Ent3p and Ent5p Exhibit Cargo-specific Functions in Trafficking Proteins between the *Trans*-Golgi Network and the Endosomes in Yeast

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The phosphoinositide-binding proteins Ent3p and Ent5p are required for protein transport from the *trans*-Golgi network (TGN) to the vacuole in *Saccharomyces cerevisiae*. Both proteins interact with the monomeric clathrin adaptor Gga2p, but Ent5p also interacts with the clathrin adaptor protein 1 (AP-1) complex, which facilitates retention of proteins such as Chs3p at the TGN. When both *ENT3* and *ENT5* are mutated, Chs3p is diverted from an intracellular reservoir to the cell surface. However, Ent3p and Ent5p are not required for the function of AP-1, but rather they seem to act in parallel with AP-1 to retain proteins such as Chs3p at the TGN. They have all the properties of clathrin adaptors, because they can both bind to clathrin and to cargo proteins. Like AP-1, Ent5p binds to Chs3p, whereas Ent3p facilitates the interaction between Gga2p and the endosomal syntaxin Pep12p. Thus, Ent3p has an additional function in Gga-dependent transport to the late endosome. Ent3p also facilitates the association between Gga2p and clathrin; however, Ent5p can partially substitute for this function. We conclude that the clathrin adaptors AP-1, Ent3p, Ent5p, and the Ggas cooperate in different ways to sort proteins between the TGN and the endosomes.

INTRODUCTION

In eukaryotic cells, the clathrin coat is involved in endocytosis as well as in the sorting and transport of proteins between the *trans*-Golgi network (TGN) and the endosomes. Clathrin heavy and light chain form the outer layer of the coat, and adaptor proteins mediate the interaction between clathrin, the membrane surface, and cargo proteins in the membrane, as well as with other accessory proteins (Owen *et al.*, 2004). Several different adaptor proteins have been identified to date, many of which interact to facilitate clathrin-mediated endocytosis. In intracellular clathrin-dependent protein trafficking, however, the relationships between the different adaptors are less well understood (Traub, 2005).

Adaptor protein 1 (AP-1) is a tetrameric clathrin adaptor complex that localizes to the TGN and/or early endosomes in yeast and in mammalian cells. It has been implicated in forward transport from the TGN, in retrograde transport from the early endosome back to the TGN, or in retention of proteins at the TGN (Meyer *et al.*, 2000; Valdivia *et al.*, 2002; Ha *et al.*, 2003; Waguri *et al.*, 2003; Foote and Nothwehr,

2006). AP-1 binds to phosphatidylinositol-4-phosphate, a phosphoinositide that is enriched on these membranes (Wang *et al.*, 2003). The small GTPase Arf-1 and specific signals on cargo proteins (Meyer *et al.*, 2005), as well as a recently discovered mammalian protein, γ -BAR (Neubrand *et al.*, 2005), are also involved in the recruitment of AP-1 to lipid membranes (Meyer *et al.*, 2005). Two consensus AP-1 binding motifs on cytosolic tails of cargo proteins have been identified, and their direct role in binding to AP-1 has been demonstrated in a few mammalian proteins (Peden *et al.*, 2001; Bonifacino and Traub, 2003; Janvier *et al.*, 2003). The recognition of a third type of AP-1 binding motif requires the participation of another mammalian protein, PACS-1 (Crump *et al.*, 2001). Finally, an unrelated AP-1 binding signal has recently been discovered in yeast (Foote and Nothwehr, 2006).

Another type of clathrin adaptor, the monomeric GGA protein, has been implicated in the transport of proteins from the TGN to the late endosome, although GGAs also interact and colocalize with some early endocytic proteins in mammalian cells. Two domains in the GGAs have a role in cargo binding: the VHS domain specifically recognizes a DXXLL signal that is present in many mammalian proteins and is not recognized by any other adaptors, whereas the GAT domain binds to ubiquitinated proteins (Bonifacino, 2004). In trafficking of mannose phosphate receptors, it has been suggested that the GGAs cooperate with AP-1, to which they convey cargo proteins after the initial recognition event (Doray *et al.*, 2002). Two GGA proteins, Gga1p and Gga2p, exist in *Saccharomyces cerevisiae* and their functions are redundant. They are required for transport of proteins such as carboxypeptidase Y (CPY) and the syntaxin Pep12p to the late endosome, and for ubiquitin-dependent sorting of the amino acid permease Gap1p (Black and Pelham, 2000; Hirst *et al.*, 2000; Costaguta *et al.*, 2001; Hirst *et*

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Abbreviations used: ALP, alkaline phosphatase; CPY, carboxypeptidase Y; DSP, dithiobis[succinimidylpropionate]; ER, endoplasmic reticulum; mRFP, monomeric red fluorescent protein; TAP, tandem affinity purification; TGN, *trans*-Golgi network; Vps, vacuolar protein sorting.

al., 2001; Scott *et al.*, 2004). However, only ubiquitin has been shown to be a direct binding partner of a yeast GGA (via the GAT domain; Scott *et al.*, 2004). Furthermore, structural analyses suggest that the yeast GGA VHS domain may not be able to accommodate the DXXLL signal peptide (Misra *et al.*, 2002).

Two novel phosphoinositide-binding proteins, Ent3p and Ent5p, were identified in a two-hybrid screen for accessory proteins that interact with AP-1 and the GGAs in yeast (Duncan *et al.*, 2003). A similar AP-1/GGA-binding protein, epsinR, exists in mammalian cells (Kalthoff *et al.*, 2002; Wasiak *et al.*, 2002; Hirst *et al.*, 2003; Mills *et al.*, 2003). Ent3p and epsinR bind to lipids via their epsin amino-terminal homology domain, whereas Ent5p contains a similar AP180 N-terminal homology domain. All three proteins also contain clathrin binding motifs in their primary sequence (Legendre-Guillemain *et al.*, 2004), and Ent5p and epsinR bind to clathrin in vitro, suggesting that they function as clathrin adaptors (Duncan *et al.*, 2003; Mills *et al.*, 2003). Furthermore, Ent3p binds to a potential cargo protein, the vacuolar soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) Vti1p, and epsinR binds to the mammalian orthologue Vti1b (Chidambaram *et al.*, 2004; Hirst *et al.*, 2004). Cells lacking both Ent3p and Ent5p display defects in trafficking of proteins between the TGN and the endosomes and the sorting of proteins into multivesicular bodies is also affected (Duncan *et al.*, 2003; Eugster *et al.*, 2004). In mammalian cells, epsinR has been implicated in retrograde sorting from the early endosome (Saint-Pol *et al.*, 2004).

To gain a better understanding of the intracellular trafficking steps mediated by clathrin, we have examined the transport of a cargo protein, the chitin synthase Chs3p, in *S. cerevisiae*. Chs3p is stored in an intracellular reservoir, from which it is transported to the cell surface in a cell cycle-dependent manner with the help of a cytosolic complex that contains Chs6p (Chuang and Schekman, 1996; Ziman *et al.*, 1998; Sanchatjate and Schekman, 2006). Chs3p proved particularly useful in discerning the role of the yeast AP-1, because an inefficient intracellular retention of Chs3p was the first easily observable phenotype associated with mutations in AP-1 subunits. Mutations in the *VPS* genes, which are involved in trafficking to the late endosome and the vacuole (Bowers and Stevens, 2005), did not affect Chs3p localization. It was therefore proposed that AP-1 mediates continuous recycling of Chs3p between the TGN and the early endosome (Valdivia *et al.*, 2002). Other proteins, such as the protease Kex2p, also follow this recycling route (Ha *et al.*, 2003; Foote and Nothwehr, 2006).

In this study, we address the role of Ent3p and Ent5p in the trafficking of Chs3p, and we compare their function to the function of the other intracellular clathrin adaptors, AP-1 and the GGAs. We show that like AP-1, but independently of it, Ent5p directly binds to Chs3p. Furthermore, we find that Ent3p is important for the function of the GGAs and may mediate the binding of Gga2p to cargo proteins.

MATERIALS AND METHODS

Strain and Plasmid Construction

Strains (Table 1) were constructed either by tetrad dissection of sporulated diploid strains, by integration of disruption cassettes replacing the whole coding region, or by integrations of insertions at the COOH-terminal codon (Longtine *et al.*, 1998). All allelic replacements or insertions were confirmed by polymerase chain reaction (PCR). *chs6Δ::HIS3*, *gga1Δ::TRP1*, and *gga2Δ::LEU2* disruption cassettes were generated by PCR amplification from total DNA isolated from JCY479, R5Y2364, and R5Y1715, respectively. The plasmid pFA6::NATMX was used to generate disruption cassettes containing the

NATMX marker (Tong *et al.*, 2004). Resistance to calcofluor (CF) was assessed by growth on YPD agar plates supplemented with 100 μg/ml Fluorescent Brightener 28 (Sigma-Aldrich, St. Louis, MO). The *pCHS3-GFP* expression plasmid was generated by subcloning *CHS3-GFP* from a plasmid generated by Valdivia *et al.* (2002) into pRS315. The plasmid pFA6a-mRFP was used to generate the COOH-terminal integration cassettes *ENT3::mRFP* and *ENT5::mRFP*.

Microscopy

For direct imaging of Chs3-green fluorescent protein (GFP), strains were transformed with *pCHS3-GFP* (CEN *LEU2*) along with a *CHS7* CEN expression plasmid pA3-2 (Valdivia *et al.*, 2002) to prevent accumulation of Chs3p in the endoplasmic reticulum (ER) (Trilla *et al.*, 1999). Cells were grown in complete synthetic dropout medium to OD₆₀₀ ~0.5. Chitin staining was performed on cells fixed with 2% formaldehyde, incubated on ice in 25 μg/ml calcofluor in water for 30 min, and washed twice before analysis. Fluorescence was visualized with an epifluorescence microscope (Nikon, Tokyo, Japan), and images were captured with a charge-coupled device (CCD) camera and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Subcellular Fractionations

Total cell lysates were fractionated on 5-ml 20–60% (wt/wt) continuous sucrose gradients as described previously (Kaiser *et al.*, 2002). Continuous gradients were generated by diffusion for 2 h at room temperature (Kolling and Hollenberg, 1994). Gradients were centrifuged for 17 h at 4°C at 100,000 × *g*. Fractions (16) were collected manually from the top of a gradient and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with antibodies against Chs3p, Sec61p, Vph1p, Pma1p (F. Portillo, Universidad Autonoma de Madrid, Spain), Anp1p (S. Munro, Medical Research Council, Cambridge, United Kingdom), Tlg1p (H. Pelham, Medical Research Council, Cambridge, United Kingdom), and Pep12p (Invitrogen, Carlsbad, CA). Protein bands were quantified as described below.

Tandem Affinity Purification (TAP) from Yeast

A tandem affinity purification tag (Rigaut *et al.*, 1999) composed of an S tag (Kim and Raines, 1993), a TEV protease cleavage site, and a ZZ tag (minimal protein A binding domain) was amplified from pKW804 (a gift from K. Weis, University of California, Berkeley, CA). The tag was integrated at the COOH termini of *APS1*, *ENT3*, *ENT5*, or *GGA2* in DDY1810 to generate TSY127, ACY134, ACY133, or ACY182, respectively. For TAP, cells were grown to OD₆₀₀ 0.6–0.8, washed with H₂O, and incubated 10 min in pre-spheroplasting buffer (100 mM Tris, pH 9.4, and 40 mM β-mercaptoethanol), followed by 30-min incubation in spheroplasting buffer (20 mM HEPES, pH 7.4, 0.7 M sorbitol, 0.5X YPD, and lyticase at 30 U/10⁷ cells) at 30°C. Spheroplasts were washed once in cross-linking buffer (20 mM HEPES, pH 7.4, 0.7 M sorbitol, and 100 mM KOAc) and resuspended in 0.02 ml of cross-linking buffer/10⁷ cells. Cross-linking was conducted with 5 mM dithiobis[succinimidylpropionate] (DSP) (Pierce Chemical, Rockford, IL) for 30 min at room temperature, and the reaction was quenched for 15 min in 100 mM Tris, pH 7.4. Cells were then resuspended in the same volume of ice-cold lysis buffer A (50 mM HEPES, pH 7.4, 350 mM NaCl, and 1% Triton X-100) with 2 mM phenylmethylsulfonyl fluoride and 2X protease inhibitors, and they were lysed with 15 strokes in a Dounce homogenizer. All subsequent steps were conducted at 4°C. Lysates were incubated for 30 min to extract membrane proteins, and the extracts were centrifuged at 3000 rpm for 6 min and at 13.5 krpm in an SS-34 rotor for 20 min. The supernatant fraction was passed over a 2-ml Sepharose CL-6B column (Sigma-Aldrich), and the flow-through was incubated for 3 h with immunoglobulin G (IgG)-Sepharose (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Beads were washed with lysis buffer A and incubated overnight in a small volume of buffer containing 80 U of AcTev protease (Invitrogen). The supernatant was then removed from the IgG-Sepharose and incubated with 60 μl of 50% S-protein agarose (Novagen, Madison, WI) for 3 h. S-protein agarose beads were washed with lysis buffer, and 2X SDS protein sample buffer containing 100 mM dithiothreitol was added to elute the bound proteins and cleave the cross-linker DSP. Samples were analyzed by SDS-PAGE, followed by Sypro red staining or immunoblotting with antibodies against Chs3p, Clc1p (affinity-purified using glutathione S-transferase-Clc1p as described previously; Harsay and Schekman, 2002), Chc1p (S. Lemmon, University of Miami), Pep12p (Invitrogen), Apl2p and Gga2p (G. Payne, UCLA, Los Angeles, CA), Vps27p (T. Stevens, University of Oregon, Eugene, OR), and dsRed (Clontech, Mountain View, CA). S-protein-horseradish peroxidase (HRP) (Novagen) was used for detection of purified TAP-tagged proteins. Sypro-stained gels and nitrocellulose membranes were scanned on a Typhoon 9400 Imager (GE Healthcare). Protein bands were quantified using ImageQuant software (GE Healthcare).

For purification of Ent3-TAP and Ent5-TAP under more native conditions, the cross-linking step was omitted, and lysis buffer B (50 mM HEPES, pH 6.8, 350 mM K-acetate, 5 mM MgCl₂, 1 mM EDTA, 1 mM β-mercaptoethanol, and 0.5% octyl glucoside) was used for lysis and subsequent purification steps.

Table 1. Strains used in this study

Name	Genotype	Source
BY4742	<i>MATα his3-Δ0 leu2-Δ0 lys2Δ0 ura3Δ0</i>	Euroscarf (Frankfurt, Germany)
ACY4	<i>MATα chs6Δ::HIS3 his3-Δ1 leu2-Δ0 met15 ura3Δ0</i>	
ACY31	<i>MATα apl2Δ::KANMX chs6Δ::HIS3 his3-Δ0 lys2Δ0 ura3Δ</i>	Euroscarf
ACY147	<i>MATα ent3Δ::KANMX his3-Δ1 leu2-Δ0 lys2Δ0 ura3Δ</i>	
ACY82	<i>MATα ent3Δ::KANMX chs6□::HIS3 his3-Δ0 leu2-Δ0 lys2Δ0 ura3Δ0</i>	Euroscarf
ACY83	<i>MATα ent5Δ::KANMX his3-Δ1 leu2-Δ0 lys2Δ0 ura3Δ0</i>	
ACY84	<i>MATα ent5Δ::KANMX chs6Δ::HIS3 his3-Δ0 leu2-Δ0 lys2Δ0 ura3Δ0</i>	Euroscarf
ACY86	<i>MATα ent3Δ::KANMX ent5Δ::KANMX his3-Δ0 leu2-Δ0 ura3Δ0</i>	
ACY87	<i>MATα ent3Δ::KANMX ent5Δ::KANMX his3-Δ0 leu2-Δ0 lys2Δ0 ura3Δ0</i>	Euroscarf
ACY135	<i>MATα gga2Δ::LEU2 his3-Δ0 leu2-Δ0 lys2Δ0 ura3Δ0</i>	
ACY137	<i>MATα gga2Δ::LEU2 chs6Δ::HIS3 his3-Δ0 leu2-Δ0 lys2Δ0 ura3Δ0</i>	Euroscarf
ACY138	<i>MATα gga2Δ::LEU2 ent3Δ::KANMX his3-Δ0 leu2-Δ0 lys2Δ0 ura3Δ0</i>	
ACY140	<i>MATα gga2Δ::LEU2 ent3Δ::KANMX chs6Δ::HIS3 his3-Δ0 leu2-Δ0 ura3Δ0</i>	Euroscarf
ACY142	<i>MATα gga2Δ::LEU2 ent5Δ::KANMX his3-Δ0 leu2-Δ0 lys2Δ0 ura3Δ0</i>	
ACY143	<i>MATα gga2Δ::LEU2 ent5Δ::KANMX chs6Δ::HIS3 his3-Δ0 lys2Δ0 leu2-Δ0 ura3Δ0</i>	Euroscarf
YPH499	<i>MATα ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52</i>	
TSY49	<i>MATα chs6Δ::HIS3 ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52</i>	Sikorski and Hieter (1989)
TSY199	<i>MATα gga1Δ::HIS3 gga2Δ::TRP1 ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52</i>	
TSY210	<i>MATα chs6Δ::HIS3 gga1Δ::HIS3 gga2Δ::TRP1 ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52</i>	Sikorski and Hieter (1989)
DDY1810	<i>MATα prb1-1122 pep4-3 prc1-407 gal2 leu2Δ trp1Δ ura3-52</i>	
TSY127	DDY1810 <i>APS1::S-Tag-Tev-ZZ::KANMX</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY168	DDY1810 <i>APS1::ZZ-Tev-S-Tag::KANMX apl4Δ::NATMX</i>	
ACY170	DDY1810 <i>APS1::ZZ-Tev-S-Tag::KANMX ent3Δ::KANMX ent5Δ::LEU2</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY133	DDY1810 <i>ENT5::ZZ-Tev-S-Tag::KANMX</i>	
ACY151	DDY1810 <i>ENT5::ZZ-Tev-S-Tag::KANMX apl4Δ::NATMX</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY152	DDY1810 <i>ENT5::ZZ-Tev-S-Tag::KANMX ent3Δ::LEU2</i>	
ACY156	DDY1810 <i>ENT5::ZZ-Tev-S-Tag::KANMX gga1Δ::TRP1 gga2Δ::LEU2</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY134	DDY1810 <i>ENT3::ZZ-Tev-S-Tag::KANMX</i>	
ACY158	DDY1810 <i>ENT3::ZZ-Tev-S-Tag::KANMX ent5Δ::LEU2</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY162	DDY1810 <i>ENT3::ZZ-Tev-S-Tag::KANMX gga1Δ::TRP1 gga2Δ::LEU2</i>	
ACY191	DDY1810 <i>ENT5::ZZ-Tev-S-Tag::KANMX ENT3::mRFP::URA3</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY192	DDY1810 <i>ENT3::ZZ-Tev-S-Tag::KANMX ENT5::mRFP::URA3</i>	
ACY182	DDY1810 <i>GGA2::ZZ-Tev-S-Tag::KANMX</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY183	DDY1810 <i>GGA2::ZZ-Tev-S-Tag::KANMX ent3Δ::LEU2</i>	
ACY184	DDY1810 <i>GGA2::ZZ-Tev-S-Tag::KANMX ent5Δ::URA3</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY188	DDY1810 <i>GGA2::ZZ-Tev-S-Tag::KANMX ent3Δ::URA3 ent5Δ::LEU2</i>	
ACY189	DDY1810 <i>GGA2::ZZ-Tev-S-Tag::KANMX apl4Δ::TRP1</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY190	DDY1810 <i>GGA2::ZZ-Tev-S-Tag::KANMX apl4Δ::TRP1 ent3Δ::LEU2</i>	
ACY180	<i>MAT? ent3::LEU2 ent5::KANMX apl2::TRP1 chs6::HIS3 ade2-101oc his3-Δ200 leu2-Δ1 trp1-Δ63 ura3-52</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
RSY3341	BY4742 <i>pho8Δ::NATMX</i>	
RSY3342	ACY147 (<i>ent3Δ::KANMX</i>) <i>pho8Δ::NATMX</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
RSY3343	ACY82 (<i>ent5Δ::KANMX</i>) <i>pho8Δ::NATMX</i>	
RSY3344	ACY87 (<i>ent3Δ::KANMX ent5Δ::KANMX</i>) <i>pho8Δ::NATMX</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY193	<i>MATα pho8Δ::NATMX ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52</i>	
ACY194	<i>MAT? ent3Δ::LEU2 pho8Δ::NATMX ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)
ACY195	<i>MATα gga1Δ::HIS3 gga2Δ::TRP1 pho8Δ::NATMX ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52</i>	
ACY196	<i>MAT? ent3Δ::LEU2 gga1Δ::HIS3 gga2Δ::TRP1 pho8Δ::NATMX ade2-101oc his3-Δ200 leu2-Δ1 lys2-801am trp1-Δ63 ura3-52</i>	D. Drubin (University of California, Berkeley, Berkeley, CA)

For mass spectrometry analysis, SDS-PAGE gels were stained with colloidal blue Coomassie stain (Invitrogen), and protein bands were excised from the gel. Gel bands were digested with Trypsin Gold (Promega; Jimenez *et al.*, 1998), and proteins were identified at the Howard Hughes Medical Institute mass spectrometry laboratory at the University of California, Berkeley, by peptide mass fingerprinting (using MSFit at <http://prospector.ucsf.edu>).

Metabolic Labeling and Immunoprecipitation

To analyze the processing of A-alkaline phosphatase (ALP) mutants, we transformed strains in which *PHO8* (encoding wild-type ALP) was deleted with pSN100 (Nothwehr *et al.*, 1993) or pSH46 (Foote and Nothwehr, 2006) encoding A(F→A)-ALP or A(Δ2-11, F→A)-ALP, respectively. Cells were

grown in SC-Ura medium to mid-logarithmic phase, harvested, labeled with [³⁵S]methionine/cysteine, and chased for different amounts of time as described previously (Seeger and Payne, 1992). Approximately 5 × 10⁶ cells were used for each time point. All experiments were performed at 30°C. A-ALP mutants were immunoprecipitated from cell lysates by using rabbit polyclonal antibodies against ALP (G. Payne). Radioactively labeled proteins were quantified from SDS-PAGE gels by using Typhoon 9400 Imager (GE Healthcare) and ImageQuant software (GE Healthcare). For calculation of the half-time of processing of A-ALP mutants, the log of the ratio of the precursor versus the mature form at each time point was plotted as a function of time, and the plots were analyzed by linear regression analysis. The precursor and mature forms of A(F→A)-ALP and

Figure 1. Simultaneous disruption of *ENT3* and *ENT5* suppresses the *chs6Δ* phenotype. (A) CF sensitivity of *chs6Δ* is restored to wild-type levels when *ENT3* and *ENT5* are mutated but not when either gene alone is mutated. (B) Staining of chitin with calcofluor.

A(Δ2–11, F→A)-ALP migrated on SDS-PAGE in a manner consistent with their predicted sizes.

RESULTS

Ent3p and Ent5p Are Involved in Intracellular Trafficking of Chs3p

We sought a test of the role of proteins that interact with AP-1 in the intracellular retention of the chitin synthase Chs3p. Ent5p was shown to interact with the γ subunit of AP-1 and to colocalize with internal clathrin puncta in yeast (Duncan *et al.*, 2003). Mutations in AP-1 subunits restore the transport of Chs3p to the cell surface in a *chs6Δ* background, in which the normal export route for Chs3p is blocked (Valdivia *et al.*, 2002). We asked whether *ent5Δ* also suppressed the *chs6Δ* phenotype by constructing an *ent5Δ chs6Δ* strain and testing its sensitivity to the chitin-binding drug calcofluor (Figure 1A). The amount of chitin in the yeast cell wall depends on the activity and localization of Chs3p. We found that *ent5Δ chs6Δ* cells were as resistant to calcofluor as *chs6Δ* cells, indicating that the transport of Chs3p to the cell surface is blocked and that unlike the AP-1 subunits, Ent5p is not required for the intracellular retention of Chs3p. Ent3p is related to Ent5p, and it also colocalizes with intracellular clathrin, but it does not interact with AP-1 *in vivo* (Duncan *et al.*, 2003). As expected, *ent3Δ* did not suppress the calcofluor resistance of *chs6Δ* cells. However, when mutations in *ENT3* and *ENT5* were combined with *chs6Δ*, the calcofluor sensitivity was restored to wild-type levels. Abundant chitin rings could be observed when *ent3Δ ent5Δ chs6Δ* cells were stained with calcofluor; these cells could not be distin-

guished from wild-type cells (Figure 1B). The results presented in Figure 1 were obtained with strains constructed in the Euroscarf background (Brachmann *et al.*, 1998), but they were reproduced with strains in the YPH499 background (Sikorski and Hieter, 1989; data not shown). These results suggest that transport of Chs3p to the cell surface is restored in *ent3Δ ent5Δ chs6Δ* cells and that both Ent3p and Ent5p may act in the AP-1 pathway but their functions may be redundant.

To directly assess the subcellular distribution of Chs3p in *ent3Δ ent5Δ chs6Δ* cells, we fractionated whole cell lysates on continuous sucrose gradients ranging from 20 to 60%. On these gradients, the heavier plasma membrane is efficiently separated from lighter internal membranes (Kaiser *et al.*, 2002). In addition to the plasma membrane marker Pma1p, we followed the distribution of the early Golgi-localized Anp1p, the endosomal target (t)-SNARE Tlg1p, and the vacuolar Vph1p, which all equilibrated at ~38% sucrose. Sec61p, which resides in the endoplasmic reticulum, was found in even lighter sucrose fractions. On the sucrose gradients prepared from wild-type cells, Chs3p had a bimodal distribution, with one peak colocalizing with the Golgi and endosomal markers, and the other peak colocalizing with Pma1p (Figure 2A). When *chs6Δ* cells were fractionated, all Chs3p was found in the lighter internal membranes, reflecting the block in transport of Chs3p to the plasma membrane in these cells. The distribution of Chs3p in the membranes from *ap-1Δ chs6Δ* cells, however, was indistinguishable from wild type (Figure 2B). Interestingly, whereas neither *ent3Δ* nor *ent5Δ* by itself significantly affected Chs3p distribution, fractionation of membranes from *ent3Δ ent5Δ* or *ent3Δ ent5Δ chs6Δ* strains yielded a Chs3p distribution profile that was different from the wild-type profile (Figure 2C). A larger fraction of the total Chs3p signal was found in the plasma membrane-containing peak, suggesting that the ratio of internal to cell surface-localized Chs3p was affected by the combined *ent3 ent5* mutations. In addition, there was a noticeable shift of the internal Chs3p peak to sucrose fractions of higher density. We speculate that this shift was due to a change in density of the late Golgi or endosomal membranes in *ent3Δ ent5Δ* cells, because the distribution of other proteins such as Tlg1p (Figure 2C), was similarly affected. We could not resolve the different late Golgi and endosomal compartments by sucrose gradient fractionation; thus, we were unable to determine whether the distribution of internal Chs3p was changed by the *ent3* and *ent5* mutations. Nonetheless, we conclude from these fractionation experiments that *ent3 ent5* mutations have a stronger effect on Chs3p trafficking than AP-1 mutations, possibly because Ent3p and Ent5p have an additional role in the intracellular retention of Chs3p.

To complement our data from fractionation experiments, we analyzed the localization of Chs3-GFP by fluorescence microscopy. As reported previously, in wild-type cells Chs3-GFP is observed in internal puncta and at the bud-neck of small-budded cells (Chuang and Schekman, 1996). The internal punctate localization is preserved in *ap-1Δ chs6Δ* cells, but cell surface-localized Chs3-GFP is not detected unless endocytosis is blocked (Valdivia *et al.*, 2002). In contrast, bud-neck-localized Chs3-GFP was observed in a number of *ent3Δ ent5Δ chs6Δ* cells, whereas the intracellular Chs3p seemed more dispersed (Figure 3). This analysis is in agreement with our fractionation results and supports the idea that together, Ent3p and Ent5p may have a broader role in Chs3p trafficking than AP-1.

Figure 2. Intracellular distribution of Chs3p in *ent3Δ ent5Δ* cells. (A) Wild-type cells were fractionated on continuous sucrose gradients as described in *Materials and Methods*. The distribution of Chs3p and of the marker proteins Pma1p (plasma membrane), Vph1p (vacuole), Anp1p (early Golgi), Tlg1p (TGN and endosomes), and Sec61p (ER) was assessed by immunoblot analysis. (B) The distribution of Chs3p on sucrose gradients from wild type, *chs6Δ*, and *chs6Δ apl2Δ*. Apl2p is the β -subunit of AP-1 in yeast. Insets show the distribution of Tlg1p and Pma1p in these strains. (C) The distribution profile of Chs3p in *ent3Δ ent5Δ* and *ent3Δ ent5Δ chs6Δ* cells is different from in wild-type cells, whereas its distribution profile in *ent5Δ chs6Δ* cells is the same as in *chs6Δ* cells. Insets show the distribution of Tlg1p and Pma1p in these strains.

Binding of AP-1 to Chs3p Does Not Depend on the Presence of Ent3p and Ent5p

To detect adaptor protein interactions with Chs3p, we chromosomally tagged the σ -subunit of AP-1, Aps1p, at the COOH terminus with a tandem affinity (TAP) tag, and purified it from yeast spheroplasts that had been incubated with the membrane-permeable cross-linking agent DSP. Tagged Aps1p is fully functional, because *chs6Δ APS1::TAP* cells are as resistant to calcofluor as *chs6Δ* cells (data not shown). We used a buffer containing salt and Triton X-100 for the purification of Aps1-TAP to extract both peripheral and integral membrane proteins from the lysed membranes. Intact AP-1 was efficiently purified by this method and could be detected in protein stains of the final samples (Figure 4A). In addition, we detected clathrin heavy chain in these samples, and the presence of clathrin light chain was confirmed by immunoblotting (Figure 4, A and B). The copurification of clathrin with Aps1p depended on the presence of cross-linker (data not shown). Interestingly, an appreciable amount of the monomer clathrin adaptor Gga2p also copurified with AP-1 (Figure 4B), confirming the obser-

Figure 3. Chs3-GFP localization in *ent3Δ ent5Δ* cells. Chs3-GFP can be visualized at the bud-neck (asterisk) in wild-type, *ent3Δ ent5Δ*, and *ent3Δ ent5Δ chs6Δ* cells but not in *apl2 chs6Δ* cells.

Figure 4. Mutations in *ENT3* and *ENT5* do not affect the binding of AP-1 to Chs3p, Gga2p or clathrin. The gene encoding the Aps1p subunit of AP-1 was tagged chromosomally at the C terminus in wild-type or *ent3Δ ent5Δ* cells. Cells were converted to spheroplasts and incubated with 5 mM cross-linker DSP before lysis. Aps1-TAP was purified by TAP on an IgG-Sepharose column followed by an S-protein agarose column. As a negative control, Aps1-TAP was purified from *apl4Δ* cells where the γ subunit is missing and the AP-1 complex cannot assemble. The same amount of cells (1500 OD₆₀₀ units) was used in each purification. DSP was then cleaved with a reducing agent, and 1/80,000 of each lysate (IgG-load) and one half of each final purified sample (S-elution) were loaded on an SDS-polyacrylamide gel. After electrophoresis, the gels were stained for total protein with Sypro red (A). The subunits of AP-1 are marked, and an asterisk marks the position of the band that corresponds to Chc1p. (B) The same samples were blotted with antibodies against Chs3p, Gga2p, and clathrin subunits Chc1p and Clc1p. An asterisk denotes residual Aps1-TAP that cross-reacts with the anti-Clc1p antibody. (C) Quantification of the protein bands in S-elution fractions. Aps1p and Apm1p were quantified from Sypro-stained gels, and the other proteins were quantified from immunoblots. The amount of a protein in each strain is expressed as a percentage of the signal in the wild-type strain that was analyzed at the same time. The values presented are the average values from three independent experiments.

vation that the yeast Gga2p interacts with AP-1 (Costaguta *et al.*, 2001). Finally, we showed by immunoblotting that Chs3p was present in the final samples (Figure 4B). The interaction between Chs3p and AP-1 was specific and reflected the association of Chs3p with AP-1 in vivo, because it depended on the addition of cross-linker and was significantly reduced in the control experiment where Aps1p was purified from *apl4Δ* cells where the AP-1 complex could not assemble (Figure 4, B and C).

We evaluated the role of Ent3p and Ent5p in the binding of AP-1 to Chs3p by using tagged Aps1p isolated from *ent3Δ ent5Δ* cells (Figure 4). No significant difference was observed when Aps1p was purified from *ent3Δ ent5Δ* compared with wild-type cells. Thus, the interaction between AP-1 and Chs3p does not require the presence of Ent3p and Ent5p, although they could mediate an event downstream of AP-1 binding to Chs3p.

Ent3p and Ent5p Both Bind to Clathrin and Cargo Proteins In Vivo

Both Ent3p and Ent5p bind to membranes in a phosphoinositide-specific manner, and Ent5p also binds to clathrin in vivo (Duncan *et al.*, 2003; Eugster *et al.*, 2004). In addition, Ent3p has been shown to interact with a vacuolar SNARE, Vti1p (Chidambaram *et al.*, 2004). These results suggest that Ent3p and Ent5p may act as bona fide clathrin cargo adaptors. To examine whether either protein might directly interact with Chs3p, we used TAP-tagged forms of Ent3p or Ent5p. Both tagged proteins are functional, as confirmed by calcofluor sensitivity assays and their correct localization in the cell (data not shown). Tagged Ent3p and Ent5p could be efficiently purified from yeast, but the amount of purified Ent3p was reduced compared with Ent5p, probably due to a four- to fivefold lower expression level of Ent3p (Figure 5A; data not shown). To identify all stable binding partners of Ent3p and Ent5p, we stained the final purified samples on an

SDS gel with Coomassie blue, excised the protein bands, and analyzed them by mass spectrometry. Interestingly, clathrin heavy chain (Chc1p) was identified in both purified samples. In addition, the heat-shock proteins Ssa1p and Ssa2p, common contaminants in protein purifications, were identified in our samples. Other Coomassie-stained bands were Ent3p in Ent3p purification, Ent5p in Ent5p purification, their degradation products, and IgG. The unidentified species of 95 kDa (marked with asterisks) was not detected in subsequent purifications.

By immunoblot analysis, the previously identified binding partners of Ent5p (Chc1p, Clc1p, AP-1 subunit Aps1p, and Gga2p; Duncan *et al.*, 2003) could be identified in the Ent5-TAP-purified fraction, and their presence depended on the addition of the cross-linker DSP before cell lysis (Figure 5B). Notably, Chs3p also copurified with Ent5p in cross-linker-dependent manner (Figure 5B). This result suggests that like AP-1, Ent5p may associate with Chs3p in vivo. The fraction of total Chs3p copurifying with either Aps1p or Ent5p was small but similar in both cases. Most notably, the interaction between Chs3p and Ent5p may be direct, because it was not interrupted by deletions of the known interactors of Ent5p: *apl4Δ*, *gga1Δ gga2Δ*, *ent3Δ* (Figure 5C). However, our attempts to confirm the interaction between Chs3p and Ent5p by an alternative method were hampered by the fact that Chs3p is a transmembrane protein with many membrane-spanning domains, and our attempts were thus unsuccessful (data not shown).

In contrast to the results obtained with Ent5p, no Chs3p was detected in the sample of purified Ent3-TAP by immunoblot. This was not due to less efficient cross-linking of Ent3p because clathrin and Gga2p copurified with Ent3p just as efficiently as with Ent5p (Figure 5D). Additionally, the late-endosomal t-SNARE Pep12p was very abundant in the purified Ent3p sample, indicating an efficient association of Ent3p with Pep12p in the membrane. We conclude that

Figure 5. Purification of Ent3p and Ent5p from wild-type and mutant cells. *ENT3* and *ENT5* were chromosomally tagged at their COOH termini with the S-Tev-ZZ tag. TAPs were performed as described in *Materials and Methods*. (A) Ent3p and Ent5p were purified from ACY134 or ACY133 cells (1200 OD₆₀₀ units), respectively. Cells were converted to spheroplasts and split into two aliquots for purifications with or without DSP. The final purified samples were incubated with reducing agent, and one third was loaded on an SDS-polyacrylamide gel. The gel was stained with colloidal Coomassie blue; protein bands were excised from the gel and analyzed by mass spectrometry. The proteins that were identified are noted in the image. (B) Ent5p was purified from spheroplasts prepared from wild-type cells (ACY133), which were incubated with 5 mM DSP or with dimethyl sulfoxide alone. An aliquot (1/60,000) of each lysate (IgG-Load) and one third of each final purified sample (S-elution) were analyzed by immunoblotting for the presence of the indicated proteins. (C) Ent5p was purified from wild-type, *apl4*Δ, *ent3*Δ, and *gga1*Δ *gga2*Δ cells (2000 OD₆₀₀ units) treated with 5 mM DSP. Samples were analyzed by immunoblotting. S-protein-HRP conjugate was used for the detection of purified Ent5p in 1/50 of each S-elution sample. (D) Ent3p was purified from wild-type, *ent5*Δ, and *gga1*Δ *gga2*Δ cells (3000 OD₆₀₀ units) treated with 5 mM DSP. The final purified samples (one half of each sample; S-elution) as well as 1/90,000 of each lysate (IgG-Load) were analyzed by immunoblotting with antibodies against Chc1p, Gga2p, Pep12p, and Clc1p. Ent3p in the "S-elution" samples was detected by Sypro stain.

both Ent3p and Ent5p bind to proteins in the membrane, but their binding specificities differ, and only Ent5p may associate with Chs3p.

Ent3p does not interact with clathrin *in vitro* (Duncan *et al.*, 2003), although it associates with clathrin *in vivo* (Friant *et al.*, 2003). We therefore tested whether a third protein, Ent5p or Gga1p/Gga2p, might facilitate the cross-linking between Ent3-TAP and clathrin. We purified Ent3p from *ent5*Δ or *gga1*Δ *gga2*Δ cells (Figure 5D). Under both conditions, Ent3p was cross-linked to clathrin as efficiently as in wild-type cells, suggesting that like Ent5p, Ent3p may directly bind to clathrin *in vivo*. However, Ent3p binds less efficiently to clathrin than Ent5p. Clathrin copurified with Ent5-TAP, but not with Ent3-TAP, even in the absence of cross-linker when a milder lysis buffer was used during the purification (data not shown). In contrast to clathrin, the amount of Pep12p that copurified with Ent3p from *gga1*Δ *gga2*Δ cells may have decreased (Figure 5D).

It was suggested that Ent3p and Ent5p also interact with Vps27p and are involved in the formation of multivesicular

bodies (Eugster *et al.*, 2004); however, by immunoblotting, we did not observe a significant amount of Vps27p copurifying with Ent3-TAP or Ent5-TAP. We also did not observe Ent3p and Ent5p associating with each other when we purified Ent3-TAP from cells expressing Ent5-monomeric red fluorescent protein (mRFP) and Ent5-TAP from cells expressing Ent3-mRFP (data not shown).

Our finding that Ent5p but not Ent3p binds to Chs3p presents an obvious question: if the functions of Ent3p and Ent5p in Chs3p trafficking are not redundant, why does *ent5*Δ by itself not suppress the *chs6*Δ trafficking defect? To address this discrepancy, we examined other possible differences in the functions of Ent3p and Ent5p.

Ent3p, but Not Ent5p, Facilitates the Function of Gga2p

Because Ent3p and Ent5p interact with Gga2p (Duncan *et al.*, 2003), we examined the role of these interactions in the context of Chs3p traffic. We crossed *ent3*Δ *chs6*Δ or *ent5*Δ *chs6*Δ cells with a *gga2*Δ strain and tested haploid mutants from these crosses for calcofluor sensitivity. We observed a

Figure 6. *ENT3* displays synthetic interaction with *GGA2*. (A) The CF resistance of *chs6Δ* cells is partially suppressed by the combined *ent3Δ gga2Δ* mutations, whereas *ent5Δ gga2Δ* has no effect. (B) Combined *gga1Δ gga2Δ* mutations also suppress *chs6Δ*.

genetic interaction between *ENT3* and *GGA2* but not between *ENT5* and *GGA2* (Figure 6A). The triple mutant *ent3Δ gga2Δ chs6Δ* displayed an increased sensitivity to calcofluor compared with the *chs6Δ* cells, suggesting that some Chs3p trafficked to the cell surface in this mutant. However, this was not the case in *ent5Δ gga2Δ chs6Δ* or in *gga2Δ chs6Δ* cells. A similar but less pronounced difference in calcofluor sensitivity was observed between *ent3Δ gga1Δ chs6Δ* and *ent5Δ gga1Δ chs6Δ* cells, whereas both *ent3Δ* and *ent5Δ* displayed some synthetic growth phenotype when combined with *gga1Δ gga2Δ*, and thus the calcofluor sensitivity of such mutants could not be determined (data not shown). In contrast to our previous findings (Valdivia *et al.*, 2002), *gga1Δ gga2Δ chs6Δ* triple mutants were also calcofluor sensitive (Figure 6B). The genetic interaction between *ENT3* and *GGA2* points to a role of Ent3p in Gga-mediated trafficking, and we suggest that the Gga proteins also contribute to the intracellular retention of Chs3p.

To further explore the possible role of Ent3p in Gga-mediated protein transport, we tagged and purified Gga2p from *ENT3* or *ent3Δ* yeast by using our established cross-linking and TAP protocol. As in AP-1, Ent3p, and Ent5p purifications, clathrin was detected in the purified Gga2p fraction (Figure 7, A and B). In some purifications, a weak Chs3p signal could also be detected in the sample of purified Gga2p; however, this result was not reproducible. Pep12p is transported from the TGN to the late endosome via the Gga pathway (Black and Pelham, 2000), and Pep12p copurified with Gga2p independently of the presence of AP-1. Interestingly, the amount of Pep12p copurified with Gga2p was substantially reduced (to background levels) when the purification was performed from *ent3Δ* cells, whereas the *ent5Δ* mutation had no effect (Figure 7, B and C). The absence of Ent3p also affected the binding of Gga2p to clathrin, albeit to a lesser extent than its binding to Pep12p. An additional decrease in the clathrin signal was observed when Gga2p was purified from *ent3Δ ent5Δ* cells, whereas deletion of the γ -subunit of AP-1 had no effect (Figure 7). Together with the results from Ent3p purifications, the Gga2p purifications suggest that Ent3p and Gga2p both function as clathrin adaptors in vivo and may cooperate in transporting some cargo to the late endosome.

We used fractionation on sucrose gradients to check whether the intracellular distribution of Pep12p was affected

in *ent3Δ* or in *ent5Δ* cells (Figure 7D). In accordance with our Gga2p purification results, the fractionation profile of Pep12p was changed when *ENT3* alone was deleted, whereas *ent5Δ* had no effect (Figure 7D). In *ent3Δ ent5Δ* cells, Pep12p was further shifted to fractions of higher sucrose density, which are enriched for plasma membrane, and its distribution profile resembled the distribution of Chs3p (Figures 2 and 7D). This result suggests that like in *gga1Δ gga2Δ* cells (Black and Pelham, 2000), some Pep12p is rerouted to the cell surface in the *ent3Δ ent5Δ* background.

Interplay between the AP-1, Ent3p/Ent5p, and Gga Pathways

We have shown that Ent3p, and to a lesser extent Ent5p, function in the Gga transport pathway, but they also direct the trafficking of Chs3p in a similar manner as AP-1. To better understand the relationship between these adaptors, we analyzed how mutations in them affect the processing of a model TGN protein, A(F→A)-ALP. A(F→A)-ALP consists of the transmembrane and the luminal domains of the vacuolar ALP, and the cytosolic domain of the TGN protein Ste13p containing a mutation in the aromatic amino acid motif FXFXD (Nothwehr *et al.*, 1993; Bryant and Stevens, 1997). Like Chs3p, A(F→A)-ALP requires AP-1 for its efficient retention at the TGN (Ha *et al.*, 2003; Foote and Nothwehr, 2006). In wild-type cells, A(F→A)-ALP is delivered to the vacuole, where it is proteolytically cleaved with a half-life of ~60 min (Nothwehr *et al.*, 1993; Foote and Nothwehr, 2006; Figure 8, A and B). The processing time is reduced to ~45 min in *ap-1* mutant cells (Ha *et al.*, 2003). In *ent3Δ ent5Δ* cells, the half-life of A(F→A)-ALP was even more significantly reduced, to ~27 min. Deletion of *ENT3* alone caused a small but reproducible reduction in the processing time, whereas *ent5Δ* had no statistically significant effect (Figure 8, A and B).

Deletion of amino acids 2–11 in A(F→A)-ALP mimics loss of AP-1 function, because this region of A(F→A)-ALP directly interacts with AP-1 (Foote and Nothwehr, 2006). In agreement with previous reports, mutation $\Delta 2-11$ increases the rate of delivery of A(F→A)-ALP to the vacuole in wild-type cells (Figure 8B). We analyzed the processing of A($\Delta 2-11$, F→A)-ALP in *ent3Δ/ent5Δ* mutants (Figure 8B). Deletion of *ENT3*, *ENT5*, or both *ENT3* and *ENT5* further increased the rate of processing of the A(F→A)-ALP mutant. Additive effects of *ent3Δ*, *ent5Δ*, and the AP-1-interacting motif mutation suggest that Ent3p and Ent5p function in a pathway parallel to the AP-1 pathway. Ent3p and Ent5p can partially but not fully substitute for each other.

Mutations in *Gga1p* and *Gga2p* also slightly increase the rate of processing of A(F→A)-ALP (Foote and Nothwehr, 2006), although this difference is less consistent (Ha *et al.*, 2003). We have shown that Ent3p has a role in Gga-mediated protein transport. We therefore asked whether the effect of *ent3Δ* on the processing of A(F→A)-ALP is due to a block in the Gga pathway by comparing the processing of A(F→A)-ALP in *ent3Δ*, *gga1Δ gga2Δ*, and *ent3Δ gga1Δ gga2Δ* cells (Figure 8C; note that the strains used in Figure 8C are different from the strains used in Figure 8, A and B, but the results are similar). Mutations in *GGA1* and *GGA2* increased the rate of A(F→A)-ALP processing at a comparable or slightly higher level than *ent3Δ*, and there was no added effect of *ent3Δ* in the *gga1Δ gga2Δ* background, suggesting that the effect of *ent3Δ* on the processing of A(F→A)-ALP may indeed be due to a role of Ent3p in the Gga pathway, independent of its role along with Ent5p in the AP-1-parallel pathway. In contrast to *ent3Δ* and *ent5Δ*, *gga1Δ gga2Δ* do not affect the processing of A($\Delta 2-11$, F→A)-ALP (Figure 8C),

Figure 7. The function of Gga2p depends on the presence of Ent3p. GGA2 was tagged chromosomally at the COOH terminus with the S-Tev-ZZ tag in wild-type, *ent3Δ*, *ent5Δ*, *ent3Δ ent5Δ*, *apl4Δ*, and *ent3Δ apl4Δ* cells. Gga2p was purified from each strain (1600 OD₆₀₀ units) by TAPs as described in *Materials and Methods*. The cross-linker DSP at 5 mM was used in all purifications. (A) Representative purified sample (S-eluate) from indicated strain backgrounds was analyzed by SDS-PAGE and stained for total protein with Sypro stain. In addition to Gga2p, clathrin heavy chain (Chc1p) could be detected in the samples. (B) The same samples as in A as well as the starting lysates (IgG-Load) were analyzed by immunoblotting with antibodies against clathrin light chain (Clc1p) and Pep12p. (C) Quantification of the amount of Pep12p and Clc1p that copurify with Gga2-TAP expressed in different strains. Protein bands from immunoblots were quantified and are expressed as a percentage of the amount purified from wild-type cells for each protein. IgG-Load represents the starting lysates, and S-elution is the final purified sample. The n value below each strain is the number of independent experiments that were quantified. Wild-type cells were analyzed in each experiment. (D) Distribution profile of Pep12p in WT, *ent3Δ*, *ent5Δ*, and *ent3Δ ent5Δ* cells. Total cell membranes were fractionated on continuous sucrose gradients as described in Figure 2. Fractions were probed for Pep12p by immunoblotting.

and in *ent3Δ gga1Δ gga2Δ* cells A(Δ2-11, F→A)-ALP was processed at the same rate as in *ent3Δ* cells (data not shown). This result suggests that the Ggas function downstream of AP-1 in the case of this cargo protein (Figure 8C and Figure 9).

DISCUSSION

The Role of Clathrin Adaptors in the Intracellular Retention of Chs3p

Chs3p is retained at the TGN/early endosome in an AP-1-dependent manner (Valdivia *et al.*, 2002). We now find that the monomeric clathrin-binding proteins Ent3p and Ent5p are also required to retain Chs3p in the TGN/endosome system, and we propose that they function in a pathway parallel to the AP-1 pathway. In addition, we show that the Gga proteins, which mediate protein transport from the

TGN to the late endosome, also contribute to the maintenance of the intracellular pool of Chs3p. We show that Ent3p, and to a lesser extent Ent5p, is required for the function of the Gga proteins. Thus, the effect of *ent3Δ ent5Δ* mutations on the intracellular distribution of Chs3p may represent combined effects of blocking its retention at the TGN and its transport to the late endosome.

Loss of Ent3p and Ent5p resulted in a pronounced accumulation of Chs3p at the cell surface (Figures 2 and 3). In a population of asynchronous *ent3Δ ent5Δ* cells, we found both by cell fractionation experiments and by microscopy that a larger fraction of total Chs3p may have been localized to the cell surface, compared with wild-type cells or *ap-1Δ* cells. Furthermore, the internal compartments populated by Chs3p in *ent3Δ ent5Δ* cells seemed different: smaller and more dispersed by microscopy and of a different buoyant

Figure 8. Mutations in *ENT3* and *ENT5* stimulate the transport of the TGN protein A(F→A)-ALP to the vacuole. (A and B) Strain RSY3341 (wild type), RSY3342 (*ent3Δ*), RSY3343 (*ent5Δ*), and RSY3344 (*ent3Δ ent5Δ*) were transformed with plasmids expressing A(F→A)-ALP (pSN100) or A(Δ2-11, F→A)-ALP (pSH46). Cells were metabolically labeled for 10 min and subjected to chase for the indicated time (in minutes) at 30°C. A-ALP mutants were immunoprecipitated from cell lysates and analyzed by SDS-PAGE. Processing of A(F→A)-ALP is shown in A, and precursor (p) and mature (m) form of A(F→A)-ALP are indicated. Half-times ($t_{1/2}$) of processing were indicated for each strain from the four time points and are plotted in B for A(F→A)-ALP (■), and for A(Δ2-11, F→A)-ALP (□). A(Δ2-11, F→A)-ALP does not interact with AP-1; therefore, its processing is equivalent to the processing of A(F→A)-ALP in *ap-1Δ* cells. The n value under each bar indicates the number of times that each experiment was repeated. (C) Processing of A(F→A)-ALP (■), and of A(Δ2-11, F→A)-ALP (□) in strains ACY193 (wild type), ACY194 (*ent3Δ*), ACY195 (*gga1Δ gga2Δ*), and ACY196 (*ent3Δ gga1Δ gga2Δ*), was analyzed as shown in A, and the $t_{1/2}$ values of processing were calculated. Each experiment was repeated two times.

density on sucrose gradients. Unlike *ap-1* mutant cells (Yeung and Payne, 2001), *ent3Δ ent5Δ* cells display defects in trafficking of proteins to the late endosome and the vacuole. However, endocytosis is not affected in *ent3Δ ent5Δ* mutants (Duncan *et al.*, 2003). The apparent retention of Chs3p at the cell surface may be due to its increased recycling from the early endosome or the TGN back to the cell surface.

Ent3p and Ent5p Have the Properties of Clathrin Adaptors

Clathrin adaptors mediate the interaction among clathrin, cargo proteins, and the lipid membrane. Ent3p and Ent5p contain phosphoinositide binding domains that can associate with the lipid membrane. Ent5p, but not Ent3p, binds to clathrin *in vitro*, probably via a type I clathrin binding box (Duncan *et al.*, 2003). *In vivo*, however, Ent3p can also associate with clathrin (Friant *et al.*, 2003; this study). This interaction is probably direct, because it is not affected by deletion of the other binding partners of Ent3p (Figure 5), but it may be weaker than the interaction between Ent5p and clathrin. A DLL clathrin binding motif was identified in the primary sequence of Ent3p (Legendre-Guillemain *et al.*, 2004).

Ent3p but not Ent5p interacts with a vacuolar SNARE Vti1p (Chidambaram *et al.*, 2004). We find that Ent5p may also bind to cargo proteins, because it associates with Chs3p and this interaction is independent of other known interacting partners of Ent5p. In addition, the endosomal t-SNARE Pep12p copurified with Ent3p and to a lesser extent with

Figure 9. Model of how clathrin-dependent pathways could contribute to the intracellular retention of Chs3p and A(F→A)-ALP. AP-1, Ent5p, and Ent3p function in parallel to retain Chs3p and A(F→A)-ALP at a compartment that may represent the TGN or the early endosome. Separate arrows indicated independent functions of these proteins and are not meant to represent different transport routes. Ent3p and Ent5p can partially substitute for each other, which is indicated by dashed arrows. In addition, Ent3p functions in the Gga pathway, which in A(F→A)-ALP operates downstream of the AP-1 pathway. This may still be the same compartment, which has been reorganized by AP-1, Ent3p, and Ent5p.

Ent5p (Figure 5). Thus, both Ent3p and Ent5p possess all the characteristics of clathrin adaptors. However, Ent3p and Ent5p also associate with other clathrin adaptors—Gga2p and AP-1 (Duncan *et al.*, 2003; Figure 5). In addition, AP-1 and Gga2p also interact (Costaguta *et al.*, 2001; Figure 4). We therefore asked whether these adaptors function independently or in cooperation with each other during clathrin-mediated protein sorting.

Ent3p Has an Important Role in the Gga Transport Pathway

In addition to the cargo binding differences noted above, *ENT3* displayed a genetic interaction with *GGA2*, whereas *ENT5* did not (Figure 6), and the loss of Ent3p but not of Ent5p affected the Gga2p cross-linking pattern. Most importantly, the interaction between Gga2p and Pep12p, an endosomal t-SNARE that must be transported from the TGN to function at the late endosome (Becherer *et al.*, 1996), was lost in an *ent3Δ* background (Figure 7). Pep12p is rerouted to the cell surface when its transport from the TGN to the late endosome is blocked by mutations in *GGA1* and *GGA2* (Black and Pelham, 2000). We also observed a change in the intracellular localization of Pep12p in *ent3Δ* but not in *ent5Δ* cells (Figure 7D), and mutations in *GGA1* and *GGA2* had an effect similar to *ent3Δ* (data not shown). The mislocalization of Pep12p was even more pronounced in *ent3Δ ent5Δ* cells, but this may be due to defects in multiple pathways (Figure 7D). These data implicate Ent3p in Gga1p/Gga2p-mediated cargo sorting. Ent3p and Gga2p colocalize in the cells to a larger extent than do Ent5p and Gga2p (Costaguta *et al.*, 2006). Conversely, the interaction between Ent3p and Pep12p may also be reduced in a *gga1Δ gga2Δ* background; thus, we suggest that Ent3p cooperates with Gga1p and Gga2p in sorting of at least a subset of cargo in the TGN-to-late endosome trafficking pathway.

In mammalian cells, two domains of the Gga proteins, the GAT domain and the VHS domain, have been shown to bind directly to signals in the cargo proteins, monoubiquitin and DXXLL, respectively; however, only the former interaction has been demonstrated in yeast (Bonifacino, 2004; Scott *et al.*, 2004). The primary sequence of the yeast VHS domain differs somewhat from the mammalian sequence and may not be able to accommodate the VHS consensus signal peptide (Misra *et al.*, 2002). An unrelated signal, FSDSPEF, in the cytosolic tail of Pep12p is important for the sorting of Pep12p to the late endosome, but a direct interaction between this signal and the Ggas has not been demonstrated (Black and Pelham, 2000). Perhaps this signal binds to Ent3p instead.

Interestingly, we also found that Ent3p, and to a lesser extent Ent5p, facilitated the binding of Gga2p to clathrin. A lower level of clathrin copurified with Gga2p from *ent3Δ* cells (Figure 7). Even less clathrin associated with Gga2p purified from *ent3Δ ent5Δ* cells, whereas the *ent5Δ* mutation by itself had no effect. However, we showed that the binding of AP-1 to clathrin was not affected by *ent3Δ ent5Δ* (Figure 4); thus, the decreased interaction between Gga2p and clathrin in these cells is probably not due to a general mislocalization of clathrin. We propose that an additional role of Ent3p in the Gga trafficking pathway is to mediate the interaction between the Gga proteins and clathrin but that Ent5p can partially substitute for this function of Ent3p. The Ggas possess two clathrin-binding motifs that when mutated decrease the amount of clathrin binding in vitro. A residual interaction with clathrin depends on the GAE domain of Gga2p or Gga1p (Mullins and Bonifacino, 2001), which corresponds to the site bound by Ent3p and Ent5p.

Nonetheless, the clathrin-binding function of Ent3p and Ent5p is probably not essential for the function of yeast Ggas, because processing of CPY and α -factor are minimally affected by deletion of the GAE domain within Gga2p (Mullins and Bonifacino, 2001).

The Relationship among AP-1, Ent3p, and Ent5p Function

In contrast to Gga2p, the absence of Ent3p and Ent5p had no effect on the interaction of AP-1 with its cargo or clathrin (Figure 4), suggesting that they may not be required for the function of AP-1. We studied the effect of *ent3Δ ent5Δ* mutations on the trafficking of another model TGN protein, A(F→A)-ALP (Nothwehr *et al.*, 1993). A(F→A)-ALP is slowly transported from the TGN to the late endosome and the vacuole, probably via the early endosome (Foote and Nothwehr, 2006). Mutations in AP-1 increase the rate of transport of A(F→A)-ALP to the vacuole (Ha *et al.*, 2003; Foote and Nothwehr, 2006), and we now show that *ent3Δ ent5Δ* has a similar but more pronounced effect on trafficking of this chimeric protein (Figure 8). A similar acceleration in A(F→A)-ALP processing was observed when a lipid-re modeling enzyme, the polyinositide phosphatase Inp53p, was mutated (Ha *et al.*, 2001). Interestingly, the effects of mutations in the AP-1-interacting motif of A(F→A)-ALP and in *ENT3* and *ENT5* are additive, suggesting that these proteins function in parallel at the same intracellular compartment (Figure 9). Independent parallel functions of these proteins are also suggested by the fact that *apl2Δ ent3Δ ent5Δ* cells have a severe growth phenotype (Costaguta *et al.*, 2006; our unpublished data), whereas *ent3Δ ent5Δ* cells do not.

Ent5p and AP-1, but not Ent3p, interact with Chs3p to a similar extent. However, mutations in AP-1 suppress the *chs6Δ*-dependent block in Chs3p export, whereas *ent5Δ* does not. It is possible that Ent3p can to some extent substitute for the loss of Ent5p. Likewise, the effect of *ent5Δ*, and to a lesser extent *ent3Δ*, on the processing of A(F→A)-ALP is only revealed when both mutations are present or when the interaction with AP-1 is also abrogated (Figure 8B), suggesting that there is some cooperativity among AP-1, Ent3p, and Ent5p. Similarly, *ent3Δ* or *ent5Δ* affect the processing of α -factor only in an *ap-1Δ* background (Costaguta *et al.*, 2006).

We attribute the small increase in the rate of processing of A(F→A)-ALP in *ent3Δ* cells to the role of Ent3p in the Gga pathway, because a similar increase was observed in *gga1Δ gga2Δ* (or in *gga1Δ gga2Δ ent3Δ*) cells (Foote and Nothwehr, 2006; Figure 8C). However, the *gga* mutations do not affect the processing of A(Δ 2-11, F→A)-ALP, a mutant protein that cannot bind to AP-1, suggesting that the Gga proteins function downstream of AP-1 in the processing of A(F→A)-ALP (Figure 9). In contrast, it was shown that in mammalian cells AP-1 acts downstream of the Ggas in the transport of mannose phosphate receptor to the lysosome (Doray *et al.*, 2002).

It is possible that this discrepancy reflects differences between yeast and mammalian cells; the localization of AP-1 is also affected by the loss of Gga function in mammalian but not in yeast cells (Ghosh *et al.*, 2003; Costaguta *et al.*, 2006). Alternatively, it may reflect differences in how the clathrin adaptors sort the different cargo proteins (see below).

In contrast to the TGN resident protein A(F→A)-ALP, whose vacuolar processing is accelerated in *ent3Δ ent5Δ* or in *gga1Δ gga2Δ* mutants, delivery of the vacuolar protein Cps1p from the TGN to the vacuole is delayed in these same mutants (Costaguta *et al.*, 2001; Duncan *et al.*, 2003). Cps1p trafficking, however, is not affected by mutations in AP-1 alone. Pep12p is another example of an AP-1-independent Gga cargo protein. Thus, the role of the Gga proteins, along with Ent3p, may be cargo-specific, and they may function

in more than one pathway in yeast. In mammalian cells, the GGAs distribute more broadly than the TGN markers; some GGA protein is also detected at the early endosome (Puertollano *et al.*, 2003). Furthermore, Ggas localize to an exaggerated endosomal compartment that is present in some (class E) *vps* mutants in yeast (Hirst *et al.*, 2001; Boman *et al.*, 2002), suggesting that their function is not limited to the TGN.

Our analysis of the *in vivo* physical interactions between the different internal clathrin adaptor proteins in yeast points to a more complex network of trafficking pathways than was previously envisioned, and it is in good agreement with a recent report by Costaguta *et al.* (2006). In mammalian cells, GGA and AP-1 colocalized on coated buds and vesicles in the area of the TGN, and extended tubules containing GGA1, AP-1, and epsinR have also been observed (Doray *et al.*, 2002; Hirst *et al.*, 2003; Puertollano *et al.*, 2003). Likewise, there is a considerable colocalization of AP-1, Gga2p, Ent3p, and Ent5p in yeast (Costaguta *et al.*, 2006). These adaptors may therefore cooperate to recruit cargo into different types of clathrin vesicles or tubular carriers at the TGN or at the early endosome, but it is also possible that they retain some proteins (such as Chs3p and A(F→A)-ALP) at the TGN by binding to them and thus excluding them from a transport carrier. A better understanding of the nature of the TGN and the endosomal compartments in yeast is required to distinguish between these possibilities.

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