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Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast

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The major coat proteins of clathrin-coated vesicles are the clathrin triskelion and heterotetrameric associated protein (AP) complexes. The APs are thought to be involved in cargo capture and recruitment of clathrin to the membrane during endocytosis and sorting in the *trans*-Golgi network/endosomal system. AP180 is an abundant coat protein in brain clathrin-coated vesicles, and it has potent clathrin assembly activity. In *Saccharomyces cerevisiae*, there are 13 genes encoding homologs of heterotetrameric AP subunits and two genes encoding AP180-related proteins. To test the model that clathrin function is dependent on the heterotetrameric APs and/or AP180 homologs, yeast strains containing multiple disruptions in AP subunit genes, as well as in the two *YAP180* genes, were constructed. Surprisingly, the AP deletion strains did not display the phenotypes associated with clathrin deficiency, including slowed growth and endocytosis, defective late Golgi protein retention and impaired cytosol to vacuole/autophagy function. Clathrin-coated vesicles isolated from multiple AP deletion mutants were morphologically indistinguishable from those from wild-type cells. These results indicate that clathrin function and recruitment onto membranes are not dependent upon heterotetrameric adaptors or AP180 homologs in yeast. Therefore, alternative mechanisms for clathrin assembly and coated vesicle formation, as well as the role of AP complexes and AP180-related proteins in these processes, must be considered.

Keywords: adaptors/AP180/autophagy/clathrin/cytosol to vacuole transport/yeast

Introduction

The movement of membrane and proteins through the secretory and endocytic pathways is facilitated by transport vesicles. The formation of these vesicles and collection of vesicle cargo are mediated by coat proteins that are recruited from the cytosol to the membrane. The best characterized coat protein complexes are clathrin and its associated proteins (APs) or adaptors, which are involved in endocytosis at the plasma membrane (PM) and in sorting or retention at the *trans*-Golgi network (TGN) (for reviews see Schmid, 1997; Hirst and Robinson, 1998). Soluble clathrin exists as triskelions containing three heavy chains (HCs) trimerized at their C-termini and three light chains (LCs), one non-covalently bound to each HC. The two major AP complexes, AP-1 (TGN sorting) and AP-2 (PM/endocytosis), are heterotetramers comprised of two ~100 kDa large chains (β1 and γ in AP-1; β2 and α in AP-2), an ~50 kDa medium chain (μ1 and μ2) and an ~20 kDa small chain (α1 and σ2). The AP subunits assemble into a complex with a brick-like morphology containing two protruding ears or appendages corresponding to the C-terminal regions of the large chains. There is also an AP-3 complex that shares structural similarities with AP-1 and AP-2 (Dell'Angelica *et al*., 1997; Simpson *et al*., 1997). In animal cells, AP-3 is involved in the formation of and/or targeting to lysosomes and specialized lysosome-like organelles (e.g. melanosomes and platelet granules) (Ooi *et al*., 1997; Simpson *et al*., 1997; Kantheti *et al*., 1998; Dell'Angelica *et al*., 1999; Feng *et al*., 1999). *In vitro* studies suggest that it also plays a role in the recycling of synaptic vesicles from endosomes (Faundez *et al*., 1998). Although AP-3 interacts with clathrin (Dell'Angelica *et al*., 1998), some studies suggest that this adaptor may also function in clathrin-independent vesicle budding (Simpson *et al*., 1997; Faundez *et al*., 1998).

According to conventional models, the AP complexes, AP-1 and AP-2, are central to the formation of clathrin-coated vesicles (Schmid, 1997; Hirst and Robinson, 1998). They are thought to mediate both vesicle content selection and recruitment of clathrin onto membranes. In this model, APs first bind to membranes, in part by interacting with cytoplasmic domains of proteins destined as cargo in clathrin-coated vesicles. Soluble triskelions then assemble onto membrane-associated APs, leading to invagination and vesicle budding. A number of lines of evidence have led to this model. First, AP-1 and AP-2 were isolated originally as major components of brain clathrin-coated vesicles (Keen *et al*., 1979) and were shown to stimulate the *in vitro* assembly of clathrin into cages (Zaremba and Keen, 1983). Electron microscopy of clathrin-coated vesicles and assembled lattices finds APs situated between the membrane and clathrin with the HC N-terminal domain pointing inward toward the adaptors (Vigers *et al*., 1986; Heuser and Keen, 1988; Smith *et al*., 1998). The AP β-chain ear/hinge region appears to contain the primary binding site for the interaction with the HC terminal domain (Shih *et al*., 1995; Dell'Angelica *et al*., 1998). In addition, AP-1 and AP-2 co-localize with clathrin in animal cells, and *in vitro* studies support their role in promoting clathrin assembly onto membranes (Peeler *et al*., 1993; Traub *et al*., 1996; Zhu *et al*., 1998). Finally, APs can bind directly to tyrosine or di-leucine-based sorting motifs in several membrane proteins transported via clathrin-coated vesicles, supporting the role for the APs in cargo capture and bridging of cargo to clathrin

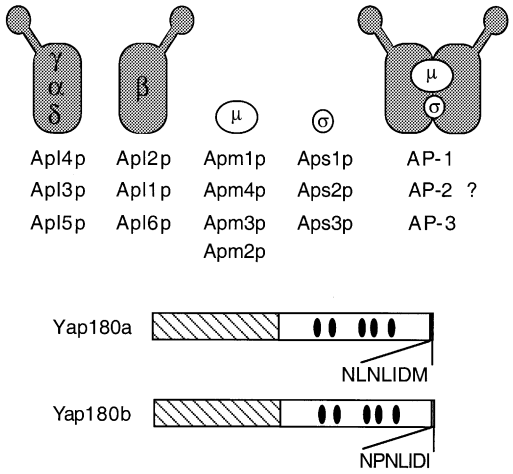

Fig. 1. Schematic of the yeast heterotetrameric AP complex subunits and the two Yap180 proteins. There are enough large (β, α, γ and δ), medium (μ) and small (σ) chains to assemble three heterotetrameric AP complexes, with one extra μ chain (Apm2p). Biochemical and/or genetic data support the grouping of the indicated subunits into AP-1 and AP-3 complexes. The grouping for AP-2 is based on homologies to the known animal AP-2 sequences. The region of Yap180 most related to animal homologs is indicated by hatched areas. The proposed clathrin-binding domains and location of NPF sequence motifs (black ovals) within the Yap180s are also indicated. See text for details.

(for reviews see Marks *et al*., 1997; Le Borgne and Hoflack, 1998, and references therein).

Although the heterotetrameric APs are thought to be most crucial for clathrin recruitment to membranes, the single chain, neuron-specific polypeptide AP180 also binds clathrin, co-purifies with brain clathrin-coated vesicles and promotes clathrin assembly *in vitro* (Ahle and Ungewickell, 1986; Kohtz and Puszkin, 1988; Murphy *et al*., 1991; Morris *et al*., 1993; Ye and Lafer, 1995). AP180 localizes with clathrin coats in synaptic nerve terminals (Maycox *et al*., 1992; Takei *et al*., 1996), and a mutation in a *Drosophila* homolog of AP180 perturbs synaptic vesicle recycling and the normal localization of clathrin in synaptic terminals (Zhang *et al*., 1998). A ubiquitously expressed human homolog of AP180, CALM, has been described (Dreyling *et al*., 1996), suggesting that non-neuronal AP180-like proteins may also promote membrane association of clathrin.

Our laboratory and others have been studying clathrin-mediated vesicular transport in the yeast *Saccharomyces cerevisiae*. Disruption of the yeast clathrin HC gene, *CHC1*, or the LC gene, *CLC1*, results in several distinct phenotypes including poor growth, temperature sensitivity, slowed endocytosis and missorting of Golgi resident proteins to the plasma membrane (Lemmon and Jones, 1987; Silveira *et al*., 1990; Seeger and Payne, 1992b; Tan *et al*., 1993; Graham *et al*., 1994; Chu *et al*., 1996; Huang *et al*., 1997). Yeast also contains genes encoding 13 heterotetrameric AP-related subunits: six large chains (α, γ, δ and three βs), four μ chains and three σ chains (Phan *et al*., 1994; Rad *et al*., 1995; Stepp *et al*., 1995; Panek *et al*., 1997; Saccharomyces Genome Database, Stanford University). These are enough subunits to form three AP complexes, with one extra μ chain (Figure 1). There are two proteins related to AP180, encoded by *YAP1801* and *YAP1802* (Wendland and Emr, 1998). The products of these genes, Yap180a and Yap180b, are 43% identical to one another and are significantly similar to the N-terminal regions of animal AP180-related proteins. Moreover, both bind Chc1p through sequences in the C-terminal region of the proteins (Wendland and Emr, 1998).

Several studies have allowed for the grouping of a number of the yeast heterotetrameric AP subunits into specific AP-like complexes (Figure 1). The disruption of *APL2*, *APL4*, *APM1* or *APS1* in cells carrying a temperature-sensitive allele of *CHC1* (*chc1-ts*) exaggerates the growth and Golgi protein retention defects of the clathrin mutants, but does not affect endocytosis (Phan *et al*., 1994; Rad *et al*., 1995; Stepp *et al*., 1995; H.Phan, B.Young and G.Payne, personal communication). Complex formation (H.Phan, B.Young and G.Payne, personal communication) and sequence homologies also suggest that these subunits function in an AP-1-like complex at the Golgi. Biochemical and genetic evidence has shown that the yeast AP-3 complex is comprised of Apl6p, Apl5p, Apm3p and Aps3p (Panek *et al*., 1997). Yeast AP-3 is required for the efficient delivery of alkaline phosphatase (ALP) from the Golgi to the vacuole by a pathway that is distinct from the well studied vacuolar protein sorting (Vps) pathway (Cowles *et al*., 1997; Stepp *et al*., 1997). Mutations in AP-3 also partially suppress the growth and Ste3p internalization defects caused by yeast plasma membrane casein kinase 1 deficiency (Panek *et al*., 1997). Disruption of any of the AP-3 component genes does not exaggerate the clathrin-minus phenotypes of *chc1-ts* strains (Panek *et al*., 1997), nor do clathrin mutants missort ALP (Vowels and Payne, 1998), indicating that the yeast AP-3 complex has clathrin-independent functions. Although Apl1p, Apl3p, Apm4p and Aps2p are most similar to AP-2 subunits (β2, α, μ2 and σ2, respectively), there is no endocytosis defect associated with deletions in these subunits (Phan *et al*., 1994; Rad *et al*., 1995; S.K.Lemmon, unpublished) and there have been no studies confirming complex formation or localization of these proteins to the plasma membrane. Finally, it is not known whether the extra μ chain (Apm2p) shares subunits with other AP complexes, but it is found in a high molecular weight complex (Stepp *et al*., 1995).

Although there is information on the function of some of the yeast heterotetrameric AP subunit proteins, deletion of any single gene does not result in clathrin-minus phenotypes (Phan *et al*., 1994; Rad *et al*., 1995; Stepp *et al*., 1995; Panek *et al*., 1997). This indicates that clathrin function is not dependent on any one AP complex and there may be functional overlap of the adaptors or AP subunits. Also, *YAP180* mutants appear relatively normal (Wendland and Emr, 1998). To explore fully the requirement of APs for clathrin function, we constructed strains carrying simultaneous disruptions in several AP genes with the goal of creating cells that have no functional heterotetrameric AP complexes or yeast AP180-related proteins. We found that these AP deletion strains did not display the major phenotypes associated with clathrin deficiency. In addition, clathrin-coated vesicles were isolated from the multiple AP deletion mutants and these coated vesicles were morphologically indistinguishable from coated vesicles isolated from wild-type cells. These results indicate that clathrin can function without adaptors and AP180-related proteins, and can assemble onto mem-

branes in the absence of these proteins. This suggests the existence of alternative mechanisms to recruit clathrin to membranes.

Results

To disrupt the function of all the yeast heterotetrameric AP complexes and/or Yap180 proteins in a single yeast cell, strains were constructed that carry simultaneous disruptions in multiple AP subunit genes. Four types of AP deletion strains were generated for this study. In the first, referred to as *apl*-Δ, all six heterotetrameric AP large chain genes were disrupted. This removes the β chains, which contain the primary clathrin-binding/assembly subunits of the AP complexes (Gallusser and Kirchhausen, 1993; Shih *et al*., 1995; Dell'Angelica *et al*., 1998) as well as the α/γ/δ chains, which, based on studies in animal cells, might interact with other accessory proteins during the coated vesicle cycle (see Hirst and Robinson, 1998, and references therein). There is also some evidence that the α-adaptin chain can bind clathrin, although it cannot promote clathrin assembly *in vitro* (Goodman and Keen, 1995). Thus, in the *apl*-Δ strain, there would be very little possibility for formation of partially functional AP complexes with clathrin recruitment activity because only the σ and μ chains remain.

The second type of strain, *apm*-Δ, had all four μ chains deleted. Yeast contains four μ chain homologs, but only enough large and σ chains for three complete AP complexes. We do not know if Apm2p, which is probably the extra μ chain, shares large and σ subunits with any of the other μ chains. Instead, it might exist in a novel complex that could compensate for the loss of the other heterotetrameric APs. We anticipated that removing all four μ chains would still result in non-functioning AP complexes, because disruption of the AP-1 or AP-3 medium chain genes alone results in identical phenotypes to those caused by the disruption of single large chain genes of yeast AP-1 or AP-3 (Stepp *et al*., 1995; Cowles *et al*., 1997; Panek *et al*., 1997; Stepp *et al*., 1997).

In addition to the β chains of the heterotetrameric AP complexes, mammalian AP180 proteins have clathrin coat assembly activity *in vitro* (Ahle and Ungewickell, 1986; Ye and Lafer, 1995) and the yeast AP180 homologs bind to Chc1p (Wendland and Emr, 1998). Therefore, we constructed strains with disruptions of both *YAP1801* and *YAP1802* (*yap180*-Δ), and another where the two *YAP180* genes and the three AP large chain β-subunit genes (*yap180*-Δ *apl*β-Δ) were deleted. We note that strains containing only the three β chain null mutations (*apl*β-Δ) were also made, and these behaved identically to the *apm*-Δ, *apl*-Δ and the *yap180*-Δ *apl*β-Δ strains (data not shown).

All of the AP deletion strains were then tested for phenotypes associated with clathrin deficiency. Since clathrin-minus strains grow slowly and display temperature sensitivity, we first examined the growth characteristics of AP deletion strains at different temperatures. As shown in Figure 2, all of the AP deletion strains grew well at all temperatures, similarly to wild-type strains. In addition, light microscopy showed that the AP deletion mutants have normal cell morphology, in contrast to clathrin-deficient cells which are enlarged, rounded and multivesiculated (not shown).

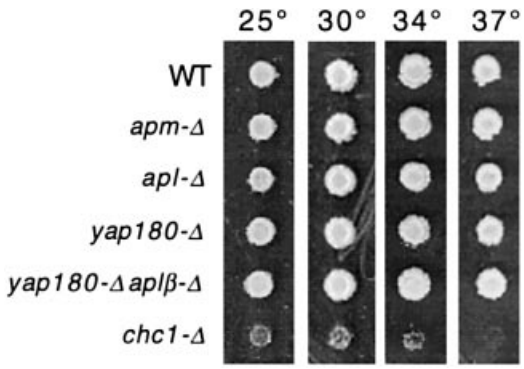

Fig. 2. AP deletion strains grow like wild-type cells. Mid-log phase cells were diluted to 10^7 cells/ml, spotted onto YEPD and grown for 2 days at the indicated temperatures. The strains examined were: SL1463 (WT), SL3106 (*apm*-Δ), SL3478 (*apl*-Δ), SL3429 (*yap180*-Δ), SL3436 (*yap180*-Δ *apl*β-Δ) and BJ3255 (*chc1*-Δ).

Late Golgi retention and vacuolar protein sorting in AP mutants

Clathrin-deficient strains are defective in the retention of proteins in the late Golgi (Seeger and Payne, 1992b). For example, the pro-α-factor-processing enzymes Kex2p, Kex1p and DPAP-A, which normally reside in the late Golgi, are mislocalized to the plasma membrane in clathrin-minus cells. As a result, α-factor precursor is not processed efficiently and a high molecular weight form of pro-α-factor is secreted instead of the 13 amino acid mature product. We examined the form of α-factor secreted by AP deletion strains to determine if these strains also had Golgi protein retention defects. As shown in Figure 3, *chc1*-Δ strains secreted >90% of their α-factor in the precursor form, reflecting the mislocalization of α-factor-processing enzymes. In contrast, wild-type and AP deletion strains secreted almost exclusively mature α-factor. Precursor α-factor was detected occasionally in some of the AP mutant cell lines (compare *apm*-Δ with *yap180*-Δ *apl*β-Δ), but the amounts were always a small percentage of the total α-factor secreted (1–10%, average of 2–4 experiments). Similar strain variability in the degree of α-factor processing has been reported previously (Stepp *et al*., 1995). These results indicate that α-factor-processing enzymes are retained efficiently in the late Golgi in the absence of heterotetrameric adaptors and/or Yap180 proteins.

There are at least two protein sorting routes from the Golgi to the vacuole (Conibear and Stevens, 1998). While most soluble vacuolar hydrolases, such as CPY, and several vacuolar membrane proteins follow the Vps pathway, the vacuolar membrane proteins ALP and Vam3p, a t-SNARE, are transported through an alternative route that requires a functional AP-3 complex (Cowles *et al*., 1997; Stepp *et al*., 1997; Darsow *et al*., 1998). In strains lacking AP-3, ALP and Vam3p are rerouted slowly through the Vps pathway. This phenotype is observed by the presence of accumulated precursor ALP (pALP) at steady state in AP-3-minus cells. Although clathrin-deficient yeast do not display an ALP sorting defect (Vowels and Payne, 1998), it was not clear if the rerouting of ALP through the Vps pathway and its

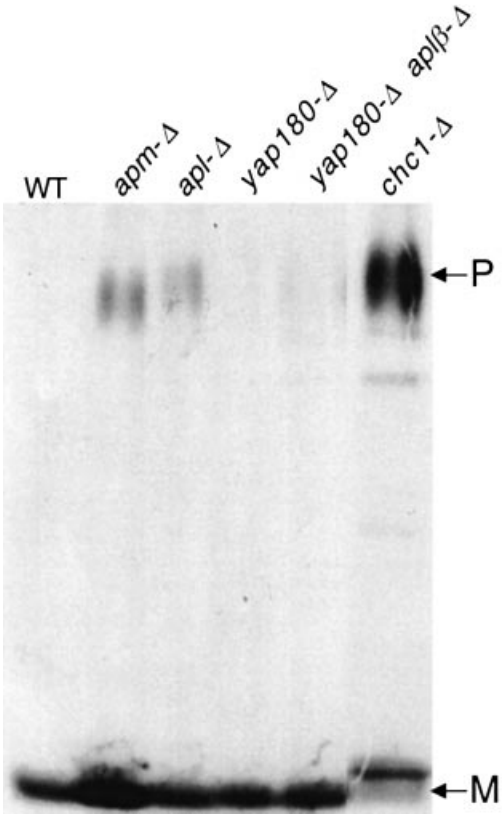

Fig. 3. AP deletion strains secrete mostly mature α-factor. Cells were ^{35}S-labeled for 30 min, and α-factor was immunoprecipitated from the medium for analysis by SDS–PAGE and autoradiography. The positions of the high molecular weight precursor (P) and mature (M) forms of α-factor are indicated. The band above the mature form, most visible in the *chc1*-Δ lane, is also an α-factor precursor form. The strains analyzed were: SL1463 (WT), SL2490 (*apm*-Δ), SL3361 (*apl*-Δ), SL3483 (*yap180*-Δ), SL3445 (*yap180*-Δ *apl*β-Δ) and BJ3255 (*chc1*-Δ).

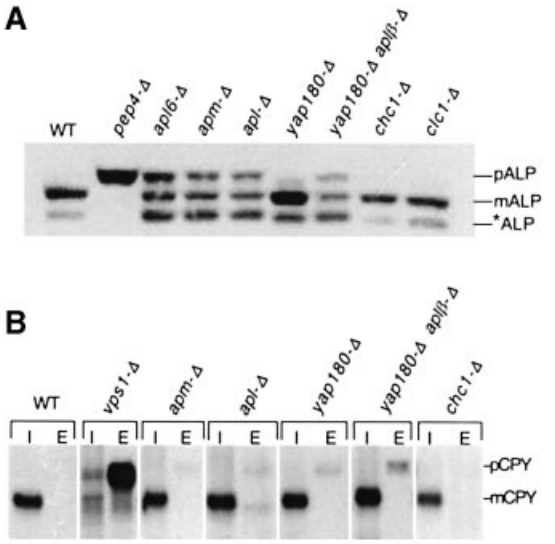

Fig. 4. (**A**) Steady-state forms of ALP in AP deletion strains. Cells were lysed and prepared for immunoblotting with affinity-purified ALP antibodies. The positions of precursor ALP (pALP), mature ALP (mALP) and soluble lumenal ALP (*ALP) are indicated. The strains examined were: SL1463 (WT), BJ3502 (*pep4*-Δ), SL3252 (*apl6*-Δ), SL3106 (*apm*-Δ), SL3478 (*apl*-Δ), SL3429 (*yap180*-Δ) and SL3436 (*yap180*-Δ *apl*β-Δ). (**B**) CPY is transported to the vacuole in AP deletion strains. Cells were ^{35}S-pulse-labeled for 10 min and chased for 30 min. CPY was immunoprecipitated from internal (I) and external fractions (E) and analyzed by SDS–gel electrophoresis and autoradiography. The strains examined were: SL1463 (WT), JHRY20-2Ca (*vps1*-Δ), SL3106 (*apm*-Δ), SL3478 (*apl*-Δ), SL3428 (*yap180*-Δ) and SL3436 (*yap180*-Δ *apl*β-Δ).

subsequent delivery to the vacuole would require the other heterotetrameric AP complexes or Yap180 proteins. Therefore, we monitored ALP processing in AP deletion mutants by immunoblotting. In wild-type cells (Figure 4), ALP exists primarily as mALP along with a small amount of soluble, lumenal *ALP. Clathrin-deficient and *yap180*-Δ strains also processed ALP normally. As expected, the AP-3-deficient *apl6*-Δ strain accumulated pALP along with mALP and *ALP. Similar amounts of pALP were detected in all of the multiple AP disruption strains containing an AP-3 mutation. This indicates that mutations in any of the other heterotetrameric AP complexes or *YAP180* genes do not enhance or suppress the AP-3 mutant phenotype.

Sorting and processing of CPY was also examined in the AP deletion strains. Newly synthesized CPY is glycosylated in the endoplasmic reticulum (p1CPY), its oligosaccharides are modified in the Golgi (p2CPY) and the protein is then delivered to the vacuole where it is proteolytically matured (mCPY). CPY processing and sorting defects have been reported for some *chc1* mutant alleles, although CPY sorting is normal in *chc1*-Δ strains (Seeger and Payne, 1992a; Chen and Graham, 1998). In a *chc1-ts* strain, p2CPY is secreted at early times after shift to 37°C, but over time the sorting defect disappears, presumably because the cells can up-regulate or begin to use an alternative pathway (Seeger and Payne, 1992a). Interestingly, a mutation in AP-1 partially suppresses the early *chc1-ts* CPY sorting defect (Rad *et al.*, 1995). This suggests that the alternative pathway is already up and running in the AP-1 mutant and that AP-1 functions in sorting to the vacuole.

Pulse–chase analysis showed that CPY is also matured efficiently and retained intracellularly in the AP deletion strains (Figure 4). Very small amounts of secreted p2CPY were sometimes observed in AP mutants and *chc1*-Δ cells, but this was minor compared with *vps1*-Δ cells where >90% of CPY was secreted as p2CPY. Since a major CPY sorting defect was not unmasked in the multiple AP mutants, this indicates that the other APs are not substituting for AP-1, nor is it likely that they are required for the postulated alternative pathway.

Endocytosis in AP deletions strains

Endocytosis in AP deletion strains was monitored by assessing the uptake of ^{35}S-labeled α-factor. α-factor binds to the Ste2p receptor on *MAT***a** cells. This binding initiates the mating signal transduction cascade and stimulates the internalization and subsequent degradation of the receptor–pheromone complex in the vacuole (Riezman, 1998). In wild-type cells, endocytosis of pre-bound α-factor was nearly complete within 15 min as compared to clathrin-deficient cells, which displayed a 2- to 3-fold reduction in their rates of α-factor uptake (Figure 5). In contrast, the initial rates of endocytosis in all of the AP deletion strains were very similar to wild-type, indicating that the internalization step was not perturbed in the absence of APs complexes and Yap180s. Interestingly, in all of the

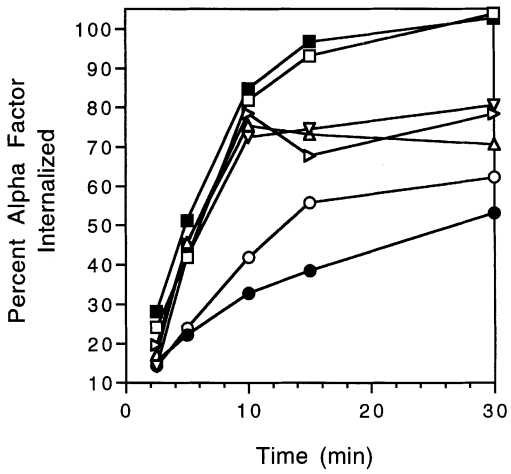

Fig. 5. The initial rates of endocytosis are normal in AP deletion strains. Radiolabeled α-factor was pre-bound to cells at 4°C. Cells were pelleted and then endocytosis was initiated by resuspension in pre-warmed YEPD at 30°C. Samples were harvested at the times indicated and the amount of internalized radiolabeled α-factor was determined. The strains shown are: WT, SL1463 (□); *yap180*-Δ, SL3428 (■); *apm*-Δ, SL3106 (▽); *apl*-Δ, SL3478 (△); *yap180*-Δ *apl*β-Δ, SL3436 (▷); *chc1*-Δ, SL3593 (○); and *clc1*-Δ, SL1929 (●).

strains that were depleted of multiple heterotetrameric adaptor subunits, the α-factor uptake leveled off between 10 and 15 min (Figure 5) and only slowly reached 100% after 60 min (data not shown). At 30 min, only ~75% of the pre-bound α-factor was internalized in adaptor deletion strains compared with ~100% in wild-type cells. The reason for this plateau effect is unknown, although an interesting possibility is that this represents recycling of α-factor/Ste2p back to the cell surface. The *yap180*-Δ strain showed normal endocytosis, consistent with a previous report (Wendland and Emr, 1998), and loss of Yap180 proteins did not affect the uptake in cells with mutations in the heterotetrameric adaptor complexes.

Previous studies showed that loss of AP-3 function could suppress the endocytic defect of the a-factor receptor, Ste3p, caused by casein kinase I deficiency (Panek *et al*., 1997). Such a suppression might mask an internalization defect associated with loss of the other AP complexes. However, we found that a strain with mutations in *apm1*-Δ, *apm2*-Δ and *apm4*-Δ, but containing the normal AP-3 μ chain gene, *APM3*, still exhibited efficient endocytosis (not shown). Overall, these studies show that cells with multiple adaptor mutations endocytose quite well compared with clathrin-deficient strains.

Cvt transport is defective in clathrin-deficient yeast but not in AP mutants

The cytoplasm-to-vacuole targeting pathway (Cvt) is a biosynthetic process that targets at least one resident hydrolase, aminopeptidase I (API) to the vacuole (Klionsky, 1998). Unlike most other characterized vacuolar hydrolases, API is not transported through the secretory pathway and delivered to the vacuole, but instead moves directly from the cytosol into the vacuole. Several yeast mutants defective in the Cvt pathway are also defective in autophagy, indicating that these pathways share common components (Klionsky, 1998). API is synthesized as a precursor protein (pAPI; 61 kDa) and is

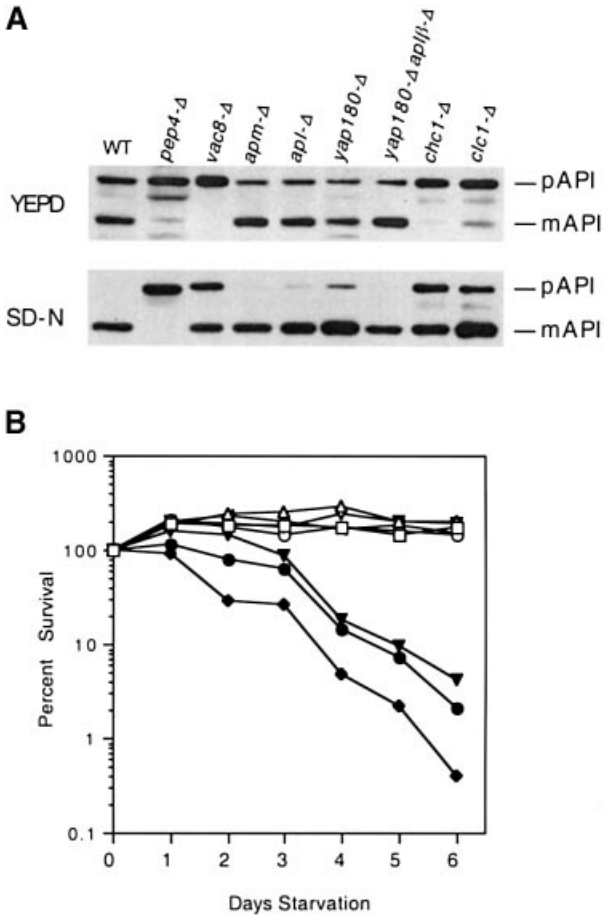

Fig. 6. (**A**) Clathrin-deficient strains, but not AP deletion strains, are defective in cytoplasm to vacuole transport of API. Mid-log phase cells were grown for 4 h in YEPD or SD-N, and cell lysates were prepared and processed for immunoblotting with anti-API antibody. The positions of precursor API (pAPI) and mature API (mAPI) are indicated. (**B**) Clathrin-deficient strains, but not AP mutants, are sensitive to nitrogen starvation. Mid-log phase cells growing in YEPD were transferred to SD-N. At the indicated times, cell aliquots were removed, diluted and plated on YEPD. The percentage survival was determined by counting the number of viable colonies from duplicate samples and dividing by the average number of colonies obtained at day 0. The strains examined in both (A) and (B) were: WT, SL1463 (□); *pep4*-Δ, BJ3502 (▼); *apm*-Δ, SL3106 (△); *apl*-Δ, SL3478 (▽); *yap180*-Δ, SL3428 (○); *yap180*-Δ *apl*β-Δ, SL3436 (◁); *chc1*-Δ, SL3593 (■); *clc1*-Δ, SL1929 (●). Analysis of LWY2887 (*vac8*-Δ) is only shown in (A).

processed proteolytically in a Pep4p-dependent manner to a mature form (mAPI; 50 kDa) upon delivery to the vacuole. For wild-type cells growing in rich medium, the Cvt pathway ensures delivery of API to the vacuole; however, after nitrogen starvation, the autophagy machinery is up-regulated, which can aid in the transport of API into the vacuole in mutants that are defective in the Cvt pathway.

To examine the Cvt pathway in AP deletion and clathrin mutants, cells were grown in either YEPD or nitrogen starvation medium (SD-N) and extracts were analyzed by immunoblotting with anti-API antibodies (Figure 6A). In wild-type cells grown in YEPD, approximately equal amounts of mAPI and pAPI were evident, and growth in SD-N resulted in complete processing of pAPI to mAPI. In contrast, clathrin-minus strains (*chc1*-Δ and *clc1*-Δ) displayed a large decrease in mAPI under both growth

conditions. The API processing defect of clathrin-deficient strains was similar to that of *vac8*-Δ cells which previously have been shown to process API more slowly than wild-type cells (Wang *et al*., 1996). Since both CPY and ALP are proteolytically matured in clathrin-deficient strains, it is unlikely that the unprocessed API in these cells is due to a vacuolar hydrolase deficiency. The processing of API was normal in each of the multiple AP deletion strains, indicating that Cvt/autophagy function is intact in these cells.

Yeast mutants that are defective in autophagy are unable to survive nitrogen starvation because they cannot utilize autophagy to scavenge nutrients from endogenous proteins (Klionsky, 1998). Therefore, clathrin and AP-deficient strains were examined for their ability to recover growth after nitrogen starvation (Figure 6B). Clathrin-deficient cells were very sensitive to nitrogen starvation and rapidly lost viability after only a few days growth under starvation conditions. Similar sensitivities to nitrogen starvation were observed in a *pep4*-Δ strain, as described previously (Lang *et al*., 1998). In contrast, wild-type and AP deletion strains survived nitrogen starvation. The resistance to nitrogen starvation was also reflected by the ability of AP-deficient homozygous diploids to sporulate, while clathrin mutants cannot (data not shown). These results suggest that clathrin has a role in the Cvt and/or autophagy pathways but functional heterotetrameric adaptors and/or Yap180 proteins are not required. Further studies will be required to elucidate the step affected in clathrin-deficient yeast.

AP-deficient strains contain clathrin-coated vesicles

The phenotypic analysis of AP deletion strains indicates that clathrin can function in the absence of AP molecules and suggests that, despite the absence of APs, clathrin can assemble efficiently onto membranes to mediate vesicle budding. To investigate this further, we attempted to isolate clathrin-coated vesicles from AP deletion strains. Cells were converted to spheroplasts and lysed for differential centrifugation, after which the 100 000 *g* microsomal pellet was resuspended and fractionated on a Sephacryl S-1000 column (Lemmon *et al*., 1988). Column fractions were then analyzed by SDS–gel electrophoresis and Coomassie Brilliant Blue staining. The position of Chc1p was confirmed by immunoblot analysis (data not shown). We found that the elution profile of clathrin was identical in samples isolated from wild-type, *apm*-Δ, *apl*-Δ and *yap180*-Δ *apl*β-Δ cells (Figure 7 and data not shown). In all cases, clathrin HC peaked in fractions 39–41 and was the major protein species seen by Coomassie Blue staining. Silver staining of column fractions revealed that some minor polypeptides co-fractionated with clathrin, but the profile of these proteins was identical in wild-type and mutant preparations (Figure 7B, data not shown). This indicates that APs are not major components of clathrin-coated vesicles in yeast.

This peak of clathrin could represent coated vesicles and/or empty cages reassembled from soluble clathrin during purification. To confirm that there were clathrin-coated vesicles present, fractions 39 and 40 were pooled, fixed and processed for examination by electron microscopy as described in Materials and methods. Figure 8 shows the results from the wild-type and *yap180*-Δ *apl*β-Δ

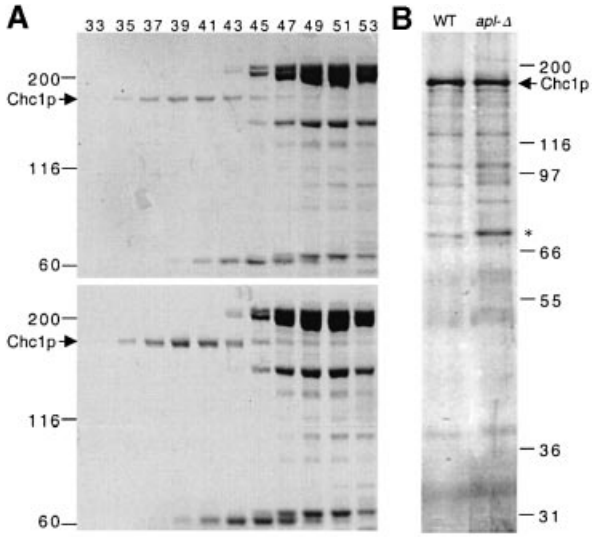

Fig. 7. (**A**) The gel filtration profile of clathrin-coated vesicles from AP deletion strains is identical to that of wild-type cells. Cells were converted to spheroplasts and osmotically lysed for differential centrifugation. The 100 000 *g* membrane pellet was resuspended and fractionated on a Sephacryl S-1000 column. Every other fraction was analyzed by SDS–PAGE and gels were stained with Coomassie Blue. The identity of Chc1p was confirmed by immunoblotting. Strains shown are wild-type (SL1463, upper panel) and *yap180*-Δ *apl*β-Δ (SL3436, lower panel). Identical results were obtained with *apm*-Δ and *apl*-Δ strains (not shown). (**B**) A similar pattern of minor proteins co-fractionate with clathrin in coated vesicle preparations from wild-type and AP mutant strains. SDS–gels of coated vesicles from wild-type and mutants were silver stained to reveal minor polypeptides. Shown are peak fractions (No. 39) from wild-type and *apl*-Δ strains. Note, the ~70 kDa band (*) peaks later in the column [see (A)]. Similar results were obtained for the *yap180*-Δ *apl*β-Δ mutant.

strains. Similar results were obtained for other multiple AP mutants. In all cases, many profiles exhibiting a clathrin coat structure were evident. Often a membrane was discernible beneath the coat, and these vesicles tended to have a dense core noted by a dark interior. Although the numbers varied in different sections and fields, the vesicles with coats typically represented 30% or more of the 70–80 nm coated structures. Therefore, these fractions contained large numbers of authentic clathrin-coated vesicles, not merely empty lattices. These experiments support our phenotypic analyses that clathrin can assemble onto membranes and carry out its functions in the absence of heterotetrameric adaptors and Yap180 proteins.

Discussion

Numerous studies have led to the prevailing model that heterotetrameric AP complexes have two functions (reviewed in Schmid, 1997; Hirst and Robinson, 1998). First, they play a role in the capture of transmembrane proteins into coated pits, at least in part by interacting with cytoplasmic domains of membrane proteins. Secondly, these complexes stimulate the association of clathrin with membranes, thus mediating the clustering of cargo with clathrin-coated vesicles. The work presented here demonstrates that heterotetrameric APs are not required for clathrin recruitment onto membranes in yeast. We found that yeast cells formed clathrin-coated vesicles despite the absence of adaptor complexes. Membrane recruitment of clathrin in the absence of AP complexes

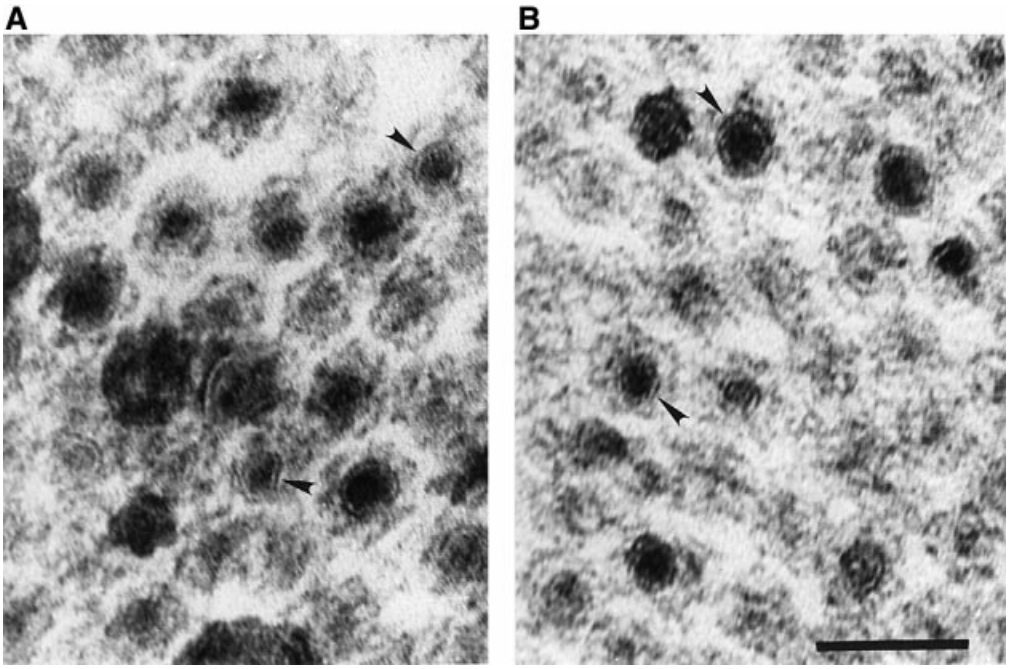

Fig. 8. Electron microscopy of isolated clathrin-coated vesicles from wild-type and AP deletion strains. Clathrin-coated vesicles were prepared as shown in Figure 7. Fractions 39 and 40 from the S-1000 column were pooled and processed for electron microscopy as described in Materials and methods. Results are shown for (**A**) wild-type (SL1463) and (**B**) *yap180*-Δ *apl*β-Δ (SL3436). Identical results were obtained for *apm*-Δ and *apl*-Δ strains (data not shown). Clathrin-coated vesicles of homogeneous size (70–80 nm diameter) were found routinely in wild-type and AP deletion samples. Arrows indicate examples of membranes beneath the clathrin coats. Bar = 0.1 μm.

was not due to yeast AP180-related proteins because clathrin-coated vesicles were also recovered from strains defective for both Yap180 and heterotetrameric adaptors. We also showed that AP deletion strains do not display the distinct phenotypes of clathrin-deficient cells, namely poor growth, mislocalization of Golgi resident proteins and slowed endocytosis of the α-factor receptor. Furthermore, this work revealed that clathrin-deficient strains are defective in the autophagy/Cvt pathways, a phenotype that also is not shared by the AP deletion strains. Taken together, these results show that in the absence of heterotetrameric APs and Yap180s, not only is clathrin able to associate with membranes, but clathrin-mediated transport remains intact.

Although our data show that APs are not required for clathrin recruitment onto membranes, heterotetrameric APs probably play a role in binding to and directing specific receptors to clathrin-coated pits under normal conditions. One of the main reasons APs have become central to the models of clathrin-mediated transport is because they are enriched in clathrin-coated vesicle preparations from animal cells and are found in stoichiometric amounts in reassembled lattices (Keen *et al*., 1979; Zaremba and Keen, 1983). The APs also bind clathrin HC, primarily through the β chain hinge/ear domain (Shih *et al*., 1995; Dell'Angelica *et al*., 1998), and they bind to the cytoplasmic tails of receptors (for reviews see Marks *et al*., 1997; Le Borgne and Hoflack, 1998), presumably forming a link between receptor cargo and the clathrin lattice. The cytoplasmic tails of many transmembrane proteins contain tyrosine-based and/or di-leucine-based sorting motifs that are critical for the correct trafficking of these proteins. Tyrosine-based sorting motifs directly interact with the μ subunits of AP complexes (Marks *et al*., 1997). Di-leucine-based motifs can also bind heterotetrameric adaptors, possibly through interacting with the β chains (Heilker *et al*., 1996; Honing *et al*., 1998; Rapoport *et al*., 1998). If AP complexes are crucial for the correct sorting of so many receptors, how can they be dispensable in yeast? Most likely, in animal cells, many more proteins have evolved to become dependent on APs to facilitate rapid and regulated traffic into clathrin-coated vesicles. Transporting these proteins with maximum efficiency may be crucial to carry out specialized and essential processes such as synaptic vesicle recycling, nutrient uptake, and regulation of signal transduction and development. The importance of heterotetrameric AP complexes in animal cells is highlighted by the findings that AP-1 γ-deficient mice and *Drosophila* AP-2 α-chain mutants are not viable (Gonzalez-Gaitan and Jackle, 1997; Zizioli *et al*., 1999).

Our studies indicate that AP complexes are not stoichiometric components of clathrin-coated vesicles in yeast. This is consistent with previous studies where yeast AP subunits could only be detected by immunoblotting of clathrin-coated vesicle preparations (Phan *et al*., 1994; Stepp *et al*., 1995). Nevertheless, yeast AP complexes are likely to function like their mammalian counterparts to direct the transport of certain membrane proteins. Yeast AP-3 is required for transport of Vam3p and ALP (via di-leucine sorting motifs) from the Golgi to the vacuole (Cowles *et al*., 1997; Stepp *et al*., 1997; Darsow *et al*., 1998; Vowels and Payne, 1998), and the AP-1 complex appears to have cargo sorting function in association with clathrin at the Golgi (Phan *et al*., 1994; Rad *et al*., 1995; Stepp *et al*., 1995). Since yeast strains depleted of APs do not display clathrin-deficient phenotypes, clathrin must be able to function in an AP-independent manner. There-

fore, if the heterotetrameric adaptors are sorting cargo via clathrin, the proteins they are sorting are probably not essential.

Another possibility is that essential proteins whose sorting is normally dependent on APs could attain their correct cellular destination by rerouting through alternative or up-regulated transport pathways in the absence of adaptors. In support of this idea, both Vam3p and ALP are rerouted through the Vps pathway in AP-3-deficient strains (Cowles *et al*., 1997; Stepp *et al*., 1997; Darsow *et al*., 1998). Likewise, in animal cells, lysosomal hydrolases and membrane proteins can be targeted directly to lysosomes from the TGN, or they can take an indirect route involving delivery to the plasma membrane followed by endocytosis (for a review see Hunziker and Geuze, 1996). Moreover, when clathrin-mediated internalization is blocked in animal cells, compensatory up-regulation of fluid-phase endocytosis occurs (Damke *et al*., 1995).

If APs are crucial in binding receptor tails and moving these receptors into coated pits, but they are not required for recruiting clathrin onto membranes, how then does clathrin become membrane associated? Structural studies on clathrin-coated vesicles as well as the clathrin HC itself provide some clues. In assembled clathrin lattices, the clathrin HC terminal domain points inward toward the membrane, suggesting that this region mediates the interaction with membrane components (Vigers *et al*., 1986; Heuser and Keen, 1988; Smith *et al*., 1998). The clathrin HC terminal domain forms a seven-blade β-propeller with obvious grooves between the first and second and the fourth and fifth blades (ter Haar *et al*., 1998). A number of proteins that interact with the HC terminal domain [including the β-subunits of heterotetrameric APs (Dell'Angelica *et al*., 1998), arrestin3 and β-arrestin (Krupnick *et al*., 1997), amphiphysin (Ramjaun and McPherson, 1998) and epsin 2 (P.De Camilli, personal communication)] contain a conserved motif where the pattern of amino acids is: hydrophobic, hydrophobic, acidic, hydrophobic, acidic. For example, in mammalian AP-3, the β-hinge/ear region interacts with clathrin via the sequence LLDLD (Dell'Angelica *et al*., 1998). There is a similar motif within the clathrin-binding site of the Yap180s (Wendland and Emr, 1998). The binding site for non-visual arrestins has been mapped to the first groove of the HC terminal domain (Goodman *et al*., 1997), leading to the proposal that different clathrin-binding proteins may align with sequences within one of the two grooves (ter Haar *et al*., 1998).

Taking into account the structure of the HC terminal domain and the number of different clathrin-binding proteins that have been identified, one can postulate at least three AP-independent mechanisms to recruit clathrin onto the membrane. In the first model, cells could utilize alternative adaptor molecules that both collect cargo and stimulate clathrin association with the membrane. For example, in animal cells, the internalization of β_2-adrenergic receptor (β_2-AR) from the cell surface is accomplished by interaction of β_2-AR with β-arrestin (or arrestin3) (Goodman *et al*., 1998). Within this complex, β-arrestin readily binds clathrin and can act as an adaptor to facilitate the clearance of the receptor from the plasma membrane through clathrin-coated pits. The existence of alternative adaptor systems distinct from AP-1 and AP-2 is also suggested by animal cell studies showing competition between some, but not all, receptors internalized in clathrin-coated vesicles via tyrosine sorting signals (Wiley, 1988; Warren *et al*., 1997). The fact that β-arrestin is found in only trace amounts in clathrin-coated vesicles (Goodman *et al*., 1997) suggests that some of these alternative adaptors may be released, rather than concentrated, when their cargo enters coated pits.

Another way in which clathrin could assemble onto membranes in the absence of adaptors is if clathrin itself could bind directly to receptor tails of membrane proteins targeted for transport through clathrin-coated vesicles. Such a direct interaction recently has been described for the clathrin terminal domain and the FxNPxY sorting signal in the cytoplasmic tail of the low density lipoprotein (LDL) receptor (Kibbey *et al*., 1998).

The third possible mechanism for clathrin recruitment brings into play recent studies from animal cells suggesting the existence of a protein network beneath the plasma membrane that is held together by the cooperative interaction of many different protein-binding domains (for reviews see Cremona and De Camilli, 1997; Wigge and McMahon, 1998). One component of this network is Eps15, which is a substrate of the epidermal growth factor receptor and is involved in clathrin-mediated internalization (Di Fiore *et al*., 1997; Benmerah *et al*., 1998). Eps15 contains several EH (or Eps15-homology) domains that bind to NPF motifs within proteins such as epsin (Di Fiore *et al*., 1997; Chen *et al*., 1998). Both Eps15 and epsin interact with the appendage domain of the AP-2 α-subunit (Benmerah *et al*., 1996; Iannolo *et al*., 1997; Chen *et al*., 1998) and epsin 2 itself interacts with clathrin (P.De Camilli, personal communication). Another component of this network is amphiphysin, a protein originally identified in synaptic termini, which has a role in endocytosis (for a review see Wigge and McMahon, 1998). Amphiphysin contains several motifs that link it to at least four members of the endocytic machinery. Amphiphysin interacts with clathrin HC via an LLDLD motif (Ramjaun and McPherson, 1998), and it binds to AP-2 (Wang *et al*., 1995; David *et al*., 1996). The SH3 domain within amphiphysin binds to the proline-rich regions of both dynamin, the coated vesicle 'pinchase' and synaptojanin, an inositol 5-phosphatase implicated in synaptic vesicle endocytosis (David *et al*., 1996; McPherson *et al*., 1996). Some synaptojanin isoforms contain NPF motifs that bind to Eps15 (Haffner *et al*., 1997). These and several other protein–protein interactions could result in a scaffold that provides a surface for clathrin lattice assembly and collects cargo into coated pits. AP-2 could normally bind cargo and then diffuse into this protein web, or AP-2 could be a component of this network and select specific cargo as they drift into the web. It is also possible that the scaffold facilitates and/or regulates the molecular rearrangements necessary to convert a planar clathrin lattice to a curved budding clathrin-coated vesicle. Recent studies visualizing endocytic clathrin-coated pit dynamics in living cells support the existence of a membrane skeleton and suggest alternative mechanisms for clathrin coat protein recruitment (Gaidarov *et al*., 1999).

Similar complexes of interacting proteins could exist in yeast (Wendland *et al*., 1998), since many of the components of the protein network described above have close

Table I. Strains used in this study

Number	Genotype	Source[a]
BJ3255	*MAT*α *chc1-*Δ*::LEU2 leu2 ura3-52 ade6*	Huang *et al*. (1997)
BJ3502	*MAT***a** *pep4-*Δ*::HIS3 prb1-*Δ*1.6R ura3-52 his3 can1*^{r}	Elizabeth Jones
JHRY20-2Ca	*MAT***a** *vps1-*Δ*::LEU2 leu2-3,112 ura3-52 his3-*Δ*200 can1*^{r}	Tom Stevens
LWY2887	*MAT*α *vac8-*Δ*::HIS3 leu2-3,112 ura3-52 trp1-*Δ*901 his3-*Δ*200 lys2-801 suc2-*Δ*9*	Wang *et al*. (1998)
SL1463	*MAT*α *leu2 ura3-52 trp1 his3-*Δ*200*	
SL1929	*MAT*α *bar1-1 clc1-*Δ*::HIS3 leu2 ura3-53 trp1 his3-*Δ*200*	Huang *et al*. (1997)
SL2940	*MAT*α *apm1-*Δ*::LEU2 apm2-*Δ*::URA3 apm3-*Δ*::HIS3 apm4-*Δ*::HIS3 leu2 ura3-52 trp1 his3-*Δ*200*	
SL3015	*MAT***a** *bar1-1 leu2 ura3-53 trp1 his3-*Δ*200*	
SL3102	*MAT***a** *bar1-1 apm1-*Δ*::LEU2 apm2-*Δ*::URA3 apm4-*Δ*::HIS3 leu2 ura3-52 trp1 his3-*Δ*200*	
SL3106	*MAT***a** *bar1-1 apm1-*Δ*::LEU2 apm2-*Δ*::URA3 apm3-*Δ*::HIS3 apm4-*Δ*::HIS3 leu2 ura3-52 trp1 his3-*Δ*200*	
SL3252	*MAT*α *bar1-1 apl6-*Δ*::HIS3 leu2 ura3-52 trp1 his3-*Δ*200*	
SL3361	*MAT*α *bar1-1 apl1-*Δ*::LEU2 apl2-*Δ*::TRP1 apl3-*Δ*::URA3 apl4-*Δ*::TRP1 apl5-*Δ*::LEU2 apl6-*Δ*::HIS3 leu2 ura3-53 trp1 his3-*Δ*200*	
SL3428	*MAT***a** *bar1-1 yap1801-*Δ*::URA3 yap1802-*Δ*::KMX leu2 ura3-52 trp1 his3-*Δ*200*	
SL3429	*MAT***a** *bar1-1 yap1801-*Δ*::URA3 yap1802-*Δ*::KMX leu2 ura3-52 trp1 his3-*Δ*200*	
SL3436	*MAT***a** *bar1-1 yap1801-*Δ*::URA3 yap1802-*Δ*::KMX apl1-*Δ*::LEU2 apl2-*Δ*::TRP1 apl6-*Δ*::HIS3 leu2 ura3-52 trp1 his3-*Δ*200*	
SL3445	*MAT*α *bar1-1 yap1801-*Δ*::URA3 yap1802-*Δ*::KMX apl1-*Δ*::LEU2 apl2-*Δ*::TRP1 apl6-*Δ*::HIS3 leu2 ura3-52 trp1 his3-*Δ*200*	
SL3478	*MAT***a** *bar1-1 apl1-*Δ*::LEU2 apl2-*Δ*::TRP1 apl3-*Δ*::URA3 apl4-*Δ*::TRP1 apl5-*Δ*::LEU2 apl6-*Δ*::HIS3 leu2 ura3-52 trp1 his3-*Δ*200*	
SL3593	*MAT***a** *bar1-1 chc1-*Δ*::LEU2 leu2 ura3-52 trp1*	

[a]All strains were generated for this study or are from this laboratory, except where indicated.

homologs in yeast. For example, the EH domain proteins End3p and Pan1p are both essential for endocytosis (Benedetti *et al*., 1994; Wendland *et al*., 1996; Tang *et al*., 1997) and, in the case of Pan1p, the EH domains bind to NPF motifs within the Yap180 proteins (Wendland and Emr, 1998). There are a number of yeast genes encoding proteins related to epsin that have yet to be characterized, but some of these contain NPF and potential clathrin LLDL-binding motifs (Chen *et al*., 1998; Kay *et al*., 1999). In addition, yeast contains two amphiphysin homologs, Rvs161p and Rvs167p, which are involved in endocytosis (Munn *et al*., 1995). Three synaptojanin (Snj) homologs have been characterized and phenotypic analysis of mutants implicates their function in the endocytic/TGN/endosomal/vacuole system (Luo and Chang, 1997; Srinivasan *et al*., 1997; Singer-Kruger *et al*., 1998; Stolz *et al*., 1998). Snj proteins have C-terminal proline-rich motifs that could associate with SH3 domains, such as that found in Rvs167p, and Snj1p has an NPF motif that could bind to an EH domain protein.

In conclusion, our studies provide strong evidence that clathrin recruitment onto membranes is not an essential function of heterotetrameric AP complexes and AP180-related proteins in yeast. Inevitably, these findings lead us to postulate that this may also be true in animal cells and that previous models of how clathrin-coated vesicles are formed need to be re-evaluated. Further investigation of the many protein–protein interactions recently implicated in this pathway should help resolve these questions.

Materials and methods

Strains, media, growth and genetic methods

Strains used in the study are listed in Table I. Multiple disruption strains were made by a combination of direct gene knockouts and crosses followed by segregation analysis. PCR analysis of genomic DNA was used to confirm the multiple knockouts in these strains. Rich YEPD medium contained 1% yeast extract, 2% peptone and 2% dextrose. Nitrogen starvation medium (SD-N) was 0.17% yeast nitrogen base without amino acids and without ammonium sulfate, 2% glucose. To test for recovery after nitrogen starvation, log phase cells growing in YEPD were washed, transferred to SD-N at 1×10^7 cells/ml and grown for several days at 30°C. Cells were removed daily, diluted and plated on YEPD. Colonies were counted after 3 days of growth to determine the number of cells surviving.

Gene disruptions

Gene disruptions in yeast were performed as described (Rothstein, 1991; Baudin *et al*., 1993). The *apm1-*Δ*::LEU2*, *apm2-*Δ*::URA3* and *apm3-*Δ*::HIS3* disruptions have been described (Stepp *et al*., 1995, 1997). Constructs to disrupt *APL1*, *APL2* and *APL4* genes were the gift of G.Payne (UCLA). In *apl1-*Δ*::LEU2*, a 2.5 bp *Bgl*II–*Sal*I *LEU2* fragment was inserted between the *Bgl*II and *Bst*XI sites in the *APL1* coding region, resulting in the removal of codons 20–577. In *apl2-*Δ*::TRP1*, a 0.9 kb *Eco*RI–*Bam*HI *TRP1* fragment was inserted between the *Bst*XI sites of *APL2* deleting codons 1–571. For *apl4-*Δ*::TRP1*, the 0.9 kb *TRP1* fragment replaced codons 8–610 of the *APL4* coding sequence.

The *apm4-*Δ*::HIS3*, *apl3-*Δ*::URA3*, *apl5-*Δ*::LEU2*, *apl6-*Δ*::HIS3*, *yap1801-*Δ*::URA3* and *yap1802-*Δ*::KMX* disruptions were all generated by integration of PCR fragments (Baudin *et al*., 1993; Wach *et al*., 1994). The PCRs contained 5′ and 3′ primers with 40–50 bp of homology to the gene targeted for disruption and 17–18 bp for the selectable marker to be amplified. Templates used in the PCRs were: pUC-HIS3 (Rothstein, 1991) for *apm4-*Δ*::HIS3* and *apl6-*Δ*::HIS3*; pUC-URA3 (Rothstein, 1991) for *apl3-*Δ*::URA3*; YDp-U for *yap1801-*Δ*::URA3* (Berben *et al*., 1991); pRS415 (Sikorski and Hieter, 1989) for *apl5-*Δ*::LEU2*; and pFA6aKanMx (Wach *et al*., 1994) for *yap1802-*Δ*::KMX*. All of the PCR-based gene disruptions removed the entire open reading frame of the target gene. Correct replacement of the targeted gene at the genomic locus was verified by analytical PCR using both internal marker gene primers and AP subunit gene-flanking primers (Wach *et al*., 1994).

Protein processing assays and endocytosis

To analyze α-factor processing, cells were labeled for 30 min with [^{35}S]methionine and cysteine (Easy Tag, New England Nuclear, Boston, MA) and α-factor was immunoprecipitated from the medium as described in Stepp *et al*. (1995). Labeling and immunoprecipitation of CPY were performed as described in Stepp *et al*. (1997). To examine ALP processing, cell lysates were generated and analyzed by immunoblotting (Stepp *et al*., 1997). Endocytosis of [^{35}S]α-factor was performed by the method of Dulic *et al*. (1991).

To examine processing of API, 2×10^7 mid-log phase cells were grown in 4 ml of SD-N (nitrogen starvation conditions) or 4 ml of YEPD for 4 h at 30°C. Cells were collected, resuspended in 400 µl of 50 mM sodium phosphate, 20 mM MES, pH 7.0, 1% SDS, 3 M urea, 1 mM

sodium azide, 2 mM phenylmethanesulfonate, and lysed with 0.15 g of glass beads by vortexing three times for 1 min at maximum speed on a vortex genie mixer with icing in between. After SDS sample buffer addition, lysates were heated for 10 min at 77°C and spun at 14 000 *g* for 10 min. Approximately 0.5×10^7 cell equivalents were analyzed by immunoblotting using a rabbit anti-API polyclonal antiserum (1/4000 dilution, gift of D.Klionsky), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Zymed Inc., San Francisco, CA) and chemiluminescent detection (ECL; Amersham, Arlington Heights, IL).

Isolation and electron microscopy of clathrin-coated vesicles

Clathrin-coated vesicles were isolated from yeast on a Sephacryl S-1000 gel filtration column (Lemmon *et al*., 1988). Fractions from the column were fixed directly by adding 37% glutaraldehyde to a final concentration of 3% and immediately centrifuged for 1 h at 100 000 *g* at 4°C. Membrane pellets were washed with 0.1 mM sodium cacodylate, 5 mM $CaCl_2$, pH 6.8 (CACO buffer), and then post-fixed in 1% osmium, 1% potassium ferrocyanide in CACO buffer for 30 min. Additional post-fixations with 1% thiocarbohydrazide in water for 5 min and again with 1% osmium, 1% ferrocyanide in CACO buffer for 30 min were performed to enhance visualization of membranes (Willingham and Rutherford, 1984). Membrane pellets were stained in 1% tannic acid in 100 mM cacodylate buffer, pH 7.4, for 30 min at 25°C and 1% uranyl acetate in water for 3–18 h at 25°C. Samples were dehydrated with graded ethanol, washed with propylene oxide and embedded in Spurr resin. Samples were sectioned (75–100 nm), stained with 1% uranyl acetate and 1% lead citrate and examined on a JOEL 100CX transmission electron microscope at 80 kV.

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