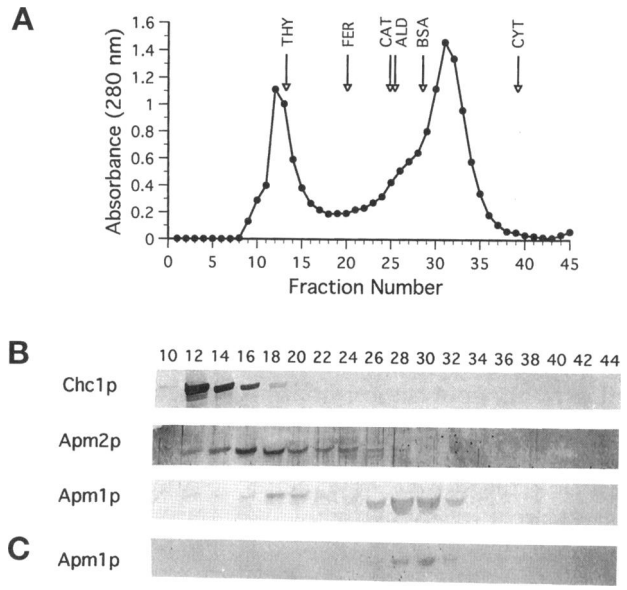

Figure 12. Apm1p and Apm2p are components of high molecular weight complexes. Cells were lysed in clathrin extraction buffer and 200 μl of the 100,000×*g* supernatant was applied to a 1 cm × 30-cm Superose-12 FPLC column as described in MATERIALS AND METHODS. Fractions of 0.25 ml were collected starting 10 min after sample injection. Protein was monitored by 280-nm absorbance and 80 μl of indicated fractions were applied to 7.5% SDS gels. After SDS-PAGE, proteins were transferred to nitrocellulose and the filter was cut into strips for probing with Chc1p, Apm2p, and Apm1p antibodies. (A) 280-nm absorbance profile from analysis of *APM1 APM2 CHC1* strain SL705. Column standards (see MATERIALS AND METHODS) are indicated as follows: THY, thyroglobulin; FER, ferritin; CAT, catalase; ALD, aldolase; BSA, bovine serum albumin; CYT, cytochrome C. (B) Immunoblot analysis of column fractions from the SL705 elution profile shown in A. (C) Immunoblot of column fractions from Superose-12 analysis of an *apm1*-Δ *APM2 CHC1* strain (SL703) probed with Apm1p antisera. The 280-nm absorbance profile for SL703 was very similar to that of SL705 (A).

(SL703) was fractionated, the 56-kDa band in the high molecular weight 63–65 Å fractions was absent, but the 56-kDa band eluting later from the column was still present (Figure 12C). This indicates Apm1p is contained in the high molecular weight complex (63–65 Å), and the crossreacting 56-kDa band elutes as a monomer of ~56 kDa, although we cannot rule out that there was also some monomeric Apm1p. Apm2p eluted earlier than Apm1p from the Superose-12 column, in a high molecular weight complex with an estimated Stokes radius of 70 Å (peak fractions 16–18). In addition, the elution patterns of Apm2p and Chc1p isolated from the *apm1*-Δ strain were identical to those shown for the *APM1* strain shown in Figure 12B. Therefore, the complex containing Apm2p is larger than that containing Apm1p, and these complexes are biochemically distinguishable.

DISCUSSION

Two genes encoding proteins related to the medium chains of animal clathrin AP complexes have been identified in yeast. In order to determine whether either of these proteins is involved in clathrin-mediated transport processes, we generated gene disruptions of *APM1* and *APM2*. Our hypothesis was that loss of Apm1p and/or Apm2p would result in phenotypes, or a subset of phenotypes (since AP-1 and AP-2 have different locations in the cell), similar to those that occur when the clathrin heavy chain is not functional. Surprisingly, we saw no effect on overall cell growth, sporulation, mating, α-factor processing or sorting to the vacuole when *APM1* and *APM2* were deleted singly or in combination in cells with normal clathrin. This indicates that neither of these gene products are required when wild type clathrin is present. However, when *apm1*-Δ was combined with a *chc1-ts* mutation, a more severe temperature sensitive phenotype and enhanced α-factor processing defect were uncovered. Since, the alpha-factor maturation defect in *chc1* cells results from mislocalization of processing enzymes, such as Kex2p or DPAP-A, from the Golgi to the cell surface (Payne and Schekman, 1989; Seeger and Payne, 1992b), our results suggest that Apm1p has a sorting or retention function at the late Golgi.

Recently, Payne and coworkers reported their analysis of deletion mutants of two AP small chain-related proteins encoded by *APS1* and *APS2* (Phan *et al.*, 1994). Aps1p resembles the mammalian Golgi AP-1 small chain, σ1 (Nakai *et al.*, 1993; Phan *et al.*, 1994), whereas Aps2p is more similar to the plasma membrane AP-2 small chain, σ2 (Kirchhausen *et al.*, 1991). Similar to our results, no phenotypic consequences for single or double deletions of *APS* genes were found in the presence of normal clathrin (Phan *et al.*, 1994; Nakai *et al.*, 1993). How can these results be explained? It is possible that AP subunits in yeast are encoded by redundant gene families whose products have partial or completely overlapping functions. We note that there are at least two μ1 genes in *C. elegans* (Lee *et al.*, 1994; T. Kirchhausen, unpublished observation) and two mammalian α-chain genes (Robinson, 1989), which encode proteins whose primary structures are highly related within each type of chain. However, it is possible that these isoforms may not be completely interchangeable due to tissue- or development-specific expression. Nevertheless, in a situation where an AP subunit is missing, the homologous subunit from another AP might be able to substitute and reconstitute function.

Alternatively, the lack of an obvious phenotype for disruptions of the *APM* or *APS* genes in the presence of wild type clathrin could be an indication that clathrin can function independently of AP complexes. However, this seems unlikely since there is ample evidence from in vitro studies that APs and clathrin interact and APs are required for clathrin binding to membranes. In addition, our studies showing enhanced defects in growth and Golgi sorting when *apm1*-Δ was combined with the *chc1-ts* mutation, and similar findings for *aps1*-Δ (Phan *et al.*, 1994), are evidence that clathrin's function, even in yeast, is dependent on AP function.

A final interpretation for the lack of phenotype of *APM* and *APS* deletions in *CHC1* strains is that AP complexes may not normally require medium or small chains for their function. However, when clathrin is impaired, such as in the *chc1-ts* mutant, medium and small chains become crucial. It is worth noting that some AP functions can be reconstituted in vitro with reassembled α and β chains that lack the medium and small subunits (Prasad and Keen, 1991). Also, β-adaptin alone can bind clathrin (Ahle and Ungewickell, 1989) and mediate coat assembly (Gallusser and Kirchhausen, 1993), and it has been reported that isolated α-adaptin is capable of interacting with membrane binding sites (Chang *et al.*, 1993). However, it is not known whether the large subunits alone are capable of the full range of AP activities in cells.

Recent studies in *C. elegans* support the requirement of AP medium chains for AP function. Null mutations in the *unc-101* gene, which encodes a homolog (87% identical) of the mammalian μ1 chain, are pleiotropic and cause major defects in behavior and development (Lee *et al.*, 1994). It is presumed that residual μ1 function in *unc-101* mutants is provided by a second μ1 gene that has been identified (Lee *et al.*, 1994; T. Kirchhausen, unpublished observation). Although it has not been directly shown that the *C. elegans* μ1 chain functions at the trans-Golgi, a chimeric protein in which the C-terminal 278 amino acids of the *C. elegans unc-101* product were replaced with the mouse AP-1 μ1 sequence was able to rescue the *unc-101* phenotype (Lee *et al.*, 1994). This suggests that the *unc-101* gene product is probably in a Golgi-associated AP-1-like complex and is important for AP function in *C. elegans*.

If Apm1p and Aps1p are components of a Golgi-localized AP-1-like adaptor complex, it is possible that they are components of the same complex. The sequence homology to their animal AP-1 counterparts, as well as the similarity of the phenotypes of *APS1* and *APM1* disruptions support this. Both *aps1*-Δ and *apm1*-Δ cause an enhanced growth defect and Golgi specific synthetic phenotype in the *chc1-ts* mutant, while neither *aps1*-Δ nor *apm1*-Δ have an effect on sorting to the vacuole. The only major phenotypic difference between *aps1*-Δ and *apm2*-Δ we have noted thus far is that the α-factor processing defect of the *chc1-ts* mutation in combination with *apm1*-Δ is more severe than that observed with *aps1*-Δ. For example, at 25°C we typically observed that 30–40% of α-factor was secreted as precursor in *apm1*-Δ *chc1-ts* strains,

while under similar conditions *aps1-Δ chc1-ts* strains secreted at most 10% precursor (Phan *et al.*, 1994). This could indicate that the μ chains are more crucial for AP function than the σ chains.

Further biochemical evidence will be required to determine whether Aps1p and Apm1p are components of the same complex, and whether this is a clathrin AP. However, we note that both Aps1p and Apm1p fractionated on gel sizing columns in high molecular weight species similar in size to native APs. In addition, both Aps1p and Apm1p coeluted with clathrin-coated vesicles on Sephacryl S-1000 chromatography, and this association with a discrete vesicle population was disrupted in cells lacking clathrin (Phan *et al.*, 1994; our studies). In the *APS1* studies, Aps1p was distributed throughout the S-1000 column when *chc1-Δ* strains were analyzed, while we observed no Apm1 polypeptide in column fractions. This disparity could indicate that Aps1p and Apm1p are not in the same protein complex; however, subtle variations in the fractionation procedure or lower sensitivity of Apm1p antisera could also account for this difference.

How, then, do we explain the result that the slow growth phenotype of cells expressing no clathrin (*GAL1: CHC1* grown on glucose or *chc1-Δ*) is more severe when *APM1* (our results) or *APS1* (Phan *et al.*, 1994) are deleted? We propose two models. First, it is possible that the Apm and Aps proteins have functions that are independent of clathrin, such as in another vesicular transport pathway. Since there was no phenotype for deletions of *APM1* and *APS1* in the presence of wild-type clathrin, in this model one would have to conclude that the Apm1p and Aps1p pathways are not limiting and only become essential when clathrin-mediated transport is reduced or eliminated.

In the second model, we propose that the AP-1-like complex containing Aps1p and Apm1p retains some of its membrane protein sorting or clustering capability, even in the absence of clathrin. Thus some directed routing of membrane proteins could take place in the presence of this AP, such as the pathway that DPAP-A and Kex2p take to the cell surface. This would be consistent with studies showing that the default pathway for cytoplasmic tail deletion mutants of DPAP-A and Kex2p is to the vacuole rather than the cell surface in *CHC1* strains (Roberts *et al.*, 1992; Wilcox *et al.*, 1992). In cells lacking both AP-1 and clathrin, further loss of trans-Golgi protein retention and random sorting would occur. This might lead to transfer of trans-Golgi proteins to a compartment where their presence is not tolerated, to loss of crucial proteins from the Golgi, or to more severe perturbations of membrane balances than seen when only clathrin is absent. In this model, Apm1p and Aps1p can function independently of clathrin, but strictly speaking, they are still performing a role in the clathrin-mediated transport pathway.

Function of Apm2p

In contrast to *apm1-Δ*, there was no synthetic effect on growth, α-factor processing, or CPY sorting when *apm2-Δ* was combined with the *chc1-ts* mutation. A number of alternatives to explain the lack of a phenotype for *apm2-Δ* mutants have been suggested above; however, we propose that the function of Apm2p is clathrin independent. This idea is appealing because Apm2p is a unique AP-medium chain-related protein. The predicted molecular weight (70 kDa) is much larger than that of any of the previously identified members of the gene family, which are 47–50 kDa. Even excluding major insertions, the identity of Apm2p to the other AP medium chains is only ~30%, and there is no preferential homology to the μ1 or μ2 classes identified in mammals, yeast or *C. elegans*. In addition, a third class of AP medium chain-related proteins (~47 kDa) that shows only ~25–30% identity to μ1, μ2, Apm1p, and Apm2p was recently identified (Pevsner *et al.*, 1994). These findings suggest that the AP medium chains are part of a larger, more diverse protein family.

Apm2p could be a component of another type of coat protein complex involved in vesicular budding, since a number of recent studies have suggested that the budding mechanisms for formation of different types of coated vesicles may be similar. The first well-characterized nonclathrin-coat protein complex was the coatamer (COP1), which has been shown to mediate intra Golgi vesicular transport and transport from the ER to the Golgi (for review see Rothman and Orci [1992]). Both coatamer and AP-1 binding to Golgi membranes requires ADP-ribosylation factor (Stamnes and Rothman, 1993; Traub *et al.*, 1993) and is inhibited by brefeldin A (Robinson and Kreis, 1992; Wong and Brodsky, 1992). In addition, the subunit composition of the coatamer complex resembles that of the combined subunits of the clathrin heavy and light chains and the AP-complex (Waters *et al.*, 1991). At least two of the mammalian coatamer subunits have weak homology to clathrin AP subunits; β-COP (110 kDa) and ζ-COP (20 kDa) are related to the mammalian β adaptins and to the AP σ-chains, respectively (Duden *et al.*, 1991; Kuge *et al.*, 1993; Serafini *et al.*, 1991). However, it is unlikely that Apm2p is a component of the yeast coatamer isolated by Hosobuchi *et al.*, (1992), even though the predicted molecular weight of Apm2p (70 kDa) is close to that of the yeast δ-COP subunit, which migrates as a 73-kDa protein on SDS gels (Hosobuchi *et al.*, 1992). Since Apm2p migrates as an 82–84 kDa protein on gels and the N-terminal sequence of yeast δ-COP does not correspond to any sequence in Apm2p (M. Hosobuchi and R. Schekman, personal communication), it is clear these two proteins are distinct. A second COP complex (COP2) that drives vesicle budding from the ER in yeast was recently characterized (Barlowe *et al.*, 1994). This coat structure

contains the Sec13p and Sec23p coat protein complexes; however, none of the component polypeptide sequences correspond to Apm2p. Nonetheless, we have shown that Apm2p is a component of a high molecular weight complex with a Stokes radius of ~70 Å and it is partially associated with a discrete small vesicle fraction in cells. This is consistent with Apm2p being a component of a vesicular transport coat protein complex. Further studies to elucidate the role of Apm2p are in progress and should provide important information concerning the general function of the larger family of proteins related to the clathrin AP medium chains.

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