The clathrin adaptor complex 1 directly binds to a sorting signal in Ste13p to reduce the rate of its trafficking to the late endosome of yeast

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Yeast trans-Golgi network (TGN) membrane proteins maintain steady-state localization by constantly cycling to and from endosomes. In this study, we examined the trafficking itinerary and molecular requirements for delivery of a model TGN protein A(F→A)-alkaline phosphatase (ALP) to the prevacuolar/endosomal compartment (PVC). A(F→A)-ALP was found to reach the PVC via early endosomes (Ees) with a halftime of ~60 min. Delivery of A(F→A)-ALP to the PVC was not dependent on either the GGA or adaptor protein 1 (AP-1) type of clathrin adaptors, which are thought to function in TGN to PVC and TGN to EE transport, respectively. Surprisingly, in cells lacking the function of both GGA and AP-1 adaptors, A(F→A)-ALP transport to the PVC was dramatically accelerated. A 12-residue cytosolic domain motif of A(F→A)-ALP was found to mediate direct binding to AP-1 and was sufficient to slow TGN→EE→PVC trafficking. These results suggest a model in which this novel sorting signal targets A(F→A)-ALP into clathrin/AP-1 vesicles at the EE for retrieval back to the TGN.

Introduction

The enzymes dipeptidyl aminopeptidase A/Ste13p and endopeptidase Kex2p, which process the α-factor mating pheromone in the yeast Saccharomyces cerevisiae, undergo repeated cycles of vesicular transport between the TGN and endosomal system (Nothwehr et al., 1993; Brickner and Fuller, 1997; Bryant and Stevens, 1997). These enzymes possess large luminal domains, a single transmembrane-spanning domain, and cytosolic domains of ~100 amino acids. Within their trafficking itinerary, the best understood step is retrieval from the prevacuolar/endosomal compartment (PVC) back to the TGN, which is mediated by the retromer, an apparent vesicle coat complex (Seaman, 2005). Retromer recognition of Ste13p and the carboxypeptidase Y receptor Vps10p occurs via binding of the Vps35p retromer subunit with aromatic amino acid–based sorting signals such as FXFXD in Ste13p (Nothwehr et al., 1993, 1999, 2000; Marcusson et al., 1994; Cooper and Stevens, 1996). However, the manner by which Ste13p and Kex2p reach the PVC is poorly understood.

Some studies suggest that Ste13p and Kex2p may traverse via the early endosome (EE) en route to the PVC. A Ste13p-based reporter protein, A(F→A)-alkaline phosphatase (ALP), reaches the PVC slowly with a half-time of ~60 min, whereas other proteins such as Vps10p and Cps1p reach the PVC within 5–15 min (Bryant and Stevens, 1997; Cowles et al., 1997). Deletion of the 2–11 region within the Ste13p cytosolic domain accelerates trafficking of A(F→A)-ALP into the PVC (Bryant and Stevens, 1997; Ha et al., 2001). A signal analogous to 2–11 exists in Kex2p (Brickner and Fuller, 1997). These data are consistent with a model in which A(F→A)-ALP reaches the PVC via the EE with the 2–11 signal conferring either EE to TGN retrieval or static retention within the TGN/EE. In support of an itinerary involving the EE, the loss of function of a yeast synaptojanin like protein Inp53p/Sjl3p, which is thought to play a role in TGN/EE traffic, accelerates the rate of trafficking of A(F→A)-ALP into the PVC but has no effect on Vps10p trafficking (Ha et al., 2001). In addition, Kex2p has been colocalized with EE markers Tlg1p and chitin synthetase III (Chs3p) as well as Snc1p, a late secretory v-SNARE that recycles from the plasma membrane back to the TGN via an early endosomal compartment (Ziman et al., 1996; Santos and Snyder, 1997; Holthuis et al., 1998; Lewis et al., 2000). Finally, the loss of function of Soi3p, which appears to be required for efficient EE to PVC trafficking, delayed trafficking of a PVC retrieval-defective form of Kex2p to the PVC but did not affect Vps10p trafficking (Sipos et al., 2004).

Clathrin-associated vesicular transport machinery clearly plays a role in trafficking between the TGN and endosomes.
of yeast. A loss of function in either clathrin heavy chain or Vps1p, a dynamin homologue that is thought to participate in the production of clathrin-coated vesicles, causes Ste13p and Kex2p to be mislocalized to the cell surface (Payne and Schekman, 1989; Seeger and Payne, 1992; Ha et al., 2003). This suggests that there is a requirement for clathrin in both TGN to EE and TGN to PVC pathways. Furthermore, clathrin-coated compartments have been shown to contain Vps10p and Kex2p (Deloche et al., 2001), and clathrin is required for in vitro TGN to PVC trafficking of Kex2p (Abazeed et al., 2005).

Two types of clathrin-associated adaptors function within the yeast TGN/endosomal system. The GGA1 and GGA2 gene products (Dell’Angelica et al., 2000; Hirst et al., 2000) are thought to function in the direct TGN to PVC pathway. Loss of GGA function causes the PVC-localized t-SNARE Pep12p to be mislocalized to the EE (Black and Pelham, 2000). Likewise, the delivery of Vps10p to the PVC and Cps1p to the PVC/vacuole, cargoes that are thought to use the direct TGN to PVC pathway, are delayed in gga1,2 mutants (Costaguta et al., 2001). No delay in A(F→A)-ALP transport into the PVC was observed in a strain lacking GGA function (Ha et al., 2003); however, a moderate delay has been reported for Kex2p (Costaguta et al., 2001). Thus, a role for GGA proteins in the trafficking of TGN resident proteins is still obscure. In contrast to the GGAs, the adaptor protein 1 (AP-1) complex appears to be involved in trafficking between the EE and TGN. Loss of AP-1 function caused the mislocalization of Chs3p to the cell surface under conditions in which it was sequestered to the EE (Valdivia et al., 2002). A severe synthetic growth defect has been observed upon the simultaneous loss of function of the GGA proteins and AP-1 (Costaguta et al., 2001). This synthetic growth defect suggests that both adaptors may mediate anterograde transport into the endosomal system, but this model has not been rigorously tested.

In this study, we tested whether A(F→A)-ALP required the AP-1 complex for transport into the PVC when the GGA-mediated pathway and the plasma membrane route were blocked. We found that when AP-1 complex function was lost under these conditions, the transport of A(F→A)-ALP into the PVC was dramatically accelerated. In addition, we demonstrate that the AP-1 complex interacts with the 2–11 sorting signal within the Ste13p cytosolic domain. These results are most consistent with a model in which clathrin/AP-1 recognizes Ste13p in the EE and directs it into a retrograde pathway to the TGN.

Results

The itinerary of A-ALP includes an EE compartment as well as the PVC

A-ALP is a model TGN membrane protein consisting of the NH2-terminal cytosolic domain of Ste13p fused to the transmembrane and luminal domains of ALP (Nothwehr et al., 1993). When TGN/endosome retention is perturbed, A-ALP is transported to the vacuole, where its COOH-terminal propeptide is proteolytically removed. For example, mutation of the FXFXD motif prevents the retrieval of A-ALP from the PVC to the TGN (Bryant and Stevens, 1997; Nothwehr et al., 2000). This mutant, A(F→A)-ALP, is proteolytically processed with a half-time of ~60 min, reflecting the kinetics of vacuolar delivery (Nothwehr et al., 1993). A-ALP has also been shown to reach the PVC in a class E vps mutant with similar kinetics (Bryant and Stevens, 1997; Ha et al., 2001), indicating that the PVC to vacuole step is quite rapid and that the rate of A(F→A)-ALP processing largely reflects the rate of trafficking into the PVC.

To test whether the A-ALP itinerary includes EEs, it was tagged with GFP and was expressed using the moderate strength CYCI promoter (Mumberg et al., 1995). Like its untagged counterpart, pulse-chase immunoprecipitation analysis of GFP–A-ALP indicated that it was unprocessed after 60 min, whereas mutation of the FXFXD motif resulted in processing with a half-time of ~60 min (unpublished data). Because the GFP tag did not interfere with the kinetics of transport into the PVC/vacuole, it is likely that the tag did not disrupt the normal trafficking patterns of A-ALP. Consistent with this, GFP–A-ALP exhibited a punctate staining pattern typical of yeast Golgi/endosomal proteins (Fig. 1 A). Cells expressing GFP–A-ALP were incubated at 0°C with the lipophilic dye FM4-64, allowing it to integrate into the plasma membrane but not be internalized (Vida and Emr, 1995). The cells were then incubated at 30°C for 2 min to allow the dye to be transported from the plasma membrane to EEs. After 2 min, FM4-64 exhibited a punctate pattern.

![Figure 1. GFP-tagged A-ALP partially colocalizes with EEs.](image-url)

Strains SHY35/pCF17 and SHY35/pG-P12-U were stained at 0°C with FM4-64, and the dye was allowed to internalize by incubating in media at 30°C for 2 or 8 min followed by the addition of NaN3 and NaF to halt traffic. (A) After 2 min of internalization, GFP-ALP, GFP-Pep12p, and FM4-64 were imaged as indicated. (B) The percentage of punctate FM4-64 structures that were positive for either GFP–A-ALP or GFP–Pep12p was quantified after 2 and 8 min of incubation at 30°C. A minimum of 150 punctate structures were analyzed for each data point.
The half-time of processing of each strain is indicated below each panel. The strains were either propagated for several doublings at 24°C before shifting to 36°C for 10 min before the initiation of the chase (B and C). After each time point, A(F→A)-ALP was immunoprecipitated and analyzed by SDS-PAGE to separate the precursor (p) and mature (m) forms. The half-time of processing of each strain is indicated below each panel.

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The vacuolar protease Cps1 is initially synthesized as an inactive precursor that is proteolytically processed to yield the mature form upon reaching the vacuole (Cowles et al., 1997). Previously, it was reported that in a gga1,2Δ strain, Cps1p transport to the vacuole is delayed (Costaguta et al., 2001). Cps1p contains a ubiquitin moiety that causes it to be sorted into multivesicular body vesicles upon reaching the PVC (Katzmann et al., 2001; Reggiori and Pelham, 2001). As GGA adaptors have been shown to recognize the ubiquitin moiety on cargo proteins at the TGN (Pelham, 2004; Scott et al., 2004), it seems likely that Cps1p enters clathrin/GGA-coated vesicles at the TGN and is directly delivered to the PVC. Using the same experimental regimen as for A(F→A)-ALP (Fig. 2 C) except with different chase times, we also observed a marked delay in Cps1p processing in a gga1,2Δ strain (Fig. 3 A), albeit not as dramatic as reported by Costaguta et al. (2001). An even stronger delay was observed in a gga1,2Δ end3-ts strain, suggesting that in the absence of GGAs, a pool of Cps1p is mislocalized to the cell surface before being transported to the vacuole via the endocytic pathway. However, even in the gga1,2Δ end3-ts strain, Cps1p was slowly processed (61-min half-time), suggesting that in the absence of GGAs, Cps1p was capable of accessing the PVC by an intracellular route, presumably via the EE. Finally, trafficking of Cps1p was blocked by the pep12-49 mutation (Fig. 3 A); thus, like A(F→A)-ALP, Cps1p must transit via the PVC to then be transported to the vacuole. In summary, these results are consistent with a model in which Cps1p uses a GGA-dependent direct TGN to PVC pathway and that A(F→A)-ALP exits the TGN via a GGA-independent pathway leading to the EE.

AP-1 is not required for A(F→A)-ALP to access the GGA-independent pathway to the endosomal system but instead slows its trafficking into the PVC.

To address whether the clathrin adaptor AP-1 might function at the TGN for the transport of A(F→A)-ALP to the EE, random mutations were introduced into the gga1,2Δ background and both the Ste13p 2–11 region and the AP-1 adaptor complex were overexpressed. Figure 4. Both the AP-1 and Ste13p 2–11 regions slow the transport of A(F→A)-ALP through the TGN–EE–PVC pathway rather than being required for anterograde transport. (A) Strains SY35 (wild type), UFY2 [apl2Δ], SY165 (gga1,2Δ), CFY6-2C/pCF2 (apl2Δ gga1,2Δ/pCEN-APL2), and CFY6-2C/pCF6 (apl2Δ gga1,2Δ/pCEN-apl2-ts) were analyzed by spotting 10-fold serial dilutions onto YPD media and incubating for 4 d at the indicated temperature. (B) SY35, CFY6-2C/pCF6, and CFY25-3B/pCF6 (apl2Δ gga1,2Δ end3-ts/pCEN-apl2-ts) strains were grown for several doublings at 24°C, shifted to 36°C (or left at 24°C as indicated) for 10 min, pulsed for 10 min, and chased as indicated. (C) Strain CFY6-2C/pCF6 carrying (left to right) plasmids pSN100 (A(F→A)-ALP), pHJ63 (A[S13A;F→A]-ALP), or pSH46 (A(F→A)-ALP) was analyzed after shifting from 24 to 36°C as in B. (D) Strains SY35, SNY94 (end3-ts), SY165, and SNY171-4D carrying pSN100 (top) or pSH46 (bottom) were analyzed after shifting from 24 to 36°C as in B. (B–D) A(F→A)-ALP or its derivatives were immunoprecipitated and analyzed by SDS-PAGE to separate the precursor (p) and mature (m) forms. The half-time of processing of each strain is indicated below each panel.
mutagenesis was used to generate a temperature-sensitive apl2-ts mutation. In the presence of the gga1,2Δ mutations, the apl2-ts allele exhibited near normal growth at 24°C but little or no growth at the nonpermissive temperature of 36°C (Fig. 4 A), which is consistent with the near synthetic lethality previously observed for mutations in these three genes (Costaguta et al., 2001).

We next asked whether the trafficking kinetics of A(F→A)-ALP was altered in this strain lacking both AP-1 and GGA function. In the event that TGN to EE trafficking of A(F→A)-ALP was blocked, including the gga1,2Δ mutations would prevent any A(F→A)-ALP from spilling into the GGA-mediated direct TGN to PVC pathway. At 36°C, the gga1,2Δ apl2-ts strain exhibited markedly accelerated processing kinetics compared with wild type (20 vs. 56 min; Fig. 4 B). In contrast to the growth characteristics of the gga1,2Δ apl2-ts strain (Fig. 4 A), the accelerated processing of A(F→A)-ALP was nearly as pronounced at 24 as at 36°C. Thus, the protein encoded by the apl2-ts allele is partially defective for trafficking at the permissive temperature. Furthermore, the accelerated trafficking of A(F→A)-ALP in this strain was also observed in a gga1,2Δ apl2-ts end3-ts strain in which both the GGA-mediated direct TGN to PVC and plasma membrane routes are blocked, leaving only the TGN to EE pathway intact. These results imply that AP-1 is not required for A(F→A)-ALP transport to the EE and argue instead that AP-1 functions to slow transport into the PVC, presumably via EE to TGN retrieval.

A(F→A)-ALP is obligated to reach the PVC before being transported to the vacuole (Fig. 2 B). However, we considered the possibility that A(F→A)-ALP might be transported to the vacuole in the gga1,2Δ apl2-ts strain by a route not normally used in wild-type cells. One possibility is that in the gga1,2Δ apl2-ts strain, regions of the TGN could be transported directly to the vacuole by an autophagic pathway. However, this is unlikely because we did not observe an expected side effect of such an event: a reduction in the amount of protein secretion of A(F→A)-ALP (Ste13p) that could be necessary for TGN to EE anterograde transport and that deletion of 2–11 might cause accelerated transport into the PVC because A(A→F)-ALP is forced into alternative pathways (i.e., a direct TGN to PVC pathway or plasma membrane pathway). To address the latter scenario, we assessed the trafficking of A(Δ2–11; F→A)-ALP in a gga1,2Δ end3-ts strain in which the alternative pathways were blocked (Fig. 4 D). Deletion of 2–11 caused the acceleration of transport to a similar extent in all four strains examined, including the gga1,2Δ end3-ts strain. The absence of a block or delay in transport in the gga1,2Δ end3-ts strain caused by deletion of the 2–11 region argues that this signal plays a role either in EE to TGN retrieval or in static TGN retention.

If the 2–11 region and AP-1 act at the same step, the deletion of 2–11 and the loss of AP-1 function should not cause an additive effect on the acceleration of A(F→A)-ALP trafficking. Consistent with 2–11 and AP-1 acting at the same step, A(Δ2–11; F→A)-ALP was found to exhibit trafficking kinetics in the gga1,2Δ apl2-ts strain (26-min half-time; Fig. 4 C) that were similar to A(F→A)-ALP in gga1,2Δ apl2-ts (20 min; Fig. 4 B) and A(Δ2–11; F→A)-ALP in gga1,2Δ (30 min; Fig. 4 D).

An in vitro binding assay was used to assess whether the heterotetrameric AP-1 complex associates with the Ste13p cytosolic domain. The Ste13p cytosolic domain (residues 1–118) was fused to the NH2 terminus of GST and the resulting fusion protein (Ste13-GST) purified from Escherichia coli onto glutathione-agarose beads. The beads were incubated with protein extracts from a yeast strain containing an epitope-tagged Ste13p and then washed extensively. The sample was then analyzed by Western blotting for Apm1p and Apl2p (Fig. 5). Both AP-1 subunits were found to associate with Ste13-GST but not with GST alone (Fig. 5 A). Interestingly, we observed little or no association of Apm1p-HA or Apl2p with Ste13-GST purified from E. coli, whereas Apm1p and Apl2p were bind to Ste13-GST (Ste13ΔΔ–11)-GST, indicating that the interaction is highly dependent on the 2–11 region. Fusions containing only residues 1–20 and 1–12 of Ste13p also bound to AP-1 with similar affinity to that of full-length Ste13-GST. Thus, residues 1–12 are necessary and sufficient for association with Apm1p-HA and Apl2p. There appeared to be little, if any, difference in the binding of these AP-1 subunits to the wild-type, S13A, and S13D versions of Ste13-GST. Data from a previous study was consistent with the idea that phosphorylated S13 might antagonize the 2–11 signal (Johnston et al., 2005); however, this binding data coupled with the observation that the S13A block is downstream of AP-1 would appear to argue against this. Finally, the data suggested that more Apm1p-HA than Apl2p was bound to the Ste13-GST beads based on comparison with the amounts of these proteins detected in the input sample. This suggested that Apm1p was binding as a monomer in addition to binding within the context of intact AP-1. Thus, it could be the subunit that Ste13p interacts with directly.

In animal cells, the μ, or less commonly, the β subunit of adaptor complexes recognizes cargo proteins (Bonifacino and Traub, 2003; Owen et al., 2004). Given our results suggesting that yeast μ1, or Apm1p, may be the AP-1 subunit that recognizes Ste13p, we asked whether Apm1p or Apm1p expressed in rabbit reticulocyte lysates in the absence of other yeast AP-1 subunits.
The clathrin adaptor complex AP-1 directly interacts with amino acids 1–12 of Ste13p. The following proteins were expressed in E. coli and purified onto glutathione-agarose beads: Ste13-GST (WT), Ste13(S13A)-GST, Ste13(S13D)-GST, Ste13(1–20)-GST, Ste13(1–12)-GST, and GST (Vect or Vect). (A) The bead samples were incubated with an SNY190 yeast protein extract followed by washing, elution, and purification onto glutathione-agarose beads: Ste13-GST (WT), Ste13(S13A)-GST, Ste13(S13D)-GST, Ste13(1–20)-GST, Ste13(1–12)-GST, and GST (Vect or Vect). (B) Rabbit reticulocyte lysates were boiled with an SNY190 yeast protein extract followed by washing, elution, and analysis of the eluted proteins by SDS-PAGE. Identical gels were immunoblotted with anti-Apl2p or anti-HA antibodies to detect Apm1-HA as indicated. Yeast extract equivalent to 4% of that incubated with each bead sample was also analyzed (input). The same samples analyzed by Western blotting were also analyzed by staining with Coomassie brilliant blue (CBB) to indicate the relative quantity of each GST fusion. (B) Rabbit reticulocyte lysates were also analyzed by staining with Coomassie brilliant blue (CBB) to indicate the relative quantity of each GST fusion. (B) Rabbit reticulocyte lysates were boiled with an SNY190 yeast protein extract followed by washing, elution, and analysis of the eluted proteins by SDS-PAGE. Identical gels were immunoblotted with anti-Apl2p or anti-HA antibodies to detect Apm1-HA as indicated. Yeast extract equivalent to 4% of that incubated with each bead sample was also analyzed (input). The same samples analyzed by Western blotting were also analyzed by staining with Coomassie brilliant blue (CBB) to indicate the relative quantity of each GST fusion. 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The same samples analyzed by Western blotting were also analyzed by staining with Coomassie brilliant blue (CBB) to indicate the relative quantity of each GST fusion. (B) Rabbit reticulocyte lysates were boiled with an SNY190 yeast protein extract followed by washing, elution, and analysis of the eluted proteins by SDS-PAGE. Identical gels were immunoblotted with anti-Apl2p or anti-HA antibodies to detect Apm1-HA as indicated. Yeast extract equivalent to 4% of that incubated with each bead sample was also analyzed (input). The same samples analyzed by Western
the GGA-mediated pathway to the PVC and does not undergo any aberrant folding or transport delays in the early secretory pathway. However, in gga1,2Δ cells, Ste13(1–23)-Cps1p is processed significantly more slowly (54 ± 5 min half-time) than Cps1p in the gga1,2Δ strain (34 ± 2 min). This difference is comparable with the difference between A(F→gga1,2→∆ (53 min) and A(∆2–11; F→A)-ALP (30 min) observed in gga1,2Δ cells (Fig. 4 D). Thus, this Ste13p NH2-terminal region slows the trafficking of Ste13-Cps1 most likely by mediating its retrieval from the EE. Importantly, a S13A mutation in the Ste13(1–23)-Cps1 context markedly slowed trafficking in the gga1,2Δ strain (98 ± 11 min). Because the S13A block appears to occur at the level of the EE (Johnston et al., 2005), this result indicates that the Ste13-Cps1 fusions do indeed traffic to the PVC via the EE as expected. A similar reduction in trafficking was observed when just residues 1–12 of Ste13p were fused to Cps1p (48 ± 3 min). Collectively, the data indicate that the first 12 residues of Ste13p are both necessary and sufficient to slow trafficking into the PVC.

Discussion

A major goal of this study was to address the respective roles of the clathrin adaptors AP-1 and GGA in the trafficking of A(F→A)-ALP, a model TGN protein based on Ste13p. Our results suggest that AP-1 functions in retrograde EE to TGN transport of A(F→A)-ALP. In addition, we demonstrate a physical interaction between a Ste13p cytosolic domain region and AP-1 and describe, for the first time, a cargo-sorting signal recognized by the yeast AP-1 adaptor.

A(F→A)-ALP is delivered to the PVC via the EE

Three independent experiments suggested that the pathway by which A(F→A)-ALP reaches the PVC involves EEs. First, GFP-tagged A-ALP was found to partially colocalize with the endocytic tracer dye FM4-64 at a time point when it was primarily localized to EEs. Second, the loss of function of Soi3p necessary for efficient EE to PVC trafficking marked delayed the trafficking of A(F→A)-ALP to the PVC. Finally, the loss of function of GGA clathrin adaptors, which appear to function in the direct TGN to PVC pathway (see Introduction), did not delay the trafficking of A(F→A)-ALP to the PVC or cause it to be mislocalized to the plasma membrane. This was in contrast to Cps1p that was delayed in strains lacking GGA function (Fig. 3; Costaguta et al., 2001). In gga1,2Δ cells, Cps1p eventually reached the PVC/vacuole in part via the plasma membrane.

Whereas our data argues that little, if any, A(F→A)-ALP traffics through the direct TGN to PVC pathway, the situation may be a bit different for Kex2p. In contrast to A(F→A)-ALP, Kex2p trafficking into the PVC was delayed somewhat because of a loss of GGA function (Costaguta et al., 2001). In addition, Kex2p is transported into the PVC significantly more rapidly than A-ALP as judged by processing/tturnover in a vps27 class E mutant (Ha et al., 2001; Sipos et al., 2004). This is consistent with the partitioning of Kex2p between a direct TGN to PVC pathway and the TGN→EE→PVC route.

In cells lacking both GGA and End3p function, Cps1p was able to reach the PVC, albeit slowly, suggesting that it is able to access the TGN to EE pathway. It seems unlikely that Cps1p would contain a cryptic targeting signal for transport to the EE. Thus, it is possible that that TGN to EE trafficking can occur by default rather than in a strictly signal-mediated fashion. According to the cisternal maturation view of Golgi trafficking, the EE may be a mature post-TGN compartment depleted of secretory as well as PVC-bound cargo that has fused with endocytic vesicles (Pelham, 1998). This would explain why Cps1p that is unable to undergo TGN to PVC transport would be swept along to the EE and, to a limited degree, the plasma membrane.

Role of AP-1 in cargo sorting

One of our major objectives was to address whether AP-1 is needed for anterograde TGN to EE trafficking of A(F→A)-ALP. It was shown previously that the loss of function of the AP-1 β-subunit Ap2p did not delay the trafficking of A(F→A)-ALP to the PVC and, in fact, accelerated it slightly (Ha et al., 2003). Because it was possible that in apl2 mutants A(F→A)-ALP could reach the PVC by alternative routes, we assessed trafficking in gga1,2Δ apl2-ts and gga1,2Δ apl2-ts end3-ts strains. In both cases, trafficking was markedly accelerated relative to a wild-type strain. A(F→A)-ALP appeared to reach the PVC by the normal TGN→EE→PVC route because a S13A mutation previously shown to prevent EE to PVC transport of A(F→A)-ALP (Johnston et al., 2005) was shown to also block trafficking in the gga1,2Δ apl2-ts strain. Furthermore, secretory pathway function of the gga1,2Δ apl2-ts strain appeared normal, ruling out any large-scale autophagic transport of TGN membranes to the vacuole that might have explained the accelerated transport.

Collectively, our results indicate that AP-1 is not required for anterograde TGN to EE transport of A(F→A)-ALP. However, we cannot entirely exclude the possibility that AP-1 is involved in anterograde trafficking and that in the gga1,2Δ apl2-ts strain, another protein complex redundant with AP-1 was able to fill in for its absence. Indeed, the role of AP-1 in animal cells has been controversial, with both evidence supporting a role for AP-1 in anterograde trafficking out of the TGN to endosomes (Huang et al., 2001; Puertollano et al., 2003) and other evidence implicating AP-1 in the retrograde transport of cargo from endosomes and post-Golgi secretory granules to the TGN (Klumperman et al., 1998; Meyer et al., 2000). However, a role for yeast AP-1 in exit from the TGN would not explain why the trafficking of A(F→A)-ALP is accelerated. Rather, the results seem to point to a role for AP-1 in slowing the transport of A(F→A)-ALP through the TGN→EE→PVC pathway. In principle, this could occur by clathrin/AP-1 mediating either vesicular retrieval from the EE back to the TGN (see Fig. 7 A) or mediating static retention in the TGN (see Fig. 7 B). We tend to favor the former model because it fits better with the known role of clathrin/AP-1 as a vesicle coat. In addition, the loss of AP-1 function caused Chs3p to be mislocalized to the cell surface under conditions in which it would normally be localized to EEs. As Chs3p appears to maintain its EE localization by cycling
It is relevant that both AP-1 and GGAs are present on the same vesicles and tubules budding from the mammalian TGN (Puertollano et al., 2003) and that the interaction of AP-1 with GGAs has been observed in both yeast and animal cells (Costaguta et al., 2001; Doray et al., 2002; Bai et al., 2004). Furthermore, mammalian GGAs mediate EE to TGN recycling of memapsin 2 (BACE; He et al., 2005). It is possible that redundancy between AP-1 and the GGAs exists such that the function of both adaptors needs to be lost to observe a strong defect in EE to TGN retrieval. On the other hand, we have detected little, if any, Gga2p binding to the cytosolic domain of Ste13p in pull-down assays (unpublished data), suggesting that the GGAs do not function in Ste13p cargo recognition.

**A novel signal in Ste13p for interaction with AP-1**

Residues 2–11 of Ste13p/A-ALP are necessary for its effective retention within the TGN/EE system (Bryant and Stevens, 1997). We observed that even with both the direct TGN to PVC and plasma membrane pathways blocked in a gga1,2Δ end3-ts strain, the deletion of residues 2–11 from A(F→A)ALP accelerated its trafficking into the PVC. Furthermore, when Cps1p was forced to traffic into the PVC via the EE in a gga1,2Δ strain, its rate of transport to the vacuole significantly decreased as a result of fusing residues 1–12 of Ste13p/A-ALP to the NH2 terminus of its cytosolic domain (Fig. 6). These results imply that this NH2-terminal region contains a signal needed to slow trafficking through the EE and suggest that this signal may mediate EE to TGN retrieval. In support of this, we observed an interaction between the Ste13p cytosolic domain and AP-1 subunits in vitro that was dependent on residues 2–11. Remarkably, Apm1p-HA and Apl2p associated with a GST fused to residues 1–12 of Ste13p with an efficiency similar to that of the entire Ste13p cytosolic domain fused to GST. This data, along with the observation that 1–12 is sufficient to slow Cps1p trafficking into the PVC in a gga1,2Δ strain, suggests that residues 1–12 make most, if not all, of the specific contacts with AP-1. However, sequences COOH-terminal to this region might be required for optimal interaction by supplying additional contacts with AP-1 and/or for properly presenting the 1–12 region to AP-1.

This study provides the first glimpse into signal-mediated sorting by yeast AP-1. Previously described signals mediating AP-1–cargo interactions in animal cells include the YXXΦ signal that binds to the μ1 subunit and (D or E)XXX(L or I), which binds either to the β1 or μ1 subunits (Bonifacino and Traub, 2003). The Ste13p motif (MSASTSHHKRKN12) is a striking departure from these well-characterized signals in animal cells. One obvious feature is a cluster of positively charged residues. The residues within this region that are critical for AP-1 binding are not yet known; however, the S2, S4, and S7 residues have been mutated to alanine without any detectable acceleration in the trafficking of A(F→A)-ALP into the PVC (Johnston et al., 2005). Our data indicate that a COOH-terminal region of yeast μ1 is sufficient to bind the 12-residue Ste13p motif (Fig. 5) and, thus, must contain most or all of the critical residues that contact the 12-residue signal.
kinetics of transport in a gga1ΔΔ strain as Ste13(1–12)-Cps1, which lacks the signal (Fig. 6). In addition, A(ΔΔ2–11)-ALP, which seems to have lost critical elements of the signal based on the fact that the S13A mutation has no effect in this protein (Johnston et al., 2005), is transported into the PVC more rapidly than A-ALP. The A(ΔΔ2–11; F→A)-ALP protein appears to use the TGN→EE→PVC pathway (Fig. 7). Thus, the data do not support the view that phosphorylated S13 is needed for EE to PVC transport. Rather, the data support a model in which unphosphorylated S13 acts as a signal to somehow prevent EE to PVC transport, apparently by retaining Ste13p in the EE. Future work will be directed toward a more precise structural and functional definition of the AP-1 and S13 signals.

Materials and methods

General methods and antibodies

The production of yeast media, the genetic manipulation of yeast strains, and all general molecular biology methods were performed as described previously (Ausubel et al., 2002) or as otherwise noted. Rabbit polyclonal antibodies against AP-1 have been previously described (Nothwehr et al., 1993; Johnston et al., 2005). Anti-AP-1 antibodies were obtained from Covance and New England Biolabs, respectively. Rabbit polyclonal antibodies raised against a fusion protein consisting of GST fused to residues 46–577 of Cps1p, whereas rabbit anti-Apl2p antibodies were gifts from G. Payne [University of California, Los Angeles, Los Angeles, CA].

DNA manipulations and yeast strains

Plasmids pSN55, pSN100, and pHj63 have been previously described (Nothwehr et al., 1993; Johnston et al., 2005). The GFP–A-ALP construct pCF17 is p416-CYC (Mumberg et al., 1995) containing PCR-derived sgGFP [Qiogenie] followed by a (GlyAla)3 linker and the coding region of STE13-PHO8 (Nothwehr et al., 1993) starting with codon 2. pCF4, CEN-CPS1 was constructed by inserting a 4.97-kbp XbaI–XhoI fragment excised from pDPB3-CPS1 (Spormann et al., 1991) into the same sites in pRS316. Plasmid pCF2 was constructed by inserting a PCR fragment containing the full-length APL2 ORF into the EcoRI–Sall sites of pRS316. pHJ46 was made by swapping a 0.5-kbp EagI–BglII fragment containing the 5′ region of STE13 with the A2–11, F85A, and F87A mutations for the corresponding fragment in pSN55. The Ste13-GST fusion constructs were made by introducing PCR or oligonucleotide duplex-derived inserts into the vector pETGEXCT (Sharrocks, 1994). Fusion of the Apl1p and Apl1–A2–158 coding sequences was performed by inserting PCR-derived inserts into the vector pMal-c2 [New England Biolabs, Inc.]. Ste13-Cps1 fusion protein constructs consisted of the PCR-derived STE13 promoter and relevant coding regions fused to the 5′ end of the CPS1 coding region and 3′ untranslated regions cloned into pRS316 (Sikorski and Hieter, 1989). The fusion [unc] of the Ste13[1–23]–Cps1 and Ste13[1–12]–Cps1 fusions were ...KSSN2GSMA... and ...RKNI2GAA1L..., respectively, with the numbered residue representing the last Ste13p residue and the underlined residues indicating the Cps1p sequence.

To construct the apl2-ts allele, the APL2 ORF was amplified via PCR using an error-prone polymerase, Genemorph (Stratagene). The resulting population of PCR products was introduced into pRS313 via homologous recombination in yeast CTF6–2C/pCF2. His+ yeast transformants were then plated onto 5-FOA to lose pCF2 followed by screening for a lack of growth at 37°C and normal growth at 23°C. Finally, the mutagenized Apl2p-expressing plasmids rescued from temperature-sensitive yeast strains were retransformed back into CTF6–2C/pCF2 to test whether the growth phenotype was linked to the plasmid.

All yeast strains are described in Table I. SRY171-4D is a derivative from a diploid made by crossing SNY165 and SNY94. SHY64 was constructed by mating type-switching UYF2. CFY6-2C is a spore derived from a diploid made by crossing SNY165/pCF2 and SHY64. CFY25-3B is a spore derived from a diploid made by crossing CFY6-2C/pCF6 with SNY94. CFY30, CFY31, CFY32, and CFY33 were all constructed using PCR-mediated gene replacement (Wach et al., 1994a) of CPS1 with the Natr marker gene. The APM1::HA–URA3 allele was integrated at the APM1 locus using plasmid pAPM1-HA–URA3 [a gift from G. Payne], resulting in strain SNY190.
1,000 OD600 units of SNY190 cells were spheroplasted, pelleted at 450 g, washed once in 40 ml of ice-cold 1.2 M sorbitol. Spheroplasts were resuspended in 10 ml of buffer C (25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT, 0.1% Triton X-100, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A), Dounce homogenized, and centrifuged at 20,000 g for 15 min. The supernatant was then transferred to a fresh tube and was incubated with a 0.75-ml bed volume of glutathione-agarose beads for 1 h at 4 °C in a total volume of 400 μl. The beads were then washed five times with buffer C and were eluted with buffer C for 90 min at 4 °C. Eluted proteins were analyzed by SDS-PAGE and immunoblotting.

Radioactive labeling, immunoprecipitation, and Western blot analysis
The procedure for immunoprecipitation of wild-type and mutant A-ALP from [35S]methionine/cysteine-labeled cells and Western blotting has been previously described (Nothwehr et al., 1993; Ha et al., 2003; Johnston et al., 2005). Radioactively labeled proteins were quantified from gels using a phosphorimager system (FLA-2000; Fuji Film). For calculation of the half-time of A-ALP and Ste13-Cps1 processing, the log of the percentage of unprocessed precursor at each time point was plotted as a function of time, and the plots were analyzed by linear regression analysis. Immunoprecipitated Cps1p and derivatives were treated with endoglycosidase H before SDS-PAGE analysis according to a published protocol (Orlean et al., 1991). The precursor and mature forms of the substrate were quantified by immunoprecipitation of wild-type and mutant A-ALP as a function of time, and the plots were analyzed by linear regression analysis.

Binding assays
The various GST fusion proteins were expressed in E. coli BL21 [DE3] (Novagen) and were affinity purified onto glutathione-agarose beads (Sigma-Aldrich). MBP, MBP-Apm1, and MBP-Apm1-Δ2–158 were expressed in E. coli BL21, affinity purified onto amylose resin (New England Biolabs, Inc.), and were eluted with buffer containing 10 mM maltose.

The binding of Ste13-GST and its various mutant derivatives with AP-1 was assayed as previously described (Ghosh and Kornfeld, 2004). In brief, supernatant was split into seven equal portions, and each portion was incubated with a 100-μl bed volume of glutathione-agarose coated with Ste13-GST (or derivatives) for 3 h at 4 °C. Samples were washed five times in buffer C followed by elution with SDS-PAGE sample buffer and heating at 100 °C for 5 min. Eluted proteins were analyzed by SDS-PAGE and immunoblotting.

Pull-down of in vitro translated wild-type and mutant Apm1p was performed by first translating Apm1p and Apm1p-Δ2–158 in rabbit reticulocyte lysates (Ambion) in the presence of [35S]methionine according to the manufacturer’s instructions. Glutathione-agarose beads were incubated with aliquots of each translation reaction diluted with buffer C for 90 min at 4 °C. The beads were then washed five times with buffer C and were eluted with SDS-PAGE sample buffer at 100 °C. Eluted proteins were analyzed by SDS-PAGE and autoradiography.

To test for direct Apm1p-Ste13p interaction, binding assays were performed as described above for the in vitro translated Apm1p except that purified MBP-Apm1, MBP–Apm1-Δ2–158, and MBP alone were incubated with the bead samples.

Microscopy
Cells harvested from log-phase cultures were incubated for 30 min in YPD media containing 0.02 mg/ml FM4-64 (Invitrogen). They were then washed twice with YP media (lacking glucose) and once with SD-ura media before being resuspended in SD-ura media. Up to this point, all steps were performed at 0–4 °C. The cells were then incubated at 30 °C for 2 or 8 min, immediately placed on ice, and metabolic activity was stopped with 10 mM NaN3 and 10 mM NaF. The cells were washed on microscope slides containing 10 mM NaN3 and 10 mM NaF. The cells were then imaged at room temperature for FM4-64 and GFP staining using an epifluorescence microscope (DM5000B) equipped with a 100× NA 1.4 HCX plan-Apo lens, digital camera (DFC350X), and FW4000 software (all from Leica). Images were overlaid using the FW4000 software, adjusted slightly for brightness and contrast, and formatted using Adobe Photoshop 7.0.

We acknowledge Holly Johnston and Seon-Ah Ha for their involvement at various stages of this work and Per Stromback for critical reading of the manuscript and advice. Gregory Payne supplied constructs and antibodies.

Table I. Saccharomyces cerevisiae strains used in this study

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*The University of Texas Medical School at Houston, Houston, TX.
References


