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Adaptor Complex-independent Clathrin Function in Yeast

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Clathrin-associated adaptor protein (AP) complexes are major structural components of clathrin-coated vesicles, functioning in clathrin coat assembly and cargo selection. We have carried out a systematic biochemical and genetic characterization of AP complexes in *Saccharomyces cerevisiae*. Using coimmunoprecipitation, the subunit composition of two complexes, AP-1 and AP-2R, has been defined. These results allow assignment of the 13 potential AP subunits encoded in the yeast genome to three AP complexes. As assessed by in vitro binding assays and coimmunoprecipitation, only AP-1 interacts with clathrin. Individual or combined disruption of AP-1 subunit genes in cells expressing a temperature-sensitive clathrin heavy chain results in accentuated growth and α-factor pheromone maturation defects, providing further evidence that AP-1 is a clathrin adaptor complex. However, in cells expressing wild-type clathrin, the same AP subunit deletions have no effect on growth or α-factor maturation. Furthermore, gel filtration chromatography revealed normal elution patterns of clathrin-coated vesicles in cells lacking AP-1. Similarly, combined deletion of genes encoding the β subunits of the three AP complexes did not produce defects in clathrin-dependent sorting in the endocytic and vacuolar pathways or alterations in gel filtration profiles of clathrin-coated vesicles. We conclude that AP complexes are dispensable for clathrin function in *S. cerevisiae* under normal conditions. Our results suggest that alternative factors assume key roles in stimulating clathrin coat assembly and cargo selection during clathrin-mediated vesicle formation in yeast.

INTRODUCTION

Selective protein transport between membrane organelles is mediated by transport vesicles. Formation of such vesicles depends on recruitment of evolutionarily conserved multimeric protein complexes to the cytoplasmic aspect of organelle membranes (Schekman and Orci, 1996). Recruited complexes assemble into membrane-associated coats that propel membrane invagination and orchestrate cargo selection, leading to generation of coated transport vesicles. A major class of coated transport vesicles is distinguished by clathrin coats. Clathrin coats at the plasma membrane and *trans* Golgi network (TGN) give rise to endosome-targeted vesicles, and clathrin may also participate in vesicle formation at endosomes (Schmid, 1997).

The major structural components of clathrin coats are two protein complexes, clathrin and clathrin adaptor proteins (APs) (Schmid, 1997; Hirst and Robinson, 1998). Clathrin is a tripod-shaped molecule, with each leg composed of a heavy chain and an associated light chain (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981; Pishvaee and Payne, 1998; Musacchio *et al.*, 1999). Clathrin assembles into a polyhedral lattice that forms the outer shell of the coat (Vigers *et al.*, 1986a,b; Smith *et al.*, 1998). The heterotetrameric APs bridge the clathrin lattice to the membrane. Purification of mammalian clathrin-coated vesicles revealed two related AP complexes, AP-1 and AP-2 (Pearse and Robinson, 1984; Keen, 1987). AP-1 localizes to the TGN and endosomes, whereas AP-2 localizes to the plasma membrane (Robinson, 1987; Ahle *et al.*, 1988). Each complex contains two large subunits (~100 kDa; γ and β1 in AP-1, α and β2 in AP-2), one medium subunit (~50 kDa; μ1 in AP-1, μ2 in AP-2) and one small subunit (~20 kDa; σ1 in AP-1, σ2 in AP-2) (Schmid, 1997; Hirst and Robinson, 1998). The highly similar β subunits bind to clathrin and promote clathrin coat assembly (Gallusser and Kirchhausen, 1993). The μ and β subunits interact with sorting signals in the cytoplasmic domains of transmembrane proteins, thereby collecting appropriate vesicle cargo (Ohno *et al.*, 1995; Rapaport *et al.*, 1998). The AP-2 α subunit, and by analogy the AP-1 γ subunit, appear to be important in recruiting additional factors necessary for clathrin-coated vesicle formation (Ben-

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Table 1. Yeast AP subunits

AP complex	Gene	Subunit	Most similar mammalian subunit[a]	
AP-1	*APL2*	β1	β1/2[b]	(38/61)[c]
	APL4	γ	γ	(32/50)
	APM1	μ1	μ1	(49/66)
	APS1	σ1	σ1	(53/73)
	(*APM2*)	?	?	
AP-2R	*APL1*	β2R	β1/2	(32/56)
	APL3	αR	α	(25/43)
	APM4	μ2R	μ2	(33/53)
	APS2	σ2R	σ2	(49/70)
AP-3	*APL6*	β3	β3	(28/48)
	APL5	δ	δ	(29/52)
	APM3	μ3	μ3	(33/41)
	APS3	σ3	σ3	(46/64)

[a] Cowles *et al.* (1997); Panek *et al.* (1997).

[b] β1 and β2 are too similar to distinguish in sequence comparisons to the yeast proteins. Sequence similarity refers to comparison to human β2.

[c] Percentage of identical/similar amino acids in comparison of yeast sequences with human sequences. GenBank accession numbers: β2, 179333; γ, 2765190; μ1B, 4885427; σ1, 3641678; α, 4240287 (designated an α subunit based on sequence comparisons (Payne, unpublished data); μ2, 1665725; σ2, 1296607; β3A, 2199512; δ, 2290770; μ3A, 4426603; σ3A, 1923270. In all cases but Apm3p sequence similarity extends over at least 80% of the yeast protein. The similarity between Apm3p and μ3A extends over the C-terminal 60% of the protein, beginning at residue 199 in Apm3p.

merah *et al.*, 1995; Wang *et al.*, 1995; David *et al.*, 1996; Wigge *et al.*, 1997a,b; Chen *et al.*, 1998; Owen *et al.*, 1999). Through these combined activities, AP complexes are thought to play a central role in clathrin-coated vesicle formation by coupling coat assembly and cargo collection.

A more widespread role for AP complexes in protein sorting is evident from recent discoveries of mammalian heterotetrameric complexes related to AP-1 and AP-2. AP-3, which is associated with endosomes and/or the TGN, plays a role in membrane protein sorting to lysosomes and synaptic vesicle formation (Le Borgne and Hoflack, 1998; Odorizzi *et al.*, 1998). Whether AP-3 acts with clathrin has not been resolved (Simpson *et al.*, 1996, 1997; Dell'Angelica *et al.*, 1998). Initial characterization of AP-4 indicates that this complex localizes to the vicinity of the TGN but does not appear to interact with clathrin (Dell'Angelica *et al.*, 1999a). The function of AP-4 has not been addressed.

The complete genome sequence of *Saccharomyces cerevisiae* allows a systematic approach to investigate AP function. Database searches using mammalian AP subunit sequences indicate that the *S. cerevisiae* genome has the potential to encode three AP β subunits, three non-β large subunits, four μ subunits, and three σ subunits (Table 1) (Cowles *et al.*, 1997a; Panek *et al.*, 1997). Based on the degree of primary sequence similarity between each yeast protein and the different mammalian AP subunits, the yeast proteins can be grouped into three potential AP complexes, leaving Apm2p unassigned (Cowles *et al.*, 1997a; Panek *et al.*, 1997). Biochemical analyses and phenotypic characterization of strains carrying gene disruptions defined a yeast AP-3 complex involved in clathrin-independent traffic from the Golgi apparatus to vacuoles (Table 1) (Cowles *et al.*, 1997a; Panek *et al.*, 1997; Stepp *et al.*, 1997; Vowels and Payne, 1998a). Surprisingly, deletion of several other AP subunit genes yielded no detectable phenotypes, even though the subunits exhibit substantial evolutionary conservation with their mammalian counterparts (up to 50% identity) (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). However, disruption of *APS1*, *APM1*, or *APL2* specifically enhanced growth and Golgi-related protein sorting defects in cells carrying a temperature-sensitive allele of the clathrin heavy chain gene (*chc1-ts*). These results offer genetic evidence for an AP-1-like complex, consisting of Aps1p, Apm1p, and Apl2p, that is involved in clathrin-dependent function at the Golgi apparatus. The fractionation properties of selected AP proteins, including the putative AP-1 subunits, is consistent with organization into multimeric complexes, but the composition of such complexes has not been addressed except for AP-3 (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995).

Here we present a more comprehensive biochemical and genetic characterization of yeast AP complexes. Our results assign subunits to one of three distinct complexes, defined by the three β subunits. Only the AP-1 complex physically and genetically interacts with clathrin. Surprisingly, in cells expressing wild-type clathrin, combined deletion of genes encoding all four AP-1 subunits, or deletion of the three β-encoding genes, does not affect clathrin-dependent trafficking processes or reduce the level of clathrin-coated vesicles. These findings suggest that clathrin function in yeast does not depend on AP complexes.

MATERIALS AND METHODS

Materials

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

Plasmids and Nucleic Acid Techniques

Plasmid constructions were carried out using standard molecular biology techniques (Sambrook *et al.*, 1989). pBKS-URA3 contains a 1.1-kb *Hin*dIII fragment of *URA3* (Rose *et al.*, 1984) inserted as a blunt-ended fragment into the *Sma*I site of pBluescript KS+ (pBKS+; Stratagene, La Jolla, CA). pBKS-TRP1 contains a 1.0-kb *Ssp*I–*Stu*I fragment of *TRP1* (Tschumper and Carbon, 1980) inserted as a blunt-ended fragment into the *Sma*I site of pBKS+. YEp352-APL2 contains a 3.8-kb *Eco*RI–*Sna*BI fragment of *APL2* (Rad *et al.*, 1995) in YEp352 (Hill *et al.*, 1986). PCR amplifications were carried out with either Deep Vent (New England Biolabs, Beverly, MA) or Elongase (Life Technologies, Rockville, MD). Primers are listed in Table 2. All PCR products were sequenced to confirm accurate amplification.

HA-tagged Constructs

HA-Apl1p. The 5′ region of *APL1* (bp 1–347; bp 1 corresponds to the A in the initiating ATG) was amplified by PCR from pAPL1–100 (Rad *et al.*, 1995) using a 5′ primer (primer 5) homologous to *APL1* bp 1–16 and containing an *Nco*I site and a 3′ primer (primer 6) homologous to *APL1* bp 349–363 with an *Xho*I site. The resulting product was subcloned into the *Nco*I and *Xho*I sites in pGEX-KG. A 2.5-kb *Bgl*II–*Hin*dIII fragment containing the remaining 3′ coding region and downstream sequences of *APL1* was transferred from pAPL1-100 to create pGEX-KG-APL1. *HA-APL1* was generated by

Table 2. Primer list

Primer number	Primer sequence
1	5′-GCTAGTCTAGACATGCCACCATTGGATAAAAG-3′
2	5′-GCCGTCGACCACTGCGGTTTTTCTAAC-3′
3	5′-GTCGGGATCCATGGGGTACCCATACGATGTTCC-3′
4	5′-GGGCTCGAGTCTAGACCTGCATAGTCCGGGACGTCATA-3′
5	5′-GGGCGGCCGCAGCTGAAGAATTCTTTTCACCAAG-3′
6	5′-CGGGGATCCAGTTGAAACTGTTTTTTAAGT-3′
7	5′-CCGGCATGCCATGGGTATGTCCGATCAAAAAG-3′
8	5′-CCCGCTCGAGCGGGACTAATACCAG-3′
9	5′-GGGGAATTCCCATGGCTGCATAGTCCGGGAC-3′
10	5′-GGGCGGCCGCAAGCTAACTATTCTCAATTAAA-3′
11	5′-CGGGGATCCAAGCGAAATACGTCCTATTAATG-3′
12	5′-CCCGCTCGAGCGGAATTCGAATCAGAC-3′
13	5′-CCGGCATGCCATGGGTATGGTAGATTCAATTCACC-3′
14	5′-GGGAAGCTTCTCGAGTACAATGCTTGGATC-3′
15	5′-GGGGGATCCTGCTGGTTGTCCAC-3′
16	5′-GGCGGATCCCAATACGAGATATTTTTC-3′
17	5′-CGGGATATCCATAGAGTTTCATCGAA-3′
18	5′-GGCGGATCCGTTGATGGCACCATAGATATCACC-3′
19	5′-CGGCAGCTGCAGTACCTCACCTCGTAAGAACCGGC-3′
20	5′-GGGTAATAGATGGTTCTATTC-3′
21	5′-TGCCCTTTCATCTGCCAG-3′
22	5′-CGGGGATCCAAAGATGAAGATATTTCAATG-3′
23	5′-GCGGCAGCTGGTCAGCCTTATTGTATAATC-3′
24	5′-GGAGGCGTTTGTGTACAAAC-3′
25	5′-CTTCCGCAGAGAGTATACAG-3′
26	5′-GGCGGATCCACAACAGCCATACGTG-3′
27	5′-CGCGATATCCGCTTCGTAAGCACATT-3′
28	5′-CCCGGAATTCCGGGTCCTGAGACAAGATGTCAG-3′
29	5′-CGGCCATGGGATCTTGCTGAGTTGTTGG-3′
30	5′-CCCGCCATGGGTGACCTCTTGCCAGTT-3′
31	5′-GGCCGAGCTCGAGTATATTTAAATACAGG-3′
32	5′-CATGCCATGGGTGTCTGGTTGCTAGGACAG-3′
33	5′-CGGCCTCGAGGCACATCATCGTTAGCCT-3′
34	5′-GGGCATATGATGCACCACCACCACCACCACGATCACGAAAATAATCC-3′
35	5′-CGCGGATCCAAGCTTGAGGGTTTCCGTGAT-3′

amplifying a tandem repeat of the hemagglutinin (HA) epitope from pGDA-4HA (a gift from Jennifer Vowels, University of California, Los Angeles, CA) with a 5′ primer (primer 3) that contains a *Bam*HI site and a 3′ primer (primer 7) carrying *Eco*RI and *Nco*I sites. The tandem tag fragment was subcloned into the *Bam*HI and *Eco*RI sites of pBKS+. The tandem HA fragment was then transferred to the *Bam*HI and *Nco*I sites in pGEX-KG-APL1, creating pGEX-KG-HA-APL1. *HA-APL1* was excised as a 2.5-kb *Bam*HI–*Hin*dIII fragment and introduced into pRS305 (Sikorski and Hieter, 1989) to create pRS305-HA-APL1. A 360-bp region upstream of the *APL1* ATG was amplified from YAP100-1 with primer 8, containing a *Not*I site and (primer 9) with a *Bam*HI site. This fragment was subcloned into pRS305-HA-APL1 with *Not*I and *Bam*HI to create pRS305-ProHA-APL1.

HA-Apl2p. The 5′ region of *APL2*, (bp 1–464; same numbering as *APL1*) was amplified by PCR from YEp352-APL2 using a 5′ primer (primer 1) homologous to *APL2* bp 1–20 and containing a 5′ *Xba*I recognition site and a 3′ primer (primer 2) homologous to *APL2* bp 445–465 with a 5′ *Sal*I site. The resulting product was subcloned into the *Xba*I and *Sal*I sites in pEG-KG (Mitchell *et al.*, 1993). A 2.9-kb *Nde*I–*Sal*I fragment containing the remaining 3′ coding region and downstream sequences of *APL2* was transferred from YEp352-APL2 to create pEG-KG-APL2. A 3.3-kb *Xba*I fragment from pEG-KG-APL2 containing full-length *APL2* was subcloned into pGEX-KG (Guan and Dixon, 1991). *HA-APL2* was generated by amplifying a tandem repeat of the HA epitope from pGDA-4HA with a 5′ primer (primer 3) carrying a *Bam*HI site and a 3′ primer (primer 4) with *Xho*I and *Xba*I sites. The tandem tag fragment was subcloned into the *Bam*HI and *Xho*I sites of pBKS+. The HA fragment was then transferred to the *Bam*HI and *Xba*I sites in pGEX-KG-APL2, creating pGEX-KG-HA-APL2. *HA-APL2* was excised as a 3.3-kb *Bam*HI–*Xho*I fragment and introduced into pRS305 to create pRS305-HA-APL2. A 428-bp region upstream of the *APL2* ATG was amplified from YEp352-APL2 with primer 5 containing a *Not*I site and primer 6 containing a *Bam*HI site. This fragment was subcloned into pRS305-HA-APL2 with *Not*I and *Bam*HI to create pRS305-ProHA-APL2.

Apl4p-HA. The 3′ region of *APL4*, bp 1825–2496, was amplified with primer 14 (*Bam*HI site) and primer 15 (*Eco*RV site). The resulting fragment was subcloned into pBS14, which carries the HA epitope coding sequence (a gift from T. Kirchhausen, Harvard University Medical School, Boston, MA), creating pAPL4-HA. A *Bam*HI–*Hin*dIII (filled in) fragment from pAPL4-HA was subcloned into the *Bam*HI and *Sac*II (filled in) sites of pBKS-URA3, creating pAPL4-HA-URA3. pAPL4-HA-URA3 was cleaved with *Mun*I to integrate *APL4-HA* into the chromosome copy of *APL4*.

HA-Apl6p. The 5′ region of APL6 (bp 1–396; same numbering as *APL1*) was amplified by PCR from *YKS5* (Panek *et al.*, 1997) using a 5′ primer (primer 10) homologous to *APL6* bp 1–19 and containing a *Nco*I site and a 3′ primer (primer 11) homologous to *APL6* bp

381–396 and containing a *Xho*I site. The resulting product was subcloned into the *Nco*I and *Xho*I sites of pGEX-KG. A 3.1-b *Sac*II–*Sac*I *YKS5* fragment was then introduced, creating pGEK-KG-APL6. *HA-APL6* was generated by transferring the tandem HA tag from pBKS+ (see above) to the *Bam*HI and *Nco*I sites in pGEX-KG-APL6, creating pGEX-KG-HA-APL6. *HA-APL6* was excised as a *Bam*HI–*Hin*dIII fragment and introduced into pRS315 (Sikorski and Hieter, 1989) to create pRS315-HA-APL6. A 360-bp region upstream of the *APL6* ATG (bp −360 to 1) was amplified from *YKS5* with a 5′ primer (primer 12) containing an *Xho*I site and a 3′ primer (primer 13) containing a *Bam*HI site. This fragment was subcloned into pRS315-HA-APL6 with *Xho*I and *Bam*HI to create pRS315-ProHA-APL6.

Apm1p-HA. A *Bam*HI–*Sma*I fragment from pBKS-URA3 was subcloned into *Bam*HI–*Eco*RV sites of pAPM1 (described below), creating pAPM1-HA-URA3. pAPM1-HA-URA3 was cleaved with *Eco*RI to integrate the *APM1*-HA into the chromosome copy of *APM1*.

Apm4p-HA. A 3′ fragment of *APM4*, beginning 775 bp region upstream of the stop codon was amplified with primer 16 homologous to bp 701–720 and containing a *Bam*HI site and primer 17 homologous to bp 1476–1542 that was designed to lack the endogenous stop codon and contain a *Pvu*II site. The PCR product was cleaved with *Bam*HI and *Pvu*II and subcloned into *Bam*HI–*Eco*RV site of pBS14, creating pAPM4-HA. To generate pAPM4-HA-URA3, a *Bam*HI–*Hin*dIII (filled in) fragment was cloned into pBKS-URA3 cut with *Sac*II (filled in) and *Bam*HI. pAPM4-HA::URA3 was cut with *Mun*I to integrate the APM4-HA into the chromosome copy of *APM4*.

Deletion Constructs

apm1Δ. *APM1* was amplified from genomic DNA with primer 20 homologous to 288 bp upstream of *APM1* and containing a *Bam*HI site and primer 21 homologous to *APM1* bp 1405–1422 and containing a *Pvu*II site. The product was cut with *Bam*HI–*Pvu*II and cloned into *Bam*HI and *Eco*RV sites of pBS14, creating pAPM1. The *URA3* gene was transferred from pBKS-URA plasmid as a *Bam*HI (filled in) and *Eco*RI (filled in) fragment into *Pst*I (filled in) and *Eco*RV (filled in) sites of pAPM1, creating papm1::URA3.

apl3Δ. The 5′ region of *APL3*, 693 bp upstream of the ATG to 197 bp upstream of the ATG, was amplified with primers 22 and 23. The resulting fragment was cut with *Kpn*I and *Cla*I to release a 322-bp fragment that was subcloned into pBKS-URA3, creating pBKS-URA3–5′APL3. The 3′ region of *APL3*, bp 2367–3075, was amplified with primer 20 (*Bam*HI site) and primer 21 (*Eco*RV site). The amplified fragment was subcloned into the *Bam*HI and *Eco*RV sites of pBKS-URA3–5′APL3 creating papl3::TRP1.

apl4Δ. The 5′ region of *APL4*, from −557 to + 69 bp with bp 1 corresponding to the A in the initiating ATG, was amplified with primers 18 and 19. The product was digested with *Hin*dIII (filled in) and subcloned into pBKS-TRP1 at the *Eco*RI site (filled in), creating pBKS-TRP-5′APL4. A 3′ *APL4 Bam*HI–*Hin*dIII (filled in) fragment from pAPL4-HA-URA3 was subcloned into *Bam*HI and *Sac*II (filled in) sites of pBKS-TRP-5′APL4, creating papl4::TRP.

GST Fusions

GST-Apl1p. The C-terminal portion of *APL1*, bp 1858–2060, was amplified from YAP100-1 with primer 26 (*Nco*I site) and primer 27 (*Sac*I site). The resulting product was cloned into pGEX-KG, creating pGEX-KG-APL1 C-term (Apl1p amino acids 620–701).

GST-Apl2p. The C-terminal portion of *APL2*, bp 1411–1910, was amplified from YEp352-APL2 with primer 28 (*Nco*I site) and primer 29 (*Xho*I site). The resulting fragment was subcloned into pGEX-KG. A 889-bp *Hin*dIII fragment containing the remaining 3′ coding region and downstream sequences of *APL2* was transferred from YEp352-APL2, creating pGEX-KG APL2 C-term (Apl2p amino acids 471–727).

GST-Apl6p. The C-terminal portion of *APL6*, bp 1621–2120, was amplified from *YKS5* with primer 24 (*Eco*RI site) and primer 25 (*Nco*I site). The resulting product was cloned into pGEX-KG. A 1.54-kb *Nde*I–*Sac*I fragment containing the remaining 3′ coding region and downstream sequences of *APL6* was transferred from *YKS5*, creating pGEX-KG-APL6 C-term (Apl6p amino acids 541–810).

Strains, Genetic Methods, and Media

Genotypes of strains used in this study are listed in Table 3. 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).

GPY1100 was generated from GPY1100**a** by mating type switching with plasmid-borne *HO* (Payne and Schekman, 1989). Similarly, GPY404.1 was generated from SEY6210. All mutant or plasmid-carrying strains are congenic with either GPY1100 or SEY6210. GPY1599–23D is a meiotic progeny from a cross of GPY1422 and GPY1357. GPY1627-2C is a meiotic progeny from a cross of GPY1422 and GPY1354. GPY1783-21D, 1783-10A, 1783-25A, and 1783-21C are meiotic progeny from a cross of GPY1705.1 and GPY404.1. Single-step gene replacement (Rothstein, 1991) was carried out with papl4::TRP1 cleaved with *Kpn*I and *Sac*I, papm1::URA3 cleaved with *Bam*HI and *Eco*RI, and papl3::TRP1 cleaved with *Cla*I and *Sac*I. All gene replacements were verified by Southern blotting or immunoblotting. With all HA constructs, immunoblotting with HA-specific antibodies detected species of the expected size, which were absent in strains lacking HA tags.

YP medium is 1% Bacto-yeast extract and 2% Bacto-peptone. YPD medium is YP with 2% dextrose. SD medium is 0.67% yeast nitrogen base (Difco, 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. SDYE is SD with 0.2% yeast extract. Cell densities in liquid culture were measured in a 1-cm plastic cuvette using a Beckman Instruments (Palo Alto, CA) DU62 spectrophotometer. One OD_{500} unit is equivalent to 2.3×10^7 cells/ml.

To assess growth on agar plates, cells were grown in YPD to stationary phase, diluted to 1×10^6 cells/ml, and further diluted 1:10 or 1:100. Three microliters of each of these dilutions were spotted onto YPD plates and incubated at 24, 30, or 37°C.

Native Coimmunoprecipitations

Cells were grown to OD_{500} of 0.5–1.0. Fifty OD_{500} units of cells were harvested and resuspended in 1 ml of 100 mM Tris-SO_4 pH 9.5, and 10 mM DTT and incubated for 10 min at 30°C. Cells were pelleted and resuspended in 1 ml of YP, 1.0 M sorbitol, and 0.5% glucose and converted to spheroplasts by addition of 16 μl of 1 mg/ml oxalyticase (Enzogenetics, Eugene, OR) and incubation for 30 min at 30°C. Spheroplasts were lysed by resuspension in 0.5 ml of ice-cold PBS, 1% Triton X-100, 1 mM EDTA, and 2× PIC (1000× PIC contains 100 mM *N*-tosyl-L-phenyl-alanine-chloromethyl ketone, 1 M benzamidine-HCl, 25 mM pepstatinA, 4 mM leupeptin, and 1 M 4-(2 aminoethyl)-benzene sulfonyl-fluoride). The lysate was clarified by centrifugation at $16{,}000 \times g$ for 10 min at 4°C and then transferred to a fresh tube containing 25 μl of a 20% suspension of protein A-Sepharose (Pharmacia, Piscataway, NJ) and appropriate antibody. For precipitations with antibodies against Apl1p, Apl2p, or Apl6p, 125 OD_{500} cell equivalents/ml of lysate were used. For APM1-HA coimmunoprecipitations, 12CA5 antibody was coupled to protein A-Sepharose with dimethylpimelimidate (Harlow and Lane, 1988).

Radiolabeling and Immunoprecipitations

For metabolic labeling of α-factor, cells were grown to midlogarithmic phase in SDYE at 24°C. Cultures were shifted to 24° or 30°C for

Table 3. Yeast strains used in this study

Strain	Genotype	Source
GPY 418	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521*	Phan *et al.* (1994)
GPY 906	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521 apl2::URA3*	Rad *et al.* (1995)
GPY 1100	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1*	Payne and Schekman (1989)
GPY 404	*MATa ura3-52, leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Payne and Schekman (1989)
GPY 719	*MATα chc1-521 leu2-3,112 ura3-52 his4-519 trp1 can1 aps1-Δ1::LEU2*	Phan *et al.* (1994)
GPY 1049	*MATα ura3-52 lys2-801 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 apl1::LEU2 apl2-Δ6::TRP1*	This study
GPY 1329	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 apl3::TRP1*	This study
GPY 1352	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521 apl4::TRP1*	This study
GPY 1353	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521 apl2::URA3 apl4::TRP1*	This study
GPY 1354	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521 apl2::URA3 apl4::TRP1 aps1::LEU2*	This study
GPY 1357	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 apl2::URA3 apl4::TRP1aps1::LEU2*	This study
GPY 1359	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 APL4-HA::URA3*	This study
GPY 1415	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 APM1-HA::URA3*	This study
GPY 1422	*MATa leu2-3,112 ura3-52 his4-519 trp1 can1 gal2 apm1::URA3*	This study
GPY 1423	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521 apm1::URA3*	This study
GPY 1599-23D	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 apm1::URA3 apl2::URA3 apl4::TRP1 aps1::LEU2*	This study
GPY 1627-2C	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 chc1-521 apl2::URA3 apl4::TRP1 aps1::LEU2 apm1::URA3*	This study
GPY 1705	*MATα ura3-52 lys2-801 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 apl1::LEU2 apl2-Δ6::TRP1 apl6::URA3*	This study
GPY 1783-21D	*MATα ura3-52 leu2-3 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apl1::LEU2*	This study
GPY 1783-10A	*MATα ura3-52 leu2-3 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apl2::TRP1*	This study
GPY 1783-25A	*MATα ura3-52 leu2-3 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apl6::URA3*	This study
GPY 1783-21C	*MATα ura3-52 leu2-3 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 apl1::LEU2 apl2::TRP1 apl6::URA3*	This study
GPY 2109	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1* + pRS305-ProHA-APL1	This study
GPY 2110	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1* + pRS305-ProHA-APL2	This study
GPY 2171	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1* + pRS315-HA-APL6	This study
GPY 2210	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1* + pRS305-ProHA-APL1 + pRS426-APM2	This study
GPY 2211	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1* + pRS305-ProHAAPL2 + pRS426-APM2	This study
GPY 2212	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1* + pRS315-HA-APL6 + pRS426-APM2	This study
GPY 2213	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1* + *APM1HA::URA* + pRS424-APM2	This study
GPY 2231	*MATα leu2-3,112 ura3-52 his4-519 trp1 can1 APM4-HA::URA3*	This study
SEY 6210	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.* (1988)
TVY 614	*MATα ura3-52 his3-Δ200 trp1-Δ901 leu2-3,112 lys2-801 suc2-Δ9 pep4::LEU2 prb1::HISG prc1::HIS3*	Vida and Emr (1995)

2 h. Labeling and immunoprecipitation were performed as described previously (Seeger and Payne, 1992a), except that labeling was for 45 min instead of 10 min. For metabolic labeling of CPY, cells were grown to midlogarithmic phase in SDYE at 30°C. Cultures were resuspended in supplemented SD and shifted to 30°C for 5 min. Labeling and immunoprecipitation was performed as described previously (Seeger and Payne, 1992b).

Antibodies and Immunoblotting

HA-specific monoclonal antibody 12CA5 was a gift from G. Weinmaster (University of California School of Medicine, Los Angeles, CA); monoclonal antibodies to yeast Chc1p and polyclonal antibodies to Apm2p and Apm3p were a gift from S.K. Lemmon (Case Western Reserve University, Cleveland, OH); antibodies to carboxypeptidase Y (CPY) and Apl6p were a gift from S.D. Emr (University of California, San Diego, CA); and antibodies to aminopeptidase I (API) were a gift from D. Klionsky (University of California, Davis, CA).

To generate Apl1p antibody, the C terminus of *APL1*, bp 1564–2060, was amplified by PCR from pAPL1–100. The 5′ primer (primer 30) contains an *Nde*I site, a start codon (ATG), and six additional histidines (CAC) fused in frame with *APL1*. The 3′ primer (primer 31) contains a *Bam*HI site. The resulting product was subcloned into pET3c (Studier *et al.*, 1990). The *APL1* C terminus was then introduced as an *Afl*II–*Hin*dIII fragment to generate pHIS-APL1 Cterm (Apl1p amino acids 523–701). Expression of pHIS-APL1 Cterm in *Escherichia coli* strain BL21 (DE3) was induced with 0.1 mM isopropyl thiogalactoside and lysed as previously described (Phan *et al.*, 1994). Histidine-tagged protein was purified by nickel-nitrolotriacetic acid affinity chromatography (Qiagen, Chatsworth, CA) as described by Bush *et al.* (1991), with the following modifications. The bacterial cell pellet was resuspended in 30 ml of buffer A; the lysate was centrifuged at 10,000 × *g* for 20 min, and buffers B and C contain 0.5% Triton X-100. The elutions with 10 ml of buffer D were followed with elutions of 10 ml of buffer E (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris-HCl, pH 4.5). Two-milliliter fractions were collected, and fractions 2 and 3 from the buffer E elution were pooled and dialyzed stepwise from 8 M urea into 6 M urea, 4 M

urea, 2 M urea, and finally into PBS, 10% glycerol, and 10 mM DTT. The sample was used as antigen for commercial production of antibody in rabbits (Cocalico Biologicals, Reamstown, PA).

To generate Apl2p antibody, pGEX-KG-APL2 C-term was expressed in BL21 (DE3) strain as described above. Cell lysis and fusion protein affinity purification with glutathione-Sepharose (Pharmacia) were carried out as recommended by the manufacturer. Purified fusion protein was used as antigen for commercial production of antibody in rabbits (Cocalico Biologicals).

Antibodies against Apl1p were affinity purified using GST-APL1 fusion protein coupled to cyanogen bromide-Sepharose 4B (Pharmacia) according to the method of Harlow and Lane (1988).

Immunoblotting was carried out according to the method of Burnette (1981) with secondary antibodies coupled to alkaline phosphatase (ALP; Bio-Rad, Richmond, CA) or coupled to horseradish peroxidase (HRP; Bio-Rad). Antibodies were visualized using color development for ALP (Bio-Rad) or epichemiluminescence (New England Nuclear, Boston, MA) for HRP (Pharmacia).

Affinity Chromatography with GST Fusion Proteins

pGEX-KG-APL1 C-term, pGEX-KG-APL2 C-term, and pGEX-KG-APL6 C-term were expressed in BL21 (DE3), and GST fusion proteins were affinity purified with glutathione-Sepharose. For preparation of yeast extract, wild-type strain TVY 614 was grown to midlogarithmic phase in YPD. Eight hundred fifty OD_{500} units of cells were converted to spheroplasts and resuspended at 85 OD_{500}/ml in ice-cold 20 mM HEPES, pH 7.2, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM DTT, 1% Trition X-100, and 2× PIC. Cells were further lysed by 20 strokes of a Dounce homogenizer. After centrifugation at 27,000 × *g* for 30 min, the supernatant was applied to 250 μl of a 50% suspension of glutathione-Sepharose carrying GST fusion proteins and incubated for 2 h at 4°C with rotation. Fusion proteins and associated proteins were eluted by three consecutive treatments with 125 μl of reduced glutathione buffer (20 mM reduced glutathione, 100 mM Tris-HCl, pH 9.0, 200 mM NaCl, 5 mM DTT, and 0.1% Triton X-100).

Fractionation Procedure

Clathrin-coated vesicles were enriched by differential centrifugation and gel filtration chromatography of the high-speed pellet fraction (100,000 × *g* for 60 min) as previously described (Chu *et al.*, 1996). Fractions were precipitated by addition of 10% trichloroacetic acid and subjected to SDS-PAGE followed by immunoblotting with monoclonal antibodies to detect Chc1p and polyclonal antibodies to detect Kex2p.

RESULTS

AP-1 Complex

Specific genetic interactions with *chc1-ts* have led to the proposal that Aps1p, Apm1p, and Apl2p (β1) are associated in an AP-1 complex that functions with clathrin at the Golgi apparatus (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). To investigate the physical association of these AP subunits and identify the non-β large subunit of the presumptive AP-1 complex, selected subunits were immunoprecipitated under nondenaturing conditions and probed for associated AP proteins by immunoblotting. We were particularly interested in monitoring Apl4p, because this protein is most similar in sequence to the γ large subunit of mammalian AP-1. For this purpose a strain was constructed in which a functional version of Apl4p tagged with the influenza HA epitope was integrated at the chromosomal *APL4* locus. Whole-cell extracts were prepared by lysis with 1% Triton X-100 to provide a population of AP complexes representative of both soluble and membrane-associated pools. Extract was incubated with antibodies specific for Apl2p (β1), and the resulting immunoprecipitate was analyzed by SDS-PAGE and immunoblotting. Both Aps1p and Apl4p-HA were coprecipitated with Apl2p (β1) (Figure 1A, compare lane 3 with the total extract in lane 1). In contrast, Aps2p was not precipitated, providing evidence for the specificity of coprecipitation (Figure 1A, lane 3). These experiments were carried out with antigen in excess, resulting in immunoprecipitation of ~10% of the AP subunits. Under conditions of antibody excess, essentially all of the Aps1p present in the extract was coprecipitated with Apl2p (Yeung, unpublished results). The specificity of small subunit interaction with Apl2p (β1) was sufficiently stringent that even in cells completely lacking the Aps1p subunit because of deletion of *APS1*, Aps2p was not detected in precipitates of Apl2p (Yeung, unpublished results).

To monitor interactions with Apm1p, a strain expressing functional Apm1p-HA was lysed, and Apm1p was immunoprecipitated with HA-specific monoclonal antibody. Immunoblotting of the resulting precipitates revealed association with Apl2p (β1) and Aps1p (Figure 1B, lane 3) but not Apl1p (β) or Aps2p (Figure 1B, lane 6). Detection of Apl2p (β1) and Aps1p was dependent on precipitation of Apm1p, because neither protein was precipitated by HA antibodies when extracts were used from cells expressing Apm1p without the HA tag (Figure 1B, lanes 2 and 5). These results suggest that the AP-1 complex consists of Aps1p (σ1), Apm1p (μ1), Apl4p (γ), and Apl2p (β1).

AP-2R Complex

A similar coimmunoprecipitation strategy was applied to characterize the complex containing the Apl1p (β) subunit. Aps2p, but not Aps1p or Apl4p-HA, was coprecipitated with Apl1p (β) (Figure 1A, lane 2). To determine whether Apl3p is the non-β large subunit associated with Apl1p (β), a variation of the coprecipitation strategy was adopted. The approach was based on analyses of mammalian and yeast AP complexes, which demonstrate that loss of single AP subunits can dramatically reduce the ability of the remaining subunits to form a stable complex (Panek *et al.*, 1997; Dell'Angelica *et al.*, 1999b). Accordingly, we monitored the effect of deleting *APL3* (*apl3*Δ) on association of Aps2p with immunoprecipitated Apl1p (β). The absence of Apl3p eliminated Aps2p interaction with Apl1p (β) observed in wild-type cells (Figure 1C, compare lane 3 with lane 1). The effect of *apl3*Δ was specific for the Apl1p (β)-Aps2p association, because the AP-1 complex was unaffected by the deletion as assessed by coprecipitation of Aps1p (σ1) with Apl2p (β1) (Figure 1C, lanes 2 and 4). Apm4p was assigned to the Apl1p (β) complex based on coprecipitation of Apl1p (β) and Aps2p with an HA-tagged version of Apm4p (Figure 1D, lane 1). The specificity of these interactions was evident from the absence of AP-1 subunits (Apl2p and Aps1p) in the immunoprecipitate of Apm4p-HA and the corresponding absence of Apl1p (β) and Aps2p in immunoprecipitates of Apm1p-HA (Figure 1D, lane 2, also see B). The lower-molecular-weight band in the Figure 1D, lane 2, upper panel, is most likely a degradation product of Apl2p (β1) (see Figure 1E, lane 2). These results group Aps2p (σ2R), Apm4p (μ2R), Apl3p (αR), and Apl1p (β2R) in a distinct complex that we term AP-2R, because of the prevailing sequence similarity of

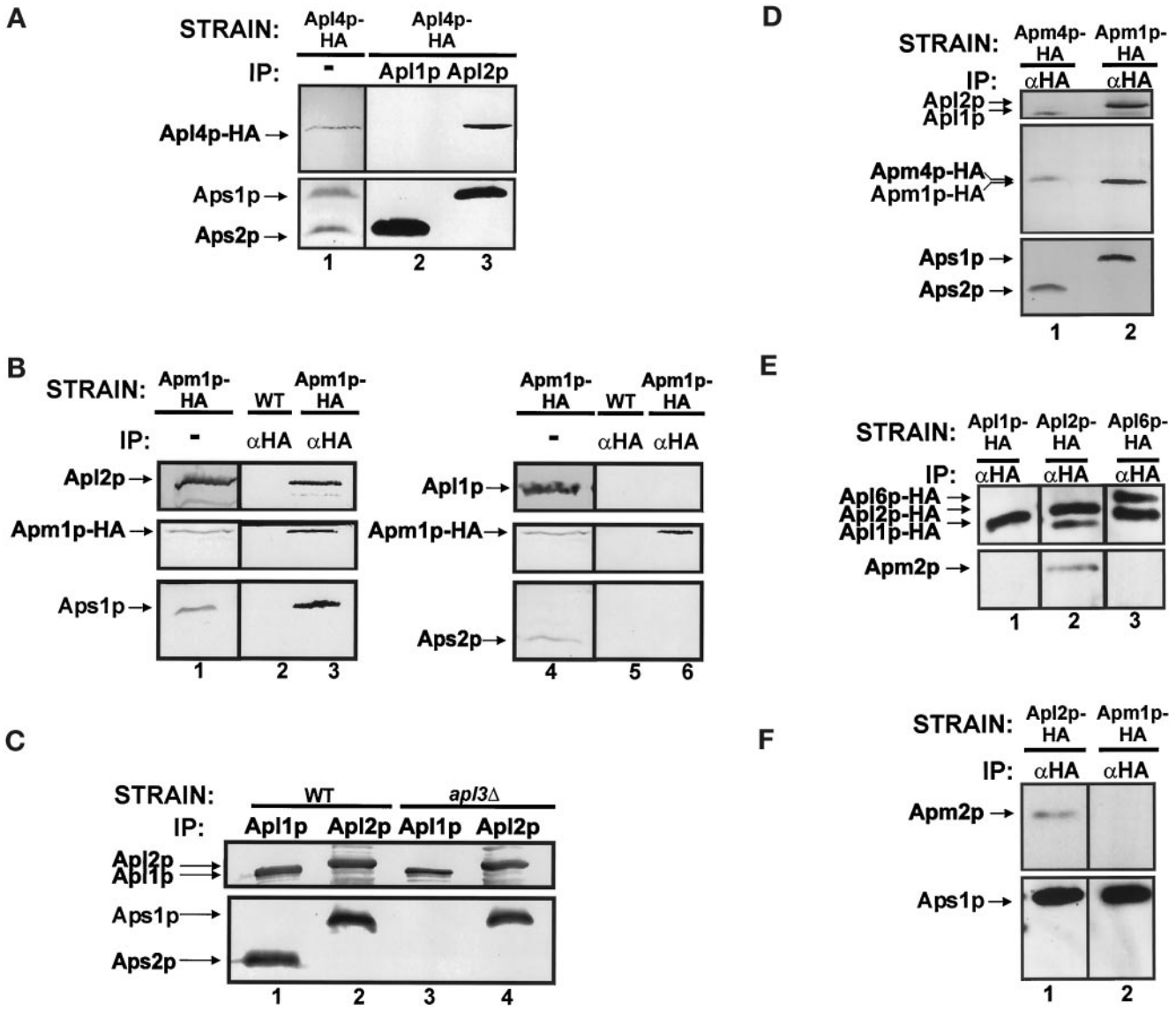

Figure 1. Physical association of AP subunits in AP-1 and AP-2R complexes. (A) Immunoprecipitation of Apl1p- and Apl2p-containing complexes. Polyclonal antibody was used to precipitate the β subunits, Apl1p (lane 2) and Apl2p (lane 3), from nondenatured lysates of Apl4p-HA-expressing cells (GPY 1359). Also analyzed was a sample of total extract from Apl4p-HA cells corresponding to 1/100 of the extract used for the immunoprecipitations (lane 1). Precipitates and total extract were subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was cut into strips and immunoblotted with 12CA5 antibody to detect Apl4p-HA (upper panels) and polyclonal antibodies to Aps1p and Aps2p (lower panels). (B) Immunoprecipitation of Apm1p-containing complexes. Monoclonal 12CA5 antibody was used to precipitate Apm1p-HA (lanes 2, 3, 5, and 6) from lysates of wild-type (GPY 1100; lanes 2 and 5) or Apm1p-HA-expressing (GPY 1415; lanes 3 and 6) cells. A sample of total extract from Apl4p-HA cells corresponding to 1/100 of the extract used for the immunoprecipitations was also analyzed (lanes 1 and 4). Samples were treated as described in A. 12CA5 antibody was used to detect Apm1p-HA, and polyclonal antibodies were used to detect Apl1p, Apl2p, Aps1p, and Aps2p. (C) Effect of *apl3*Δ on immunoprecipitation of AP-2R complexes. Coimmunoprecipitations using polyclonal antibody to precipitate Apl1p (lanes 1 and 3) or Apl2p (lanes 2 and 4) from lysates of wild-type (GPY 1100; lanes 1 and 2) or *apl3*Δ (GPY 1329; lanes 3 and 4) strains were analyzed as in B. (D) Immunoprecipitation of Apm4p- and Apm1p-containing complexes. 12CA5 antibody was used to precipitate Apm4p-HA (lane 1) and Apm1p-HA (lane 2) from lysates of Apm4p-HA-expressing (GPY 2231; lane 1) or Apm1p-HA-expressing (GPY 1415; lane 2) strains. Samples were analyzed as in B. (E) Coimmunoprecipitation of Apm2p with Apl2p. 12CA5 antibody was used to precipitate Apl1p (lane 1), Apl2p (lane 2), or Apl6p (lane 3) from Apl1p-HA-expressing (GPY 2210), Apl2p-HA-expressing (GPY 2211), or Apl6p-HA-expressing (GPY 2212) strains, all of which carry the multicopy *APM2* plasmid. Samples were analyzed as in B. The lower-molecular-weight HA-containing bands in lanes 2 and 3 are degradation products. (F) Immunoprecipitation of Apm1- and Apm2-containing complexes. 12CA5 antibody was used to precipitate Apl2p-HA (lane 1) and Apm1p-HA (lane 2) from Apl2p-HA-expressing (GPY 2211) or Apm1p-HA-expressing (GPY 2213) strains, both of which carry the multicopy *APM2* plasmid. Samples were analyzed as in B.

the yeast subunits with mammalian AP-2 subunits. The "R" is attached to signify "related" because, unlike the established connection between the mammalian AP-2 complex and clathrin-mediated endocytosis, there is no physical or genetic evidence linking AP-2R to clathrin or endocytosis (see below).

Apm2p Associates with Apl2(β1)

Apm2p is unusual because it is significantly larger (~70 kDa) than the other three μ subunits (~50 kDa), and it is less conserved with mammalian μ chains (Cowles *et al.*, 1997a; Panek *et al.*, 1997). Association of Apm2p with AP β subunits was investigated by coimmunoprecipitation using HA antibody to precipitate HA-tagged versions of Apl1p (β2R), Apl2p (β1), or Apl6p (β3). Using extracts from wild-type cells we were unable to detect reproducible association with any of the β subunits, but occasionally Apm2p appeared to be coprecipitated with Apl2p (β1). To increase the sensitivity of the assay, the immunoprecipitation was repeated with extracts from cells expressing Apm2p at elevated levels from a multicopy plasmid. Apm2p in these extracts was specifically coprecipitated with Apl2p (β1) (Figure 1E). Conversely, Apl2p (β1) and Aps1p were preferentially precipitated with Apm2p antibodies (Yeung, unpublished results). Because of the unique properties of Apm2p, we considered the possibility that the protein was peripherally associated with intact AP-1 (containing Apm1p) rather than an integral part of a separate Apl2p-containing complex. As an approach to distinguish between these alternatives, we investigated whether overexpressed Apm2p could be coprecipitated with Apm1p-containing AP-1 complexes. For this purpose, the multicopy *APM2* plasmid was introduced into a strain expressing Apm1p-HA, and AP-1 was immunoprecipitated from extracts of these cells with HA antibody. Although AP-1 subunit Aps1p (σ1) was coprecipitated with Apm1p-HA, no associated Apm2p was detected (Figure 1F, lane 2). In contrast, a parallel precipitation of Apl2p (β1)-HA coprecipitated Apm2p and Aps1p (Figure 1F, lane 1). This finding suggests that Apm2p is able to interact with β1, potentially as part of an alternative AP-1-like complex lacking Apm1p.

AP-1 Interacts with Clathrin

In mammalian cells, β subunits of AP-1, AP-2, and AP-3 interact with clathrin in vitro (Gallusser and Kirchhausen, 1993; Dell'Angelica *et al.*, 1998). However, in yeast, deletions of AP-1 β or σ subunits, but not cognate AP-2R or AP-3 subunits, display genetic interactions with *chc1-ts*, raising the possibility that only yeast AP-1 interacts with clathrin (Phan *et al.*, 1994; Rad *et al.*, 1995; Panek *et al.*, 1997). To address this possibility we examined physical interactions of yeast AP complexes with clathrin using in vitro binding assays and coimmunoprecipitation.

For in vitro binding experiments, N-terminal truncations of β subunits were fused to GST and expressed in *E. coli.* These truncated versions were selected because clathrin-binding sites in mammalian β subunits are located toward the C termini (Kirchhausen, 1990; Shih *et al.*, 1995; Dell'Angelica *et al.*, 1998), and initial attempts to express GST fused to full-length yeast β subunits resulted in insoluble proteins. Each GST-yeast β fusion was bound to glutathione-Sepharose and then incubated with extract from a wild-type yeast strain. Bound proteins were eluted with reduced glutathione and were analyzed by SDS-PAGE followed by immunoblotting with clathrin heavy chain antibodies or staining with Coomassie brilliant blue. As shown in Figure 2A, GST-Apl2p (β1) bound clathrin heavy chain (Figure 2A, lane 2). Specificity of binding was apparent from Coomassie blue staining of the bound fraction, which re-

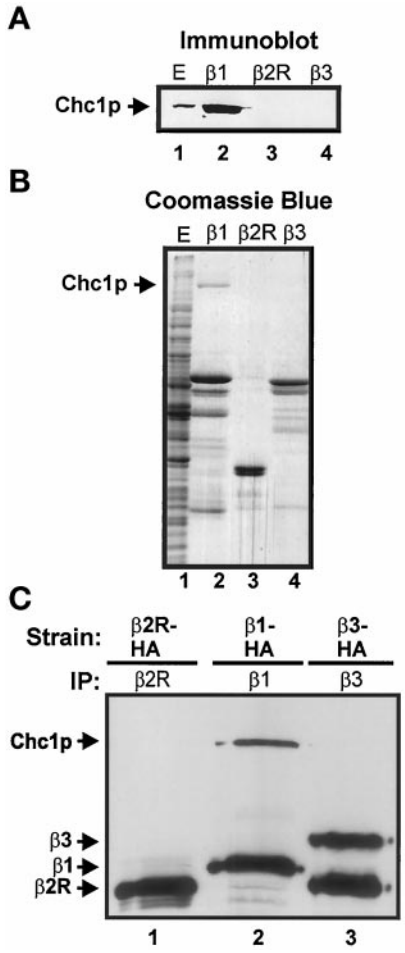

Figure 2. AP-1 interacts with clathrin. (A and B) C-terminal regions of β sbunits were fused to GST and expressed in *E. coli.* Fusion proteins were bound to glutathione-Sepharose beads and then incubated with extract from strain TVY 614. Bound proteins were eluted and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with antibodies to detect Chc1p (A) or stained with Coomassie brilliant blue (B). Lane 1 in both panels contains a sample of the starting extract corresponding to 1/10,000 of the extract used for incubations with GST fusions. The most prominent species in lanes 2–4 are the GST fusion proteins. (C) Immunoprecipitations of AP β subunits. Polyclonal antibodies were used to precipitate Apl1p (β2R; lane 1), Apl2p (β1; lane 2), or Apl6p (β3; lane 3) from native extracts of Apl1p(β2R)-HA-expressing (GPY 2109), Apl2p(β1)-HA-expressing (GPY 2110), or Apl6p(β3)-HA-expressing (GPY 2171) strains. Precipitates were subjected to SDS-PAGE, transferred to nitrocellulose, and immunblotted with monoclonal antibodies to detect Chc1p and HA epitopes. The lower-molecular-weight band in lane 3 is a β3 degradation product.

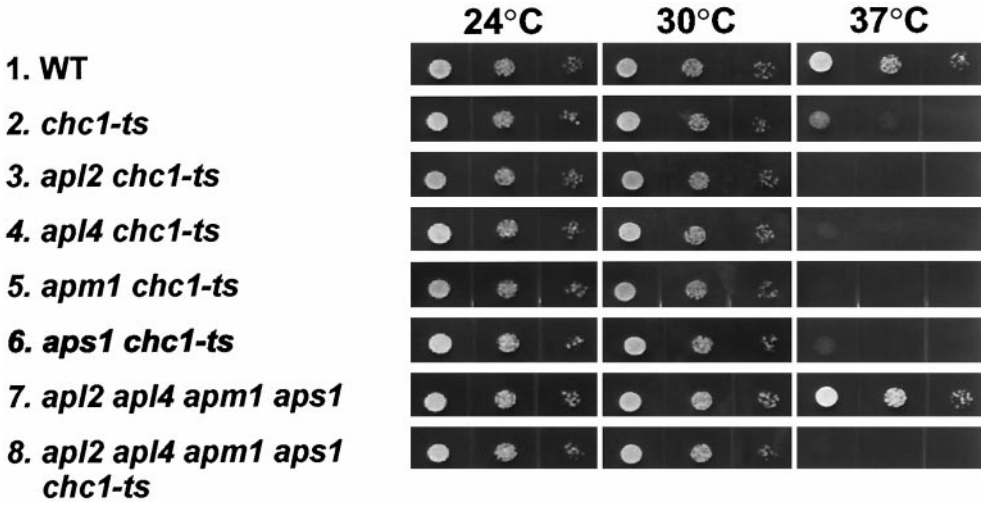

Figure 3. AP-1 subunit gene deletions accentuate the growth defect of *chc1-ts* cells but not *CHC1* cells. Wild-type (GPY1100), *chc1-ts* (GPY418), *apl2*Δ *chc1-ts* (GPY906), *apl4*Δ *chc1-ts* (GPY1352), *apm1*Δ *chc1-ts* (GPY1423), *aps1*Δ *chc1-ts* (GPY719), *apl2*Δ *apl4*Δ *apm1*Δ *aps1*Δ (GPY1599–23D), and *apl2*Δ *apl4*Δ *apm1*Δ *aps1*Δ *chc1-ts* (GPY 1627-2C) strains were grown overnight to saturation at 24°C in YPD. Cells were diluted to 10^6 cells/ml and further serially diluted 1:10 and 1:100. Three microliters of each dilution were spotted onto YPD agar and incubated at 24, 30, or 37°C.

vealed the clathrin heavy chain to be the single major protein larger than the 58.5-kDa fusion protein when compared with the starting extract (Figure 2B, lanes 1 and 2). Neither GST-Apl1p (β2R) nor GST-Apl6p (β3) was found to bind clathrin (Figure 2, A and B, lanes 3 and 4). No other major high-molecular-weight species larger than the fusion proteins were detected in the bound fractions by Coomassie blue staining (Figure 2B, lanes 3 and 4). The identity of bands migrating faster than the fusions have not been addressed but could represent degradation products from the fusions. These results suggest that only Apl2p (β1) interacts with clathrin.

As an alternative approach to assess clathrin binding by AP complexes, native immunoprecipitations of each AP complex were probed for associated clathrin heavy chain. AP complexes were immunoprecipitated from extracts of cells expressing HA-tagged β subunits with polyclonal antibodies directed against the β subunits. These antibodies are known to recognize the native AP complexes (Figure 1; Yeung, unpublished results). Immunoblotting with HA-specific antibodies indicated that approximately equal amounts of each AP complex were precipitated, but clathrin was associated only with AP-1 (Figure 2C). Thus, clathrin interacts selectively with AP-1 by both coimmunoprecipitations and GST fusion binding assays. Together with results from studies of genetic interactions between AP subunit deletions and *chc1-ts* (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995) our findings argue that AP-1 is the sole clathrin-associated adaptor of the three yeast AP complexes.

Disruption of AP-1 Enhances Effects of chc1-ts

Earlier studies failed to detect growth or protein trafficking defects in strains carrying deletions of AP-1 β, μ, or σ subunits or a combination of β and σ subunits (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). However, the same AP-1 subunit deletions accentuate growth and protein trafficking defects in *chc1-ts* cells. The effects are specific to AP-1 subunit deletions; AP-2R and AP-3 mutations do not interact with *chc1-ts* (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995; Panek *et al.*, 1997). To address the possibility that the subtle effects of AP-1 single and double subunit deletions are attributable to residual activity of partial complexes, we examined growth, protein sorting, and clathrin-coated vesicle formation in cells (*ap1-null*) lacking the four AP-1 subunits, Apl4p (γ), Apl2p (β1), Apm1p (μ1), and Aps1p (σ1).

Growth was monitored by incubating serial dilutions of cells on agar plates at 24, 30, or 37°C. Wild-type and *ap1-null* strains grew at the same rate at all three temperatures, indicating that the absence of AP-1 does not perturb growth (Figure 3, rows 1 and 7). We also compared the effects of AP-1 single subunit deletions with the AP-1-null combination in a congenic set of *chc1-ts* strains. In agreement with previous findings (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995), *apl2* (β1), *apm1*, or *aps1* reduced the ability of *chc1-ts* cells to grow at 37°C but not at lower temperatures (Figure 3, rows 2, 3, 5, and 6). Deletion of *APL4* (γ) in the *chc1-ts* strain caused a similar defect (Figure 3, row 4). Although not readily apparent in Figure 3, limited growth of *apl4* (γ) *chc1-ts* and *aps1 chc1-ts* cells was observed at the highest cell densities at 37°C. No growth of *apl2* (β1) *chc1-ts* and *apm1 chc1-ts* cells was observed at 37°C, suggesting that loss of β or μ AP-1 subunits is slightly more deleterious to growth of *chc1-ts* cells than loss of γ or σ subunits. The growth properties of the *ap1-null chc1-ts* strain mirrored those of the *apl2* (β1) *chc1-ts* and *apm1 chc1-ts* strains (Figure 3, rows 3, 5, and 8).

Cells with mutations in clathrin subunits secrete a highly glycosylated precursor form of the α-factor mating pheromone (Payne and Schekman, 1989; Seeger and Payne, 1992b; Chu *et al.*, 1996, 1999; Huang *et al.*, 1997). This defect is attributed to mislocalization of the Golgi membrane protein Kex2p, which normally initiates proteolytic maturation of the pheromone precursor in the TGN (Fuller *et al.*, 1988). In the absence of clathrin function, Kex2p is mislocalized to the plasma membrane, and the resulting depletion of TGN Kex2p allows some fully glycosylated precursor to avoid proteolytic maturation (Payne and Schekman, 1989; Seeger and Payne, 1992b). Thus, the level of secreted highly glycosylated α-factor precursor provides a convenient measure of Kex2p localization. Previous studies indicated that effects of

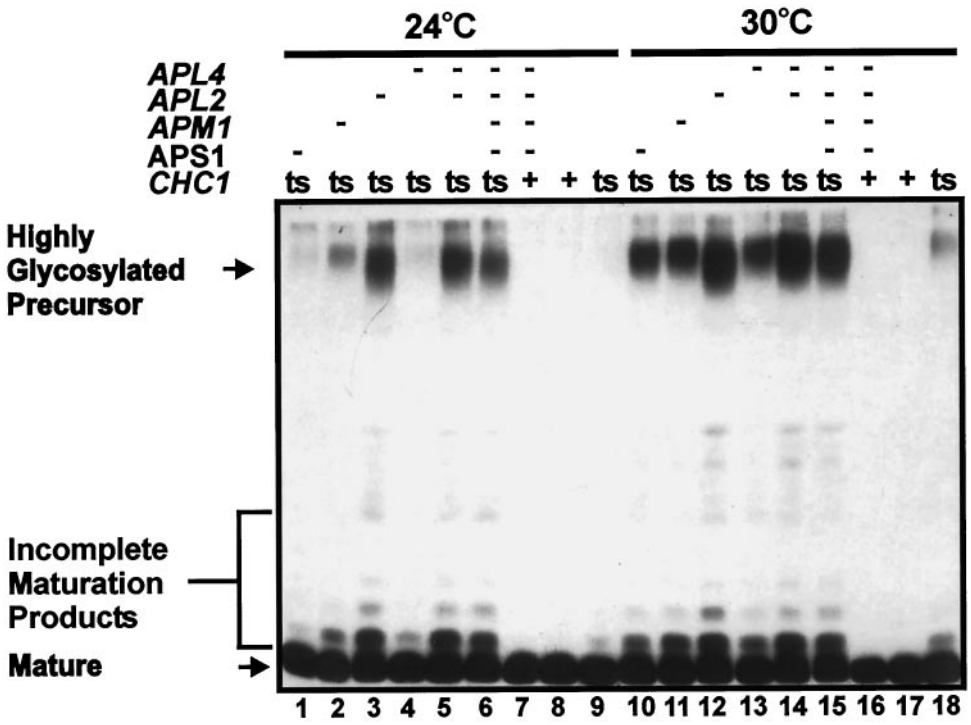

Figure 4. AP-1 subunit gene deletions enhance the α-factor maturation defect in *chc1-ts* cells. Strains *aps1*Δ *chc1-ts* (GPY719; lanes 1 and 10), *apm1*Δ *chc1-ts* (GPY 1423; lanes 2 and 11), *apl2*Δ *chc1-ts* (GPY 906; lanes 3 and 12), *apl4*Δ *chc1-ts* (GPY 1352; lanes 4 and 13), *apl2*Δ *apl4*Δ *chc1-ts* (GPY 1353; lanes 5 and 14), *apl2*Δ *apl4*Δ *apm1*Δ *aps1*Δ *chc1-ts* (GPY 1627–2C; lanes 6 and 15), *apl2*Δ *apl4*Δ *apm1*Δ *aps1*Δ (GPY 1599-23D; lanes 7 and 16), wild type (GPY 1100; lanes 8 and 17), and *chc1-ts* (GPY 418; lanes 9 and 18) were grown overnight at 24°C and then shifted to 24 or 30°C for 2 h. Cells were metabolically labeled with [^{35}S]methionine and cysteine for 45 min at 24 or 30°C. α-Factor was immunoprecipitated from the culture supernatant and subjected to SDS-PAGE and autoradiography. −, deletion of a gene; + or blank, presence of a gene; ts, presence of *chc1-ts*.

single and double AP subunit deletions on α-factor maturation generally parallel effects on growth; in both cases defects are apparent only when AP-1 subunits are deleted in combination with *chc1-ts* cells (Phan *et al*., 1994; Rad *et al*., 1995; Stepp *et al*., 1995). However, α-factor maturation is a more sensitive assay than growth, because effects of AP-1 subunit deletions on maturation in *chc1-ts*cells can be detected at a temperature (24°C) at which growth is unaffected. Accordingly we assessed α-factor maturation in cells carrying various combinations of AP-1 subunit deletions and *chc1-ts*.

Cells were labeled with [^{35}S]methionine and cysteine at 24 or 30°C, α-factor was immunoprecipitated from the medium, and the immunoprecipitate was analyzed by SDS-PAGE. Maturation was complete in wild-type cells at either temperature (Figure 4, lanes 8 and 17) and virtually complete in *chc1-ts* cells at 24°C (Figure 4, lane 9). No maturation defect was detected in the *ap-1-null* mutant at either temperature (Figure 4, lanes 7 and 16) or in cells with single AP-1 subunit deletions (Phan *et al*., 1994; Rad *et al*., 1995; Stepp *et al*., 1995; Phan and Yeung, unpublished results). However, even at the permissive temperature for *chc1-ts*, elimination of individual AP-1 subunits in *chc1-ts* cells resulted in secretion of precursor α-factor (Figure 4, lanes 1–4 and 9). Deletion of the *APS1* or *APL4* (γ) resulted in slight maturation defects (Figure 4, compare lanes 1 and 4 with lane 9). Deletion of *APM1* had a greater effect, and deletion of *APL2* (*β1*) produced the most severe defect (Figure 4, lanes 2 and 3). Analysis of Kex2p in the *apl2* (*β1*) *chc1-ts* strain at 24°C confirmed that the α-factor maturation defect was accompanied by Kex2p mislocalization (Phan, unpublished results). We considered two interpretations of the observation that the *apl2* (*β1*) *chc1-ts* strain exhibited the most pronounced defect. Either β1 is the most important subunit for AP-1 function (at least in α-factor maturation), or the absence of β1 results in a partial complex with inhibitory activity. To distinguish between these possibilities, we examined *apl2*Δ (*β1*) *apl4*Δ *chc1-ts*, and *ap-1-null chc1-ts* strains. If the β1-deficient partial complex is inhibitory, then elimination of the other subunits should alleviate inhibition and result in minor defects comparable with the effects of *aps1*Δ or *apl4*Δ. However, the double and quadruple AP-1 subunit deletion combinations in *chc1-ts* cells caused severe α-factor maturation defects (Figure 4, lanes 5 and 6), supporting the interpretation that β1 is particularly important for Kex2p localization. Similar results were obtained with cells incubated at 30°C, a temperature at which clathrin heavy chain expressed from the *chc1-ts* allele is partially defective (Figure 4, lane 18). However, at 30°C, accentuation of α-factor maturation defects by *aps1*Δ and *apl4*Δ was more apparent (Figure 4, lanes 10 and 13 compared with lane 18). These data are generally consistent with results from the growth assays and support the conclusion that AP-1 is not required for growth or α-factor maturation (and Kex2p localization) in cells expressing wild-type clathrin. In cells with compromised clathrin function, roles for AP-1 in growth and α-factor maturation can be detected, and the β subunit appears to be especially important.

The innocuous effects of AP-1 subunit deletions on clathrin-dependent processes suggest that clathrin-coated vesicle formation does not rely on AP-1. To address the role of AP-1 in clathrin coat assembly, we determined whether clathrin-coated vesicles could be identified in extracts of *ap-1-null* cells. Extracts from *ap-1-null* cells or wild-type cells were fractionated by differential centrifugation, and the high-speed pellet fraction was subjected to gel filtration chroma-

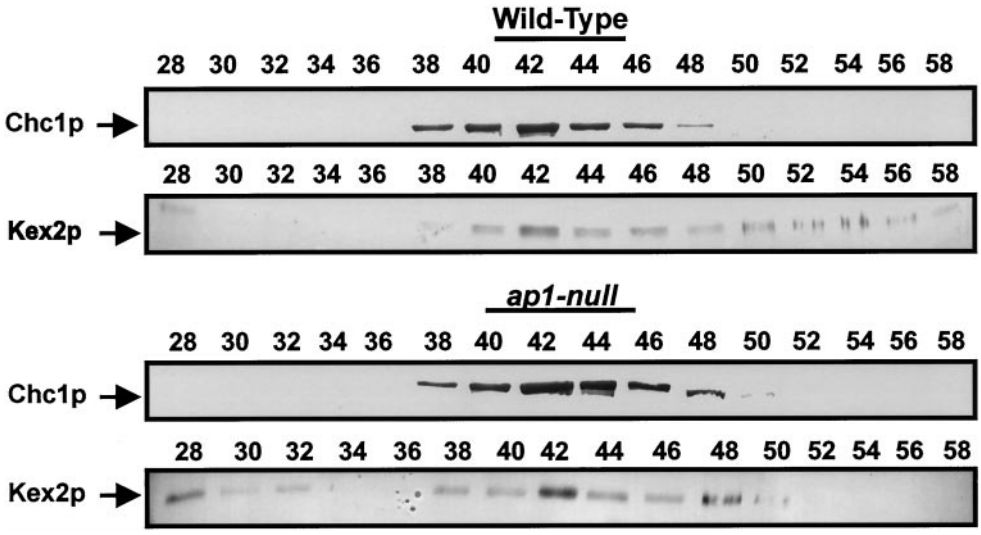

Figure 5. AP-1 subunit gene deletions do not affect clathrin-coated vesicles. A high-speed pellet fraction from wild-type (GPY 1100) or *apl2*Δ *apl4*Δ *apm1*Δ *aps1*Δ (GPY1599–23D) cells was chromatographed through a column of Sephacryl S-1000 as described in MATERIALS AND METHODS. Fractions were precipitated with TCA, and the precipitates were analyzed by SDS-PAGE and immunblotting with monoclonal antibodies to Chc1p and polyclonal antibodies to Kex2p.

tography through Sephacryl S-1000. Fractions from the S-1000 column were analyzed by SDS-PAGE and immunblotting for clathrin heavy chain and the likely clathrin-coated vesicle cargo protein, Kex2p (Payne and Schekman, 1989; Seeger and Payne, 1992a). The peaks of clathrin heavy chain and Kex2p from wild-type cells occurred in fraction 42 (Figure 5), corresponding to the profile expected from previous analyses of yeast clathrin-coated vesicles (Phan *et al.*, 1994; Chu *et al.*, 1996). Material from *ap-1-null* cells yielded essentially the same elution peaks of clathrin heavy chain and Kex2p, indicating that elimination of AP-1 does not affect clathrin coated vesicles (Figure 5). The levels of Kex2p in nonpeak fractions often varies in different preparations from the same strain, suggesting that the minor differences in Kex2p distribution in Figure 5 are not significant. Together, our analyses of *ap-1-null* cells argue that AP-1 is not required for clathrin-coated vesicle formation or function.

Deletion of all Three AP β Subunits Does Not Reveal Functional Redundancy

Although AP-1 specifically displays physical and genetic interactions with clathrin, limited functional redundancy between AP-1 and AP-2R and/or AP-3 could account for the absence of defects in cells expressing wild-type clathrin in combination with AP-1 subunit deletions. Our analysis of AP-1 function identifies the β subunit as a particularly important subunit, suggesting that deletion of the β subunit is an effective strategy to abolish the activity of an AP complex. Characterization of cells lacking AP-3 subunits also supports this approach (Cowles *et al.*, 1997a; Panek *et al.*, 1997; Stepp *et al.*, 1997). We therefore generated a strain carrying deletions of all three AP β subunits (referred to as 3βΔ) and carried out phenotypic analyses to assess functional redundancy between AP complexes. Growth of the 3βΔ strain was equivalent to wild type at 24, 30, and 37°C (Yeung, unpublished results). Maturation of α-factor was compared in wild-type, an *apl1* (*β2R*) *apl2* (*β1*) double mutant, the 3βΔ strain, and a *chc1-ts* strain. At 30°C, no defects in α-factor maturation were observed except the expected mild maturation defect in *chc1-ts* cells (Figure 6).

Trafficking through the endocytic pathway was evaluated by measuring turnover of the α-factor mating pheromone receptor Ste3p. Ste3p is normally consititutively internalized and transported to the vacuole where it is degraded (Davis *et al.*, 1993). In cells with defects in the endocytic pathway, either at the internalization step or at subsequent steps, delivery of Ste3p to the vacuole is delayed or blocked, thereby enhancing Ste3p stability (for examples see Davis *et al.*, 1993; Tan *et al.*, 1993). To determine the rate of Ste3p degradation, wild-type and 3βΔ cells were subjected to a pulse–chase regimen followed by lysis and immunoprecipitation of Ste3p. No change in the kinetics of Ste3p turnover was apparent in the 3βΔ strain compared with wild type (Figure 7A, lanes 1–8). Phosphorimage quantitation of the data in Figure 7 yielded a $t_{1/2}$ for Ste3p degradation of 18 min for wild-type cells and 20 min for 3βΔ cells. As a control, the same procedure was applied to *chc1-ts* cells labeled at 24°C and then shifted to the nonpermissive temperature (37°C) upon initiation of the chase period. Imposition of the endocytic defect in the *chc1-ts* cells resulted in a 2.5-fold decrease ($t_{1/2}$ = 46 min) in the rate of Ste3p turnover (Figure 7A, lanes 9–12).

Three distinct trafficking pathways to the vacuole were examined in 3βΔ cells. The first pathway is the well-characterized route from the TGN to the vacuole followed by the vacuolar hydrolase CPY (Bryant and Stevens, 1998). CPY is synthesized as an inactive precursor that is core-glycosylated in the endoplasmic reticulum to yield p1CPY (67 kDa). Transport via the secretory pathway to and through the Golgi apparatus results in further glycosylation to the p2 form (69 kDa). At the TGN p2CPY is sorted into vesicles targeted to a prevacuolar endosome compartment. From endosomes p2CPY is delivered to the vacuole, where proteolytic activation produces the mature form, mCPY (61 kDa). Sorting and transport from the TGN through endosomes to the vacuole (referred to here as the CPY pathway) requires the activity of a large number of proteins including clathrin and the products of the vacuolar protein sorting genes (*VPS*) (Bryant and Stevens, 1998). Defects in the pathway are manifested as secretion or intracellular accumula-

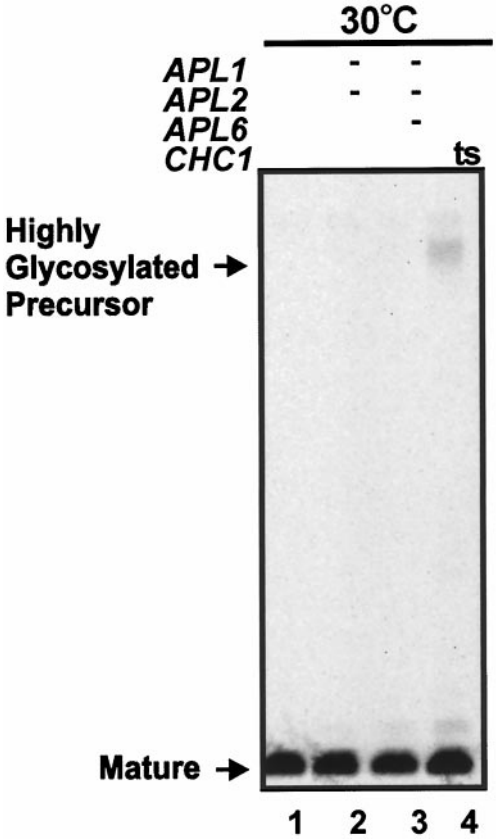

Figure 6. Deletion of three β subunits does not affect α-factor maturation. Wild-type (SEY 6210; lane 1), *apl1*Δ *apl2*Δ (GPY 1049; lane2), *apl1*Δ *apl2*Δ *apl6*Δ (GPY 1705.1; lane3), and *chc1-ts* (GPY 982; lane 4) cells were grown and labeled, and α-factor maturation was analyzed as described in the legend to Figure 4. −, deletion of a gene; + or blank, presence of a gene; ts, presence of *chc1-ts*.

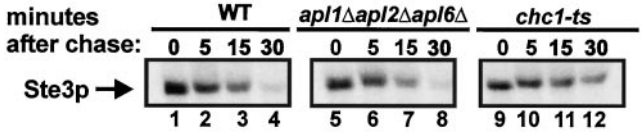

Figure 7. Deletion of three β subunits does not affect trafficking through the endocytic pathway. Ste3p degradation was analyzed in wild-type (SEY 6210; lanes 1–4), *apl1*Δ *apl2*Δ *apl6*Δ (GPY 1783–21C; lanes 5–8), and *chc1-ts* (GPY 982; lanes 9–12) strains. Strains were grown to logarithmic phase at 24°C. Wild-type and *apl1*Δ *apl2*Δ *apl6*Δ cells were shifted to 30°C, and *chc1-ts* cells were shifted to 24°C for 5 min before a 10-min labeling period at the shift temperature. Labeling was quenched with unlabeled amino acids, and cells were incubated at 30°C (WT and *apl1*Δ *apl2*Δ *apl6*Δ) or 37°C (*chc1-ts*) and harvested at the designated times after institution of the chase. After cell lysis, Ste3p was immunoprecipitated and analyzed by SDS-PAGE and autoradiography.

tion of p2CPY, readily detected by pulse–chase immunoprecipitation analysis of CPY. To assess CPY sorting and delivery to the vacuole in 3βΔ cells, mutant and wild-type cells were subjected to a pulse–chase regimen, and then CPY was immunoprecipitated from intracellular and extracellular fractions. This protocol revealed no difference in the kinetics of conversion of p1 to p2 to mCPY, or in the amount of secreted p2CPY, indicating normal CPY pathway function in 3βΔ cells (Figure 8A). To investigate the possibility that AP complexes might provide cargo-selective function in this pathway, we also examined two other soluble vacuolar proteins that follow this route, proteinase B (PrB) and proteinase A (PrA) (Bryant and Stevens, 1998). Vacuolar delivery of both proteins was unaffected in the mutant cells, as judged by maturation kinetics and levels of secretion (Yeung, unpublished results). The second pathway connects the Golgi apparatus to the vacuole by a route that bypasses prevacuolar endosomes (Bryant and Stevens, 1998). This pathway relies on AP-3 and is independent of clathrin and those Vps proteins involved in transport to and from endosomes (Cowles *et al*., 1997a,b; Piper *et al*., 1997; Stepp *et al*., 1997; Bryant and Stevens, 1998; Vowels and Payne, 1998a). Similar to the CPY pathway, integrity of the AP-3-dependent pathway can be evaluated through pulse–chase immunoprecipitation of an appropriate cargo protein such as the vacuolar membrane protein ALP. Because ALP is a membrane protein, it is not necessary to monitor secretion; so whole cell lysates were used for immunoprecipitation. By analysis of wild-type, *apl6*Δ (*β3*), and 3βΔ strains, we found the extent of the ALP maturation defect in 3βΔ cells to be no greater than that in cells lacking only the AP-3 β subunit (Figure 8B). Residual ALP maturation in the β3-deficient cells is due to missorting to the CPY pathway (Cowles *et al*., 1997a; Stepp *et al*., 1997; Vowels and Payne, 1998a), and the same is likely to be the case in the 3βΔ cells. The similar extent of ALP processing in 3βΔ- and β3-deficient cells indicates that eliminating all three AP β subunits does not enhance sorting defects attributable to the absence of AP-3 β alone. The third pathway delivers the cytoplasmic protein API to the vacuole by a process related to autophagy (Klionsky, 1998). This cytoplasmic-to-vacuole (Cvt) pathway involves formation of double-membrane vesicles, which selectively sequester cytoplasmic API. Cvt vesicles fuse directly with the vacuole leading to proteolytic maturation of API. Pulse–chase immunoprecipitation demonstrated no defect in API maturation in the 3βΔ cells (Figure 8C). Together, these analyses indicate that multiple transport pathways to the vacuole are unperturbed by the absence of AP complexes.

Finally, we applied the clathrin-coated vesicle isolation procedure to 3βΔ cells. As anticipated from the absence of clathrin-dependent sorting defects, gel filtration chromatography yielded matching profiles of clathrin heavy chain and Kex2p in wild-type and mutant cells, offering no evidence of defects in clathrin-coated vesicle formation (Figure 9).

DISCUSSION

We have carried out biochemical and genetic characterization of yeast AP complexes. Of the 13 potential AP subunits identified in the yeast genome, four have been previously assigned to AP-3 (Cowles *et al*., 1997a; Panek *et al*., 1997). The results reported here indicate that eight of the remaining subunits make up two distinct AP complexes, AP-1 and AP-2R. The extra medium subunit can associate with β1 when overexpressed, raising the possibility of an alternative form of AP-1. These findings argue that yeast express three

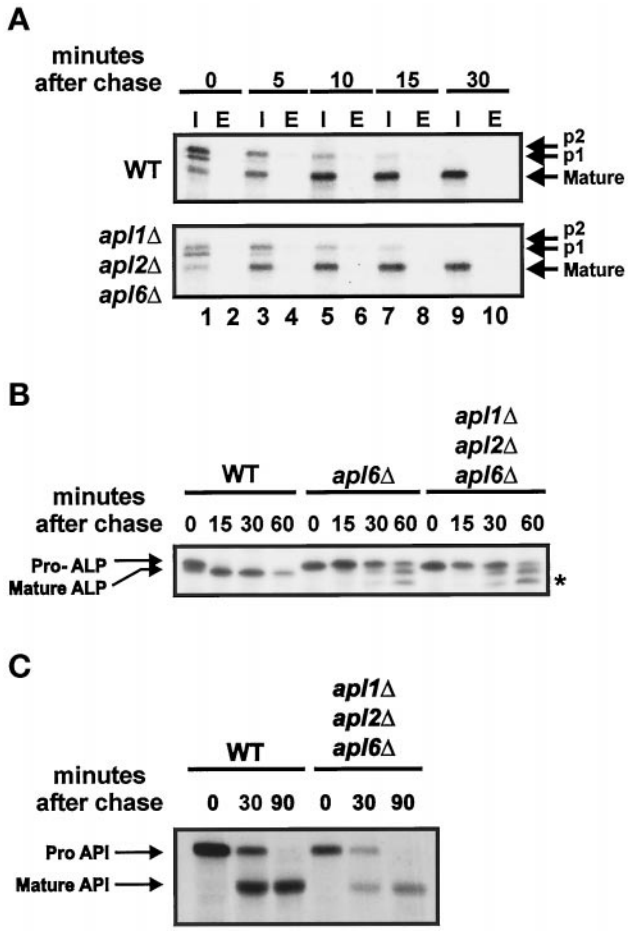

Figure 8. Deletion of the β subunits does not affect transport pathways to the vacuole. (A) CPY transport in wild-type (GPY 6210) and *apl1*Δ *apl2*Δ *apl6*Δ (GPY 1783–21C) cells was analyzed by pulse–chase immunoprecipitation. Cells were grown and labeled at 30°C for 10 min followed by a chase period of 30 min. At the designated times, culture supernatant (E) and cell lysate (I) were prepared, CPY was immunoprecipitated and analyzed by SDS-PAGE. Endoplasmic reticulum-modified (p1), Golgi-modified (p2), and mature (M) forms are indicated. (B) ALP transport in wild-type (GPY6210), *apl6*Δ (1783-25A), and *apl1*Δ *apl2*Δ *apl6*Δ (GPY 1783-21C) cells was analyzed using the pulse–chase regimen described in A. ALP was immunoprecipitated from cell lysates at the designated chase times and analyzed by SDS-PAGE. Precursor and mature forms are indicated as well as a degradation product (*). (C) API maturation in (GPY 6210) and *apl1*Δ *apl2*Δ *apl6*Δ (GPY 1783-21C) cells by pulse–chase immunoprecipitation. API was immunoprecipitated from cell lysates at the designated chase times and analyzed by SDS-PAGE. Precursor and mature forms are indicated.

principal AP complexes. Only β1-containing complexes exhibit physical and genetic interactions with clathrin, yet elimination of all four subunits of the major AP-1 form does not affect growth, clathrin-dependent maturation of α-factor precursor, or assembly of clathrin coats. Cells lacking all three β subunits were subjected to a wide survey of protein trafficking pathways. Except for anticipated defects in AP-3-dependent transport to the vacuole, mutant cells sustained normal levels of pheromone receptor endocytosis, α-factor maturation, vacuolar protein sorting, and clathrin-coated vesicles. We conclude that AP complexes are not obligatory for clathrin-coated vesicle formation and clathrin-mediated protein sorting events in yeast.

Sequence comparisons between yeast and mammalian AP complex subunits indicate that these proteins have been conserved during evolution (Cowles *et al.*, 1997a; Panek *et al.*, 1997). In view of this conservation, as high as 50% amino acid identity, it is surprising that subunit deletions cause trafficking defects solely in the case of AP-3. In earlier studies, which involved single or double subunit deletions, the innocuous consequences of AP-1 and AP-2R mutations could theoretically be attributed to activity of incomplete AP complexes (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). Consistent with this possibility, comparisons of synthetic interactions between single AP-1 subunit deletions and *chc1-ts* indicate that the extent of α-factor maturation defects varies depending on the subunit that is eliminated (Figure 4). We therefore sought to inactivate AP-1 completely by generating a strain lacking the four AP-1 subunits (β1, γ, μ1, and σ1). No defects were detected in this strain. Because Apm2p can associate with β1, at least when overexpressed, a residual contribution of this subunit to clathrin-dependent processes in the *ap1-null* strain might be envisioned. However, even when Apm2p is overexpressed, it cannot functionally replace μ1 in *apm1*Δ *chc1-ts* cells (Stepp *et al.*, 1995). Nor does deletion of *APM2* accentuate defects in *apm1*Δ *chc1-ts* cells (Stepp *et al.*, 1995). These observations, combined with the absence of other AP-1 subunits in the *ap1-null* strain, makes it improbable that Apm2p substitutes in any significant way for AP-1. Given the likelyhood that deletion of β1, γ, μ1, and σ1 abolishes AP-1 activity, the lack of phenotypes in the *ap1-null* strain indicates that AP-1 is not necessary for normal clathrin function.

The relationship of Apm2p to AP-1 remains to be established. A requirement for overexpression to detect reproducible association of Apm2p with β1 suggests that either the Apm2p-containing AP-1-like complex is much less abundant than AP-1, or Apm2p does not normally associate with β1. Two-hybrid interactions between Apm2p and Apl4p(γ) have been observed (Huang and Lemmon, personal communication), favoring the idea that Apm2p is part of an AP-1-like complex. However, the absence of phenotypes associated with disruption of *APM2* in either wild-type, *chc1-ts*, or AP-1 subunit deletion strains (Stepp *et al.*, 1995) leaves the significance of these associations uncertain.

Functional redundancy between AP complexes could obscure a role for AP-1 in clathrin-dependent transport steps in cells expressing wild-type clathrin. However, our studies provide both biochemical and genetic evidence against this idea. In vitro binding assays with GST fusions to the three β subunits showed clathrin binding only to β1. Additionally clathrin was coimmunoprecipitated with AP-1 but not AP-2R or AP-3. These findings suggest that of the three AP complexes, only AP-1 is capable of associating with clathrin. As a genetic test for functional substitution of AP-1 by AP-2R and/or AP-3, genes encoding all three β subunits were deleted. We selected β subunits as targets to disrupt AP function based on our analysis of synthetic interactions between AP-1 subunit deletions and *chc1-ts*, which demonstrate that deletion of β1 is equivalent to deletion of all four

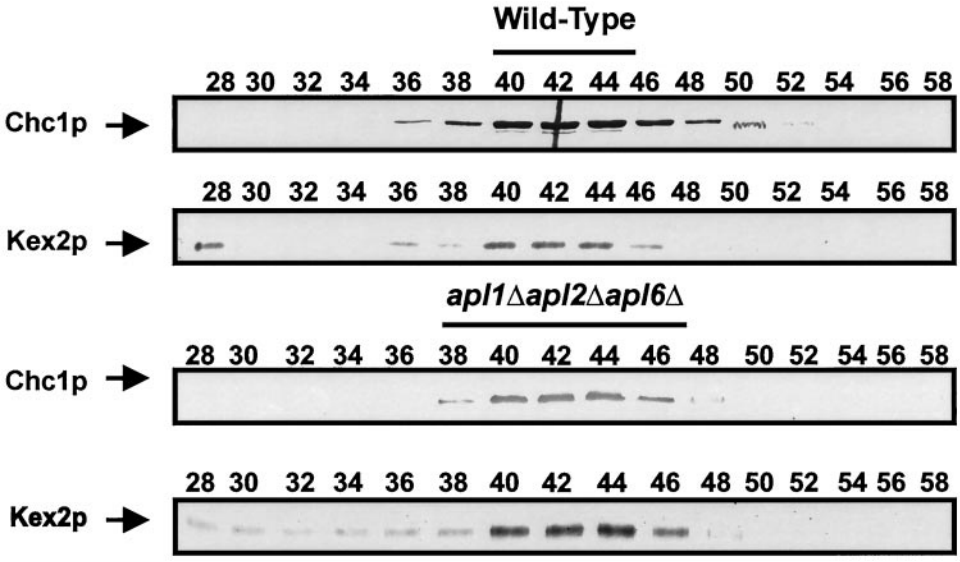

Figure 9. β subunit gene deletions do not affect clathrin-coated vesicles. High-speed pellet fractions from wild-type (SEY 6210) and *apl1*Δ *apl2*Δ *apl6*Δ (GPY 1783–21C) cells were chromatographed through Sephacryl S-1000 and analyzed as described in Figure 5.

AP-1 subunits. In agreement with the importance of β subunits in AP function, mutation of the AP-3 β subunit is effective in blocking the AP-3 pathway (Cowles *et al.*, 1997a; Stepp *et al.*, 1997). However, despite disruption of all three β subunits, we were unable to detect defects in clathrin-dependent trafficking pathways. The concordance of results from both biochemical and genetic approaches prompts us to discount the idea of redundant function between the three AP complexes.

Could there be another, uncharacterized AP complex capable of substituting for AP-1? Analysis of the yeast genome sequence suggests that this possibility is remote. When mammalian or yeast AP subunits are used to search the yeast genome sequence, the most highly related sequences constitute the known set of 13 AP subunits (Cowles *et al.*, 1997a; Panek *et al.*, 1997). Beyond this group, sequence matches are of limited length and marginal statistical significance. Thus, the 13 AP-related proteins probably represent the complete contingent of AP subunits in yeast.

The prevailing paradigm for clathrin coat formation, established primarily through studies of mammalian clathrin, assigns key roles for AP complexes in assembly of the clathrin lattice at appropriate membranes and in cargo collection (Schmid, 1997; Hirst and Robinson, 1998). In contrast to our results, AP subunit mutations in filamentous fungi, nematodes, fruit flies, and mice have readily discernable phenotypes, supporting the central importance of AP complexes in clathrin-mediated protein transport in these organisms (Lee *et al.*, 1994; Keon *et al.*, 1995; González-Gaitán and Jäckle, 1997; Zizioli *et al.*, 1999). If AP complexes are unnecessary for clathrin function in yeast, then it is likely that other factors subserve clathrin assembly and cargo selection functions. Among the expanding list of proteins associated with clathrin coats, there are a number of candidates that could provide appropriate activities. Mammalian neuronal AP180 binds clathrin and stimulates lattice assembly in vitro (McMahon, 1999). Two recently identified yeast homologues of AP180 also interact with clathrin and could be assembly factors (Wendland and Emr, 1998). However, deletion of both yeast AP180-encoding genes together with *apl2* (*β1*) has no deleterious effects on growth, pheromone receptor endocytosis, or α-factor maturation (Yeung, Payne, and Wendland, unpublished results). Similar results have been obtained in analyses of cells lacking the six AP large subunits and the two yeast AP180s (Huang *et al.*, 1999). Other newly discovered clathrin-interacting proteins such as Epsin (Chen *et al.*, 1998) and its yeast homologues Ent1p and Ent2p (Wendland *et al.*, 1999) interact with clathrin and may promote coat assembly. Further genetic analysis of these proteins will be needed to assess their role in clathrin coat assembly. Precedents for cargo collection by proteins other than AP complexes have been established through studies of mammalian cells. In the case of β-adrenergic receptor endocytosis, nonvisual arrestins bind both the receptor and clathrin heavy chain, thereby functioning as adaptors to direct receptors into clathrin-coated vesicles (Goodman *et al.*, 1996, 1997). Although a clear homologue of arrestin has not been identified in yeast, analogous adaptors could exist. Alternatively, there may not be a need for a unique adaptor protein. For example, a peptide containing the endocytosis targeting signal from the low-density lipoprotein receptor interacts with the N-terminal globular domain of clathrin heavy chain (Kibbey *et al.*, 1998), suggesting that clathrin might act directly to collect certain cargo. These examples suggest a diversification of clathrin assembly and cargo collection activities even in mammalian cells, where the importance of AP complexes is well established. Perhaps under the optimal growth conditions used in laboratory experiments, alternatives to AP-1 assume a more significant role in clathrin-mediated transport in yeast.

Our results clarify structural and functional distinctions between yeast AP complexes and offer additional insights into the relationship between yeast and mammalian APs. Previously, the synthetic growth and α-factor maturation defects caused by combination of *chc1-ts* with AP-1 subunit deletions were interpreted as evidence for AP-1 association with clathrin (Phan *et al.*, 1994; Rad *et al.*, 1995; Stepp *et al.*, 1995). The specific physical interaction of clathrin with AP-1

and β1 in vitro now provides more direct evidence that AP-1 is a clathrin-associated complex. Thus, yeast AP-1 mimics mammalian AP-1 in both the primary sequence of subunits and physical interaction of the β subunit with clathrin. Although the consequences of subunit deletion appear to be substantially more severe in animal cells (Zizioli *et al.*, 1999), the genetic and physical interactions between yeast AP-1 and clathrin suggest that the similarity between mammalian and yeast AP-1 extends to a functional level. As proposed above, the more subtle functional contribution of yeast AP-1 may be attributable to the artificial nature of laboratory growth conditions. In contrast to AP-1, yeast AP-3 does not bind clathrin in our assays. This finding is consistent with genetic experiments indicating that AP-3 acts in a clathrin-independent pathway for membrane protein transport from the Golgi apparatus to vacuoles (Vowels and Payne, 1998a). Although mammalian AP-3 resembles its yeast cognate by acting in membrane protein sorting to lysosomes, the relationship to clathrin is less clear. Mammalian β3 interacts with clathrin in vitro, and AP-3 can be colocalized with clathrin coats in vivo (Dell'Angelica *et al.*, 1998). However, AP-3 does not copurify with clathrin-coated vesicles (Simpson *et al.*, 1996, 1997). Resolution of these apparent discrepancies should establish the extent of similarity between yeast and mammalian AP-3 complexes. The third yeast AP complex that we defined, AP-2R, displays the highest primary sequence similarity to mammalian AP-2. However, in other ways AP-2R is clearly distinct from AP-2. Unlike mammalian AP-2, which shares a highly similar clathrin-binding β subunit with AP-1 (84% identity; Kirchhausen *et al.*, 1989), AP-2R contains a β subunit that is only 24% identical to yeast β1 and does not appear to bind to clathrin. Furthermore, mammalian AP-2 associates with endocytic clathrin-coated vesicles, whereas a role for AP-2R in endocytosis has not been detected, nor have we observed synthetic interactions between AP-2R subunit deletions and *chc1-ts*. Identification of a role for yeast AP-2R awaits additional experiments.

In summary, the first comprehensive description of AP complexes in a single organism is now emerging from studies of *S. cerevisiae*. Three major, functionally distinct complexes have been described: AP-1, and perhaps an alternative form with a different medium subunit, acts in a clathrin-dependent protein sorting pathway from the TGN; AP-2R probably acts in a clathrin-independent pathway, but the identity of this pathway has not been uncovered; and AP-3 acts in clathrin-independent traffic of membrane proteins from the Golgi apparatus to vacuoles. Elimination of AP function results in AP-3 pathway defects but otherwise appears to be insignificant for clathrin-dependent events. Our results imply the existence of factors other than AP complexes, which play central roles in clathrin coat assembly and cargo selection.

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