The Role of the AP-1 Adaptor Complex in Trafficking between the trans-Golgi Network and Endosomal System

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By Christopher Foote

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

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Presented by Christopher Foote

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TABLE OF CONTENTS

ABSTRACT

In *Sacchromyces cerevisiae* it is generally accepted that there are two routes for trafficking of proteins from the trans-Golgi network (TGN) to the vacuole. One involves direct transport from the TGN to the vacuole. The second involves transport from the TGN to the prevacuolar compartment (PVC) via GGA coated vesicles, followed by PVC to vacuole transport. We propose that there is an alternative third route. This route entails transit from the TGN to the early endosome (EE), followed by delivery to the PVC and subsequent transit to the vacuole.

To test this hypothesis, the processing kinetics of the protein A(F→A)-ALP was examined. Its processing only occurs in the vacuole. Here it is shown that processing of A(F→A)-ALP is contingent upon delivery to the PVC. Processing is blocked in strains lacking functional Pep12p, a PVC t-SNARE required for vesicle docking at the PVC. In support of an alternative route, the processing kinetics of A(F→A)-ALP is not affected by mutations in the GGA proteins. This is in contrast to other proteins that use the GGA pathway, as their delivery to the vacuole is significantly slowed when GGA function is ablated. Further support of an EE itinerary is the observation that A(F→A)-ALP colocalizes with the lipophilic dye, FM4-64 at a time when the dye is predominantly associated with the EE. Disruption of the AP-1 vesicle coat complex leads to an accelerated processing of A(F→A)-ALP. Additionally, a pull down assay reveals that there is a physical interaction between two of the four AP subunits with A(F→A)-ALP. Deletion of this region results in accelerated processing time of A(F→A)-ALP.

Appending the region of A(F→A)-ALP that interacts with both subunits of AP-1 to Cps1p, a protein that does not normally transit through the EE delays its progress to the vacuole when it is forced to use the TGN-EE-PVC pathway. These results are consistent with a model in which A(F→A)-ALP traffics through the EE in transit to the vacuole. It physically interacts with AP-1, and this interaction delays its delivery to the vacuole. In mammalian systems, AP-1 has been implicated in transport from the EE to the TGN. Data presented in this thesis are consistent with this model and suggests that in *Sacchromyces cerevisiae* AP-1 functions as a retrieval mechanism from the EE to the TGN.

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
AP	Adaptor protein
CPS	Carboxypeptidase S
CPY	Carboxypeptidase Y
EE	Early endosomes
ER	Endoplasmic reticulum
ERGIC	ER Golgi intermediate compartment
GFP	Green fluorescent protein
GGA	Golgi-localized, γ-ear-containing, ARF-binding protein
GST	Glutathione-S-transferase
HA	Influenza A virus haemagglutinin
MPR	Mannose-6-phosphate receptor
PtdIns	Phosphatidylinositol
PM	Plasma membrane
PVC	Prevacuolar compartment
TGN	*trans*-Golgi network
ts	Temperature sensitive
VTC	Vesicular tubular cluster

CHAPTER I
INTRODUCTION

Endomembrane System

All cells must acquire nutrients from the outside world. Eukaryotic cells have developed a complex internal membrane system that allows for the uptake and subsequent delivery of nutrients to the lysosomes, termed vacuoles in yeast. These nutrients, macromolecules, enter the cell via endocytosis and transit to the vacuole through the endocytic pathway. In addition to acquiring nutrients, the internal membrane system provides eukaryotes with the ability to release components into the extracellular space. These components traverse the internal membrane system via the biosynthetic-secretory pathway and are targeted to the plasma membrane where they are released into the extracellular space. A third route for the trafficking of proteins through the internal membrane system involves the synthesis of proteins in the endoplasmic reticulum (ER) followed by transit through the biosynthetic/secretory pathway, but instead of delivery to the cell surface, proteins are delivered to the lysosome/vacuole as well as the various compartments that comprise both the biosynthetic/secretory pathway and the endocytic pathway (see Fig. 1-1 for a model of the internal membrane system).

Proteins destined for secretion as well as resident proteins for the various compartments in the biosynthetic and endocytic pathways are all synthesized in the endoplasmic reticulum (ER). Synthesis is initiated in the cytoplasm, but after the emergence of an ER signal sequence, the nascent protein and ribosomal machinery are targeted to the ER for translocation of the protein into the ER lumen (von Heijne, 1985). It should be noted that there also exists in yeast a mechanism for post translational

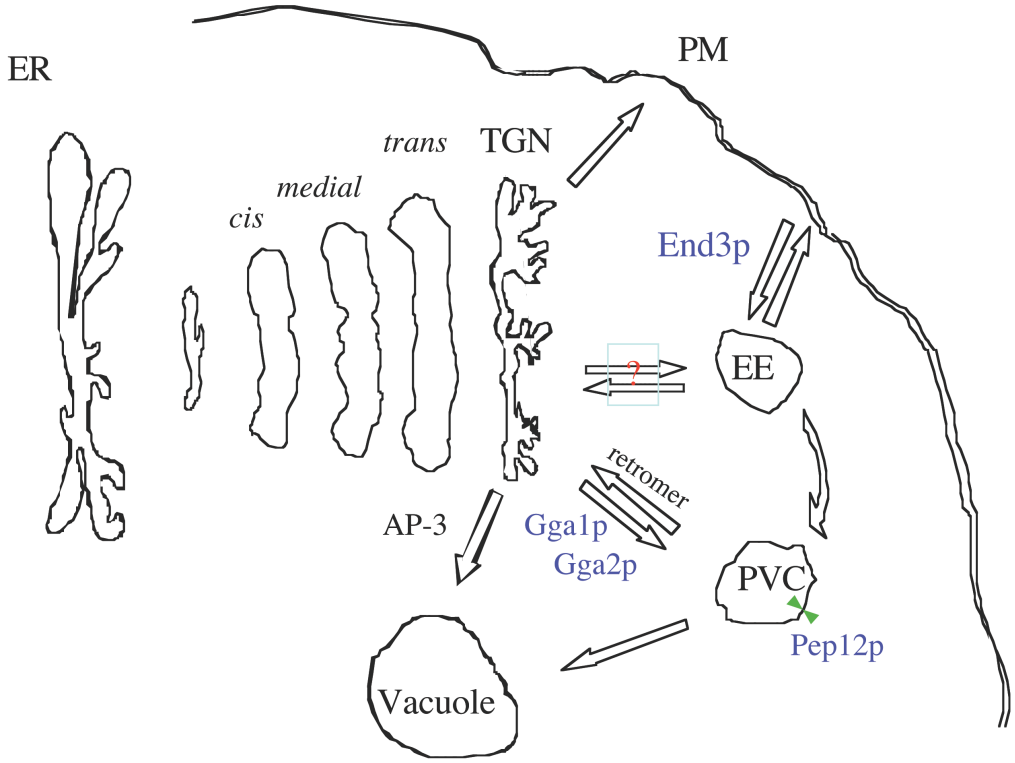


Fig. 1-1: Internal membrane system

translocation as yeast lacking the signal recognition particle (SRP), a molecule required for cotranslational translocation, are viable (Mutka and Walter, 2001). For most proteins, following translocation into the lumen, the signal sequence is cleaved and the protein is released from the membrane. An exception to this is the translocation mechanism for transmembrane proteins that span the membrane either once or a number of times. A second sequence, termed the stop transfer sequence (Davis and Model, 1985) is present in these proteins and it arrests translocation into the ER lumen. This results in a portion of the protein residing in the ER lumen and a portion residing in the cytosol. These can be divided into two types, I and II. Type I transmembrane proteins are oriented with the N-termini in the lumen of the ER and the C-termini in the cytosol. Type II transmembrane proteins are the reverse, the C-termini is translocated into the lumen of the ER and the N-termini is in the cytosol. In type II transmembrane proteins the signal anchor sequences function for both insertion as well as anchoring. Entry into the ER, either fully as in the case of lumenal proteins or partially as is the case with transmembrane proteins, is the first step for passage through the endomembrane system that includes the ER, the Golgi apparatus, endosomes, lysosomes (vacuole), intermediate transport vesicles and the plasma membrane.

Transit between the ER and Golgi

Transit of proteins from the ER to the Golgi involves trafficking through an intermediate compartment termed ER Golgi Intermediate Compartments (ERGIC) or alternatively vesicular tubular clusters (VTC). It appears that these compartments are distinct from the ER, though is has been shown in special circumstances that they remain

attached to the ER (Krijnse-Locker et al., 1994). Transit of cargo to these compartments requires the COPII coat complex. This complex is involved in cargo sorting and vesicle budding from specific sites on the periphery of the ER, termed ER exit sites (Barlowe and Schekman, 1993; Kaiser and Schekman, 1990; Bannykh and Balchm,1997). After vesicles bud from the ER, the COP II complex disassociates allowing the uncoated vesicles to fuse and form the ERGIC. The next step in transit to the Golgi is not well defined. In animal cells, it has been shown that ERGICs travel along microtubules to the *cis* Golgi (Lippincott and Schwartz, 1998). Next they fuse with one another to form new *cis* Golgi and receive distal cargo from more mature cisternae. Retrieval of ER proteins swept along aberrantly in this process are recycled back to the ER via COPI coat vesicles (Bannykh and Balchm, 1997; Lowe and Kreis, 1998).

Cisternae maturation

Transit of cargo through the Golgi stacks has been somewhat controversial. As an organelle, the Golgi is generally represented as a series of stacked compartments that are stable and discrete from one another. One view, the vesicle transport model, is that cargo is transported from one stack to another via anterograde trafficking vesicles and proteins that aberrantly traffic to distal stacks are retrieved via retrograde COPI vesicles (Dunphy and Rothman, 1985; Rothman and Wieland, 1996). The problem with this model is threefold. First, it would appear that the stacks themselves are transitory structures. Freshly made stacks appear on the *cis* side of the Golgi and are believed to originate via the fusion of ERGICs. In addition, on the *trans* side of the Golgi it would appear that the *trans* Golgi network (TGN) vesiculates into secretory vesicles as well as other membrane

structures that fuse with the endosomal system. These observations support a model in which newly formed cisternae progress through the stacks via a maturation process in which resident proteins that are swept along as cisternae mature are trafficked to *cis* proximal stacks via retrograde vesicles. Over time, as newly formed *cis* cisternae acquire more distal cargo and are depleted via retrograde trafficking of *cis* Golgi residents they mature into the *medial* and *trans* stacks. The second problem with the vesicle transport model is that a number of glycoprotein aggregates (algal scales, vesicular stomatitis membrane glycoprotein (VSV-G) and collagen) that move through the Golgi stacks have been identified that are larger than the transport vesicles meant to carry them. Furthermore these aggregates have not been found in vesicles that bud from the Golgi stacks. These aggregates are secreted to the cell surface and therefore must pass through the Golgi in what appears to be a vesicle independent process (Becker et al., 1995). The third factor weighing against the vesicle transport theory is that the vesicles that would mediate the forward progression have not been identified. Studies by the Rothman lab (Orci et al., 2000) and others have suggested that COPI vesicles mediate forward transport through the stacks in addition to the retrograde retrieval from the *cis*-Golgi to the ER as well as intra-Golgi retrograde transport. However, other groups using similar assays and techniques have determined that COPI plays little if any role in the forward progression of cargo through the Golgi stacks (Mironov et al., 2001). Furthermore, since COP I mediates retrograde trafficking between the stacks it is difficult to envision a model in which it has a dual role mediating anterograde trafficking as well (See Fig. 1-2).

The issue of how cargo passes through the Golgi remains murky, and *a priori* one model does not necessarily preclude the other from occurring. In summary there is

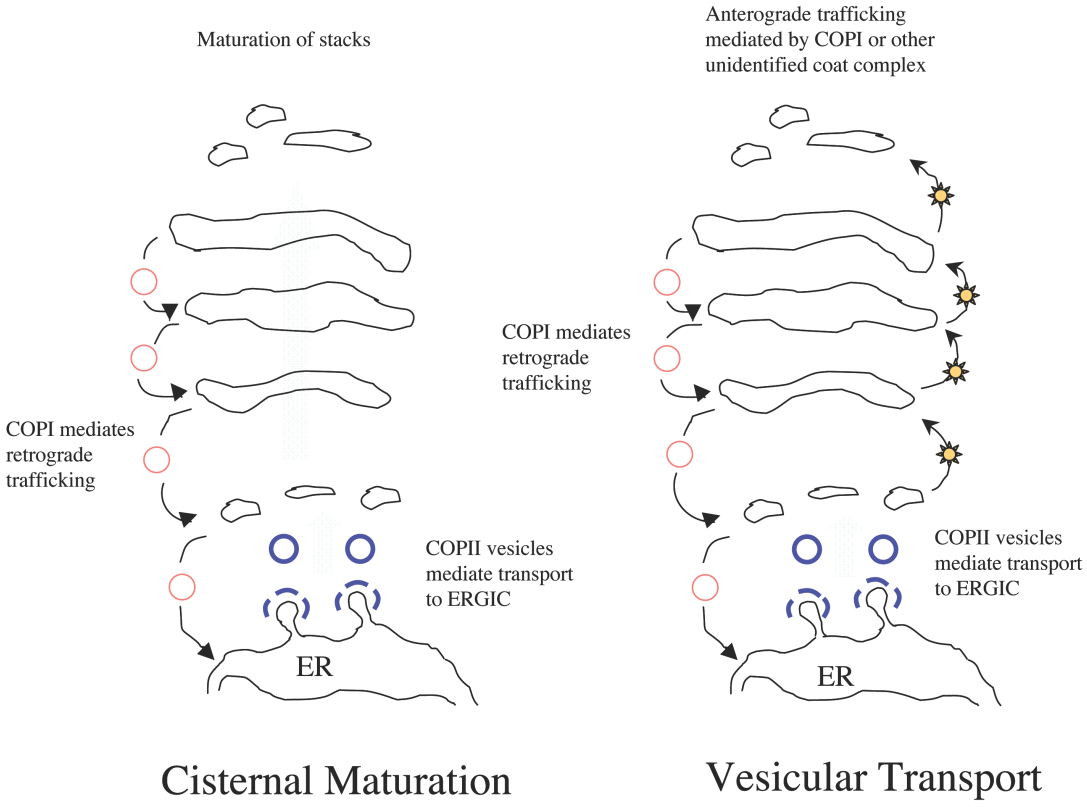


Fig. 1-2: Two models for progression of cargo through the Golgi

evidence for and against both models, and it may be the case that forward transport of cargo is mediated by vesicular transport as well as cisternae maturation.

Vesicular Transport

Transport of proteins via vesicles occurs at a number of steps along the biosynthetic/endocytic pathway. Specifically, COPII coated vesicles mediates transport from the ER to the ERGIC, COPI coated vesicles mediate retrieval from the Golgi back to the ER as well as intra-Golgi retrograde trafficking. At the TGN a number of distinct clathrin-coated vesicles mediate transport to the vacuole, the endosomal system as well as the plasma membrane. Lastly, endocytosis involves vesicular trafficking from the plasma membrane to the endosomal system.

Certain requirements must be met in order for specific cargo to be packaged in a vesicle and transported to target organelles. First, the cargo needs to be selected and sorted for vesicular transport. This occurs by the presence of sorting signals within the cargo protein that results in its association with specific coat protein complexes. For example, alkaline phosphatase (ALP) contains a dileucine sorting signal in its cytosolic domain that allows it to associate with adaptor protein-3 (AP-3) and be transported directly to the vacuole from the TGN via AP-3 vesicles (Vowels and Payne, 1998). Proteins that mediate the recruitment of cargo to clathrin coated membrane domains are called adaptor proteins (AP). To date, three adaptor protein complexes in *S. cerevisiae* have been identified; AP-I, AP-2 and AP-3. These are comprised of four subunits that play a role in cargo recruitment as well as recruitment of coat protein to the complex (see Fig. 1-3). It is believed that AP-1 and AP-2 in yeast recruit clathrin to form the vesicle

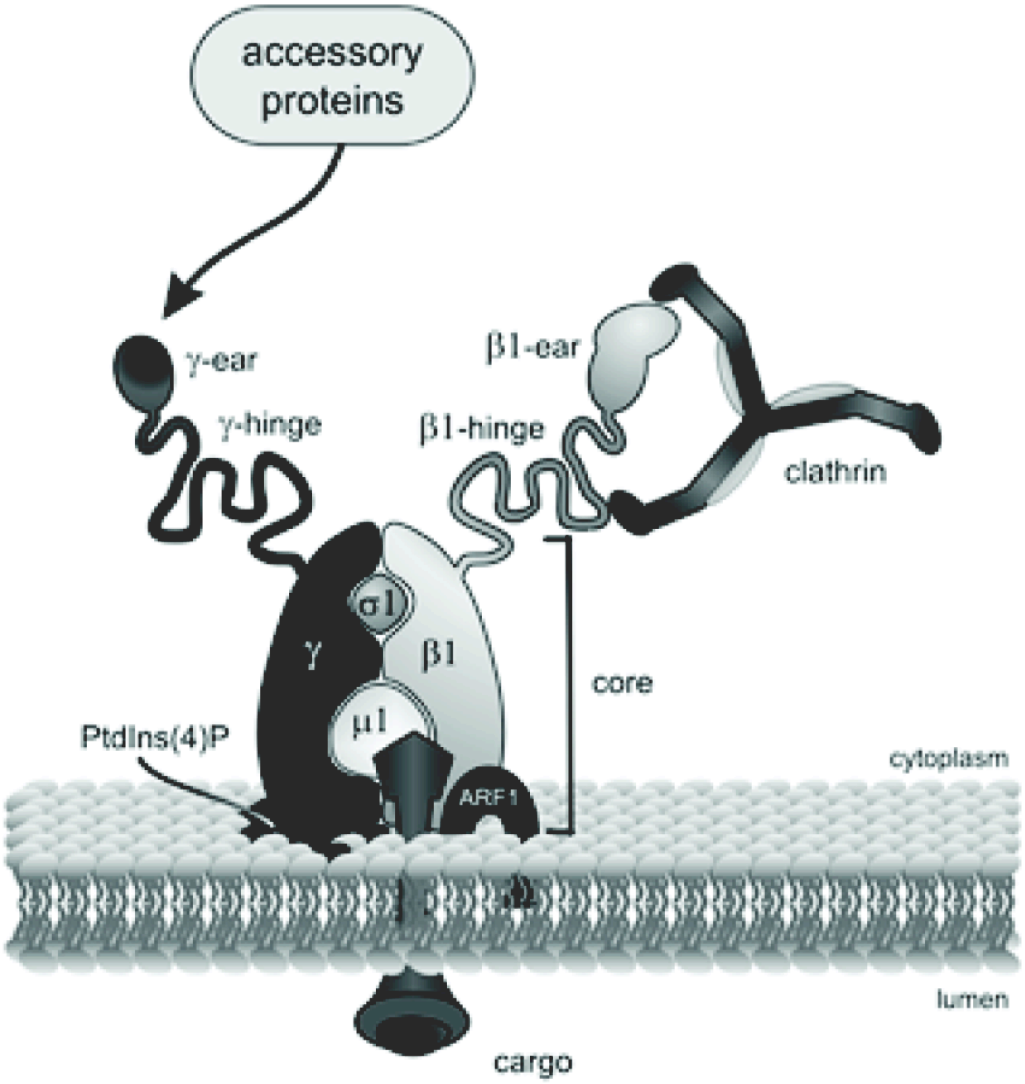
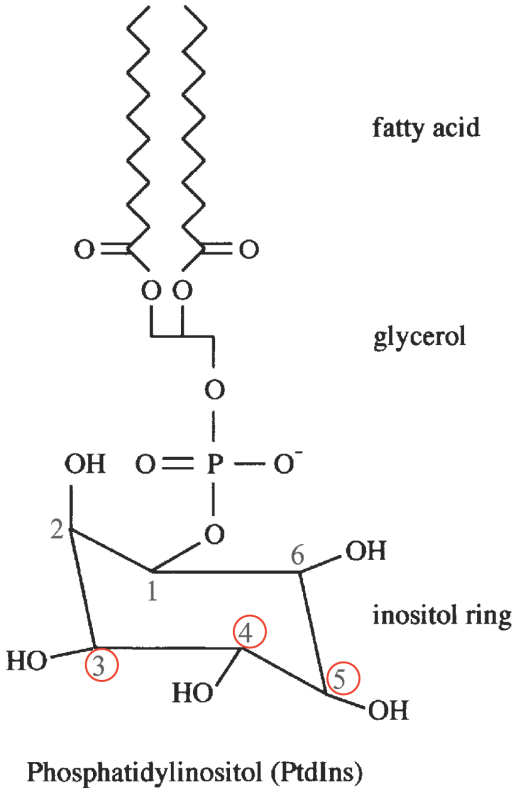


Fig. 1-3: Formation of AP-1/clathrin coated vesicle (McPherson et al., 2005)

coat. It is unclear if AP-3 recruits clathrin. It has been demonstrated that AP-3 can function independently of clathrin and uses Vps41p in lieu of clathrin (Darsow et al., 2001; Vowels and Payne, 1998).

In addition to the three AP complexes, another family of coat associated proteins called GGAs (for Golgi localized, gamma ear-containing, ARF binding proteins) have been implicated in protein trafficking. The yeast GGAs are encoded by *GGA1* and *GGA2* genes, and are monomeric, involved in protein sorting at the TGN and recruitment of clathrin (Boman, 2001; Hirst et al., 2001).

It is unclear if it is cargo receptors that recruit adaptors to the site of vesicle formation, or the other way around in which adaptors help to concentrate receptors and cargo at the point of vesicle formation. In the case of clathrin-coated vesicles that function at the TGN, additional factors have been identified that recruit the adaptors. The main protein involved in this recruitment is ADP-ribosylation factor (ARF). ARF is a small GTPase that is localized to the cytosol when bound to GDP. Following exchange of GDP for GTP it associates with membranes and in turn recruits adaptors to those membranes (Shiba et al., 2003; Wang et al., 2003). Adaptors may also be recruited by binding directly to transmembrane cargo or cargo receptors, i.e. type I or type II proteins, which contain cytosolic sorting signals such as dileucine motifs or conjugated ubiquitin (Bonifacino and Traub, 2003). A third mechanism for recruitment of adaptors to membranes points to a role for phosphoinositides (Ford et al., 2001; Itoh et al., 2001) These are membrane lipids that can be converted to seven different phosphorylated derivatives via specific kinases and phosphatases (see Fig. 1-4). Phosphatidylinositol (4,5)-bisphopshate [Ptdins(4,5)P_2] has been shown to interact with clathrin coats



Phosphatidylinositol (PtdIns)

Fig. 1-4: Phosphatidylinositol structure

(Martin, 2001) as well as specific adaptors; AP-2, AP180 and epsin (Evan et al., 1985).

Once adaptors are recruited to membranes (Fig. 1-3), cargo is enriched at these sites through the direct interaction of sorting signals in the cytosolic domains with adaptors in the case of transmembrane proteins, or alternatively for soluble cargo they must first bind a transmembrane receptor that in turn associates with specific adaptors via sorting signals within the cytosolic domain. At the *S. cerevisiae* TGN, the most widely characterized example of this latter group is CPY and its receptor is Vps10p (Cooper and Stevens, 1996; Marcusson et al., 1994).

The next step in vesicle formation is budding and release from the membrane. The deposition of coat proteins, clathrin in the case of TGN trafficking, onto membranes enriched with adaptors leads to membrane curvature that in turn facilitates budding. Clathrin and clathrin adaptor complexes both form spherical, cage-like structures (Kirchhausen, 2000) that are necessary for the budding of nascent vesicles. Scission, or release from the membrane is accomplished via direct action of the coat proteins to sever the neck between the vesicle and the donor compartment and in the case of clathrin coated vesicles further mediated by accessory proteins such as dynamin (Sever, 2003).

Once the vesicle is released into the cytosol it must lose its coat before it can fuse with its target membrane. The uncoating of clathrin vesicles occurs via a series of events. First the Swa2p, *S. cerevisiae* auxilin homolog, binds clathrin and recruits the 70kD heat shock cognate protein (Hsc70) to the vesicle. In addition to recruitment, Swa2p stimulates the ATPase activity of Hsc70 (Gall et al., 2000). Once activated, Hsc70 functions to disassemble the clathrin coat complex (Mayer and Bukau, 2005). Phosphoinositides also appear to play a role in uncoating, as mammalian systems

deficient in the synaptojanin class of phosphoinositide phosphatases accumulate clathrin coated vesicles (Cremona and De Camilli, 2001; Harris et al., 2000; Verstreken et al., 2003). These results suggest that Ptdins(4,5)P_2 dephosphorylation is required for proper uncoating of clathrin vesicles.

Once uncoated, vesicles are able to dock and fuse with the appropriate target membrane. The soluble NSF attachment protein receptor (SNARE) super family mediates docking and membrane fusion. This family can be divided into two categories, v-SNAREs that are incorporated into vesicles during budding, and t-SNAREs which reside in the membrane of target organelles. Most SNAREs are C-terminally anchored in the membrane with their N-termini free in the cytosol. These free N-termini interact specifically with other SNAREs via a coiled coil conformation. Structural studies indicate that the N-termini interaction involves a four helical bundle, one helix contributed by a monomeric v-SNARE and the other three by the oligomeric t-SNARE **(**Sutton et al., 1998**)**. Interactions between a specific v-SNARE and its cognate t-SNARE are what determine target membrane specificity for the uncoated vesicle (Rothman, 1994). Additional proteins are required for both docking and fusion with target membranes. These include rabs (small GTPases) and tethering factors in addition to the SNARE interactions.

Trafficking from the TGN

The *trans* Golgi network (TGN) is the last stop for cargo trafficking through the Golgi apparatus be it via vesicular transport or cisternae maturation. At the TGN, proteins have three possible trafficking routes; transport to the plasma membrane,

transport to the endosomal system or direct transport to the vacuole. The vesicles that mediate cargo transport at the TGN are distinct from the COPI vesicles involved in intra-Golgi transport. High resolution electron microscopy indicates that the TGN produces clathrin coated vesicles that mediate transport to the endosomal/vacuolar system (Ladinsky et al., 1999). In addition, secretory vesicles are formed from the TGN that deliver cargo to the cell surface. This latter group is clathrin independent and may not even involve vesicular budding. One hypothesis is that secretory carriers are remnants of TGN compartments that have been depleted of resident Golgi, endosomal and vacuolar cargo (Ladinsky et al., 1999). Evidence that disputes this model is that secretory vesicles accumulate in a number of late acting *sec* mutants. Additionally, there is evidence that TGN to plasma membrane transport is signal mediated, specifically delivery of Fus1p to the plasma membrane requires O-glycosylation (Proszynski et al., 2004).

Delivery of cargo from the TGN to the vacuole can occur via a number of discrete pathways. The most direct route is transport from the TGN to the vacuole. This is mediated via the packaging of cargo into vesicles containing the adaptor complex AP-3. Studying the trafficking itinerary of alkaline phosphatase (ALP) led to the discovery of AP-3, and this route has historically been called the ALP pathway. ALP is synthesized as a high molecular weight precursor containing a single signal-anchor signal, and is thus a type II integral membrane protein. Upon delivery to the vacuole its C-terminus is proteolytically cleaved in a Pep4p dependent manner (Klionsky et al., 1990). In the absence of any one of the four subunits that comprise AP-3, ALP is not delivered to the vacuole and mislocalized to cytoplasmic vesicles and tubules (Cowles et al., 1997a).

Proteins utilizing alternative routes to the vacuole are unaffected in AP-3 mutants. The most widely studied alternative route follows a trafficking itinerary of TGN to late endosome also called the prevacuolar compartment (PVC) to the vacuole. The components of this alternative pathway have been elucidated through the examination of carboxypeptidase Y (CPY) trafficking, and the route is called the CPY pathway. CPY is a soluble vacuolar protein. At the TGN it is packaged into clathrin-coated vesicles destined for the PVC, following its release into the PVC it is subsequently delivered to the vacuole. The machinery required for its transport to the vacuole was determined through a number of different genetic screens (Jones, 1977; Robinson et al., 1988; Rothman and Stevens, 1986). In the absence of properly functioning traffic machinery, CPY is aberrantly secreted. These genetic screens took advantage of this phenotype and uncovered 55 genes that when rendered nonfunctional resulted in CPY secretion. These genes are classified as *v*acuolar *p*rotein *s*orting (*vps*) mutants. The GGA adaptor protein(s) that mediate the transport of CPY were not found within this collection of *vps* mutants, presumably due to genetic redundancy (see below).

The adaptor protein(s) that mediate transit from the TGN to the PVC are beginning to be identified. The likely suspects are the Gga1 and Gga2 proteins. Studies examining the trafficking of CPY indicate that its delivery to the vacuole is delayed and a substantial aberrantly secreted in a *gga1Δ gga2Δ* background (Mullins and Bonifacino, 2001). In addition, in the same background the t-SNARE for the PVC, Pep12p, is mislocalized to the early endosome (EE) (Black and Pelham, 2000). These phenotypes were contingent on both proteins being deleted, suggesting that they are redundant. This

would explain why they were not initially identified in previous genetic screens, as both would have had to be deleted to yield a phenotype.

Transit to the vacuole is not limited to the ALP or CPY pathways. The endocytic pathway also delivers proteins to the vacuole. This pathway includes the plasma membrane, the EE, the PVC and the vacuole. The adaptor proteins that mediate transit to the EE have not been identified in *S. cerevisiae*. AP-2 has been well characterized in mammalian systems for mediating this process. Endocytic proteins accumulate on the plasma membrane when AP-2 function is ablated (McCormick PJ, 2005).

It should be noted that in *S. cerevisiae* when internal trafficking routes are blocked a number of integral membrane proteins transit to the vacuole via the endocytic pathway after they are aberrantly trafficked to the plasma membrane (Nothwehr et al., 1995; Ha et al., 2003) It is therefore apparent that the endocytic pathway and the biosynthetic pathway share a number of structural components in addition to trafficking machinery.

Recent studies also suggest that there is a fourth pathway for exit out of the TGN that delivers cargo to the EE, further evidence of the convergence of the biosynthetic and endocytic pathways. The vesicular machinery that mediates this process has not been identified in *S. cerevisiae*. In mammalian systems clathrin/AP-1 vesicles have been implicated in both forward trafficking to the EE and retrograde trafficking from the EE to the TGN. The γ subunit of AP-1 has been shown to colocalize with the TGN and the EE, as well as with the cargo, mannose 6-phosphate receptor (MPR), exiting the TGN (Waguri et al., 2003). In addition, in a mouse model, AP-1 has been shown to mediate the retrieval of MPRs from the EE to the TGN (Meyer et al., 2000). The role of

clathrin/AP-1 vesicles in yeast is less well established. The few studies that have been done have revealed that AP-1 and clathrin may act to recycle chitin synthase III (Valdivia et al., 2002), though this data is circumstantial at best. It is unclear if AP-1 functions in anterograde trafficking from the TGN to the EE in *S. cerevisiae*.

Endosomal to TGN Trafficking

Trafficking between the EE and the TGN remains for the most part unverified. A much better characterized retrieval pathway exists between the PVC and the TGN. This pathway recycles cargo and receptors that are delivered either intentionally or aberrantly to the LE. The adaptor complex that mediates this trafficking event is called the retromer and was initially identified in genetic screens for CPY secretion. The retromer consists of five proteins: Vps35p, Vps29p, Vps5p, Vps17p, and Vps26p. One of its frequent fliers is the CPY sorting receptor, Vps10p (Seaman et al., 1998).

Vps10p is a Type I receptor that binds CPY in the TGN, and functions to escort CPY from the TGN to the PVC, where the receptor and cargo disassociate. The cargo, CPY, proceeds onward to the vacuole and the receptor is recycled back to the TGN to sort additional CPY molecules. In support of this model, CPY is synthesized at a rate 20-fold higher than Vps10p, but binds with a 1:1 ratio (Cooper and Stevens, 1996). The primary cytosolic signal involved in Vps10p retromer recruitment has been identified, and consists of an aromatic amino acid sequence ($YSSL_{1495}$). When this sequence is mutated Vps10p ends up in the vacuole and CPY is aberrantly secreted (Cooper and Stevens, 1996).

TGN resident proteins

Much evidence of the bi-directional flow of proteins between the TGN and EE came from the study of yeast TGN residents: Kex2p and Ste13p. These proteins are peptidases that mediate maturation of the sex pheromone α factor (Fuller et al., 1989). The Golgi apparatus is in constant flux, and the terminal compartment, the TGN, is continuously being turned over as it vesiculates and delivers cargo to the endosomes, vacuole and plasma membrane. To maintain residency in a compartment that is not fixed in time or space, requires cycling to distal, post TGN compartments, followed by retrieval to newly formed TGN. In support of this model, this cycling itinerary that includes distal compartments appears to be the mechanism for TGN localization of Kex2p and Ste13p (Brickner and Fuller, 1997; Bryant and Stevens, 1997; Cereghino et al., 1995; Nothwehr and Stevens, 1994).

A-ALP is a model TGN protein

In order to fully examine the trafficking itinerary of Ste13p, a model protein was constructed comprised of the 118 amino acid cytosolic domain of Ste13p fused to the transmembrane and lumenal domains of ALP. This protein, A-ALP, is a type II integral membrane protein, localized to the TGN and cycles between the PVC and the TGN in a manner dependent upon the retromer. The 118 amino acid cytosolic domain had previously been shown to be necessary and sufficient to localize Ste13p as well as A-ALP to the TGN (Nothwehr et al., 1993; Roberts et al., 1992). It was determined that for efficient TGN localization the aromatic amino acid sequence $F_{85}XF_{87}XD$ was required (Nothwehr et al., 1993). This signal is recognized by the retromer for efficient retrieval

back to the TGN (Nothwehr et al., 2000). A derivative, termed A(F→A)-ALP, contains alanine substitutions at amino acid positions 85 and 87 and is not recognized by the retromer. The absence of retrieval by the retromer results in delivery to the vacuole and subsequent proteolytically cleavage of a 3 kD propeptide at the C terminus in a *PEP4* dependent manner (Nothwehr et al., 1993). This event is used as an assay for vacuolar delivery. The last step in transit from the PVC to the vacuole occurs quite rapidly and therefore assessments of protein delivery times to the vacuole are accepted as being indistinguishable from delivery times to the PVC.

Recent evidence suggests that A-ALP and Kex2p reach the PVC via the EE. Specifically, A(F→A)-ALP reaches the vacuole relatively slowly, with a half-time of 60 min. In contrast, other proteins such as Vps10p and Cps1p reach the PVC quickly with half-times between 5 and 15 min (Bryant and Stevens, 1997; Cowles et al., 1997b). Deletion of amino acids 2-11 in A(F→A)-ALP greatly accelerates delivery to the PVC (Ha et al., 2001), suggesting that this region confers either enhanced retention in the TGN or alternatively retrieval from a distal compartment. A signal analogous to 2-11 exists in Kex2p (Brickner and Fuller, 1997). Additional support for the EE as an intermediate trafficking compartment is seen in an *inp53*Δ strain in which A(F→A)-ALP trafficking is accelerated, but Vps10p trafficking is unaffected (Ha et al., 2001). Inp53p contains two phosphoinositide phosphatase domains and regulates the concentration of phosphoinositides thought to play roles in TGN/EE vesicular trafficking (Ha et al., 2003). Kex2p has been shown to colocalize with the EE t-SNARE Tlg1p and the EE resident Chs3p, as well as Snc1p, a late secretory v-SNARE that is recycled from the plasma membrane to the TGN via the EE (Holthius et al., 1998; Lewis et al., 2000; Santos and

Snyder, 1997). Lastly, Soi3p, which is involved in PVC to EE trafficking, is necessary for efficient delivery of Kex2p to the PVC, but dispensable for Vps10p delivery to the LE (Sipos et al., 2004).

In this thesis I have sought to more firmly establish the trafficking itinerary of A(F→A)-ALP and by extension Ste13p. In addition, I have identified trafficking machinery that is responsible for the proper localization of A(F→A)-ALP to the TGN. Lastly I have identified the region within A(F→A)-ALP that mediates interaction with this trafficking machinery responsible for its TGN location.

CHAPTER II

MATERIALS AND METHODS

General Methods and Antibodies

The production of minimal (synthetic dextrose) and rich (YPD) yeast medias, the genetic manipulation of yeast strains, and all general molecular biology methods were performed as described (Ausebel et al., 2000) or as otherwise noted. Rabbit polyclonal antibodies against alkaline phosphatase (ALP) have been previously described (Nothwehr et al., 1996) (Spelbrink and Nothwehr, 1999). Rabbit anti-hemagglutinin epitope (HA) antibodies were from Covance (Richmond, CA). Rabbit polyclonal antibodies against Cps1p were generated in New Zealand white rabbits using a fusion protein consisting of GST fused to residues 46-577 of Cps1p (gift from G. Payne University of California, Los Angelos, CA). Rabbit polyclonal antibodies against Apl2p, Gga1p and Gga2p were a gift from G. Payne, University of California, Los Angelos, CA.

Plasmids and Yeast Strains

All plasmids previously described are referenced in Table 1. The GFP-A-ALP construct pCF17 is p416-CYC containing PCR derived sgGFP (Qbiogene Inc., Irvine, CA) followed by a $(GlyAla)_3$ linker and the coding region of *STE13-PHO8*. pSH46 was constructed by inserting the 0.4 kb NcoI fragment from pSN100 into the NcoI site of pAH72 resulting in plasmid pSN317. The EagI-Bgl II fragment from pSN317 was then inserted into pSN55 using the same sites resulting in plasmid pSH46. Plasmid pCF2 was

constructed by inserting a PCR fragment containing the full length *APL2* ORF into the EcoRI/SalI sites of pRS316. pCF4 was constructed by inserting a 4.97 kb XbaI/XhoI fragment excised from pDP83-CPS1 into the same sites in pRS316. pCF34, pCF35 and pCF36 were constructed by amplifying the cytosolic domain of Ste13p and its derivatives using plasmids pSN55, pAK2 and pHJ56 as templates, respectively. The resulting PCR products were subcloned into the XbaI / SacI sites of pETGEXCT. pHJ59 and pCF37 was constructed by inserting an oligo duplex containing *STE13* residues 1-20 and 1-12 respectively into pETGEXCT. pSN311 was constructed by amplifying the Ste13p cytosolic domain containing the Δ2-11 mutation. The PCR product was subcloned into pETGEXCT using the NcoI site. Ste13-Cps1 fusion protein constructs consisted of PCR derived *STE13* promoter and relevant coding sequences cloned into EagI/EcoRI sites of pRS316. The fusion junctions for Ste13(1-23)-Cps1 and Ste13(1-12)-Cps1 are…$KSSN_{23}GSM_1IAL$… and …$RKN_{12}GAI_2AL$…respectively.

To construct the *apl2-ts* allele the *APL2* ORF was amplified using an error prone polymerase, Genemorph, from Stratagene (La Jolla, CA). The resulting population of PCR products was introduced into pRS313 via homologous recombination in CFY6-2C/pCF2. His+ yeast transformants were selected and plated onto 5-FOA in order to lose pCF2. Resulting clones were screened for growth defects at 37º C but not 24ºC. Finally, the mutagenized Apl2p-expressing plasmid rescued from the temperature sensitive yeast strain was retransformed back into CFY6-2C/pCF2 to ensure that the growth defect was in fact plasmid mediated.

All strains previously described are referenced in Table 2. SNY171-4D is a spore derived from a diploid made by crossing SNY165 and SNY94. SHY64 was constructed

by mating type switching UFY2. 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., 1994) of CPS1 with the NatR marker gene. The parental strains were SHY35, SNY156, SNY165 and SNY171-4D, respectively. CFY8, CFY9 and CFY10 were all constructed using PCR mediated gene replacement, (Wach et al., 1994) of Snx4p, Snx41p and Snx42p respectively. CFY11 is CFY10 with its mating type switched. CFY18-4C was constructed by mating CFY11 and CFY 9 and the resulting diploid was sporulated and dissected giving rise to spore 4C. CFY12 is CFY9 mating type switched. CFY19-2C was constructed by mating CFY8 and CFY12 and the resulting diploid was sporulated and dissected giving rise to spore 2C. CFY26-4D was constructed by mating CFY18-4C and CFY19-2C the resulting diploid was sporulated and dissected giving rise to spore 2D.

Radioactive labeling, immunoprecipitation, and western blot analysis

The procedure for immunoprecipitation of wild type and mutant A-ALP from [^{35}S]-methionine/cysteine-labeled cells was performed as previously described (Nothwehr *et al.*, 1993). Radioactively labeled proteins were quantified from gels using a Phosphorimager system (Fuji Photo Film Co., Tokyo, Japan). For calculation of the half-time of protein delivery to the vacuole, the log of the percentage of unprocessed protein at each time point was plotted as a function of time and the plots were analyzed by linear regression analysis. Differences in half-times that fell within one standard deviation were deemed statistically insignificant. Immunoprecipitated Cps1p and derivatives were

subjected to endoglycosidase H treatment prior to separation via SDS PAGE electrophoresis.

Binding assays

The various GST fusion proteins were expressed in *E. coli* BL21(DE3) (Novagen, Madison, WI) and were affinity purified onto glutathione-agarose beads (Sigma, St. Louis, MO). The binding of Ste13-GST and its various mutant derivatives with AP-1 was assayed using a method previously published, (Ghosh and Kornfeld, 2004). Yeast extracts were prepared in parallel from strains SHY35 and pCF35. Briefly 500 $O.D._{600}$ units of cells were harvested from each strain, washed once in ice cold H_2O and resuspended in 47.5 ml spheroplast buffer (1.4 M Sorbitol, 50 mM Tris pH 7.5, 2 mM $MgCl_2$, 10 mM DTT). Cells were spheroplasted with 12.5mg of Zymolyase 20T (Seikagaku, Tokyo, Japan), were pelleted at 450 *g*, and then washed once in 40 ml ice cold 1.2 M Sorbitol. Spheroplasts were pelleted at 450 *g* and resuspended in 10 ml 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, 0.5mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin A). Spheroplasts were dounced homogenized, and spun at 20,000 *g* for 15min, the supernatant was transferred to a fresh tube, and was brought up to 45 ml with Buffer C. Lysates were incubated with 300 μl bed volume of glutathione-agarose beads for 1hr at 4°C. The agarose beads were pelleted at 750 *g*. The supernatant was split into equal portions and incubated with 150 μl bed volume of immobilized fusion proteins for 2 hr at 4°C. Samples were washed five times in Buffer C. Proteins were eluted with the addition of 10mM glutathione in 50mM Tris pH 8, 100mM NaCl, 1 mM DTT.

Fluorescent Microscopy

Cells harvested from log-phase cultures were incubated for 30 min in YEPD media containing 0.02 mg/ml FM4-64 (Molecular Probes Inc., Eugene, OR). They were then washed twice with YEPD media and once with SD-ura media before being resuspended in SD-ura media. These preparations were carried out at 4ºC. Cells were incubated at 30ºC for 2 and 8 min, immediately placed on ice. Metabolic activity was arrested by the addition of 10 mM NaN_3 and 10 mM NaF. Cells were mounted onto 2% agarose pads containing 10 mM NaN_3 and 10 mM NaF. Cells were imaged at room temperature for FM4-64 and GFP staining using a DM5000B epifluorescent microscope equipped with a 100x HCX Plan-Apo lens, aperture = 1.4, DFC350X digital camera, and FW4000 software (Leica Microsystems, Inc., Bannockburn, IL). Images were overlayed using the FW4000 software and formatted with Adobe Photoshop 7.0. Percent colocalization was determined by counting the number of punctate structures that stained positively for FM4-64 and dividing that by the number of FM4-64 punctate structures that also stained positively for GFP.

Subcellular Fractionation

SHY35 and SHY38 were propagated overnight in YPD, the following day, 50 $O.D._{600}$ were pelleted at 460 *g* from cells in log phase. The supernatant was then spun at 15,000g. The resulting pellet was resuspended in Thorner buffer (8 M urea, 5% sodium dodecyl sulfate SDS, 40 mM Tris [pH 6.8], 0.1 mM EDTA, ß-mercaptoethanol added fresh to 1%). The 15,000 *g* supernatant was subjected to a 200,000 *g* spin for 2 hr. The

200,000 *g* pellet was resuspended in Thorner buffer. Equivalent $O.D._{600}$ (.063 $O.D._{600}$) were loaded for each fraction. Samples were separated on 12% SDS-PAGE gels, proteins were than transferred to nitrocellulose membranes. Blots were probed with the indicated antibodies. Protein quantification was assessed using a LAS-1000 Luminescent Image Analyzer (Fuji Photo Film Co., Tokyo, Japan). The percentage of protein in each fraction was normalized to the unfractionated lysate signal that was set at 100%.

Table 1. Plasmids used in this study

Strain/plasmid	Description	Origin or Reference
pRS306	*URA3* parental plasmid	(Sikorski and Hieter, 1989)
pRS313	CEN-*HIS3* parental plasmid	(Sikorski and Hieter, 1989)
pRS316	CEN-*URA3* parental plasmid	(Sikorski and Hieter, 1989)
p416-CYC	*CEN-URA3* parental plasmid with *CYC1* promoter	(Mumberg et al., 1995)
pETGEXCT	*E. coli* expression vector for expressing N-terminal protein fusions linked to GST	(Sharrocks, 1994)
pCF34	*STE13* cytosolic domain cloned into pETGEXCT	This study
pSN311	*STE13* cytosolic domain (containing Δ2-11 mutation) cloned into pETGEXCT	This study
pCF35	*STE13* cytosolic domain (containing mutation $S_{13}A$) cloned into pETGEXCT	This study
pCF36	*STE13* cytosolic domain (containing mutation $S_{13}D$) cloned into pETGEXCT	This study
pHJ59	*STE13* residues 1-20 cloned into pETGEXCT	This study
pCF37	*STE13* residues 1-12 cloned into pETGEXCT	This study
pSN55	*STE13-PHO8* gene fusion encoding A-ALP in pRS316	(Nothwehr et al., 1993)
pSN100	*STE13-PHO8* with $F_{85}A$, $F_{87}A$ mutations in pRS316	(Nothwehr et al., 1993)
pSN317	*STE13-PHO8* with Δ2-11, $F_{85}A$, $F_{87}A$ mutations in pRS314	This study
pAH72	*STE13-PHO8* with Δ2-11 mutations in pRS314	(Ha et al., 2001)
pSH24	*PEP12-ts* in pRS306	This study
pSH46	*STE13-PHO8* with Δ2-11, $F_{85}A$, $F_{87}A$ in pRS316	This study

pHJ63	*STE13-PHO8* with $S_{13}A$, $F_{85}A$, $F_{87}A$ mutations in pRS316	(Johnston et al., 2005)
pDP83-CPS1	*CPS1* in pDP83	(Spormann et al., 1991)
pCF2	*APL2* in pRS316	This study
pCF4	*CPS1* in pRS316	This study
pCF6	*apl2-ts* in pRS313	This study
pCF17	sgGFP followed by $(GlyAla)_3$ fused to *STE13-PHO8* in p416-CYC	This study
pG-P12-U	GFP fused to *PEP12*	(Black and Pelham, 2000)
pCF30	*STE13* (residues 1-23) fused to full length *CPS1* in pRS316	This study
pCF32	*STE13* (residues 1-23 containing $S_{13}A$) fused to full length *CPS1* in pRS316	This study
pCF33	*STE13* (residues 1-23 containing $S_{13}D$) fused to full length *CPS1* in pRS316	This study
pCF38	*STE13* (residues 1-12) fused to full length *CPS1* in pRS316	This study

Table 2. *S. cerevisiae* strains used in this study

Strain/plasmid	Description	Origin or Reference
SHY35	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2*	(Ha et al., 2001)
SHY38	SHY35 *inp53Δ*	(Ha et al., 2001)
UFY2	SHY35 *apl2::KanR*	(Ha et al., 2003)
SHY64	UFY2 mating type switched	This study
SNY36-9A	*MAT*a *leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2*	(Nothwehr et al., 1995)
SNY94	SNY36-9A *end3ts*	(Spelbrink and Nothwehr, 1999)
SNY156	SNY36-9A *pep12-49 (ts)*	(Bruinsma et al., 2004)
SNY165	*MATα leu2-3,112 ura3-52 his3-200 trp1-90 lys2-801 suc2-9 pho8-X gga1Δ::TRP1 gga2Δ::KanR*	(Ha et al., 2003)
SNY171-4D	*MATα leu2-3,112 ura3-52 his3-200 trp1-90 lys2-801 suc2-9* (*pho8-ΔX* or *pho8Δ::ADE2*) *gga1Δ::TRP1 gga2Δ::KanR end3-ts*	This study
LSY2	SNY36-9A *pep4Δ::TRP1*	(Spelbrink and Nothwehr, 1999)
CFY6-2C/pCF2	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 pho8Δ::ADE2 apl2Δ::KanR gga1Δ::TRP1 gga2Δ::KanR* + pCF2	This study
CFY25-3B/pCF6	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 apl2Δ::KanR gga1Δ::TRP1 gga2Δ::KanR end3-ts (pho8-ΔX or pho8Δ::ADE2)* +pCF6	This study
CFY30	SHY35 *cps1Δ::NatR*	This study
CFY31	SNY156 *cps1Δ::NatR*	This study
CFY32	SNY165 *cps1Δ::NatR*	This study
CFY33	SNY171-4D *cps1Δ::NatR*	This study
CFY35	*MATα ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 prb1Δ::HISG prc1Δ::HIS3 APM1::HA-URA3*	This study

CFY37	SNY36-9A *vps8*Δ*::KanR*	This study
CFY38	SNY36-9A *soi3*Δ*::KanR*	This study
CFY8	SHY35 *snx41*Δ*::KanR*	This study
CFY9	SHY35 *snx42*Δ*::KanR*	This study
CFY10	SHY35 *snx4*Δ*::KanR*	This study
CFY11	CFY10 mating type switched	This study
CFY12	CFY9 mating type switched	This study
CFY18-4C	SHY35 *snx4*Δ*::KanR snx42*Δ*::KanR*	This study
CFY19-2C	SHY35 mating type switched *snx41*Δ*::KanR snx42*Δ*::KanR*	This study
CFY26-4D	SHY35 *snx4*Δ*::KanR snx41*Δ*::KanR snx42*Δ*::KanR*	This study

CHAPTER III

The trafficking itinerary of A(F→A)-ALP includes the EE

Background

Recent studies suggest that Ste13p and Kex2p may traverse through the EE in transit to the vacuole. First, the Ste13p based reporter A(F→A)-ALP, reaches the PVC slowly with a half-time of approximately 60 min., in contrast Vps10p and Cps1p reach the PVC much more quickly with a half-time of 5 min. (Cowles et al., 1997b) (Bryant and Stevens, 1997). Deletion of residues 2-11 within A(F→A)-ALP accelerates its trafficking into the PVC (Bryant and Stevens, 1997; Ha et al., 2001), and an analogous signal exists in Kex2p (Brickner and Fuller, 1997). This data is consistent with a model in which both A(F→A)-ALP and Kex2p are routed through the EE and slowly delivered to the PVC. Residues 2-11 could function as a sorting signal either in a static retention mechanism at the TGN, or in a retrieval mechanism at the EE. In the absence of static retention or EE retrieval the proteins would be quickly delivered to the PVC. Further evidence in support of this model is the trafficking phenotype of A(F→A)-ALP in an *inp53*Δ mutant. Inp53p functions to dephosphorylate phosphoinositides, specifically Ptdins(4,5)P_2, that have been implicated in both vesicle formation as well as vesicle uncoating (Cremona and De Camilli. 2001). In the absence of Inp53p function, trafficking of A(F→A)-ALP to the PVC is accelerated, but trafficking of Vps10p is unaffected (Ha et al., 2001). This is suggestive of two distinct populations of vesicles,

one regulated by Inp53p that mediates A(F→A)-ALP transport and the other independent of Inp53p, that mediates Vps10p as well as Cps1p transport. Additional evidence for a EE itinerary is the observation that Kex2p colocalizes with the EE t-SNARE, Tlg1p as well as chitin synthase III (Chs3p) which is stored in the EE, and Snc1p, a secretory protein that recycles from the plasma membrane to the TGN via the EE (Holthuis et al., 1998; Lewis et al., 2000; Santos and Snyder, 1997; Ziman et al., 1996). Finally, Soi3p is required for transport from the EE to the TGN (Sipos et al., 2004). In a *soi3*Δ strain trafficking of a retrieval defective Kex2p derivative is delayed. This is strong evidence that Kex2p transits through the EE, as blocking a downstream trafficking event, Soi3p-mediated transport to the PVC, significantly slows Kex2p delivery to the PVC.

A-ALP is a Ste13p based reporter protein. It consists of the 118 amino acid cytosolic domain of Ste13p fused to the lumenal and transmembrane domains of ALP. The cytosolic domain is believed to be the sole determinant in mediating trafficking events (Nothwehr et al., 1993). Once delivered to the PVC, it is rerouted back to the TGN via the retromer. A retromer retrieval signal is required for this event and consists of the aromatic sequence $F_{85}XF_{87}XD$ for A-ALP (where X represents any amino acid) (Bryant and Stevens, 1997; Nothwehr et al., 2000). In the absence of retromer retrieval, A-ALP is quickly delivered from the PVC to the vacuole. Once in the vacuole, its C-terminus is processed to release an approximately 3 kD propetide (Nothwehr et al., 1993). Processed versus unprocessed forms of the protein are observable on a polyacrylamide gel, and used as an assay to determine kinetic delivery to the vacuole. Mutations of F_{85} and F_{87} to alanines disrupts retromer retrieval and results in delivery of this A-ALP

derivative [A(F→A)-ALP] to the vacuole with a half-time of 60 min (Nothwehr et al., 1993).

This study seeks to more firmly establish the EE as the destination of A(F→A)-ALP following its exit from the TGN.

Results

To test if A-ALP transits through the EE on its way to the PVC, the protein was tagged with super glow Green Fluorescent Protein (sgGFP) and expressed using the *CYC1* promoter (Mumberg et al., 1995). Addition of the tag to either A-ALP or A(F→A)-ALP did not affect their processing kinetics, implying that the tagged derivatives follow the same trafficking itinerary as untagged proteins (Holly Johnston unpublished data). The staining pattern of GFP-A-ALP was punctate which is typical of yeast TGN/endosomal proteins (Fig. 3-1). Cells expressing GFP-A-ALP were incubated with the lipophilic dye FM4-64. This dye strongly interacts with lipids upon introduction to yeast cells and integrates with the plasma membrane where it is internalized and delivered to the vacuole via the endocytic pathway. Delivery of the dye to the vacuole is energy, time and temperature dependent (Vida and Emr, 1995). Initial incubation was carried out at 0ºC to allow for integration with the plasma membrane but not internalization of the dye. Cells were shifted to 30º C for 2min. to allow the dye to enter the endocytic pathway and occupy the EE. The staining pattern of the dye after 2 min was punctate, typical of yeast endosomes, with little or no vacuolar staining (Fig 3-1). Pictures were taken of FM4-64 punctate structures using a RFP filter and GFP punctate structures using a GFP filter. Overlaying the fluorescent field of FM4-64 with that of

Fig. 3-1: GFP-tagged A-ALP partially colocalizes with early endosomes. 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. At the end of each time point trafficking was stopped by the addition of NaN_3 and NaF. *(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 was analyzed for each data point.

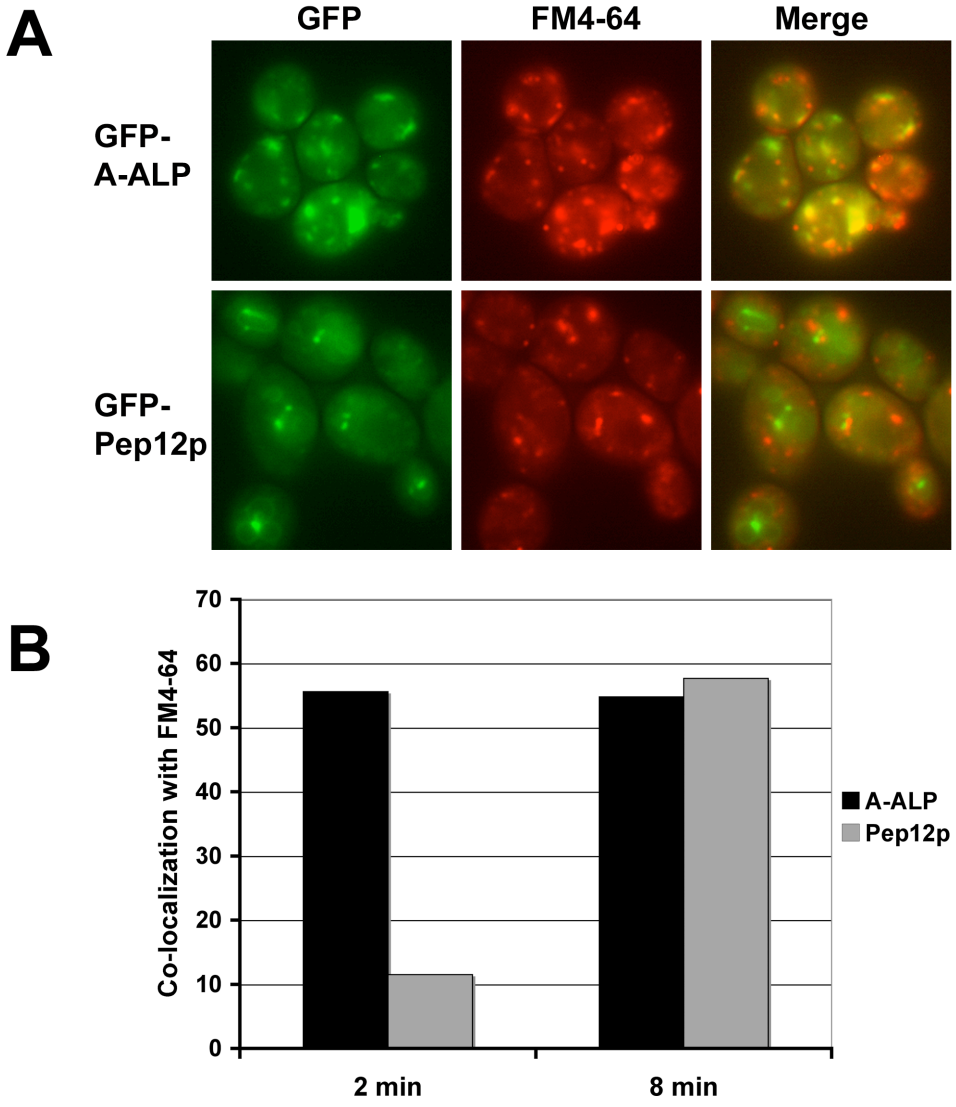

A
GFP
FM4-64
Merge
GFP-
A-ALP
GFP-
Pep12p
B
Co-localization with FM4-64
70
60
50
40
30
20
10
0
2 min
8 min
A-ALP
Pep12p

Fig. 3-1

GFP-A-ALP resulted in 56% of the punctate structures staining positive for both FM4-64 and GFP. This colocalization was compared with a control endosomal protein, GFP-Pep12p. This protein is a t-SNARE for the PVC and does not reside in the EE (Black and Pelham, 2000). It exhibits a punctate staining pattern typical of proteins in the TGN/endosomal system, however only 11% of the FM4-64 structures were positive for GFP-Pep12p. The GFP-Pep12 results strongly suggest that the vast majority of FM4-64 punctate structures are EE and not PVC, following 2 min of internalization. One would expect as the dye proceeds to the vacuole that over time the EE negative control, GFP-Pep12p, will show an increased colocalization as the dye enters the PVC. Following 8 min internalization of the dye the amount of colocalization does in fact jump to 58% for GFP-Pep12p and remains fairly constant for GFP-A-ALP with 55% colocalization. This is consistent with a model in which GFP-A-ALP is in both the EE as well as the PVC, and GFP-Pep12p is restricted to the PVC. At the two min time point, the dye is limited to the EE and therefore only colocalizes with GFP-A-ALP. Over time, as FM4-64 progresses toward the vacuole it occupies the PVC and therefore colocalizes with both GFP-A-ALP and GFP-Pep12p. To determine if A(F→A)-ALP accesses the PVC via the EE, its processing was examined in *soi3*Δ and *vps8*Δ strains. Soi3p and Vps8p are both implicated in trafficking between the EE and the PVC (Luo and Gallwitz, 2003; Sipos et al., 2004). In addition Vps8p also appears to function in delivery from the PVC to the vacuole (Subramanian et al., 2004). Processing of A(F→A)-ALP was significantly slowed in both mutants, and the delay was greatest in the *soi3*Δ strain (Fig 3-2A). These results are consistent with the delay observed in Kex2p delivery to the vacuole in a *soi3*Δ

Fig. 3-2: A(F→A)-ALP uses a TGN-to-EE-to-PVC pathway before reaching the vacuole. Wild type (SHY35), *soi3*Δ (CFY38), *vps8*Δ (CFY37), *pep12-ts* (SNY156), *gga1*Δ *gga2*Δ (SNY165), and *gga1*Δ *gga2*Δ *end3-ts* (SNY171-4D) strains carrying a plasmid expressing A(F→A)-ALP (pSN100) were analyzed. Cells were pulsed for 10 min with [^{35}S]methionine/cysteine and chased with an excess of unlabeled amino acids for the indicated times. The strains were either incubated at 30˚C throughout the time course *(A)* or were propagated for several doublings at 24˚C before shifting to 36˚C for 10 min prior to the initiation of the chase *(B, 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 the panels in *A* and *C*.

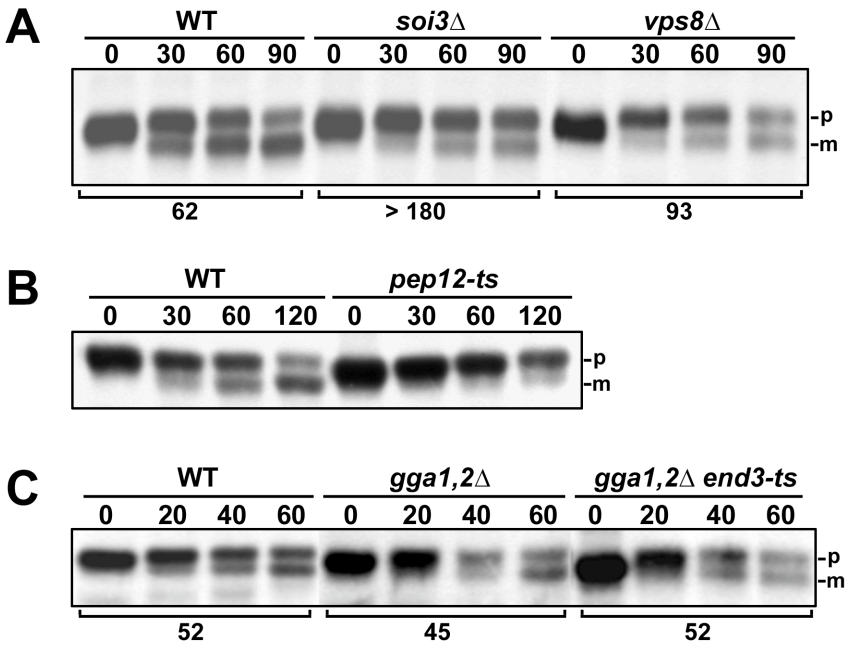

A
WT
soi3Δ
vps8Δ
0 30 60 90 0 30 60 90 0 30 60 90
-p
-m
62
> 180
93
B
WT
pep12-ts
0 30 60 120 0 30 60 120
-p
-m
C
WT
gga1,2Δ
gga1,2Δ end3-ts
0 20 40 60 0 20 40 60 0 20 40 60
-p
-m
52
45
52

Fig. 3-2

strain (Sipos et al., 2004), strongly suggesting that both A(F→A)-ALP as well as Kex2p access the PVC via the EE.

To ensure that delivery of A(F→A)-ALP to the vacuole requires transit to the PVC and does not occur via an alternative pathway, its processing was examined in a strain lacking functional Pep12p. Vesicles targeted for the PVC must interact with the t-SNARE, Pep12p, in order to fuse with and release cargo into the PVC. In the absence of Pep12p, PVC bound vesicles accumulate in the cytosol (Becherer et al., 1996). Vacuolar processing of A(F→A)-ALP was assessed in a strain temperature sensitive for Pep12p. Wild type and *pep12-ts* strains were propagated at 24ºC then shifted to 30ºC for 10 min and chased for the indicated times (Fig 3-2B). The processing half-time in a wild type strain was approximately 60 min. In contrast, processing in the *pep12-ts* temperature sensitive strain was almost completely blocked. This result indicates that in order for A(F→A)-ALP to access the vacuole it must traverse through the PVC, and can not utilize in alternative pathway when trafficking to the PVC is perturbed. This data points to a trafficking itinerary of TGN-EE-PVC-vacuole for A(F→A)-ALP.

The CPY pathway involves transit from the TGN to the vacuole via clathrin-coated vesicles. GGA adaptor proteins mediate the formation of these vesicles in the CPY pathway. It has previously been shown that A(F→A)-ALP is mislocalized to the plasma membrane in clathrin mutants and than delivered to the vacuole via the endocytic pathway (Nothwehr et al., 1995; Ha et al., 2003). It is possible that A(F→A)-ALP could utilize the CPY pathway to access the PVC. To test that it does not access the PVC via this GGA-mediated direct TGN to PVC route, A(F→A)-ALP processing time was examined in strains deficient for GGA function and temperature sensitive for End3p.

This latter protein is required for internalization of proteins at the plasma membrane (Bénédetti et al., 1994). Strains were propagated at 24º C then shifted to 30º for 10 min, followed by a 10 min pulse and chased for the indicated times (Fig 3-2C). There was no change in the processing kinetics of A(F→A)-ALP in a *gga1*Δ *gga2*Δ strain or *gga1*Δ *gga2*Δ *end3-ts* strain compared to wild type (Fig 3-2C). If the GGA proteins mediate trafficking of A(F→A)-ALP to the vacuole, then a delay would have been expected in strains deficient for these proteins. This data refutes a model in which A(F→A)-ALP accesses the PVC via the GGA-dependent CPY pathway, as trafficking is not slowed in strains lacking GGA function. Furthermore, A(F→A)-ALP is not aberrantly transported to the plasma membrane in a *gga1*Δ *gga2*Δ strain. Therefore, the phenotype of A(F→A)-ALP delivery to the plasma membrane in a clathrin mutant implies that there is another GGA-independent, clathrin dependent pathway between the TGN and the endosomal system and it does not include the plasma membrane.

The observation that ablating GGA function does not affect the trafficking itinerary of A(F→A)-ALP strongly suggests there is an alternative pathway to the endosomal system. To strengthen our assertion that GGA proteins do not mediate forward trafficking of A(F→A)-ALP, we wanted to juxtapose the lack of trafficking defects seen with A(F→A)-ALP in a *gga1*Δ *gga2*Δ strain with trafficking defects observed with proteins that use the GGA/CPY pathway in a *gga1*Δ *gga2*Δ strain. Cps1p is a vacuolar protease that is transported to the vacuole via the CPY pathway. Once in the vacuole it is proteolytically cleaved yielding the mature form (Cowles et al., 1997b). Its trafficking kinetics should be dramatically affected in strains lacking GGA function. A recent study examined Cps1p delivery to the vacuole in a *gga1*Δ *gga2*Δ strain

(Costaguta et al., 2001), and found that processing was completely blocked. We attempted to repeat these results. In addition to a *gga1*Δ *gga2*Δ strain, trafficking was also examined in a *gga1*Δ *gga2*Δ *end3-ts* strain and in a *pep12-ts* strain by pulse chase immunoprecipitation. Cells were propagated at 24ºC then shifted to 36ºC for 10 min, pulsed for 10 min and chased for the indicated times (Fig 3-3A). In a *gga1*Δ, *gga2*Δ strain Cps1p was significantly delayed but not blocked as reported by Costaguta et. al. 2001. Furthermore, addition of the *end3-ts* mutation increased the delay suggesting that a pool of Cps1p does go to the plasma membrane in a *gga1*Δ, *gga2*Δ strain. The lack of a complete block in the *gga1*Δ *gga2*Δ *end3-ts* strain further reinforces a model in which there is an internal GGA-independent pathway for delivery from the TGN to the endosomal system. In addition, access to the vacuole requires Cps1p to transit through the PVC as processing is blocked by the *pep12-ts* mutation (Fig 3-3A).

These results suggest that Cps1p uses the GGA dependent pathway to access the vacuole in a PVC dependent manner. To further support this itinerary, trafficking of Cps1p was examined by pulse chase immunoprecipitation in a *soi3*Δ and in *vps8*Δ strain. If Cps1p predominantly uses the TGN-to-PVC pathway than the *soi3*Δ mutant should have little effect on its trafficking itinerary, and the *vps8*Δ mutation should significantly affect it because Vps8p plays a role in TGN-to-PVC as well as PVC-to-vacuole trafficking. Cells were propagated at 30º C, pulsed for 10 min, and chased for the indicated times (Fig 3-3B). In support of a TGN to PVC itinerary, the *soi3*Δ mutant had little or no effect on the kinetics of vacuolar delivery of Cps1p, and this was in stark contrast to the delay seen in Cps1p vacuolar delivery in the *vps8*Δ strain.

Fig. 3-3: Cps1p uses a GGA-dependent direct TGN-to-PVC pathway prior to reaching the vacuole. Strains CFY30, CFY32, CFY33, and CFY31 (from left-to-right) carrying a CEN-*CPS1* plasmid were analyzed in *(A)* while strains SHY35, CFY38, and CFY37 were analyzed in (*B*). The strains were either propagated for several doublings at 24˚C before shifting to 36˚C for 10 min prior to the initiation of the chase *(A)* or were incubated at 30˚C throughout the time course (*B*). After each chase time Cps1p was immunoprecipitated, treated with endoglycosidase H, 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.

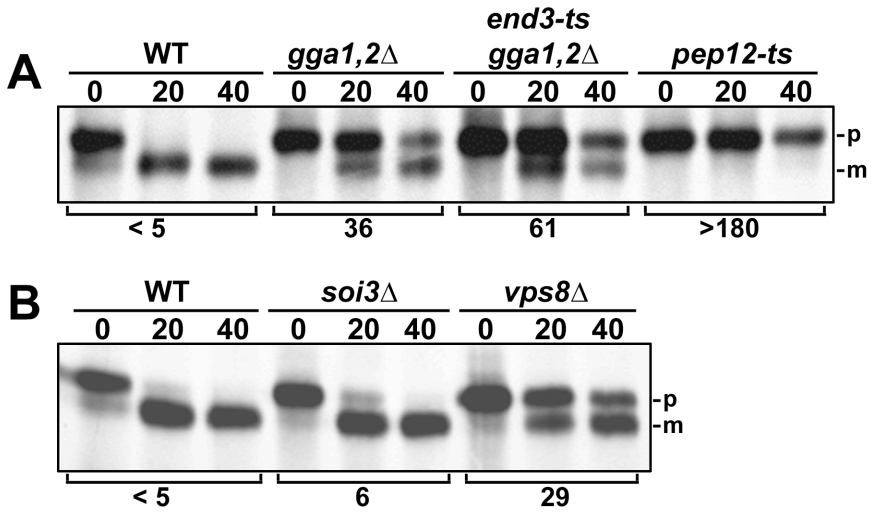

A
WT
gga1,2Δ
end3-ts
gga1,2Δ
pep12-ts
0 20 40 0 20 40 0 20 40 0 20 40
-p
-m
< 5
36
61
>180
B
WT
soi3Δ
vps8Δ
0 20 40 0 20 40 0 20 40
-p
-m
< 5
6
29

Fig. 3-3

Discussion

These results strongly point to a trafficking itinerary of TGN-to-EE-to-PVC for A(F→A)-ALP. First, it was demonstrated that a GFP tagged A-ALP colocalizes with the fluorescent dye FM4-64 at a time when the dye predominantly occupies the EE. This was in contrast to a PVC localized t-SNARE, Pep12p that did not colocalize with the dye at this early time point. Clathrin adaptors, Gga1 and Gga2 proteins are not required for anterograde trafficking of A(F→A)-ALP as the processing kinetics of A(F→A)-ALP delivery to the vacuole is unaffected in a *gga1*Δ *gga2*Δ strain. Furthermore, the protein is not mislocalized to the plasma membrane in a *gga1*Δ *gga2*Δ strain, as it is in a clathrin mutant. Transit through the PVC is required, as loss of Pep12p function blocks processing. Lastly, proteins that mediate delivery from the EE to the PVC, Soi3p and to a lesser extent Vps8p, are required for efficient delivery of A(F→A)-ALP to the PVC. These data reinforce a model in which A(F→A)-ALP exits the TGN in clathrin coated vesicles independently of GGA function and are delivered to the EE, followed by delivery to the PVC.

The trafficking kinetics of A(F→A)-ALP contrasted with those seen with Cps1p further reinforce this model. Cps1p is affected in a *gga1*Δ *gga2*Δ and even more so in a *gga1*Δ *gga2*Δ *end3-ts* strain. The lack of a complete trafficking block of Cps1p in the *gga1*Δ *gga2*Δ *end3-ts* strain suggests that the TGN-to-EE trafficking step may not be signal mediated. It seems unlikely that Cps1p would contain this signal as its normal itinerary is TGN-to-PVC via clathrin/GGA coated vesicles. It does however access this pathway in the *gga1*Δ *gga2*Δ *end3-ts* strain. One explanation is based on the cisternal maturation model in which the formation of the EE is due to vesiculation of the TGN

which has been depleted of secretory proteins as well as proteins utilizing the CPY and ALP pathways (Pelham, 1998). In the absence of TGN-to-PVC transport, Cps1p is swept along with other "signal-less" cargo in a post TGN compartment that fuses with endocytic vesicles to form the EE.

CHAPTER IV

AP-1 is not required for anterograde trafficking of A(F→A)-ALP, rather it delays its delivery to the PVC

Background

The lack of a trafficking defect observed in strains lacking GGA function suggests that A(F→A)-ALP accesses the endosomal system via a population of vesicles that form independently of the GGAs. Furthermore, this population appears to be clathrin coated, as A(F→A)-ALP is aberrantly transported to the plasma membrane in clathrin mutants (Ha et al., 2003). In *S. cerevisiae* three AP complexes have been identified; AP-1, AP-2 and AP-3. Only AP-1 has been shown to physically interact with clathrin (Pishvaee et al., 2000; Yeung and Payne, 2001; Yeung et al., 1999). The role of AP-1 in trafficking between the TGN and endosomal system has not been well established. In mammalian systems it has been implicated in both anterograde trafficking from the TGN to the EE (Huang et al., 2001) (Puertollano et al., 2003) as well as retrograde trafficking from EE to the TGN (Klumperman et al., 1998) (Meyer et al., 2000). In yeast much less is known about its function. In the absence of AP-1 in yeast, chitin synthase III (Chs3p) is mislocalized to the plasma membrane under conditions in which it is normally sequestered in the EE (Valdivia et al., 2002). Furthermore, when combined with *gga1*Δ *gga2*Δ mutations loss of AP-1 function results in a loss of viability (Costaguta et al., 2001). This data implies that AP-1 functions at the EE or at the TGN and that its function is either fully or partially redundant with GGA function. As the GGAs mediate forward progression from the TGN to the endosomal system, AP-1 does appear to be an

excellent adaptor protein candidate for mediating transport between the TGN and the EE. However, trafficking of A(F→A)-ALP in a strain lacking AP-1 function is not delayed but instead slightly accelerated (Ha et al., 2003). Although this suggests that AP-1 does not mediate anterograde trafficking, it is possible that in AP-1 mutants A(F→A)-ALP could be rerouted through the GGA pathway to access the PVC or trafficked to the plasma membrane followed by endocytosis to the EE and subsequently delivered to the PVC.

A number of distinct signals have been identified in A(F→A)-ALP that mediate its trafficking itinerary. In addition to the $F_{85}XF_{87}XD$ domain that mediates retrieval from the PVC (Nothwehr et al., 1993), and the 2-11 region that slows delivery to the PVC (Bryant and Stevens, 1997; Ha et al., 2001), there is a third signal that is involved in either retrieval from the EE or progression from the EE to the PVC. This signal involves phosphorylation of serine at residue 13 in the cytosolic domain of A(F→A)-ALP. A $S_{13}A$ mutant is blocked from accessing the PVC, though it does proceed as far as the EE (Johnston et al., 2005). Furthermore, a $S_{13}D$ mutant that mimics phosphorylation is accelerated in its delivery to the vacuole (Johnston et al., 2005). Thus the S_{13} residue when unphosphorylated appears to strongly impede trafficking to the PVC.

Formation of vesicle coats requires a number of accessory proteins in addition to the adaptors, coat and cargo receptor. Specifically, Ptdins(4,5)P_2 have been implicated in recruiting clathrin adaptors to membranes (Bonifacino and Lippincott-Schwartz, 2003). One class of yeast proteins, sorting nexins, are recruited to membranes via specific binding to phosphoinositide derivatives to in turn facilitate adaptor recruitment and protein trafficking. For example, the retromer subunit Vps5p contains a PX domain that

mediates binding to phosphatidylinositol 3-phosphate [Ptdins(3)P] at the PVC (Burda et al., 2002; Sato et al., 2001). Furthermore, the sorting nexins Snx4, Snx41 and Snx42 mediate retrieval from the EE to the TGN of the exocytic v-SNARE Snc1p (Hettema et al., 2003). Thus if A(F→A)-ALP cycles between the EE and the TGN these sorting nexins may facilitate vesicle formation at the EE and mediate retrieval of A(F→A)-ALP.

Results

To more fully investigate the role of AP-1 in A(F→A)-ALP trafficking a temperature sensitive allele of *APL2* was needed in order to construct a triple mutant lacking GGA and AP-1 function. AP-1 is a heterotetramer comprised of four proteins. The β subunit is Apl2p (see Fig 1-3). Using random mutagenesis an *apl2-ts* allele was generated and introduced into a strain that was *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*URA*-*APL2*. The wild type *APL2* plasmid was selected against and the resulting strain, *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS*-*apl2-ts* assayed for growth at 24ºC and 37ºC (Fig 4-1A). Viability was comparable to wild type at 24º C, though the rate of growth was somewhat slower. In contrast, at the nonpermissive temperature there was little if any growth. These results are in agreement with the synthetic lethality previously observed when these three genes are rendered nonfunctional (Costaguta et al., 2001).

The trafficking kinetics of A(F→A)-ALP was examined in this strain at both the permissive and nonpermissive temperatures. Cells were propagated at 24ºC, shifted to 36ºC, pulsed for 10 min and chased for the indicated times (Fig 4-1B). Interestingly, at 36ºC trafficking was significantly accelerated as well as at 24ºC. The difference in half-times at both temperatures was within one standard deviation, thus it would appear that

Fig. 4-1: Both the adaptor complex AP-1 and the Ste13p 2-11 region slow transport of A(F→A)-ALP through the TGN/EE/PVC pathway rather than being required for anterograde transport. *(A)* Strains SHY35 (wild type), UFY2 (*apl2*Δ), SNY165 (*gga1*Δ *gga2*Δ), CFY6-2C/pCF2 (*apl2*Δ *gga1*Δ *gga2*Δ*/pCEN-APL2*), and CFY6-2C/pCF6 (*apl2*Δ *gga1*Δ *gga2*Δ*/pCEN-apl2-ts*) were analyzed by spotting 10-fold serial dilutions onto YEPD media and incubating for 4 days at the indicated temperature. *(B)* Strains SHY35, CFY6-2C/pCF6, and CFY25-3B/pCF6 (*apl2*Δ *gga1*Δ *gga2*Δ *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, then chased with unlabeled amino acids for the indicated times. *(C)* Strain CFY6-2C/pCF6 carrying (left-to-right) plasmids pSN100 [A(F→A)-ALP], pHJ63 [A(S_{13}A; F→A)-ALP], or pSH46 [Δ2-11; F→A)-ALP] was analyzed after shifting from 24˚C to 36˚C as in *(B)*. *(D)* Strains SHY35, SNY94 (*end3-ts*), SNY165, and SNY171-4D carrying pSN100 (upper panel) or pSH46 (lower panel) was analyzed after shifting from 24˚C to 36˚C as in *(B)*. *(B, C,* and *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.

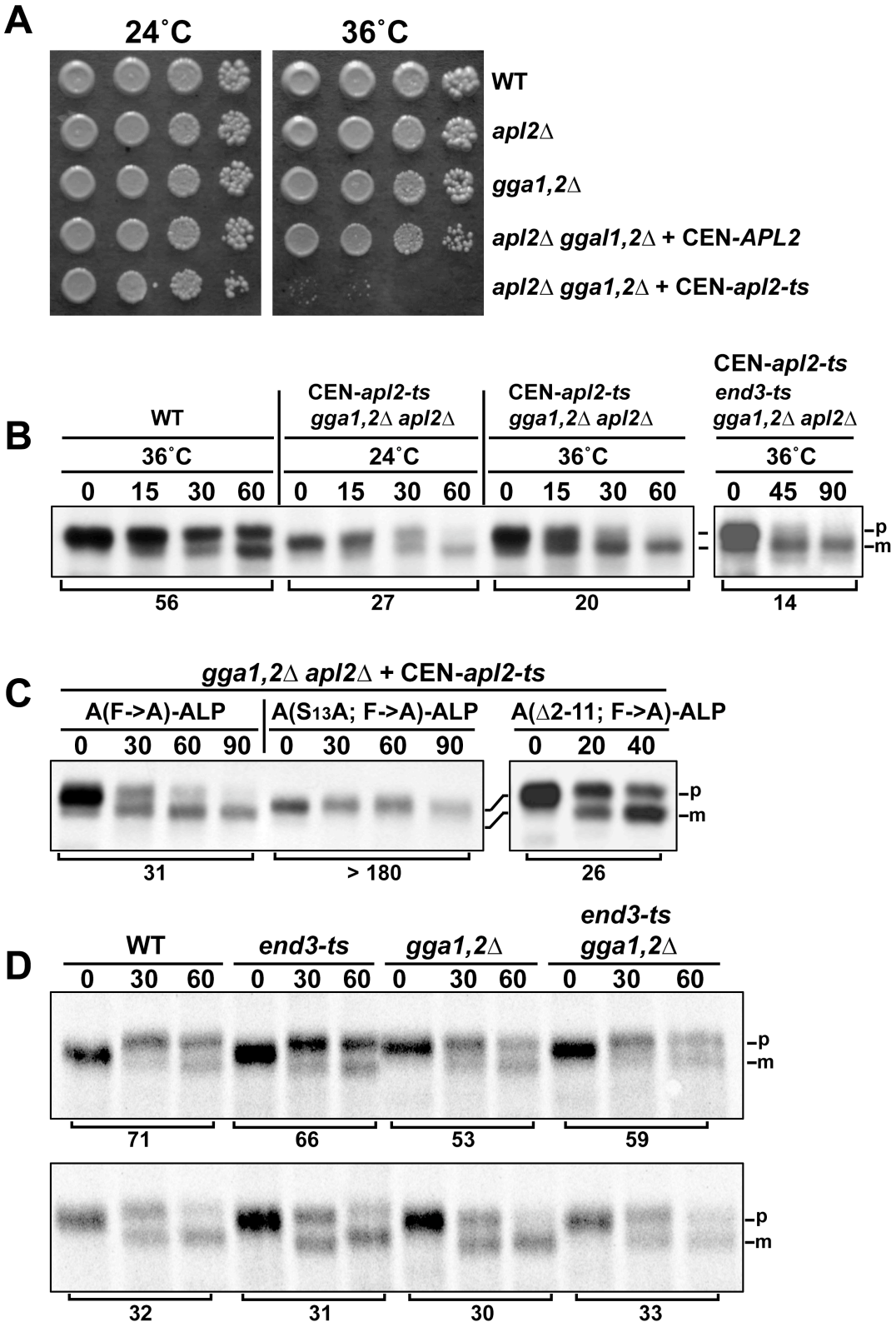

A
24˚C
36˚C
WT
apl2Δ
gga1,2Δ
apl2Δ ggal1,2Δ + CEN-APL2
apl2Δ gga1,2Δ + CEN-apl2-ts
B
WT
36˚C
0 15 30 60
CEN-apl2-ts gga1,2Δ apl2Δ
24˚C
0 15 30 60
CEN-apl2-ts gga1,2Δ apl2Δ
36˚C
0 15 30 60
CEN-apl2-ts end3-ts gga1,2Δ apl2Δ
36˚C
0 45 90
p
m
56
27
20
14
C
gga1,2Δ apl2Δ + CEN-apl2-ts
A(F->A)-ALP
0 30 60 90
A(S13A; F->A)-ALP
0 30 60 90
A(Δ2-11; F->A)-ALP
0 20 40
p
m
31
> 180
26
D
WT
0 30 60
end3-ts
0 30 60
gga1,2Δ
0 30 60
end3-ts gga1,2Δ
0 30 60
p
m
71
66
53
59
p
m
32
31
30
33

Fig. 4-1

the *apl2-ts* allele is functional for growth at 24º C, but not trafficking of A(F→A)-ALP at this permissive temperature. To ensure that the protein is not aberrantly trafficked to the plasma membrane, the trafficking kinetics of A(F→A)-ALP was examined in a *gga1*Δ *gga2*Δ *end3-ts apl2*Δ/pCEN-*HIS3-apl2-ts* strain. If processing occurred via an itinerary that included the plasma membrane, it should be blocked in this strain. Processing was not blocked, rather it was accelerated, consistent with the phenotype observed in the *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain. These results provide strong evidence that GGA and AP-1 function are dispensable for A(F→A)-ALP delivery to the EE. Furthermore, the trafficking itinerary of A(F→A)-ALP does not include the plasma membrane when GGA and AP-1 functions are simultaneously ablated.

A devil's advocate or committee member might suggest that in the absence of GGA and AP-1 function normal trafficking routes out of the TGN may be compromised, and TGN proteins could be delivered to the vacuole via an alternative aberrant pathway, i.e. the autophagic pathway. To address this concern, trafficking of A(S_{13}A; F→A)-ALP was examined in the *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain. The S_{13}A mutation in A(F→A)-ALP blocks its trafficking in wild type cells, and this block occurs at the EE (Johnston et al., 2005). Cells were propagated at 24ºC, shifted to 36ºC, pulsed for 10 min. and chased for the indicated times (Fig 4-1C). Trafficking of A(S_{13}A; F→A)-ALP was indeed blocked in the *gga1*Δ *gga2*Δ, *apl2*Δ/pCEN-*HIS3-apl2-ts* strain at the nonpermissive temperature as was the case for the wild type strain. This result suggests that delivery of A(F→A)-ALP to the PVC in a strain compromised in GGA and AP-1 function occurs via the EE. The accelerated processing is not due to delivery to the vacuole via an alternative pathway not seen in wild type cells.

Residues 2-11 of A(F→A)-ALP function to slow delivery of the protein to the PVC (Bryant and Stevens, 1997; Ha et al., 2001; Johnston et al., 2005). It is unclear at what point in the trafficking itinerary of A(F→A)-ALP that this signal comes into play. It could function at the TGN in static retention, or it could mediate retrieval at the EE, thereby slowing delivery to the PVC. Alternatively, the signal could be required for entry into the TGN-EE pathway and in the absence of 2-11 the protein is routed through an alternative pathway.

To determine its itinerary, the trafficking kinetics of A(Δ2-11, F→A)-ALP were examined using pulse chase immunoprecipitation. We first examined its trafficking in a *gga1*Δ *gga2*Δ *end3-ts* strain as well as the parental strains; wild type, *gga1*Δ *gga2*Δ and *end3-ts*. Cells were propagated at 24ºC, shifted to 36ºC, pulsed for 10 min and chased for the indicated times (Fig 4-1D). Processing half-times were the same as wild type in all three strains. This result suggests that A(Δ2-11, F→A)-ALP is not shuttled into an alternative pathway, as processing is the same as wild type as in a *gga1*Δ *gga2*Δ *end3-ts* strain in which both alternatives are blocked. Therefore, residues 2-11 appear to function at either the TGN in static retention or at the EE by preventing retrieval.

To address this latter issue, processing was examined in a *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain. If the 2-11 signal functions at the same step as AP-1, then there should not be an additive effect with respect to accelerated delivery to the vacuole. In contrast, if the signal functions at the TGN then the effects should be additive as both 2-11 function (TGN retention) and AP-1 function (EE retrieval) are operable. Cells were propagated at 24ºC, shifted to 36ºC, pulsed for 10 min and chased for the indicated times (Fig 4-1C). Processing half-times were similar for A(Δ2-11, F→A)-ALP

in the *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain (26 min half-time; Fig 4-1C) and the wild type strain (32 min half-time; Fig 4-1D). These results were also similar to the processing time of A(F→A)-ALP in the *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain (20 min half-time; Fig 4-1B). Therefore, the results are consistent with a model in which the 2-11 signal functions in concert with AP-1.

These results indicate that A(F→A)-ALP cycles between the EE and the TGN to maintain TGN residency. AP-1 appears to be the adaptor that mediates retrieval via clathrin coated vesicles from the EE to the TGN. We next asked if other proteins played a role in this retrieval. It has previously been observed that sorting nexins Snx4p, Snx41p and Snx42p mediate retrieval of the exocytic v-SNARE, Snc1p, from the EE to the TGN. Is retrieval of A(F→A)-ALP from the EE to the TGN mediated by these sorting nexins?

The trafficking kinetics of A(F→A)-ALP was examined in *snx4*Δ, *snx41*Δ, and *snx42*Δ strains. Cells were propagated at 30ºC, pulsed for 10 min and chased for the indicated times (Fig 4-2A). There was no discernible difference in any of these three strains as compared to wild type. The possibility exists that these sorting nexins may be redundant and the function of one may be able to compensate for another. To assess this, a triple mutant was constructed, *snx4*Δ *snx41*Δ *snx42*Δ, that lacked all three sorting nexins. The processing half-time for A(F→A)-ALP was determined in this strain via pulse chase immunoprecipitation (Fig 4-2B). There was no discernible difference in processing half-times in the *snx4*Δ *snx41*Δ *snx42*Δ strain compared to a wild type strain.

Fig. 4-2: Snx4p, Snx41p and Snx42p do not mediate trafficking of A(F→A)-ALP. (*A*) Wild type (SHY35), *snx4*Δ (CFY10), *snx41*Δ (CFY8) and *snx42*Δ (CFY9) strains or (*B*) Wild type (SHY35) and *snx4*Δ *snx41*Δ *snx42*Δ (CFY26-2D) strains carrying a plasmid expressing A(F→A)-ALP (pSN100) were analyzed. Cells were pulsed for 10 min with [^{35}S]methionine/cysteine and chased with an excess of unlabeled amino acids for the indicated times. The strains were incubated at 30˚C throughout the time course. 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 the panels in *A* and *B*.

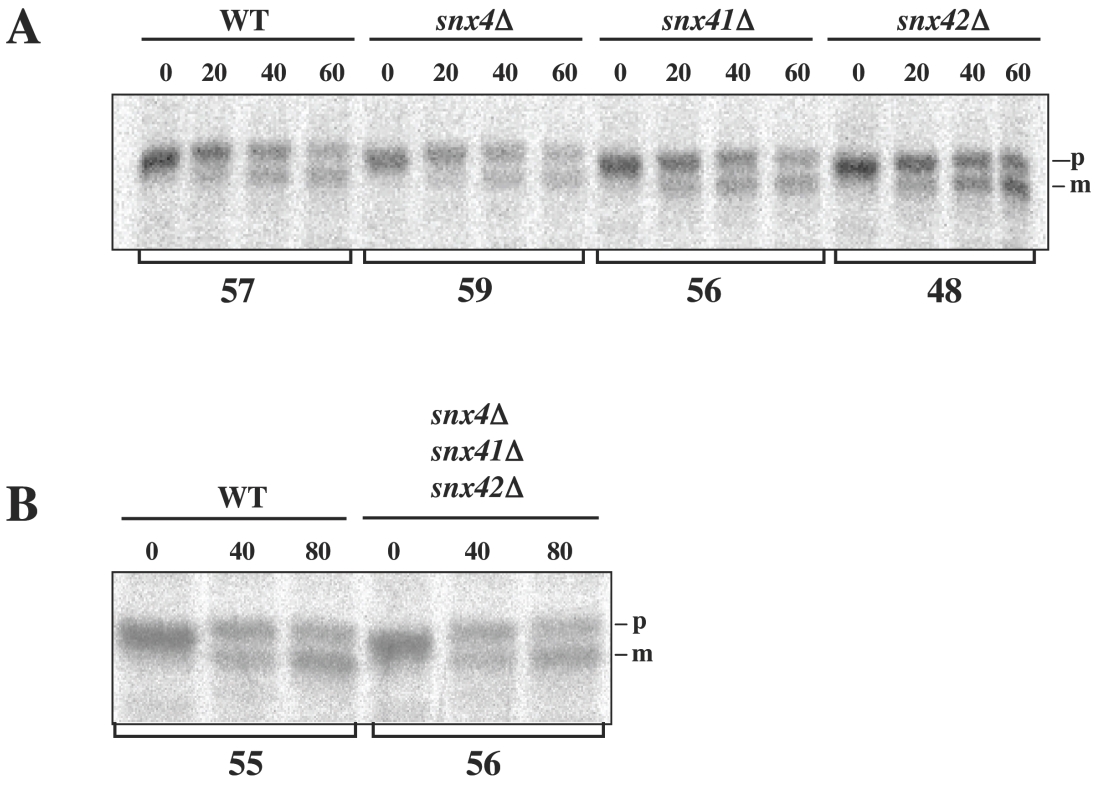

A
WT
snx4Δ
snx41Δ
snx42Δ
0 20 40 60 0 20 40 60 0 20 40 60 0 20 40 60
p
m
57
59
56
48
B
snx4Δ
snx41Δ
snx42Δ
WT
0 40 80 0 40 80
p
m
55
56

Fig. 4-2

Discussion

A triple mutant, *gga1*Δ *gga2*Δ *apl2*Δ, was constructed containing a temperature sensitive allele of *APL2* (this gene encodes one of the four proteins that comprises the AP-1 complex). This mutant displayed significant growth defects at the nonpermissive temperature and near normal growth at the permissive temperature. All of the parental strains (wild type, *gga1*Δ *gga2*Δ and *apl2*Δ) exhibited normal growth at the nonpermissive temperature. These results suggest that the GGA proteins and AP-1 share overlapping functions. This is further reinforced by the observation that trafficking of A(F→A)-ALP to the vacuole is greatly accelerated in a *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain, but not in an *apl2*Δ strain. Furthermore, this accelerated phenotype suggests that AP-1 functions in slow delivery of the protein to the PVC. It was also demonstrated that processing of an A(F→A)-ALP derivative, A(S_{13}A; F→A)-ALP, is blocked in this triple mutant. A(S_{13}A; F→A)-ALP processing is also blocked in a wild type strain, and the block occurs at the EE (Johnston et al., 2005). Therefore, it stands to reason, that the internal GGA-independent TGN-to-EE pathway is functional in a *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain at the nonpermissive temperature. Processing of A(F→A)-ALP in a *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain is most likely via a TGN-EE-PVC-vacuole route, and not by an aberrant alternative pathway not seen in wild type cells.

These results also establish the order of trafficking events at the EE. AP-1 functions in retrieval of A(F→A)-ALP from the EE or in static retention at the TGN. In the absence of AP-1, the unphosphorylated S_{13} signal is still functional based on the

S_{13}A phenotype. Therefore the signaling event that is mediated by S_{13} is downstream of AP-1 function. S_{13} does not regulate AP-1 retrieval.

The 2-11 region of A(F→A)-ALP mediates slow delivery to the vacuole (Ha et al., 2001; Johnston et al., 2005). To ensure that the accelerated trafficking phenotype is specific to the TGN-EE-PVC-vacuole route, A(Δ2-11, F→A)-ALP processing was examined in mutant strains in which the alternative routes were blocked. In the absence of GGA function as well as that of End3p, the processing half-time for A(Δ2-11, F→A)-ALP was accelerated to a similar degree as in wild type cells. Therefore the accelerated trafficking phenotype is due to the absence of the 2-11 signal within the TGN-EE-PVC-vacuole route.

In the context of a *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain at the nonpermissive temperature, the delivery of A(Δ2-11, F→A)-ALP is not accelerated compared to its delivery in a wild type strain. Furthermore, the half-time of A(F→A)-ALP processing in a *gga1*Δ *gga2*Δ *apl2*Δ/pCEN-*HIS3-apl2-ts* strain at the nonpermissive temperature, is similar to the processing time of A(Δ2-11, F→A)-ALP in a wild type strain. These data suggest that the 2-11 signal and AP-1 function at the same step. If they did not, one would expect the accelerated phenotypes to be additive. These results suggest a model in which the 2-11 signal and AP-1 mediates slow delivery presumably by cycling A(F→A)-ALP between the EE and the TGN. The role of 2-11 may be recognition with AP-1 subunits, thereby facilitating packaging into clathrin/AP-1 vesicles bound for the TGN.

One of the functions of sorting nexins in yeast is to recycle the exocytic v-SNARE, Snc1p, back to the TGN from the EE. Snx4p, Snx41p and Snx42p have all been

implicated in retrieval of Snc1p from the EE to the TGN. In the absence of any one of these proteins, Snc1p is missorted to the vacuole (Hettema et al., 2003). Data presented here suggests that A(F→A)-ALP cycles between the EE and TGN as well. It seems plausible that these sorting nexins may also mediate the retrieval of A(F→A)-ALP to the TGN. The processing half-time of A(F→A)-ALP in strains deficient for each sorting nexin as well as a strain lacking all three sorting nexins was the same as in a wild type strain. If these sorting nexins played a role in the retrieval of A(F→A)-ALP one would expect an accelerated processing phenotype in these mutant strains. Since the half-times were all similar it seems unlikely that Snx4p, Snx41p and Snx42p assist in retrieval of A(F→A)-ALP from the EE to the TGN.

CHAPTER V

AP-1 binds to the 2-11 region of Ste13 and slows its progression to the PVC

Background

Recruitment of adaptor proteins is in part mediated by binding to receptors/transmembrane cargo embedded in the membrane. AP-1 is a heterotetramer comprised of four protein subunits; β1, γ1, σ1 and μ1. In mammalian systems, AP-1 and AP-2 have been shown to bind *in vitro* to cytoplasmic domains of membrane receptors (Pearse, 1988; Beltzer and Spiess, 1991). Mannose-6-phosphate receptor (MPR) cycles between the TGN and endosomes via clathrin coated-vesicles. Trafficking of MPR is signal mediated; requiring a dileucine and tyrosine motif that is thought to be a binding site for AP-1 (Klumperman et al., 1998; Honing et al., 1997; Le Borgne and Hoflack, 1997; Heilker et al., 1996). The tyrosine signal binds to the μ1 subunit and the dileucine motif binds to the β1 subunit (Bonifacino and Traub, 2003). These signals are important for proper transport of MPR from the TGN to the EE. The dileucine and tyrosine motif are also found on many cell surface proteins, and these signals interact with AP-2 to mediate endocytosis via clathrin-coated vesicles (Bonifacino and Dell'Angelica, 1999; Heilker et al., 1999). In yeast, transport of Pep12p to the PVC requires a sorting signal, FSD, in its N-terminal domain. Proper sorting to the PVC requires this signal as well as functional GGA proteins (Black and Pelham, 2000), another type of clathrin adaptor. Retrieval of A(F→A)-ALP from the PVC requires the sorting signal $F_{85}XF_{87}XD$. This signal interacts with Vps35p of the retromer, which in turn mediates retrograde

trafficking back to the TGN (Nothwehr et al., 2000). The 2-11 signal in A(F→A)-ALP mediates slow delivery to the vacuole (Bryant and Stevens, 1997). The function of the 2-11 signal could be either retention at the TGN thereby preventing transport to the EE resulting in a slow delivery phenotype. Alternatively, it could function at the EE to facilitate retrieval back to the TGN, thereby preventing A(F→A)-ALP's progression to the PVC. Sorting signals have previously been demonstrated to recruit adaptors to form vesicles, therefore the latter explanation seems most plausible.

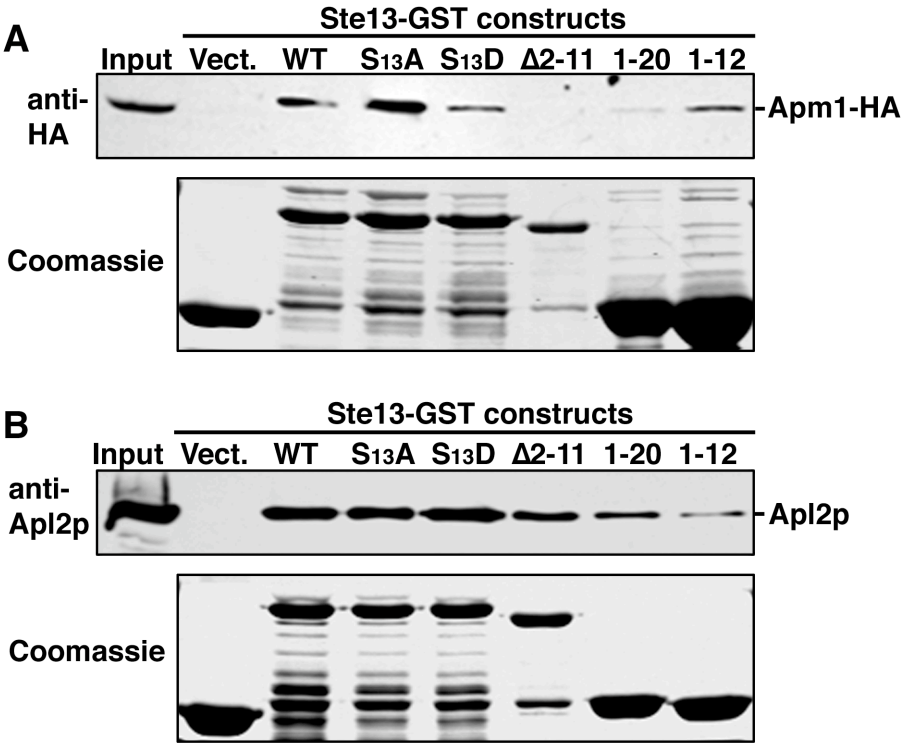
Cps1p transits to the vacuole via the CPY pathway in a GGA dependent manner (Costaguta et al., 2001) and Fig 3-1. In addition, Cps1p contains a cytosolic ubiquitin attachment site, and when tagged with ubiquitin is delivered from the PVC to the vacuole (Katzmann et al., 2001; Reggiori and Pelham, 2001). GGA adaptors recognize ubiquitin moieties on transmembrane cargo proteins at the TGN (Pelham, 2004; Scott et al., 2004), thus it appears that ubiquitin plays a role in sorting Cps1p into the GGA dependent CPY pathway at the TGN. It is unclear if there are additional sorting signals within the cytosolic domain of Cps1p that assist in determining its trafficking itinerary.

Results

Data in the previous chapter (Fig 4-1) suggests that the 2-11 signal and AP-1 function at the same step. The role of both is to slow delivery of A(F→A)-ALP to the vacuole. The data supports a model in which the 2-11 signal recruits AP-1 out of the cytosol and facilitates packaging of A(F→A)-ALP into retrograde clathrin/AP-1 vesicles. In order to more fully examine this model, an *in vitro* binding assay was used to determine if sorting signals within the cytosolic domain of A(F→A)-ALP interact with

the AP-1 complex. Residues 1-118 of Ste13p (cytosolic domain of A(F→A)-ALP), were fused to glutathione S-transferase (GST). The resulting fusion protein, Ste13-GST, was expressed in *E. coli* and purified on glutathione agarose beads. Fusion proteins were also constructed containing full length Ste13p with mutations; Δ2-11, $S_{13}A$ and $S_{13}D$. The $S_{13}A$ mutation prevents A(F→A)-ALP from exiting the EE, and the $S_{13}D$ mutation accelerates delivery of A(F→A)-ALP to the vacuole (Johnston et al., 2005). Additional GST fusions were constructed containing residues 1-20 of Ste13p and residues 1-12 of Ste13p. Glutathione beads bound to these fusion proteins, and GST alone as a negative control, were incubated with yeast protein extracts from a strain containing a functional HA-tagged allele (*APM1::HA*) of the μ1 subunit of AP-1 (Yeung et al., 1999). The beads were washed and bound extracts were eluted via the addition of reduced glutathione and analyzed by SDS-PAGE and western blot analysis. Blots were probed with an anti-HA antibody that was specific for Apm1-HA (Fig. 5-1A). A total of 2% of the yeast extract incubated with each bead sample was loaded directly to give an assessment of the input of AP-1 subunits incubated with the beads. Positive association was observed with GST-Ste13 as well as with the $S_{13}A$ and $S_{13}D$ derivatives. Interestingly, binding was also observed with the GST-Ste13 (residues 1-20) as well as GST-Ste13 (residues 1-12), though this interaction was not as strong as with the full length Ste13p cytosolic domain. No association was seen with GST alone or GST-Ste13(Δ2-11). In a separate pull-down experiment, western analysis was also performed with antibodies against Apl2p, the β1 subunit of AP-1 (Fig 5-1B). The binding results were similar to those for Apm1-HA experiment, except the GST-Ste13(Δ2-11) construct associated with Apl2p. However, the binding affinity was diminished compared to GST-Ste13. These results provide

Fig. 5-1: The clathrin adaptor complex AP-1 interacts with amino acids 1-12 of Ste13p. (*A*) The following proteins were expressed in *E. coli*, purified onto glutathione-agarose beads, and incubated with a yeast CFY35 protein extract: GST (*Vect.*), Ste13-GST (*WT*), Ste13(S_{13}A)-GST ($S_{13}A$), Ste13(S_{13}D)-GST ($S_{13}D$), Ste13(1-20)-GST (*1-20*), and Ste13(1-12)-GST (*1-12*). The bead samples were washed, eluted, and eluted proteins analyzed by SDS-PAGE and immunoblotting for Apm1-HA. The Apm1-HA immunoblot (*upper panel*) and an identical gel coomassie that was stained rather than immunoblotted (*lower panel*) are shown. The latter gel indicates the relative sizes and abundance of the GST fusion proteins. (*B*) Shown is a separate experiment carried out in same fashion as in (*A*) except that Apl2p was detected on the immunoblot. In the immunoblots shown in both (*A*) and (*B*) yeast extract equivalent to 2% of that incubated with each of the bead samples was also analyzed (*input*). (*C*) Amino acid sequence 1-12 of Ste13p.



C

MSASTHSHKRKN…

Fig. 5-1

strong evidence that the 2-11 region does function to recruit AP-1. Furthermore, it would appear that Apm1p binding is absolutely contingent upon the 2-11 signal. In contrast, the 2-11 signal is dispensable for Apl2p binding, though it does appear to increase the binding affinity between Apl2p and the cytosolic domain of Ste13p, suggesting that Apl2p binds to the 2-11 signal in addition to other unidentified structural features.

The previous results demonstrated that residues 1-12 of Ste13p were sufficient and necessary for binding of AP-1 *in vitro*. The 2-11 signal is required for slow delivery of A(F→A)-ALP to the PVC. However, it has never been shown to be sufficient for slow delivery *in vivo*. Cps1p uses the CPY pathway to access the PVC, as its trafficking itinerary is delayed in a *gga1*Δ *gga2*Δ (Fig 3-3A). Furthermore, Cps1p does not appear to use the TGN-EE-PVC pathway as its processing is not significantly affected in a *soi3*Δ strain (Fig 3-3B). To test if the N-terminus of Ste13p is sufficient to mediate slow delivery, it was appended to the N-terminus of full length Cps1p and the resulting construct was assayed via pulse chase immunoprecipitation for the slow delivery phenotype in a *gga1*Δ *gga2*Δ strain. This strain was chosen because Cps1p normally uses the GGAs to access the PVC. We wanted to block its normal route and force it into a route that included the EE; either the TGN-EE-PVC or TGN-PM-EE-PVC pathway. A construct containing residues 1-23 of Ste13p fused to Cps1p, Ste13(1-23)-Cps1, was processed very rapidly in a wild type strain. This result is similar to Cps1p in a wild type background and indicates that the fusion protein exits the ER with the same kinetics as Cps1p and appears to access the GGA dependent pathway. In a *gga1*Δ *gga2*Δ strain processing is significantly slowed. Moreover, it is delayed to a much greater degree than the delay seen in Cps1p in a *gga1*Δ *gga2*Δ strain, 50 min versus 32 min half-time,

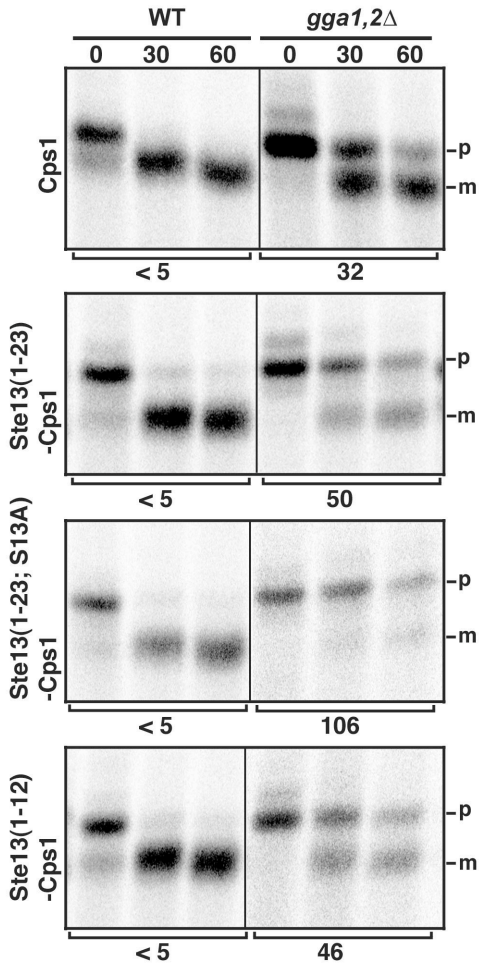
respectively (Fig 5-2). This difference is comparable to the difference between A(F→A)-ALP (53 min half-time) and A(Δ2-11, F→A)-ALP (30 min half-time) observed in a *gga1*Δ *gga2*Δ strain (Fig. 4-1D). Thus, the N-terminal 23 residues of Ste13p are sufficient to mediate the slow delivery phenotype.

To assess whether the chimera was trafficking via a pathway that includes the EE, a construct was made that contained the first 23 residues of Ste13p with the $S_{13}A$ mutation and the full length Cps1p, Ste13(1-23, $S_{13}A$)-Cps1, and assayed for delivery to the vacuole via pulse chase immunoprecipitation. The $S_{13}A$ mutation had previously been shown to block progression of A(F→A)-ALP passed the EE (Johnston et al., 2005). Once again, the half-time for Ste13(1-23, $S_{13}A$)-Cps1 was extremely fast in a wild type background, indicating that it was delivered to the PVC via the GGA pathway, independently of the EE. In the *gga1*Δ *gga2*Δ strain, its processing half-time was significantly delayed (106 min), though not completely blocked. This result demonstrates that the chimera was accessing the EE, though the $S_{13}A$ block was not as tight as when $S_{13}A$ was introduced in the A(F→A)-ALP context. Furthermore, the quick processing half-time (< 5 min) observed in a wild type strain when Ste13(1-23, $S_{13}A$)-Cps1 is trafficked via the TGN-PVC route reinforces our model in which the S_{13} signal only comes into play at the EE (Johnston et al., 2005).

In an attempt to whittle down the slow delivery/ AP-1 recruitment signal to its smallest possible region, the first 12 residues of Ste13p were appended to Cps1p and the processing half-time of this construct, Ste13(1-12)-Cps1 was assessed via pulse chase immunoprecipitation. Consistent with the other chimeras, processing was extremely fast (< 5 min half-time) in a wild type background, and significantly delayed (46 min half-

Fig. 5-2: Residues 1-12 of Ste13p are sufficient to slow trafficking of Cps1p into the PVC/vacuole. (A) Yeast strains CFY30 (*cps1*Δ) and CFY32 (*cps1*Δ *gga1*Δ *gga2*Δ) carrying plasmids expressing Cps1p, Ste13(1-23)-Cps1, Ste13(1-23; S_{13}A)-Cps1, and Ste13(1-12)-Cps1 were subjected to pulse-chase analysis at 30˚C and immunoprecipitation as described in the legend to Fig. 3. The Cps1p construct was driven by the endogenous *CPS1* promoter while the other constructs were driven by the *STE13* promoter. The half-time of processing is indicated below each panel. (B) Schematic of trafficking route.

A


WT
gga1,2Δ
0
30
60
0
30
60
Cps1
p
m
< 5
32
Ste13(1-23)
-Cps1
p
m
< 5
50
Ste13(1-23; S13A)
-Cps1
p
m
< 5
106
Ste13(1-12)
-Cps1
p
m
< 5
46

B

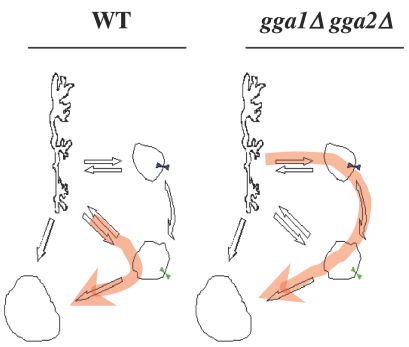

WT
gga1Δ gga2Δ

Fig. 5-2

time) in a *gga1*Δ *gga2*Δ strain. Thus, in agreement with our *in vitro* AP-1 binding results, residues 1-12 are sufficient and necessary to mediate the slow trafficking phenotype.

Discussion

Data presented here strongly supports a model in which Ste13p residues 1-12 mediate slow delivery of A(F→A)-ALP by recruiting AP-1 which in turn mediates retrograde trafficking from the EE to the TGN. It was demonstrated that the cytosolic domain of Ste13p physically interacts with two of the four subunits of the AP-1 complex; β1 and μ1. Furthermore, the sorting signal mediating the μ1 subunit interaction is within Ste13p residues 1-12, as it bound to GST-Ste13(1-12) but not to GST-Ste13(Δ2-11). Apl2p binds to Ste13p residues 1-12 as it interacted with GST-Ste13(1-12), however it also appears to bind to other residues since it also interacted with GST-Ste13(Δ2-11). Taken together these results demonstrate that an N-terminal motif within the Ste13p cytosolic domain physically interacts with the AP-1 complex.

A previous study had demonstrated that the S_{13} residue was important in mediating trafficking events. Specifically, an S_{13}A mutation in the A(F→A)-ALP context blocked exit out of the EE for delivery to the PVC. In contrast, an S_{13}D mutation accelerated the processing half-time of A(F→A)-ALP (Johnston et al., 2005). One model that would explain this result is that the S_{13}D antagonizes the 2-11 signal that in turn mediates AP-1 retrieval, thereby leading to an accelerated PVC delivery phenotype. The S_{13}A would in contrast positively regulate the 2-11 signal, thereby increasing the efficiency of EE-TGN recycling. This would result in continuous cycling between the

TGN and EE and explain the block in exiting the EE to the PVC. Data presented here appear to refute that model. First, an $S_{13}A$ mutation caused a block in processing of A(F→A)-ALP in the *gga1*Δ *gga2*Δ *apl2-ts* strain (Fig. 4-1C). If the role of $S_{13}A$ was solely to regulate 1-12 and in turn retrieval via AP-1, then there should not have been a block in processing; instead one would expect a 20-30 min half-time. Second, *in vitro* binding of AP-1 to Ste13p cytosolic domain was not significantly affected either positively in the $S_{13}A$ derivative or negatively in the $S_{13}D$ derivative. Though figure 5-1A does show a difference in Apm1-HA binding affinity for $S_{13}A$ and $S_{13}D$, this difference is not reproducible. Taken together, these results support a model in which the 2-11 signal/AP-1 recruitment functions upstream of the S_{13} signal, and the two signal are independent of one another.

The *in vitro* data provides compelling evidence that the N-terminus of Ste13p contains sorting signals that recruit AP-1. To extend this observation and place it in a physiological context, we appended the N-termini sorting signals onto Cps1p expecting to see a delay in processing indicative of EE-TGN cycling. In order for this to succeed, Cps1p and the chimeras needed to be rerouted to an alternative pathway that included the EE. This was accomplished by eliminating GGA function, thereby blocking the TGN-to-PVC route. Under wild type conditions, Cps1p as well as the chimeras were all processed very quickly. Thus, the chimeras all exit the ER and transit through the Golgi with the same kinetics as Cps1p. However, the processing half-times of Ste13(1-23)-Cps1 and Ste13(1-12)-Cps1 in a *gga1*Δ *gga2*Δ strain were delayed with respect to Cps1p. The delay was consistent with the delay seen in A(F→A)-ALP in the same background. This result provides *in vivo* evidence that residues 1-12 of Ste13p are sufficient to

mediate recruitment of AP-1. Furthermore, this delay is only realized when the chimeras are trafficking through the EE. In the absence of an EE itinerary, when the chimeras are transported via the CPY pathway, the 2-11 sorting signal has no effect on the processing kinetics.

It had previously been demonstrated that A(S_{13}A, F→A)-ALP does not progress passed the EE (Johnston et al., 2005). This phenotype was used as an indicator that the chimeras are trafficking through the EE in transit to the vacuole. The Ste13(1-23, S_{13}A)-Cps1 construct is significantly delayed in a *gga1*Δ *gga2*Δ strain. Though processing was not completely blocked, this result demonstrates that the S_{13} signal is functional and therefore the chimeras are trafficking through the EE. Further support of the idea that the S_{13} signal functions at the EE is that the Ste13(1-23, S_{13}A)-Cps1 processing half-time is comparable to Cps1p in a wild type background when it is not trafficking through the EE. Instead it is accessing the vacuole via the CPY route; TGN-to-PVC-to-vacuole, and therefore the S_{13} signal does not come into play.

The trafficking itinerary of Kex2p is thought to be similar to that of Ste13p. Both are resident TGN proteins that function to process α-factor. Kex2p contains a signal analogous in function to the 2-11 signal found in Ste13p, although it lacks obvious sequence similarities with the Ste13p 2-11 signal (Brickner and Fuller, 1997). Furthermore, in a *soi3*Δ background, a retrieval-defective Kex2p is significantly delayed in delivery to the vacuole (Sipos et al., 2004). This is indicative of an EE itinerary. Kex2p is retrieved from the PVC via the retromer (Voos and Stevens, 1998). Lastly, Kex2p is phosphorylated to a similar degree as A-ALP and its derivatives (Johnston et al., 2005), suggestive of a signal analogous to S_{13} found in Ste13p. There is evidence,

though, that Kex2p may use the TGN-to-PVC pathway, whereas our data implies that there is little if any trafficking of A(F→A)-ALP via this pathway. First, Kex2p trafficking to the PVC is somewhat delayed in a *gga1*Δ *gga2*Δ strain (Ha et al., 2001). In addition, Kex2p delivery to the PVC is about twice as fast as A(F→A)-ALP (Ha et al., 2001; Sipos et al., 2004). This suggests that Kex2p does not cycle between the EE and the TGN. But importantly, this does not preclude it from trafficking through the EE to access the PVC.

CHAPTER VI

Clathrin and adaptor proteins accumulate on membranes in *inp53*Δ mutants

Background

Phosphoinositides are a class of membrane lipids that mediate a number of trafficking events. They are comprised of a fatty acid tail, connected by a glycerol backbone to an inositol ring. Within the inositol ring there are three potential phosphorylation sites; 3, 4 and 5 (see Fig 1-4). The removal or addition of phosphates by various kinases and phosphatases at these three positions allows for seven different phosphoinositide derivatives to be generated.

Recruitment of adaptor proteins to donor membranes facilitates vesicle formation (Bonifacino and Lippincott-Schwartz, 2003). Recruitment requires the activity of a number of different proteins. In mammalian systems, the addition of the drug Brefeldin A has been demonstrated to prevent AP-1 assembly onto TGN membranes via the inactivation of ADP ribosylation factor (ARF) (Robinson and Kreis, 1992). It was also shown in yeast that Brefeldin A prevents ARF from becoming activated, by blocking exchange of GDP for GTP (Peyroche et al., 1999). Additional studies in mammalian systems, has demonstrated that ARF is required for recruitment of AP-3, AP-4 and the GGAs and it physically interacts with these adaptors (Nie et al., 2003). ARF has also been shown to stimulate phosphoinositide production, specifically phosphatidylinositol 4-phosphate [Ptdins(4)P] and phosphatidylinositol (4,5)-bisphosphate [Ptdins(4,5)P_2] (Godi

et al., 1999). It has subsequently been shown that AP-2 specifically binds to Ptdins(4,5)P_2 to mediate endocytosis at the plasma membrane in animal cells (Collins et al., 2002). Further studies in an mammalian cells, has shown Ptdins(4)P to be primarily localized to the TGN and to bind *in vitro* to AP-1. Additionally, down regulating the kinase that generates Ptdins(4)P causes AP-1 to become cytosolic and the addition of exogenous Ptdins(4)P restores AP-1 TGN association (Wang et al., 2003). These results demonstrate that ARF, and the phosphoinositides produced by its activation, play key roles in recruitment of adaptor proteins to donor membranes.

It would also appear that uncoating of clathrin/adaptor proteins from vesicles is mediated to some degree by dephosphorylating phosphoinositides. In mammalian systems deletion of the Ptdins(4,5)P_2 phosphatase, synaptojanin, results in the accumulation in the cytosol of coated vesicles. This suggests that dephosphorylation of Ptdins(4,5)P_2 facilitates uncoating of newly formed vesicles (Cremona et al., 1999; Harris et al., 2000; Verstreken et al., 2003).

There are three functional domains in synaptojanin; two mediate the removal of phosphates from phosphoinositides and the third is a C-terminal proline rich domain. One of the catalytic domains resembles that of the yeast polyphosphoinositde phosphatase Sac1p, and is capable of hydrolyzing Ptdins(3)P, Ptdins(4)P, Ptdins(3,5)P_2. The other domain is a phosphoinositide 5-phosphatase domain and only catalyzes the removal of the phosphate at the 5' position of the inositol ring primarily in Ptdins(4,5)P_2 (Guo et al., 1999).

S. cerevisiae contains four proteins with phosphoinositide 5-phosphatase activity, Inp51p, Inp52p, Inp53p and Inp54p. The first three also contain a *SacI* domain, though

this domain is inactive in Inp51p (Guo et al., 1999). Deletion of Inp51p results in an increase in cellular levels of Ptdins(4,5)P_2 (Stolz et al., 1998). Deletions of both Inp52p and Inp53p leads to an increase in Ptdins(3,5)P_2 (Guo et al., 1999). No detectable change in phosphoinositide levels was observed in an *inp53*Δ single mutant strain (Ha et al., 2001). A triple mutant lacking functional Inp51p, Inp52p and Inp53p is lethal, apparently due to an accumulation of Ptdins(4,5)P_2, as viability can be restored via the addition of a *Sac1* deficient derivative of Inp52p (Stefan et al., 2002). With respect to trafficking, an *inp51*Δ *inp52*Δ strain, but not an *inp52*Δ *inp53*Δ strain, displays endocytic defects, implying a role for Inp51p in endocytosis (Singer-Krüger et al., 1998). Trafficking of A(F→A)-ALP is accelerated in *inp53*Δ strains. The defect appears to be specific to trafficking between the TGN and EE, as the AP-3 pathway is unaffected as well as the retromer and CPY pathways (Ha et al., 2001). Additionally, the half-time for processing of A(F→A)-ALP is the same in an *inp53*Δ strain as it is in an *apl2*Δ *inp53*Δ strain, 29 min and 26 min respectively, suggesting that both Inp53p and AP-1 act in the TGN/EE leg of the pathway (Ha et al., 2003). Further evidence of a role for Inp53p at the TGN/EE is that *INP53* genetically interacts with *CHC1* (Bensen et al., 2000) and the proline rich domain of Inp53p physically interacts with clathrin heavy chain (Ha et al., 2003). Clathrin coated vesicles have been implicated in trafficking predominantly at the TGN. The CPY pathway is mediated by the GGAs, both of which have been shown to bind clathrin (Nakayama and Wakatsuki, 2003). In addition the EE-to-TGN AP-1 retrieval step is thought to be clathrin mediated, as AP-1 binds clathrin (Yeung and Payne, 2001). Finally, clathrin also appears to function at the plasma membrane in receptor mediated endocytosis of α-factor receptor Ste2p (Newpher et al., 2005).

However, endocytosis appears to be normal in an *inp53*Δ mutant, therefore it is likely that the function of Inp53p is to mediate trafficking events at the TGN or EE.

Results

The processing half-time of A(F→A)-ALP is accelerated in an *inp53*Δ strain. This acceleration is consistent with a model in which the slow delivery phenotype mediated by EE-to-TGN retrieval is no longer functional. Polyphosphoinositide phosphatases have been implicated in vesicle formation as well as vesicle uncoating. Thus to assess whether the accelerated delivery of A(F→A)-ALP is due to an aberrant distribution of phosphoinositides on membranes, we used subcellular fractionation to determine if adaptor proteins and clathrin accumulated on membranes in an *inp53*Δ strain.

Wild type and *inp53*Δ cell lysates were subjected to centrifugation at 15,000 *g* and 200,000 *g*. The pellets were resuspended in SDS-PAGE loading buffer. Unfractionated lysate, the 15,000 *g* pellet, the 200,000 *g* pellet and the 200,000 *g* supernatant were analyzed by SDS-PAGE and western blot for Chc1p (Fig. 6-1A). Chc1p was enriched in the 200,000 *g* pellet in the *inp53*Δ strain compared to the wild-type strain (12.4% versus 4.8% respectively). Furthermore, the percentage that localized to the 15,000 *g* pellet was 22% in wild type and 15% in the *inp53*Δ strain. Enrichment in the 200,000 *g* pellet in the *inp53*Δ strain suggests that membranes within this subcellular fraction are not releasing clathrin as efficiently as in a wild type strain. The predominant membranes in this fraction are EE, PVC, TGN and vesicles.

Fig. 6-1: Clathrin, Apl2p and Gga2p accumulate on membranes in *inp53*Δ mutants. Lysates from strains SHY35 and SHY38 were subjected to centrifugation at 15,000 *g* to generate pellet (P15) and supernatant (S15) fractions. The S15 fraction was centrifuged at 200,000 *g* to generate P200 and S200 fractions. An equivalent percentage of each fraction was separated via SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with (*A*) anti-Chc1p (*B*) anti-Apl2p (*C*) anti-Gga2p and (*D*) anti-Gga1p antibodies. Protein fractions were quantified and normalized to unfractionated lysates yielding the indicated percentages.

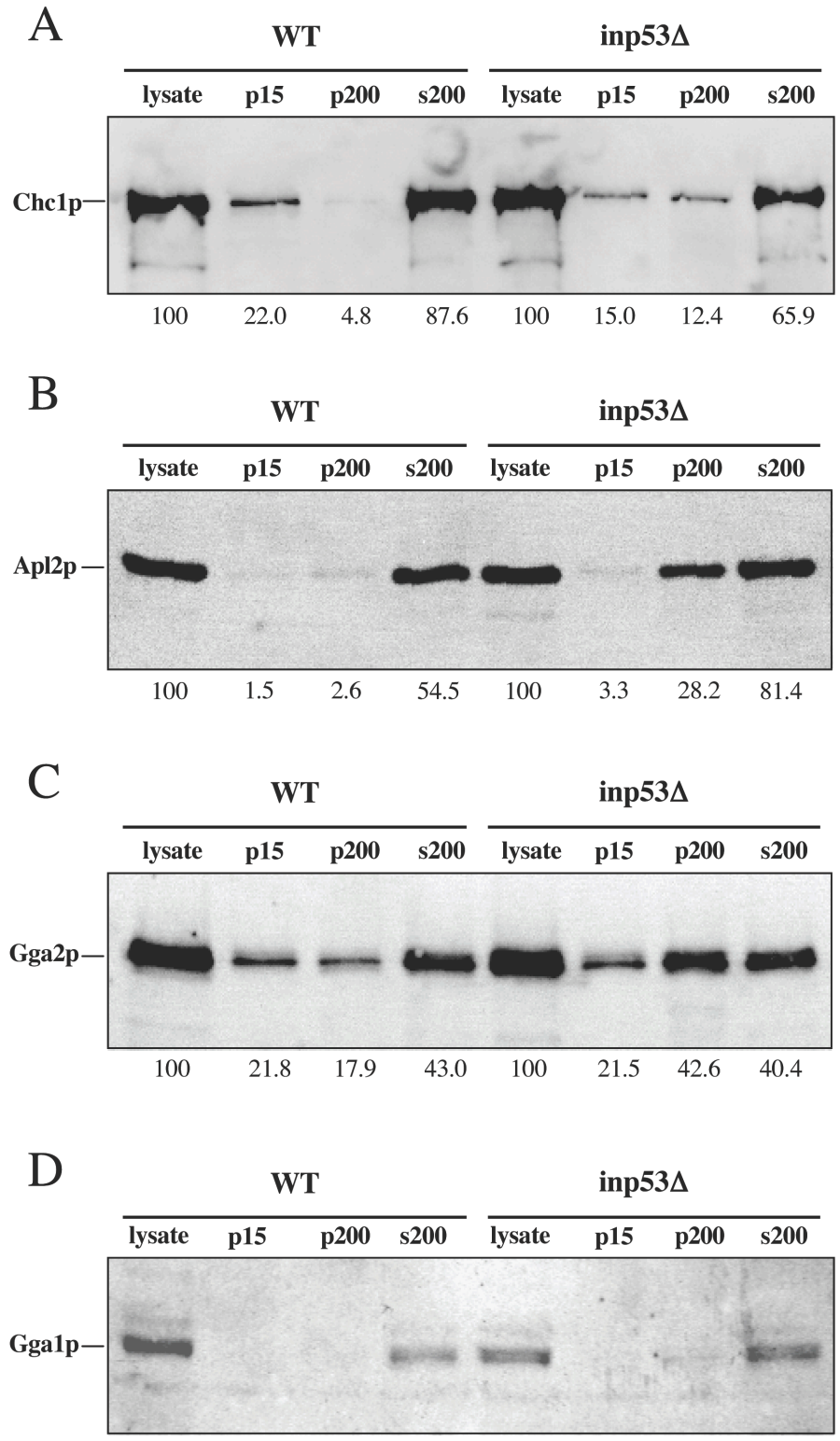

A
WT
inp53Δ
lysate
p15
p200
s200
lysate
p15
p200
s200
Chc1p
100
22.0
4.8
87.6
100
15.0
12.4
65.9
B
WT
inp53Δ
lysate
p15
p200
s200
lysate
p15
p200
s200
Apl2p
100
1.5
2.6
54.5
100
3.3
28.2
81.4
C
WT
inp53Δ
lysate
p15
p200
s200
lysate
p15
p200
s200
Gga2p
100
21.8
17.9
43.0
100
21.5
42.6
40.4
D
WT
inp53Δ
lysate
p15
p200
s200
lysate
p15
p200
s200
Gga1p

Fig. 6-1

The distribution of Apl2p was examined as well from the same fractions (Fig. 6-1B). Similar to what was observed with Chc1p, Apl2p was enriched in the 200,000 *g* pellet in the *inp53*Δ strain compared to the wild-type strain, though the enrichment was much more pronounced, 28.2% in the *inp53*Δ strain versus 2.64% in the wild type strain. Thus the increase in P200 membrane association is much more dramatic in an *inp53*Δ strain for AP-1 vesicles than it is for clathrin. Additionally, there was far less Apl2p in the 15,000 *g* pellet compared to Chc1p in both wild type and the *inp53*Δ strain.

Gga2p was also found to be enriched in the 200,000 *g* pellet, in the *inp53*Δ strain (Fig. 6-1C). However, relative to Apl2p, the enrichment was not nearly as striking, 42.6% in the *inp53*Δ strain versus 17.9% in the wild type strain. One difference between the localization of Apl2p and Gga2p was that there was far less Apl2p in the 15,000 *g* pellet compared to Gga2p in both wild type and the *inp53*Δ strain. Interestingly, Gga1p subcellular fractionation was not affected in an *inp53*Δ strain (Fig6-1D).

Discussion

Phosphoinositides have been implicated in vesicle formation as well as uncoating of vesicles. Inp53p regulates the concentration of specific phosphoinositides by dephosphorylating Ptdins(3)P, Ptdins(4)P, Ptdins(3,5)P_2 via its *Sac1* domain and Ptdins(4,5)P_2 via its phosphoinositide 5-phosphatase domain (Guo et al., 1999). A(F→A)-ALP trafficking is accelerated in an *inp53*Δ mutant. Inp53p directly interacts with clathrin. The trafficking defect observed in the *inp53*Δ strain appears to be limited

to the EE-to-TGN pathway, as AP-3, the retromer pathway and the CPY pathway were all unaffected in an *inp53*Δ background (Ha et al., 2003).

Our results demonstrate that adaptors and clathrin accumulate on membranes in a *inp53*Δ strain. Clathrin, Apl2p and Gga2p are enriched on membranes in the 200,000 *g* pellet of the *inp53*Δ strain. This pellet contains Golgi, TGN, EE, and the PVC. Enrichment was greatest for Apl2p. Furthermore, for Gga2p and clathrin there was also considerable fractionation with the 15,000 *g* pellet in both the wild type and the *inp53*Δ mutant. This is consistent with clathrin and Gga2p functioning at one or more trafficking steps distinct from the step at which AP-1 functions. The plasma membrane is present in the 15,000g pellet. Clathrin plays a role in endocytosis of receptors at the plasma membrane (Newpher et al., 2005). Therefore it seems plausible that clathrin would cofractionate with the plasma membrane. It is not clear why Gga2p cofractionates with the 15,000g. Clathrin has been implicated in TGN-to-PVC transport, TGN-to-EE transport, EE-to-TGN transport as well as endocytosis. To date, Gga2p has only been implicated in TGN-to-PVC trafficking. Therefore, in trafficking steps in which Inp53p does not play a role, clathrin and Gga2p localization should be the same in wild type and *inp53*Δ strains. Ablation of Inp53p function only appears to affect transport between the TGN and EE, and AP-1 has only been implicated in transport between the EE and TGN. It is therefore consistent that in an *inp53*Δ strain, AP-1 localization is more dramatically affected than clathrin and Gga2p.

Does Inp53p mediate formation of a vesicle or alternatively an uncoating event? The accelerated trafficking of A(F→A)-ALP would be more consistent with the former and not the latter. If loss of Inp53p function resulted in an uncoating defect one would

expect the vesicles to accumulate in the cytosol. This would not yield an accelerated trafficking phenotype. It would be analogous to what is seen with A(F→A)-ALP in which Pep12p function is eliminated. In the *pep12-ts* strain, A(F→A)-ALP trafficking is blocked and vesicles accumulate in the cytosol (Fig 3-1A). The opposite phenotype is seen in an *inp53*Δ strain. However, there is the possibility that AP-1 is limiting, and by preventing uncoating, the pathway is blocked due to a depletion of AP-1 recruitment at the donor membrane. This seems unlikely however, as even in the *inp53*Δ strain there is a considerable amount of Apl2p in the 200,000 *g* supernatant (Fig. 6-2B). The 200,000 *g* supernatant contains the cytosol and the pool of free AP-1 is still quite large in this fraction relative to the AP-1 associated with membranes in the 200,000 *g* fraction.

How can the accelerated trafficking phenotype be explained if Inp53p functions at the TGN? One explanation is that in the absence of Inp53p function, the TGN-EE-PVC pathway is blocked, and A(F→A)-ALP is aberrantly transported via the GGA pathway directly from the TGN to the PVC. In support of this model, the processing half-time of A(F→A)-ALP is significantly delayed (176 min) in a *gga1*Δ *gga2*Δ *inp53*Δ strain (Ha et al., 2003). Furthermore, the lack of an affect of an *apl2*Δ mutation on A(F→A)-ALP trafficking in the context of *inp53*Δ could be due to A(F→A)-ALP trafficking via the TGN-to-PVC route, in which the EE is bypassed (Ha et al. 2003). Unfortunately, there is evidence that refutes this model. Processing of A(S_{13}A; F→A)-ALP is blocked in an *inp53*Δ strain, this block occurs at the EE, therefore in an *inp53*Δ strain A(S_{13}A; F→A)-ALP transits to the EE (Johnston et al., 2005) and not via the GGA mediated TGN-to-PVC pathway. It is currently difficult to reconcile these discrepancies. Additional

information is necessary in order to propose a definitive role for Inp53p in A(F→A)-ALP trafficking.

CHAPTER VII

Summary

The results presented in this thesis further define the trafficking itinerary of A(F→A)-ALP. An additional sorting signal within the N-termini of Ste13p that mediates TGN/EE retention is characterized. It is demonstrated that there is a physical interaction between Ste13p and AP-1, and this is the first time a cargo-sorting signal has been shown to interact with *S. cerevisiae* AP-1. Furthermore, the functional roles of AP-1 and Inp53p are also addressed in the context of A(F→A)-ALP trafficking. This data contributes to the understanding of protein trafficking in *S. cerevisiae* and specifically addresses the endosomal cycling mechanism that affords Ste13p its TGN residency.

A(F→A)-ALP transits through the EE

Three separate experiments suggest that A(F→A)-ALP is trafficked through the EE in transit to the vacuole. First, GFP-A(F→A)-ALP colocalizes with FM4-64 when the dye is predominantly occupying the EE. As a negative control, it was demonstrated that a GFP tagged PVC resident protein, Pep12p, does not colocalize with the dye under the same conditions. This is compelling evidence that within the pool of A(F→A)-ALP a fraction is localized to the EE. Furthermore, protein trafficking from the EE to the PVC is mediated by Soi3p (Sipos et al., 2004). In a *soi3*Δ mutant, A(F→A)-ALP is severely delayed in its delivery to the PVC and vacuole. This suggests that A(F→A)-ALP transits from the EE to the PVC as part of its trafficking itinerary, as machinery that mediates

transport between the EE and PVC is required for efficient delivery of A(F→A)-ALP to the PVC. Finally, it was demonstrated that A(F→A)-ALP must transit through the PVC to access the vacuole, and delivery to the PVC is GGA-independent. The evidence for this is two-fold. In a *pep12-ts* mutant, vacuolar delivery of A(F→A)-ALP is effectively blocked. Pep12p is a t-SNARE in the PVC membrane, and is required for vesicular fusion at the PVC (Becherer et al., 1996). Therefore, A(F→A)-ALP does not use the AP-3 mediated TGN-to-vacuole pathway to access the vacuole, it has to transit through the PVC. It does not appear to use the GGA-mediated TGN-to-PVC pathway either as there was no change in the kinetics of vacuolar delivery of A(F→A)-ALP in a *gga1Δ gga2Δ* strain or in a *gga1Δ gga2Δ end3-ts* strain. This latter control indicates that in the absence of GGA function A(F→A)-ALP is not aberrantly secreted and endocytosed as an alternative route to the PVC via the plasma membrane. This was in contrast to Cps1p that does use the GGA pathway as its delivery to the PVC was significantly delayed in a *gga1Δ gga2Δ* strain and even more so in a *gga1Δ gga2Δ end3-ts* strain.

These results suggest that the trafficking itinerary for A(F→A)-ALP is TGN-to-EE-to-PVC. Identification of the machinery that mediates TGN-to-EE trafficking has not yet been identified in *S. cerevisiae*. One of the few clues as to the machinery that mediates this trafficking route is that in a *chc1-ts* mutant A(F→A)-ALP is delivered to the vacuole and this delivery can be blocked by blocking endocytosis (Ha et al., 2003). These results demonstrate that in the absence of clathrin, A(F→A)-ALP is aberrantly trafficked to the plasma membrane. Once at the plasma membrane it is subsequently endocytosed to the EE, and transported to the PVC and vacuole. Thus the TGN-to-EE

trafficking event requires clathrin and the plasma membrane-to-EE-to-PVC-vacuole route apparently does not strictly require clathrin.

It would appear that entry into TGN-to-EE pathway is not very selective. Evidence for this is that Cps1p normally uses the GGAs, but in their absence can be routed to the PVC via an internal pathway. It seems unlikely that Cps1p would contain a sorting signal required for entry onto the TGN-to-EE pathway. One model consistent with this data is based upon the cisternae maturation model. The terminal compartment of the Golgi, the TGN, may in fact vesiculate and fuse with endocytic vesicles to form EE compartments. In this model, as the TGN matures it would be depleted of cargo exiting via the two secretory pathways as well as the ALP and CPY pathways. Proteins lacking sorting signals would remain in this compartment as they have no mechanism for exit. Furthermore, proteins that utilize the GGA dependent CPY pathway would be stuck within this compartment in strains lacking functional GGAs. To a certain degree, this is what was observed with Cps1p in a *gga1Δ gga2Δ* strain. Clathrin appears to function in the trafficking itinerary of A(F→A)-ALP. One explanation for the function of clathrin, in this model, would be to mediate the vesiculation of the TGN, to form post TGN compartments to fuse with endocytic vesicles. In this model, sorting signals are dispensable for protein trafficking to the EE. In contrast, retrograde sorting signals are not precluded and in fact may be the one and only determinant for proper cargo localization. An alternative model would be the traditional view in which transport from the TGN to the EE is adaptor mediated.

AP-1 and the 2-11 signal of A(F→A)-ALP function to slow trafficking into the PVC

The adaptor protein that mediates transport between the TGN and EE in vertebrates has been identified as AP-1, though there is still some controversy as to whether it functions in anterograde or retrograde trafficking (Huang et al., 2001; Puertollano et al., 2003; Klumperman et al., 1998; Meyer et al., 2000). In *S. cerevisiae*, the role of AP-1 is even murkier. One of our objectives was to determine if AP-1 mediates anterograde trafficking from the TGN to the EE. It was previously shown in yeast that loss of AP-1 function did not dramatically affect the trafficking of A(F→A)-ALP. One possibility is that in the absence of AP-1, A(F→A)-ALP leaks into the GGA dependent, CPY pathway. To address this issue, trafficking of A(F→A)-ALP was assessed in a strain lacking both GGA function and AP-1 function. Surprisingly, delivery to the vacuole was significantly accelerated. This phenotype was not due to entry into the secretory pathway, as the addition of an *end3-ts* mutation did not block trafficking to the vacuole. Furthermore, A(S_{13}A; F→A)-ALP, which had previously been shown to be blocked at the EE, was also blocked in the *gga1Δ gga2Δ apl2-ts* strain. This latter result provides strong evidence that in a *gga1Δ gga2Δ apl2-ts* strain, A(F→A)-ALP is following its normal trafficking itinerary. In addition, the S_{13} signal is downstream of AP-1. We have interpreted these results to suggest that AP-1 does not mediate anterograde trafficking for A(F→A)-ALP, but instead mediates retrograde trafficking back to the TGN. In addition, the processing half-time of A(F→A)-ALP in the *gga1Δ gga2Δ apl2-ts* strain, was strikingly similar to the half-time of A(Δ2-11; F→A)-ALP. Furthermore, the accelerated phenotypes were not additive, suggesting that the 2-11 signal and AP-1 function at the same step. This led to a model in which trafficking through the EE allows for recycling back to the TGN and serves as a complementary

mechanism to retromer recycling at the PVC as a means of localizing A(F→A)-ALP to the TGN. Furthermore, we hypothesize that the mechanism of recycling at the EE is mediated by AP-1 and the sorting signal for entry into AP-1 vesicles is the 2-11 region of Ste13p.

The 1-12 signal physically interacts with AP-1 and is sufficient to slow delivery into the PVC

Residues 2-11 and the analogous signal in Kex2p had previously been demonstrated to be necessary for slow delivery of Kex2p and A(F→A)-ALP to the PVC (Bryant and Stevens, 1997). In the context of A(F→A)-ALP, this slow delivery phenotype was eliminated in a *gga1Δ gga2Δ apl2-ts* strain. This suggested that the 2-11 signal may interact with AP-1 to mediate a trafficking event. To assess this we examined to see if Ste13p cytosolic domain and various Ste13p cytosolic domain derivatives physically interacted with AP-1. Our results demonstrated that in fact Ste13p cytosolic domain does interact with AP-1. Furthermore, the interacting region mapped to residues 1-12. We showed that the μ1 subunit, Apm1p, specifically bound to Ste13p cytosolic domain in a 2-11 contingent fashion. The β1 subunit, Apl2p, also bound but was not absolutely 2-11 dependent, as it bound to the Ste13p(Δ2-11) construct. However the 2-11 signal did seem to increase its binding affinity, suggesting that Apl2p binds to sites C-terminal to residues 2-11 in addition to binding to residues 2-11. These results describe the first AP-1 sorting signal identified in yeast.

To determine if the N-terminus of Ste13p was sufficient for mediating slow delivery of proteins to the PVC, we appended it as well as various Ste13p cytosolic

domain derivatives to Cps1p and assessed the trafficking kinetics of the chimeras. The chimeras accessed the vacuole with the same kinetics as Cps1p in wild type strains. When rerouted to the TGN-to-EE-to-PVC pathway in strains deficient for GGA function, the chimeras all exhibited the slow delivery phenotype when compared to Cps1p. To ensure that the chimeras were in the TGN-to-EE-to-PVC pathway, we assessed if the $S_{13}A$ mutation blocked trafficking. It had previously been shown to act as a block of progression out of the EE to the PVC (Johnston et al., 2005). A $S_{13}A$ delay was observed, implying that the chimeras were in fact transiting through the EE. Furthermore, residues 1-12 of Ste13p appended to Cps1p were sufficient to mediate the slow delivery phenotype. We therefore conclude that the sorting signal for associating with AP-1 is the 2-11 signal as the *in vitro* and *in vivo* data strongly support this hypothesis.

Clathrin and adaptors accumulate on membranes in an *inp53*Δ strain

Phosphoinositides have been implicated in both vesicle formation and vesicle uncoating. The concentration of specific phosphoinositides is regulated by kinases and phosphatases. Inp53p is a phosphatase with activity for Ptdins(3)P, Ptdins(4)P, Ptdins(4,5)P_2 and Ptdins(3,5)P_2. Inp53p has been shown to mediate trafficking of A(F→A)-ALP. In *inp53*Δ mutants, processing of A(F→A)-ALP is accelerated (Ha et al., 2003). To determine if this phenotype is due to aberrant recruitment of adaptors, as well as clathrin, to membranes, we looked at the sub-cellular fractionation of Apl2p, Gga2p, Gga1p and Chc1p in wild type and *inp53*Δ strain. Gga2p and clathrin were found to be moderately enriched in the 200,000 *g* pellet. Apl2p had a much more pronounced

enrichment in the 200,000 *g* pellet. Gga1p fractionation was not altered in the *inp53*Δ strain.

This data suggests that AP-1 is much more sensitive than the GGAs to changes in the concentration of phosphoinositides regulated by Inp53p in the TGN/endosomal system. The point at which Inp53p acts is still unclear though. It could act at the TGN or EE and mediate vesicle formation, alternatively it could function in the uncoating of clathrin-coated vesicles It is difficult to reconcile the uncoating model with the accelerated trafficking phenotype of A(F→A)-ALP seen in an *inp53*Δ mutant. One would expect a delay in delivery if vesicles carrying A(F→A)-ALP were prevented from uncoating. The situation would be analogous to what is seen in a *pep12-ts* in which vesicle fusion with the PVC is blocked and trafficking of A(F→A)-ALP to the vacuole is blocked. It is possible that the uncoating defect could deplete the amount of cytosolic AP-1 in solution such that the AP-1 is not recruited to the EE. This seems unlikely to be applicable in this situation as the Apl2p signal is quite strong in the 200,00 *g* supernatant, suggesting that there is a large pool of free AP-1 in the cytosol.

The accelerated trafficking phenotype is reminiscent of A(Δ2-11, F→A)-ALP in a wild type strain and A(F→A)-ALP in a *gga1Δ gga2Δ apl2-ts* strain. It is therefore, compelling to think that the phenotype is due to a defect in AP-1/clathrin coat assembly at the EE. Unfortunately, additional data appear to refute this model in addition to refuting the uncoating model. Specifically trafficking of A(F→A)-ALP is severely delayed in a *gga1Δ gga2Δ inp53Δ* strain (Ha et al., 2003). This result suggests that in the absence of Inp53p, A(F→A)-ALP is utilizing the GGA dependent pathway. This would place the action of Inp53p at the TGN and mediate TGN-to-EE trafficking. It is difficult

to accept that A(F→A)-ALP could use the GGA dependent, CPY pathway, in the absence of Inp53p. All of our previous data suggests that A(F→A)-ALP does not enter the CPY pathway. Additional experiments are needed to determine the mechanism by which A(F→A)-ALP trafficking is mediated by Inp53p. Specifically, do clathrin coated vesicles accumulate in an *inp53Δ* strain? If so, what are the cargo? To test if depletion of AP-1 via an uncoating defects mediates the accelerated phenotype, one could see if the addition of exogenous AP-1 restores the slow delivery phenotype.

Conclusion

This thesis has addressed the trafficking itinerary of A(F→A)-ALP and the machinery that mediates its retention in the TGN/endosomal system. Evidence presented in this thesis suggests that A(F→A)-ALP cycles between the TGN and EE. This cycling is mediated by transport via AP-1/clathrin-coated vesicles in a retrograde manner. The 1-12 region acts as a sorting signal for entry into AP-1 vesicles. The anterograde trafficking step is a mystery as is the role of Inp53p. The endosomal system is quite dynamic, therefore I favor a model in which the TGN vesiculates in a clathrin dependent manner and fuses with endocytic vesicles to form the EE. This event may or may not be adaptor dependent. A(F→A)-ALP that leaks into the PVC from the EE does so in a Soi3p dependent manner. At the PVC A(F→A)-ALP is recycled back to the TGN via the retromer complex and the sorting signal for this event is the $F_{85}XF_{87}XD$ motif (Fig 7-1).

A(F→A)-ALP trafficking itinerary =
TGN - EE - PVC - Vacuole

Residues 1-12 afford entry into
AP-1 pathway at the EE

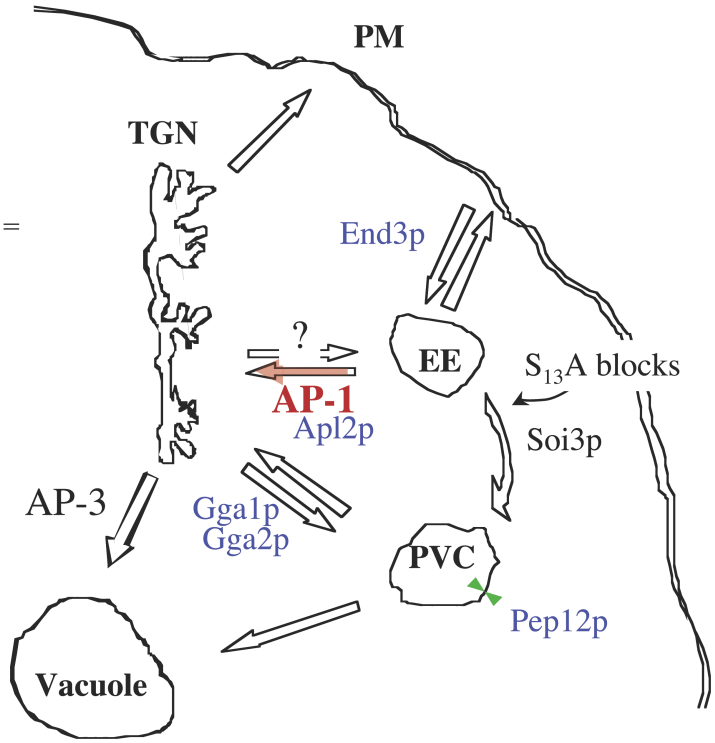


Fig. 7-1: Model of A(F→A)-ALP trafficking itinerary

REFERENCES

Ausebel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, K. Struhl, and J.A. Smith. 2000. Current protocols in molecular biology. John Wiley & Sons, Inc.

Bannykh, S., and Balchm, W. 1997. Membrane Dynamics at the Endoplasmic Reticulum-Golgi Interface. *J. Cell Biol.* 138:1-4.

Barlowe, C., and R. Schekman. 1993. *SEC12* encodes a guanine-nucleotide-exchange factor essential for transport vesicle budding from the ER. *Nature.* 365:347-349.

Becherer, K.A., S.E. Rieder, S.D. Emr, and E.W. Jones. 1996. Novel syntaxin homologue, Pep12p, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. *Mol. Biol. Cell*. 7:579-594.

Becker, B., A. Bolinger, and M. Melkonian. 1995. Anterograde transport of algal scales through the Golgi complex is not mediated by vesicles. *Trends Cell Biol.* 5:305-307.

Beltzer, J.P., and M. Spiess. 1991. *In vitro* binding of the asialoglycoprotein receptor to the beta adaptin of plasma membrane coated vesicles. *EMBO*. 10:3735-3742.

Bensen, E.S., G. Costaguta, and G.S. Payne. 2000. Synthetic genetic interactions with temperature-sensitive clathrin *Saccharomyces cerevisiae*: Roles for synaptojanin-like Inp53p and dynamin-related Vps1p in clathrin-dependent protein sorting at the trans-Golgi network. *Genetics*. 154:83-97.

Black, M.W., and H.R.B. Pelham. 2000. A selective transport route from Golgi to late endosomes that requires the yeast GGA proteins. *J. Cell Biol.* 151:587-600.

Boman, A.L. 2001. GGA proteins: new players in the sorting game. *Journal of Cell Science*. 114:3413-3418.

Bonifacino, J.S., and E.C. Dell'Angelica. 1999. Molecular bases for the recognition of tyrosine-based sorting signals. *J. Cell Biol.* 145:923-926.

Bonifacino, J.S., and J. Lippincott-Schwartz. 2003. Coat proteins: shaping membrane transport. *Nat Rev Mol Cell Biol.* 4:409-14.

Bonifacino, J.S., and L.M.w. Traub. 2003. Signals for Sorting of Transmembrane Proteins to Endosomes and Lysosomes. *Annu Rev Biochem*. 6:6.

Brickner, J.H., and R.S. Fuller. 1997. *SOI1* encodes a novel, conserved protein that promotes TGN-endosomal cycling of Kex2p and other membrane proteins by modulating the function of two TGN localization signals. *J. Cell Biol.* 139:23-26.

Bruinsma, P., R.G. Spelbrink, and S.F. Nothwehr. 2004. Retrograde transport of the mannosyltransferase Och1p to the early Golgi requires a component of the COG transport complex. *J. Biol. Chem.* 279:39814-39823.

Bryant, N.J., and T.H. Stevens. 1997. Two separate signals act independently to localize a yeast late Golgi membrane protein through a combination of retrieval and retention. *J. Cell Biol.* 136:287-297.

Burda, P., S.M. Padilla, S. Sarkar, and S.D. Emr. 2002. Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase. *J Cell Sci*. 115:3889-900.

Bénédetti, H., S. Raths, F. Crausaz, and H. Riezman. 1994. The *END3* gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. *Mol. Biol. Cell*. 5:1023-1037.

Cereghino, J.L., E.G. Marcusson, and S.D. Emr. 1995. The cytoplasmic tail domain of the vacuolar protein sorting receptor Vps10p and a subset of *VPS* gene products regulate receptor stability, function, and localization. *Mol. Biol. Cell*. 6:1089-1102.

Collins, B.M., A.J. McCoy, H.M. Kent, P.R. Evans, and D.J. Owen. 2002. Molecular architecture and functional model of the endocytic AP2 complex. *Cell*. 109:523-535.

Cooper, A.A., and T.H. Stevens. 1996. Vps10p cycles between the late-Golgi and prevacuolar compartments in its function as the sorting receptor for multiple yeast vacuolar hydrolases. *J. Cell Biol.* 133:529-541.

Costaguta, G., C.J. Stefan, E.S. Bensen, S.D. Emr, and G.S. Payne. 2001. Yeast Gga coat proteins function with clathrin in Golgi to endosome transport. *Mol. Biol. Cell*. 12:1885-1896.

Cowles, C.R., G. Odorizzi, G.S. Payne, and S.D. Emr. 1997a. The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell*. 91:109-118.

Cowles, C.R., W.B. Snyder, C.G. Burd, and S.D. Emr. 1997b. Novel Golgi to vacuole delivery pathway in yeast - identification of a sorting determinant and required transport component. *EMBO J*. 16:2769-2782.

Cremona, O., and P. De Camilli. 2001. Phosphoinositides in membrane traffic at the synapse [Review]. *Journal of Cell Science*. 114:1041-1052.

Cremona, O., G. Di Paolo, M.R. Wenk, A. Luthi, W.T. Kim, K. Takei, L. Daniell, Y. Nemoto, S.B. Shears, R.A. Flavell, D.A. McCormick, and P. De Camilli. 1999. Essential role of phosphoinositide metabolism in synaptic vesicle recycling. *Cell*. 99:179-188.

Darsow, T., D.J. Katzmann, C.R. Cowles, and S.D. Emr. 2001. Vps41p function in the alkaline phosphatase pathway requires homo-oligomerization and interaction with AP-3 through two distinct domains. *Mol. Biol. Cell*. 12:37-51.

Davis, N.G., and P. Model. 1985. An artificial anchor domain: hydrophobicity suffices to stop transfer. *Cell*. 41:607-14.

Dunphy, W.G., and J.E. Rothman. 1985. Compartmental organization of the Golgi stack [Review]. *Cell*. 42:13-21.

Evan, G.I., G.K. Lewis, G. Ramsay, and M. Bishop. 1985. Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell. Biol.* 5:3610-3616.

Ford, M.G.J., B.M.F. Pearse, M.K. Higgins, Y. Vallis, D.J. Owen, D. Gibson, C.R. Hopkins, P.R. Evans, and H.T. McMahon. 2001. Simultaneous binding of PtdIns(4,5)P_2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science*. 291:1051-1055.

Fuller, R.S., A. Brake, and J. Thorner. 1989. Yeast prohormone processing enzyme (*KEX2* gene product) is a Ca2+-dependent serine protease. *Proc. Natl. Acad. Sci.* 86:1434-1438.

Gall, W.E., M.A. Higginbotham, C.Y. Chen, M.F. Ingram, D.M. Cyr, and T.R. Graham. 2000. The auxilin-like phosphoprotein Swa2p is required for clathrin function in yeast. *Current Biology*. 10:1349-1358.

Ghosh, P., and S. Kornfeld. 2004. The cytoplasmic tail of the cation-independent mannose 6-phosphate receptor contains four binding sites for AP-1. *Arch Biochem Biophys*. 426:225-30.

Godi, A., P. Pertile, R. Meyers, P. Marra, G. Di Tullio, C. Lurisci, A. Luini, D. Corda, and M.A. De Matteis. 1999. ARF mediates recruitment of PtdIns-4-OH kinase-ß and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. *Nature Cell Biol.* 1:280-287.

Guo, S.L., L.E. Stolz, S.M. Lemrow, and J.D. York. 1999. SAC1-like domains of yeast SAC1, INP52, and INP53 and of human synaptojanin encode polyphosphoinositide phosphatases. *J. Biol. Chem.* 274:12990-12995.

Ha, S.-H., J.T. Bunch, H. Hama, D.B. DeWald, and S.F. Nothwehr. 2001. A novel mechanism for localizing membrane proteins to the yeast trans-Golgi network requires function of a synaptojanin-like protein. *Mol. Biol. Cell*. 12:3175-3190.

Ha, S.A., J. Torabinejad, D.B. DeWald, M.R. Wenk, L. Lucast, P. De Camilli, R.A. Newitt, R. Aebersold, and S.F. Nothwehr. 2003. The synaptojanin-like protein Inp53/Sjl3 functions with clathrin in a yeast TGN-to-endosome pathway distinct from the GGA protein-dependent pathway. *Mol Biol Cell*. 14:1319-33.

Harris, T.W., E. Hartwieg, R.H. Horvitz, and E.M. Jorgensen. 2000. Mutations in synaptojanin disrupt synaptic vesicle recycling. *The J. Cell Biol.* 150:589-599.

Heilker, R., U. Manningkrieg, J.F. Zuber, and M. Spiess. 1996. In Vitro Binding Of Clathrin Adaptors to Sorting Signals Correlates With Endocytosis and Basolateral Sorting. *EMBO J*. 15:2893-2899.

Heilker, R., M. Spiess, and P. Crottet. 1999. Recognition of sorting signals by clathrin adaptors. *Bioessays*. 21:558-567.

Hettema, E.H., M.J. Lewis, M.W. Black, and H.R.B. Pelham. 2003. Retromer and the sorting nexins Snx4/41/42 mediate distinct retrieval pathways from yeast endosomes. *EMBO J*. 22:548-557.

Hirst, J., M.R. Lindsay, and M.S. Robinson. 2001. GGAs: Roles of the different domains and comparison with AP-1 and clathrin. *Molecular Biology of the Cell*. 12:3573-3588.

Holthuis, J.C.M., B.J. Nichols, and H.R.B. Pelham. 1998. The syntaxin Tlg1p mediates trafficking of chitin synthase III to polarized growth sites in yeast. *Mol. Biol. Cell*. 9:3383-3397.

Honing, S., M. Sosa, A. Hillerehfeld, and K. Vonfigura. 1997. The 46-kda mannose 6-phosphate receptor contains multiple binding sites for clathrin adaptors. *J. Biol. Chem.* 272:19884-19890.

Huang, F.T., A. Nesterov, R.E. Carter, and A. Sorkin. 2001. Trafficking of yellow-fluorescent-protein-tagged mu 1 subunit of clathrin adaptor AP-1 complex in living cells. *Traffic*. 2:345-357.

Itoh, T., S. Koshiba, T. Kigawa, A. Kikuchi, S. Yokoyama, and T. Takenawa. 2001. Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science*. 291:1047-1051.

Johnston, H.D., C. Foote, A. Santeford, and S.F. Nothwehr. 2005. Golgi-to-late endosome trafficking of the yeast pheromone processing enzyme Ste13p is

regulated by a phosphorylation site in its cytosolic domain. *Mol Biol Cell*. 16:1456-68.

Jones, E.W. 1977. Proteinase mutants of *Saccharomyces cerevisiae*. *Genetics*. 85:23-33.

Kaiser, C.A., and R. Schekman. 1990. Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell.* 61:723-733.

Katzmann, D.J., M. Babst, and S.D. Emr. 2001. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell*. 106:145-155.

Kirchhausen, T. 2000. Clathrin. *Annual Review of Biochemistry*. 69:699-727.

Klionsky, D.J., P.K. Herman, and S.D. Emr. 1990. The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* 54:266-292.

Klumperman, J., R. Kuliawat, J.M. Griffith, H.J. Geuze, and P. Arvan. 1998. Mannose 6-phosphate receptors are sorted from immature secretory granules via adaptor protein ap-1, clathrin, and syntaxin 6-positive vesicles. *J. Cell Biol.* 141:359-371.

Krijnse-Locker, J., M. Ericsson, P.G. Rottier, and G. G. 1994. Characterization of the budding compartment of mouse hepatitis virus: evidence that transport from the RER to the Golgi complex requires only one vesicular transport step. *Journal of Cell Biology*. 124:55-70.

Ladinsky, M.S., D.N. Mastronarde, J.R. McIntosh, K.E. Howell, and L.A. Staehelin. 1999. Golgi structure in three dimensions: Functional insights from the normal rat kidney cell. *J. Cell Biol.* 144:1135-1149.

Le Borgne, R., and B. Hoflack. 1997. Mannose 6-phosphate receptors regulate the formation of clathrin-coated vesicles in the TGN. *J. Cell Biol.* 137:335-345.

Lewis, M.J., B.J. Nichols, C. Prescianotto-Baschong, H. Riezman, and H.R.B. Pelham. 2000. Specific retrieval of the exocytic SNARE Snc1p from early yeast endosomes. *Mol. Biol. Cell*. 11:23-38.

Lippincottschwartz, J. 1998. Cytoskeletal proteins and Golgi dynamics. *Curr. Opin. Cell Biol.* 10:52-59.

Lowe, M., and T.E. Kreis. 1998. Regulation of membrane traffic in animal cells by COPI. *Biochimica et Biophysica Acta - Molecular Cell Research*. 1404:53-66.

Luo, Z., and D. Gallwitz. 2003. Biochemical and genetic evidence for the involvement of yeast Ypt6-GTPase in protein retrieval to different Golgi compartments. *J Biol Chem*. 278:791-9.

Marcusson, E.G., B.F. Horazdovsky, J.L. Cereghino, E. Gharakhanian, and S.D. Emr. 1994. The sorting receptor for yeast vacuolar carboxypeptidase Y is encoded by the VPS10 gene. *Cell.* 77(4):579-86.

Martin, T.F.J. 2001. PI(4,5)P-2 regulation of surface membrane traffic. *Curr. Opin. in Cell Biol.* 13:493-499.

Mayer, M.P., and B. Bukau. 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci.* 62:670-684.

McCormick, P.J., M.J., Bonifacino JS. 2005. Involvement of clathrin and AP-2 in the trafficking of MHC class II molecules to antigen-processing compartments. *Proc Natl Acad Sci U S A*. 102:7910-5.

McPherson, P.S., and B. Ritter. 2005 Peptide motifs: building the clathrin machinery. *Mol Neurobiol*. 32:73-88.

Meyer, C., D. Zizioli, S. Lausmann, E.L. Eskelinen, J. Hamann, P. Saftig, K. von Figura, and P. Schu. 2000. mu 1A-adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors. *EMBO J*. 19:2193-2203.

Mironov, A.A., G.V. Beznoussenko, P. Nicoziani, O. Martella, A. Trucco, H.-S. Kweon, D.D. Giandomenico, R.S. Polishchuk, A. Fusella, P. Lupetti, E.G. Berger, W.J.C. Geerts, A.J. Koster, K.N.J. Burger, and A. Luini. 2001. Small cargo proteins and large aggregates can traverse the Golgi by a common mechanism without leaving the lumen of cisternae. *J. Cell Biol.* 155:1225-1238.

Mullins, C., and J.S. Bonifacino. 2001. Structural requirements for function of yeast GGAs in vacuolar protein sorting, alpha-factor maturation, and interactions with clathrin. *Molecular & Cellular Biology*. 21:7981-7994.

Mumberg, D., R. Müller, and M. Funk. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*. 156:119-122.

Mutka, S., and P. Walter. 2001. Multifaceted physiological response allows yeast to adapt to the loss of the signal recognition particle-dependent protein-targeting pathway. *Mol Biol Cell.* 12:577-88.

Nakayama, K., and S. Wakatsuki. 2003. The Structure and Function of GGAs, the Traffic Controllers at the TGN Sorting Crossroads. *Cell Struct Funct*. 28:431-42.

Newpher, T.M., R.P. Smith, V. Lemmon, and S.K. Lemmon. 2005. In vivo dynamics of clathrin and its adaptor-dependent recruitment to the actin-based endocytic machinery in yeast. *Dev Cell*. 9:87-98.

Nie, Z., D.S. Hirsch, and P.A. Randazzo. 2003. Arf and its many interactors. *Curr Opin Cell Biol*. 15:396-404.

Nothwehr, S.F., N.J. Bryant, and T.H. Stevens. 1996. The newly identified yeast *GRD* genes are required for retention of late-Golgi membrane proteins. *Mol. Cell. Biol.* 16:2700-2707.

Nothwehr, S.F., E. Conibear, and T.H. Stevens. 1995. Golgi and vacuolar membrane proteins reach the vacuole in *vps1* mutant yeast cells via the plasma membrane. *J. Cell Biol.* 129:35-46.

Nothwehr, S.F., S.-A. Ha, and P. Bruinsma. 2000. Sorting of yeast membrane proteins into an endosome-to-Golgi pathway involves direct interaction of their cytosolic domains with Vps35p. *J. Cell Biol.* 151:297-309.

Nothwehr, S.F., C.J. Roberts, and T.H. Stevens. 1993. Membrane protein retention in the yeast Golgi apparatus: dipeptidyl aminopeptidase A is retained by a cytoplasmic signal containing aromatic residues. *J. Cell Biol.* 121:1197-1209.

Nothwehr, S.F., and T.H. Stevens. 1994. Sorting of membrane proteins in the yeast secretory pathway. *J. Biol. Chem.* 269:10185-10188.

Orci, L., M. Ravazzola, A. Volchuk, T. Engel, M. Gmachl, M. Amherdt, A. Perrelet, T.H. Söllner, and J.E. Rothman. 2000. Anterograde flow of cargo across the Golgi stack potentially mediated via bidirectional "percolating" COPI vesicles. *Proceedings of the National Academy of Sciences of the United States of America*. 97:10400-10405.

Pearse. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *EMBO*. 7:3331-3336.

Pelham, H.R. 2004. Membrane Traffic: GGAs Sort Ubiquitin. *Curr Biol*. 14:R357-9.

Pelham, H.R.B. 1998. Getting through the Golgi complex. *Trends Cell Biol.* 8:45-49.

Peyroche, A., B. Antonny, S. Robineau, J. Acker, J. Cherfils, and C.L. Jackson. 1999. Brefeldin A acts to stabilize an abortive ARF-GDP-Sec7 domain protein complex: Involvement of specific residues of the Sec7 domain. *Molecular Cell*. 3:275-285.

Pishvaee, B., G. Costaguta, B.G. Yeung, S. Ryazantsev, T. Greener, L.E. Greene, E. Eisenberg, J.M. McCaffery, and G.S. Payne. 2000. A yeast DNA J protein required for uncoating of clathrin-coated vesicles in vivo. *Nature Cell Biology*. 2:958-963.

Proszynski, T.J., K. Simons, and M. Bagnat. 2004. O-glycosylation as a Sorting Determinant for Cell Surface Delivery in Yeast. *Mol Biol Cell*.

Puertollano, R., N.N. van der Wel, L.E. Greene, E. Eisenberg, P.J. Peters, and J.S. Bonifacino. 2003. Morphology and dynamics of clathrin/GGA1-coated carriers budding from the trans-Golgi network. *Mol Biol Cell*. 14:1545-57.

Reggiori, F., and H.R.B. Pelham. 2001. Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. *EMBO Journal*. 20:5176-5186.

Roberts, C.J., S.F. Nothwehr, and T.H. Stevens. 1992. Membrane protein sorting in the yeast secretory pathway: evidence that the vacuole may be the default compartment. *J. Cell Biol.* 119:69-83.

Robinson, J.S., D.J. Klionsky, L.M. Banta, and S.D. Emr. 1988. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* 8:4936-4948.

Robinson, M.S., and T.E. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell*. 69:129-38.

Rothman, J.E. 1994. Intracellular membrane fusion. *Adv. Sec. Mess. Phospho. Res.* 29:81-96.

Rothman, J.E., and F.T. Wieland. 1996. Protein sorting by transport vesicles. *Science*. 272:227-234.

Rothman, J.H., and T.H. Stevens. 1986. Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell.* 47:1041-1051.

Santos, B., and M. Snyder. 1997. Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. *J. Cell Biol.* 136:95-110.

Sato, T.K., M. Overduin, and S.D. Emr. 2001. Location, location, location: Membrane targeting directed by PX domains. *Science*. 294:1881-1885.

Scott, P.M., P.S. Bilodeau, O. Zhdankina, S.C. Winistorfer, M.J. Hauglund, M.M. Allaman, W.R. Kearney, A.D. Robertson, A.L. Boman, and R.C. Piper. 2004. GGA proteins bind ubiquitin to facilitate sorting at the trans-Golgi network. *Nat Cell Biol*. 6:252-9.

Seaman, M.N.J., J.M. McCaffery, and S.D. Emr. 1998. A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J. Cell Biol.* 142:665-681.

Sever, S. 2003. Dynamin and endocytosis. *Curr Opin Cell Biol*. 14:463-7.

Sharrocks, A.D. 1994. A T7 expression vector for producing N- and C-terminal fusion proteins with glutathione S-transferase. *Gene*. 138:105-108.

Shiba, T., M. Kawasaki, H. Takatsu, T. Nogi, N. Matsugaki, N. Igarashi, M. Suzuki, R. Kato, K. Nakayama, and S. Wakatsuki. 2003. Molecular mechanism of membrane recruitment of GGA by ARF in lysosomal protein transport. *Nat Struct Biol*. 10:386-93.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 122:19-27.

Singer-Krüger, B., Y. Nemoto, L. Daniell, S. Ferro-Novick, and P. De Camilli. 1998. Synaptojanin family members are implicated in endocytic membrane traffic in yeast. *J. Cell Sci.* 111:3347-3356.

Sipos, G., J.H. Brickner, E.J. Brace, L. Chen, A. Rambourg, F. Kepes, and R.S. Fuller. 2004. Soi3p/Rav1p Functions at the Early Endosome to Regulate Endocytic Trafficking to the Vacuole and Localization of TGN Transmembrane Proteins. *Mol Biol Cell*. 15:3196-3209.

Spelbrink, R.G., and S.F. Nothwehr. 1999. The yeast *GRD20* gene is required for protein sorting in the *trans*-Golgi network/endosomal system and for polarization of the actin cytoskeleton. *Mol. Biol. Cell*. 10:4263-4281.

Spormann, D.O., J. Heim, and D.H. Wolf. 1991. Carobypeptidase yscS: gene structure and function of the vacuolar enzyme. *Eur. J. Biochem.* 197:399-405.

Stefan, C.J., A. Audhya, and S.D. Emr. 2002. The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. *Mol. Biol. Cell*. 13:542-557.

Stolz, L.E., W.J. Kuo, J. Longchamps, M.K. Sekhon, and J.D. York. 1998. *INP51*, a yeast inositol polyphosphate 5-phosphatase required for phosphatidylinositol 4,5-bisphosphate homeostasis and whose absence confers a cold-resistant phenotype. *J. Biol. Chem.* 273:11852-11861.

Subramanian, S., C.A. Woolford, and E.W. Jones. 2004. The Sec1/Munc18 protein, Vps33p, functions at the endosome and the vacuole of Saccharomyces cerevisiae. *Mol Biol Cell.* 15:2593-605.

Sutton, R.B., D. Fasshauer, R. Jahn, and A.T. Brunger. 1998. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*. 395:328-9.

Valdivia, R.H., D. Baggot, J.S. Chuang, and R. Schekman. 2002. The yeast clathrin adaptor protein complex 1 is required for the efficient retention of a subset of late Golgi membrane proteins. *Dev. Cell*. 2:283-294.

Verstreken, P., T.W. Koh, K.L. Schulze, R.G. Zhai, P.R. Hiesinger, Y. Zhou, S.Q. Mehta, Y. Cao, J. Roos, and H.J. Bellen. 2003. Synaptojanin is recruited by endophilin to promote synaptic vesicle uncoating. *Neuron*. 40:733-48.

Vida, T.A., and S.D. Emr. 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* 128:779-792.

von Heijne, G. 1985. Signal sequences. The limits of variation. *J Mol Biol.* 184:99-105.

Voos, W., and T.H. Stevens. 1998. Retrieval of resident late-Golgi membrane proteins from the prevacuolar compartment of *Saccharomyces cerevisiae* is dependent on the function of Grd19p. *J. Cell Biol.* 140:577-590.

Vowels, J.J., and G.S. Payne. 1998. A dileucine-like sorting signal directs transport into an AP-3-dependent, clathrin-independent pathway to the yeast vacuole. *EMBO J.* 17:2482-2493.

Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen. 1994. New heterologous modules for classical of PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast*. 10:1793-1808.

Waguri, S., F. Dewitte, R. Le Borgne, Y. Rouillé, Y. Uchiyama, J. Dubremetz, and B. Hoflack. 2003. Visualization of TGN to Endosome Trafficking through Fluorescently Labeled MPR and AP-1 in Living Cells. *MBC*. 14:142-155.

Wang, Y.J., J. Wang, H.Q. Sun, M. Martinez, Y.X. Sun, E. Macia, T. Kirchhausen, J.P. Albanesi, M.G. Roth, and H.L. Yin. 2003. Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell*. 114:299-310.

Yeung, B.G., and G.S. Payne. 2001. Clathrin interactions with C-terminal regions of the yeast AP-1 beta and gamma subunits are important for AP-1 association with clathrin coats. *Traffic*. 2:565-576.

Yeung, B.G., H.L. Phan, and G.S. Payne. 1999. Adaptor complex-independent clathrin function in yeast. *Mol. Biol. Cell*. 10:3643-3659.

Ziman, M., J.S. Chuang, and R.W. Schekman. 1996. Chs1p and Chs3p, two proteins involved in chitin synthesis, populate a compartment of the *Saccharomyces cerevisiae* endocytic pathway. *Mol. Biol. Cell*. 7:1909-1919.

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