

A Family of Proteins with γ -Adaptin and VHS Domains that Facilitate Trafficking between the Trans-Golgi Network and the Vacuole/Lysosome[○]

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Abstract. We have cloned and characterized members of a novel family of proteins, the GGAs. These proteins contain an NH₂-terminal VHS domain, one or two coiled-coil domains, and a COOH-terminal domain homologous to the COOH-terminal “ear” domain of γ -adaptin. However, unlike γ -adaptin, the GGAs are not associated with clathrin-coated vesicles or with any of the components of the AP-1 complex. GGA1 and GGA2 are also not associated with each other, although they colocalize on perinuclear membranes. Immunogold EM shows that these membranes correspond to trans elements of the Golgi stack and the TGN. GST pulldown experiments indicate that the GGA COOH-

terminal domains bind to a subset of the proteins that bind to the γ -adaptin COOH-terminal domain. In yeast there are two *GGA* genes. Deleting both of these genes results in missorting of the vacuolar enzyme carboxypeptidase Y, and the cells also have a defective vacuolar morphology phenotype. These results indicate that the function of the GGAs is to facilitate the trafficking of proteins between the TGN and the vacuole, or its mammalian equivalent, the lysosome.

Key words: GGA • AP-1 • vesicle coat • membrane traffic • protein sorting

Introduction

The major components of clathrin-coated vesicles are clathrin and adaptor or AP complexes. The clathrin provides the scaffold that deforms the membrane into a vesicle, while the adaptor complexes select the vesicle cargo and also recruit accessory proteins to the site of vesicle formation. There are two adaptor complexes associated with clathrin: AP-1, which is found at the TGN, and AP-2, which is found at the plasma membrane. More recently, two additional AP complexes have been described, AP-3 (Simpson et al., 1996; Dell’Angelica et al., 1997) and AP-4 (Dell’Angelica et al., 1999; Hirst et al., 1999). All four AP complexes are heterotetramers: AP-1 contains the subunits γ , β 1, μ 1, and σ 1; AP-2 contains α , β 2, μ 2, and σ 2; AP-3 contains δ , β 3, μ 3, and σ 3; and AP-4 contains ϵ , β 4, μ 4, and σ 4. The four subunits in the four complexes show homology to their counterparts in the other three com-

plexes, but in the case of the γ , α , δ , and ϵ subunits, the homology is restricted to the first 600 amino acids. This conserved NH₂-terminal domain is followed by a hinge domain of 100–200 amino acids, and then by a completely divergent COOH-terminal appendage or “ear” domain of 100–300 amino acids (Hirst and Robinson, 1998). It is this ear domain that recruits accessory proteins from the cytosol onto the membrane where they facilitate coated vesicle formation. Most of the information about accessory proteins has come from studies on AP-2, where several proteins have been shown either to bind directly to the ear domain of α -adaptin or to interact with one of the ear domain binding partners (Marsh and McMahon, 1999). The functions of some of these proteins are beginning to be elucidated, and there is evidence that the proteins may act at different stages of the coated vesicle cycle to drive the process forward (Simpson et al., 1999).

Although less is known about the requirements for clathrin-coated vesicle formation at the TGN, it is likely that accessory proteins are also recruited onto the membrane by AP-1. To date, only one such protein has been identified, γ -synergin, which binds directly to the ear do-

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main of γ -adaptin (Page et al., 1999). Like Eps15, γ -synergisin is an EH¹ (for Eps15 homology) domain-containing protein, and by analogy to Eps15 and other EH domain-containing proteins, the EH domain of γ -synergisin probably binds to an as yet unidentified additional accessory protein(s) containing the tripeptide NPF (Salcini et al., 1997). Given the similarities in subunit composition and function between the four AP complexes, it seems likely that for AP-3 and AP-4 there also exists a set of accessory proteins.

While we were searching through the EST database for homologues of adaptor subunits, we found several sequences that showed significant homology to the ear domain of γ -adaptin. This homology was restricted to the last 120–130 amino acids, which includes the γ -synergisin binding site. The NH₂-terminal regions of the novel sequences contain a motif called a VHS domain (Lohi and Lehti, 1998), which has been found in a number of proteins, some of which have been implicated in membrane traffic. The first three VHS domain-containing proteins to be identified were Vps27p, Hrs, and STAM. Vps27p is a yeast protein required for vacuolar protein sorting (Piper et al., 1995). Hrs is a FVYE zinc finger-containing protein, originally identified as a hepatocyte growth factor-regulated tyrosine kinase substrate, which is associated with transferin receptor-containing endosomes (Komada et al., 1997). STAM was identified as a signal transducing adaptor molecule. It is phosphorylated in response to interleukin-2 and it is a potential binding partner for Hrs (Asao et al., 1997). Another VHS domain-containing protein, EAST, was identified as an epidermal growth factor receptor-associated protein with SH3 and TAM domains. It is a substrate for the EGF receptor and it also binds to Eps15 (Lohi et al., 1998). The function of the VHS domain is still unknown.

We have cloned and characterized three novel mammalian proteins that contain both a γ -adaptin ear homology domain and a VHS domain. While this manuscript was in preparation, we found out that Boman et al. (2000) had independently cloned these same proteins in a two-hybrid library screen for ARF-binding proteins; and after our manuscript had been submitted, we discovered that Dell'Angelica et al. (2000) had also independently cloned these proteins. Our laboratories have agreed to call the proteins GGAs (pronounced Gigas), for Golgi-localized, γ ear-containing, ARF-binding proteins. In addition to the three mammalian GGAs, we have also identified two members of this family in the budding yeast *S. cerevisiae*. Like the mammalian GGAs, the yeast proteins also contain a γ -adaptin ear homology domain and a VHS domain. Deletion of the two *GGA* genes in yeast has provided further insights into the function of the *GGA* protein family.

¹Abbreviations used in this paper: CPY, carboxypeptidase Y; EAST, epidermal growth factor receptor-associated protein with SH3 and TAM domains; EH, Eps15 homology; GGAs, Golgi-localized, γ ear-containing, ARF-binding proteins; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; MALDI, matrix assisted laser desorption ionization; NRK, normal rat kidney; STAM, signal transducing adaptor molecule; VHS domains, domains containing proteins Vps27p, Hrs, and STAM.

Materials and Methods

Cloning and Sequencing

Several sequences within the EST database were identified that had significant homology to the COOH-terminal ear domain of γ -adaptin. Clones were obtained from the IMAGE Consortium, sequenced, and expressed as recombinant fusion proteins for the production of antibodies. Most molecular biology techniques were carried out as described by Sambrook et al. (1989). Two human ESTs encoding GGA1 were identified (IMAGE Consortium Clone ID 127720 and 129926), but both were found to be missing the 5' end. Thus, a human heart cDNA library (Clontech) was screened with a PCR fragment from one of the clones, and the library clone was ligated to EST127720 through a unique SmaI site to obtain a clone encoding the full-length protein. GGA2 was identified as a human retina EST (IMAGE Consortium Clone ID 363745). Sequencing indicated that this clone contained the full open reading frame. GGA3 was identified as a human testis EST (IMAGE Consortium Clone ID 728294), which encodes the COOH-terminal end of the protein. The full-length sequence of GGA3 is now available in the nonredundant database (GenBank/EMBL/DBJ accession number D63876; Nagase et al., 1995). Sequencing of all clones was carried out by John Lester (University of Cambridge, UK) on an automated ABI sequencer. The entire coding sequence was read in both directions.

Antibody Production

To construct glutathione S-transferase (GST) fusion proteins of GGA1 and GGA2, amino acids 289–508 and 567–639 of GGA1, and 305–482 of GGA2, were amplified by PCR and ligated into pGEX-4T-1 (Pharmacia Biotech), and expression of the fusion protein was induced in MC1061 cells. The GGA1 fusion proteins were both partially soluble and were purified using glutathione-Sepharose affinity chromatography (Pharmacia Biotech). The GGA2 fusion protein was found to be insoluble and was purified from inclusion body preparations as previously described (Page and Robinson, 1995). In each case the antigens were injected into pairs of rabbits. The immunization protocol and affinity purification of the resulting antisera were performed as described by Page and Robinson (1995). Because of the possibility that the antiserum raised against the COOH-terminal portion of GGA1 might cross-react with related sequences in other proteins, after affinity purification this antiserum was adsorbed with fusion proteins made from the COOH-terminal domains of GGA2 (prepared as above and containing amino acids 454–613) and of γ -adaptin (described by Seaman et al., 1996).

Expression of Epitope-tagged GGA2

An epitope-tagged version of full-length GGA2 was constructed by the insertion of the 8 amino acid (DYKDDDDK) FLAG tag at the COOH terminus and ligation into the vector pSTAR, which contains a tetracycline-inducible promoter. Transfection of normal rat kidney (NRK) fibroblasts was performed using Fugene reagent (Life Technologies Inc.), and stably transfected cells were selected with G418 (Boehringer Mannheim Corp.). Expression of the epitope-tagged GGA2 was induced by the overnight addition of 10 mM doxycycline to the culture medium. By immunofluorescence, >90% of transfected NRK cells stained positive with M5, an mAb against the FLAG epitope (Sigma Chemical Co.).

Immunoprecipitations and Western Blotting

Immunoprecipitations were carried out on HeLa cell extracts under non-denaturing conditions, as previously described (Hirst et al., 1999; Page et al., 1999). Clathrin-coated vesicles were purified from rat liver as described by Manfredi and Bazari (1987). For gel filtration, pig brain cytosol was prepared by homogenizing 1 g pieces of pig brain in 2 ml cytosol buffer (25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM dithiothreitol and 1 mg/ml glucose) and fractionated on a Superose 6 100-ml column as described by Stamnes and Rothman (1993). The column was calibrated with β -amylase (200 kD), albumin (66.2 kD), and carbonic anhydrase (29 kD; Sigma Chemical Co.). Samples were subjected to SDS-PAGE and Western blots probed with various antibodies, followed by ¹²⁵I-protein A as previously described (Hirst et al., 1999).

Immunofluorescence and Immunoelectron Microscopy

NRK cells, either nontransfected or stably expressing FLAG-tagged human GGA2, were fixed with 3% paraformaldehyde, followed by 0.1% saponin as previously described (Seaman et al., 1993). For some experiments, the cells were treated with 100 μ g/ml brefeldin A (BFA; Sigma Chemical Co.) for 2 min before fixation. The cells were then labeled with either mouse anti-FLAG or rabbit anti-GGA1, either alone, together with each other, or together with other antibodies, including rabbit anti- γ -adaptin (Seaman et al., 1996), rabbit anti- γ -synergin (Page et al., 1999), and mouse anti-TGN38 (2F7.1, a gift from George Banting, University of Bristol, Bristol, UK). The secondary antibodies used were fluorescein-conjugated goat anti-rabbit IgG and Texas red-conjugated goat anti-mouse IgG (Molecular Probes).

For immunoelectron microscopy, NRK cells were fixed either intact or after permeabilization by immersion in liquid N₂ and incubation with pig brain cytosol plus ATP, an ATP regenerating system, and GTP γ S, as previously described (Seaman et al., 1993; Simpson et al., 1996). Frozen ultrathin sections were labeled with rabbit anti-GGA1, followed by protein A-gold and observed in a Philips CM100 transmission electron microscope.

GST Pulldown Experiments

For GST pulldown experiments, three GST fusion proteins were constructed as described above. GST-GGA1 contains amino acids 468–639 of human GGA1, GST-GGA2 contains amino acids 454–613 of human GGA2, and GST- γ contains amino acids 706–823 of mouse γ -adaptin (Seaman et al., 1996). All of the constructs were soluble and were prepared as previously described (Page and Robinson, 1995). Pulldowns were carried out essentially as described by Page et al. (1999), using pig brain cytosol prepared in PBS containing 0.1% NP-40 and a protease inhibitor cocktail (Complete Mini), at a protein concentration of 1.5 mg/ml. Bound proteins were eluted with SDS-PAGE sample buffer and subjected to SDS-PAGE. Gels were either stained with Coomassie blue for matrix assisted laser desorption ionization (MALDI) mass spectrometry or transferred to nitrocellulose for Western blotting. Further details are available at <http://www.jcb.org/cgi/content/full/149/1/67/DC1> as supplemental information.

Yeast Knockout and Rescue Experiments

The GGA-deficient strains were constructed in the YPH500 strain (Sikor-ski and Hieter, 1989) using a PCR-based method. Primers were designed to flank the open reading frame of GGA1 or GGA2 with 30 complementary base pairs, in addition to 25 base pairs of sequence complementary to a selectable marker. The resulting PCR products contained URA3 or HIS3 flanked by 30 base pairs of either GGA1 or GGA2, respectively. The PCR products were transformed into either the wild-type haploid yeast strain, YPH500, or JH2 (see Table I). Transformants were selected by growth on –ura or –his plates. Deletion of the gene was confirmed by PCR on genomic DNA prepared from the transformants.

For rescue experiments, wild-type GGA1 and GGA2 were cloned by PCR from genomic DNA prepared from YPH500. BamHI and PstI restriction sites in the primers allowed for rapid cloning into the CEN vectors pRS414 (for GGA1) and pRS415 (for GGA2), allowing the two genes to be expressed under their own promoters at approximately endogenous levels. These constructs were transformed into the JYY3 *gga1 Δ /gga2 Δ* strain, as well as control strains, and the transformants selected on –trp or –leu plates. Rescue of the JHY3 strain by a mammalian GGA was also tested, using mammalian GGA2 expressed via the *VPS5* promoter. Primers were designed to amplify the full open reading frame of human GGA2, incorporating the restriction sites NcoI and XhoI. The resulting PCR product was cloned into *VPS5*-424, replacing the *VPS5* coding region with the mammalian cDNA. This construct was transformed into the

Table I. Strains Used

Strain	Genotype	Source
YPH500	<i>MATα ura3-52 leu2 his3-Δ200 lys2 ade2 trp1</i>	R. Duden
JHY1	<i>YPH500 gga1Δ::URA3</i>	This study
JHY2	<i>YPH500 gga2Δ::HIS3</i>	This study
JHY3	<i>JHY2 gga1Δ::URA3</i>	This study
EMY18	<i>SEY6210 vps35Δ::HIS3</i>	M. Seaman

gga1 Δ /gga2 Δ strain, as well as control strains, and the transformants were selected on –trp plates.

The single and double knockout strains were first tested for their ability to sort and process carboxypeptidase Y (CPY), using the CPY sorting assay described by Seaman et al. (1997). To investigate the distribution of Vps10p in the different strains, yeast cells were transformed with the multi-myc tagged Vps10p construct (Cereghino et al., 1995), fixed, spheroplasted, and labeled with a mouse monoclonal anti-myc antibody (Sigma Chemical Co.), followed by Texas red-conjugated goat anti-mouse IgG (Molecular Probes). FM4-64 (Molecular Probes) uptake experiments were performed as described by Vida and Emr (1995).

Online Supplemental Information

The online version of this article includes text and figures that accompany the information presented here and is available at <http://www.jcb.org/cgi/content/full/149/1/67/DC1>.

Results

Identification of a Novel Family of Proteins

In a search for novel proteins related to AP components, sequences were identified within the nonredundant and EST databases that showed significant homology to the COOH-terminal ear domain of γ -adaptin, although their NH₂-terminal sequences showed no homology to any of the AP subunits. However, the NH₂ termini of these proteins showed homology to several other proteins, all of which contain a recently described motif known as a VHS domain (Lohi and Lehti, 1998), and a Pfam search revealed that the γ -adaptin-related proteins also contain VHS domains. There are at least three such proteins in humans, encoded by different genes: GGA1, GGA2, and GGA3. A full-length sequence of GGA3 is present in the nonredundant database (GenBank/EMBL/DBJ accession number D63876; Nagase et al., 1995), while we obtained full-length sequences of GGA1 and GGA2 from ESTs and library clones. Fig. 1 a shows the sequences and alignments of GGA1, GGA2, and GGA3. Their predicted sizes are 70, 67, and 74 kD, respectively. GGA1 and GGA2 are 45% identical, while GGA3 is 45% identical to GGA1 and 35% identical to GGA2. Fig. 1 b shows an alignment of the VHS domain of human GGA1 with the VHS domains of Hrs, Vps27p, and STAM, while Fig. 1 c shows an alignment of the COOH-terminal region of GGA1 with the ear domains of two mammalian γ -adaptin isoforms, γ 1 and γ 2, and the putative γ -adaptin in *S. cerevisiae*, Apl4p. Similar alignments were obtained with GGA2 and GGA3 (data not shown). GGA1 can be seen to have a typical VHS domain, and the homology with the γ -adaptin COOH terminus extends over the entire ear domain.

In addition to the VHS domain at the NH₂ terminus and the γ -adaptin ear homology domain at the COOH terminus, all three proteins contain either one or two predicted coiled coil domains downstream from the VHS domain, followed by a variable domain, where the proteins show little or no homology with each other. However, the amino acid content of the variable domains is similar to that of the adaptin hinge domains, containing a high proportion of hydrophilic amino acids, prolines, and alanines. This suggests that the variable domain may function as a flexible stalk or hinge, connecting the conserved NH₂-terminal and COOH-terminal domains to each other, in the same

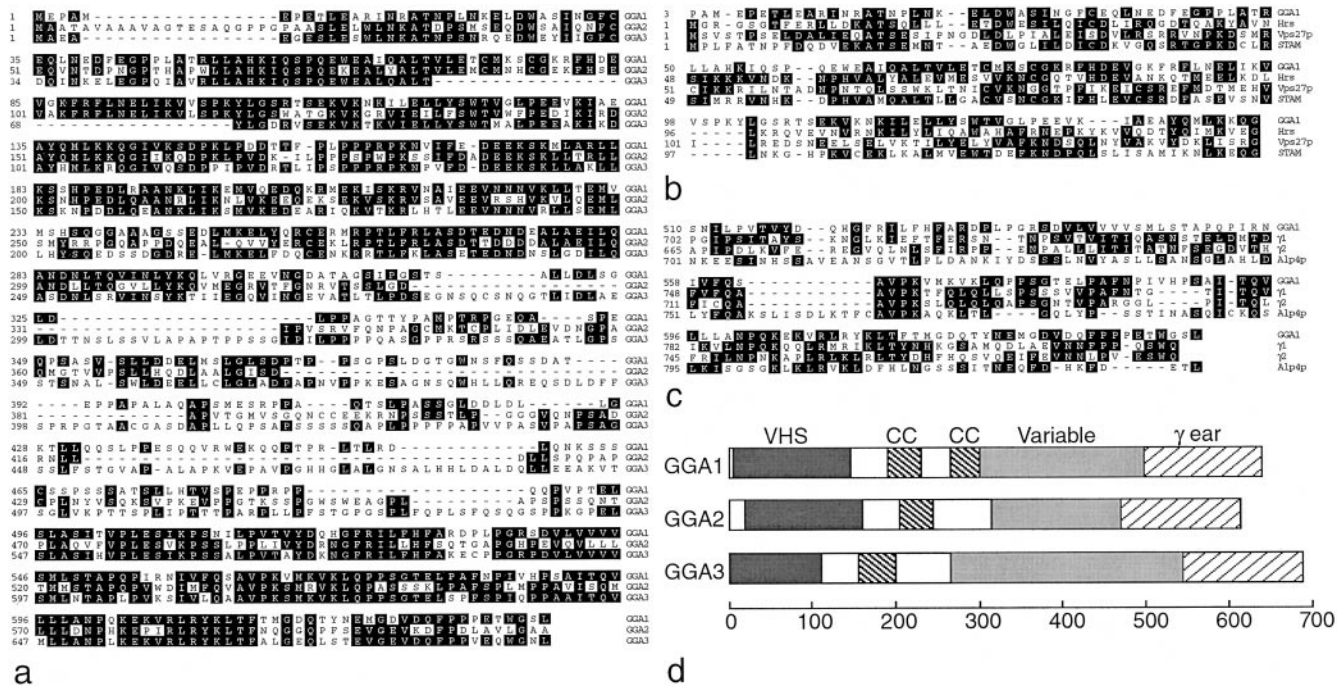


Figure 1. Sequences, alignments, and domain organization of human GGAs. **a**, The complete protein sequences of the three mammalian GGAs. GGA1 and GGA2 are 45% identical to each other, and 45 and 35% identical, respectively, to GGA3. These sequence data are available from GenBank/EMBL/DDBJ under accession numbers AF233521 (GGA1), AF233522 (GGA2), and D63876 (GGA3; the GGA3 sequence was entered into the database by Nagase et al., 1995). **b**, Comparison of the VHS domain of GGA1 with the VHS domains of Hrs, Vps27p, and STAM. GGA1 can be seen to have a typical VHS domain. **c**, Comparison of the COOH terminus of GGA1 with the COOH termini of the two mammalian γ -adaptin isoforms, $\gamma 1$ and $\gamma 2$, and the putative γ -adaptin homologue in *S. cerevisiae*, Alp4p. All four proteins can be seen to have related COOH-terminal ear domains. **d**, Schematic diagram of GGA1, GGA2, and GGA3, showing the positions of the VHS domain, the coiled coil domains (CC), the central variable region, and the γ -adaptin ear homology domain.

way that the adaptin ears are connected by hinges to the core or "head" of the AP complex. The three GGAs are shown diagrammatically in Fig. 1 d.

Biochemical Characterization of GGA1 and GGA2

To characterize GGA1 and GGA2 further, we raised polyclonal antibodies against fragments of the proteins expressed as GST fusion proteins. On Western blots of whole HeLa cell extracts, the antibodies against GGA1 recognized a band with an apparent molecular weight of 85 kD, whereas the GGA2-specific antibodies recognized a band with an apparent molecular weight of 67 kD; thus, GGA1 runs slightly more slowly than predicted from its amino acid content, while GGA2 runs in the expected position (Fig. 2 a).

Because AP-1 is associated with clathrin-coated vesicles, we next investigated whether GGA1 and GGA2 are also associated with clathrin-coated vesicles. For this experiment, equal protein loadings of clathrin-coated vesicles purified from rat liver and a crude microsomal membrane fraction from an earlier stage in the preparation were subjected to SDS-PAGE, and Western blots were probed with antibodies specific for the AP-1 subunits, γ -adaptin and $\mu 1$; for the AP-2 subunit $\mu 2$; for the AP-4 subunit ϵ ; and for the AP-1 accessory protein γ -synergin; as well as for GGA1 and GGA2 (Fig. 2 b). As expected, ϵ was de-

pleted from clathrin-coated vesicles, since AP-4 is not associated with clathrin (Hirst et al., 1999), while γ -adaptin, $\mu 1$, $\mu 2$, and γ -synergin were all enriched. However, the antisera raised against GGA1 and GGA2 were unable to detect bands in the clathrin-coated vesicle preparation, although they labeled bands of the appropriate sizes in the microsome samples. Thus, despite their homology to γ -adaptin, GGA1 and GGA2 do not appear to be associated with clathrin-coated vesicles.

To determine whether GGA1 and GGA2 are associated with the AP-1 complex or with any of its subunits, native immunoprecipitation experiments were performed. Fig. 2 c shows the results of one such experiment. HeLa cell extract was immunoprecipitated under nonreducing conditions with anti- γ , anti-GGA1, and anti-GGA2, as well as with anti- ϵ as a control. The immunoprecipitates were subjected to SDS-PAGE, blotted onto nitrocellulose, and probed with antibodies specific for ϵ , γ , $\beta 1$, $\mu 1$, $\sigma 1$, γ -synergin, GGA1, and GGA2. The results show that, as expected, anti- γ brings down all the subunits of the AP-1 complex, as well as γ -synergin. However, γ does not coprecipitate with either GGA1 or GGA2. Conversely, antibodies against GGA1 and GGA2 do not bring down the other GGA, γ -synergin, or any of the subunits of the AP-1 adaptor complex. These results indicate that GGA1 and GGA2 do not associate with each other or with the AP-1 complex.

To investigate the possibility that GGA1 and GGA2 form a complex with as yet unidentified proteins, pig brain cytosol was fractionated by gel filtration on a Superose 6 column. The column fractions were subjected to SDS-PAGE and Western blots were probed with anti- μ 1, anti-

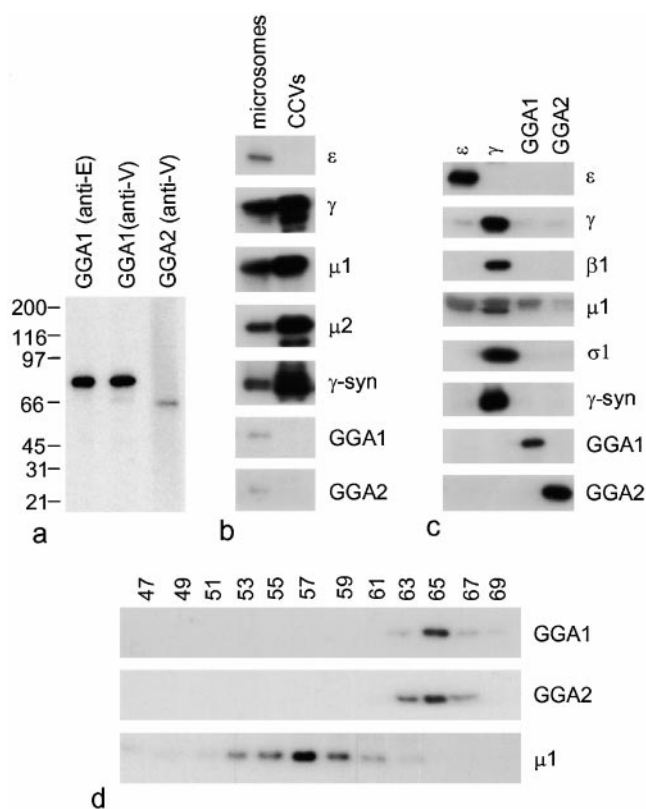


Figure 2. Biochemical characterization of GGA1 and GGA2. a, Western blots of whole HeLa cell extracts probed for GGA1 and GGA2. Two GGA1 antibodies were used, one raised against the COOH-terminal ear domain (anti-E) and one against the central variable region (anti-V). The GGA2 antibody was raised against the variable region. The GGA1 antibodies label a band with an apparent molecular weight of 85 kD, while the GGA2 antibody labels a band with an apparent molecular weight of 67 kD. b, Equal protein loadings of clathrin-coated vesicles purified from rat liver and a crude microsomal fraction from a previous stage in the preparation were subjected to SDS-PAGE, followed by Western blotting. The blot shows that the γ and μ 1 subunits of the AP-1 complex and the μ 2 subunit of the AP-2 complex, as well as the γ -adaptin binding protein γ -synerglin, are strongly enriched in clathrin-coated vesicles, whereas the ϵ subunit of the AP-4 complex, GGA1, and GGA2 are not. c, Cytosol was prepared from HeLa cells and immunoprecipitated under non-denaturing conditions using affinity-purified antibodies raised against GGA1, GGA2, the AP-4 ϵ subunit, and the AP-1 γ subunit. Western blots of the immunoprecipitates were probed with antibodies against ϵ , γ , β 1, μ 1, σ 1, γ -synerglin, GGA-1, and GGA-2. The results show that GGA1 and GGA2 do not associate with each other or with the AP-1 complex. c, Pig brain cytosol was fractionated on a Superose 6 column and Western blots of the odd-numbered fractions were probed with antibodies against the μ 1 subunit of AP-1, GGA1, and GGA2. AP-1, as marked by μ 1, eluted in fractions corresponding to >200 kD, whereas GGA1 and GGA2 eluted in similar positions in fractions corresponding to \sim 75 kD.

GGA1, and anti-GGA2 (Fig. 2 d). μ 1 was detected in fractions 51–63, peaking at fraction 57, which corresponds to >200 kD, consistent with its native molecular weight of 263 kD as a component of the AP-1 complex. In contrast, GGA1 and GGA2 were detected in fractions 63–67, with a peak corresponding to an apparent size of \sim 75 kD. These results further support the claim that the GGAs do not associate with AP-1, and also indicate that the GGAs are not part of a large protein complex.

Localization of GGA1 and GGA2

The antibodies raised against GGA1 and GGA2 were used for immunofluorescence to determine the distribution of the protein in intact cells. The GGA1-specific antibodies were found to label a discrete pattern of dots in the perinuclear region of the cell (Fig. 3 a). This distribution is similar to that of γ -adaptin or γ -synerglin; however, both γ -adaptin and γ -synerglin are found not only on the TGN, but also on more peripheral membranes that have been shown to correspond to early and/or recycling endosomes (Futter et al., 1998), while GGA1 has a more restricted perinuclear distribution. The antibodies raised against GGA2 were also found to label punctate structures in the perinuclear region of the cell, as well as a grainy background (data not shown). To improve the signal relative to background labeling, we inserted an 8 amino acid FLAG tag into the COOH-terminal end of full-length human GGA2 and expressed the construct in NRK cells. By immunofluorescence, the anti-FLAG staining was localized exclusively to punctate structures in the perinuclear region, without the grainy background (Fig. 3 b).

The AP-1 complex, like a number of other coat proteins, requires the small GTPase ARF to associate with membranes (Stamnes and Rothman, 1993; Seaman et al., 1996) and rapidly becomes cytosolic if cells are treated with BFA (Robinson and Kreis, 1992), which inhibits ARF guanine nucleotide exchange factor(s) (Peyroche et al., 1996). Similarly, both endogenous GGA1 (Fig. 3 c) and FLAG-tagged GGA2 (Fig. 3 d) become cytosolic rather than membrane-associated after treating cells with BFA for 2 min. This rapid response indicates that GGA1 and GGA2 cycle on and off the membrane in an ARF-dependent manner.

Although GGA1 and GGA2 have similar immunofluorescence patterns and are both BFA-sensitive, they do not coimmunoprecipitate, indicating that they are not associated with each other. To determine whether the two proteins colocalize, NRK cells stably transfected with FLAG-tagged GGA2 were double-labeled with anti-FLAG and anti-GGA1. Fig. 3, e and f, shows that the double-labeling patterns are largely coincident, indicating that GGA1 and GGA2 are associated with the same membranes and that they may function together in the same pathway(s).

Cells were also double-labeled with antibodies against FLAG-tagged GGA2 or endogenous GGA1, together with antibodies against γ -adaptin, γ -synerglin, and TGN38. Fig. 4 shows that GGA1 and GGA2 have very similar distributions to all three of these proteins, all of which are localized at least partially to the TGN. However, there are subtle differences in the labeling patterns. Thus, FLAG-tagged GGA2 (Fig. 4 a) is more strictly perinuclear than

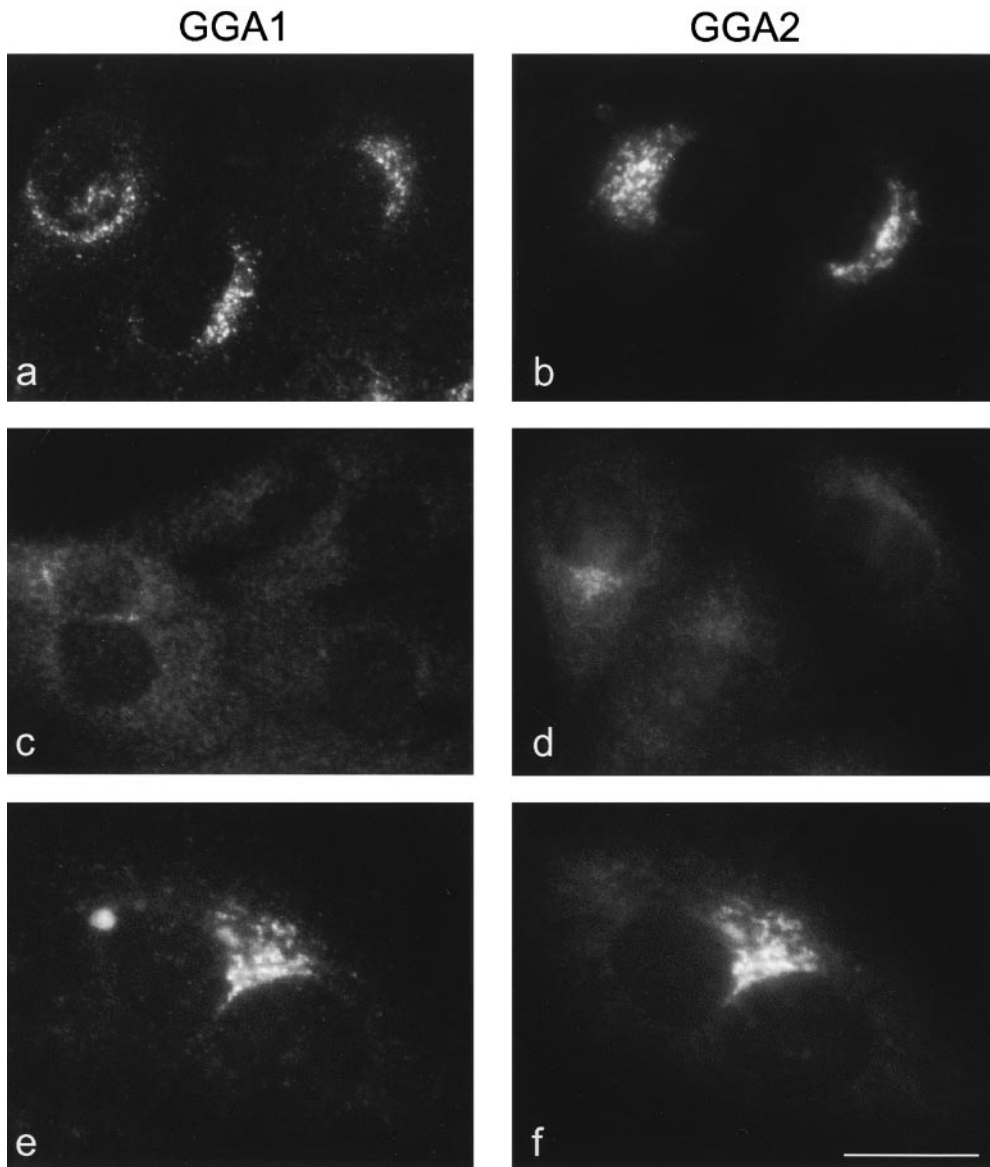


Figure 3. Immunofluorescence localization of GGA1 and GGA2. NRK cells, either nontransfected (a and c) or stably expressing FLAG-tagged GGA2 (b, d, e, and f), were labeled with either an affinity-purified rabbit polyclonal antibody against GGA1 (a, c, and e) or a mouse mAb against the FLAG tag (b, d, and f). Both proteins can be seen to have a punctate distribution in the perinuclear region of the cell (a and b, e and f). The cells shown in c and d were incubated with 100 $\mu\text{g/ml}$ BFA for 2 min at 37°C before fixation. Both proteins have been redistributed to the cytoplasm. The cells in e and f were double-labeled. The two patterns appear to be coincident. Bar: (a–d) 20 μm ; (e and f) 14 μm .

γ -adaptin (Fig. 4 b), and the actual pattern of dots is distinct from that of γ -adaptin, although the two are in close proximity (Fig. 4, a and b). Similar results were obtained when COS cells were double-labeled with anti-GGA1 and the γ -adaptin mAb 100/3 (data not shown). In contrast, double-labeling for GGA1 and GGA2 gave much more coincident patterns (see Fig. 3, e and f). Fig. 4, c and d, shows cells double-labeled for FLAG-tagged GGA2 (c) and γ -synergin (d). Again, γ -synergin is more dispersed than GGA2 and the two patterns of dots are somewhat different. By comparison, when cells are double-labeled for γ -adaptin and γ -synergin, the two patterns are virtually identical (Page et al., 1999). GGA1 and TGN38 also have distinct distributions: the GGA1 labeling (Fig. 4 e) is more punctate than the TGN38 labeling (Fig. 4 f), indicating that it is found at discrete foci rather than throughout the TGN compartment.

GGA1 was also localized at the electron microscope level, both in intact cells and in cells that had been perme-

abilized by freezing and thawing, then incubated with exogenous cytosol plus ATP and GTP γ S. Previously, we have shown that this treatment stabilizes the membrane association of proteins that are recruited onto membranes in an ARF-dependent manner, such as AP-1 and AP-3 (Robinson and Kreis, 1992; Simpson et al., 1996). Although GTP γ S can cause mistargeting of AP-2 (Seaman et al., 1993), this treatment does not affect the distribution of most proteins, and we found that the localization of GGA1 in GTP γ S-treated cells was virtually identical to that in control cells, both by immunofluorescence and by immunogold EM (data not shown). The advantage of this treatment is that cell membranes are much easier to visualize in the electron microscope. Fig. 5 a shows an example of such a cell labeled with anti-GGA1 followed by protein A coupled to 15-nm gold. The labeling is associated with Golgi membranes and with tubulovesicular membranes near the Golgi stack. Clathrin-coated budding profiles can often be seen in the same vicinity (Fig. 5 a, arrowheads).

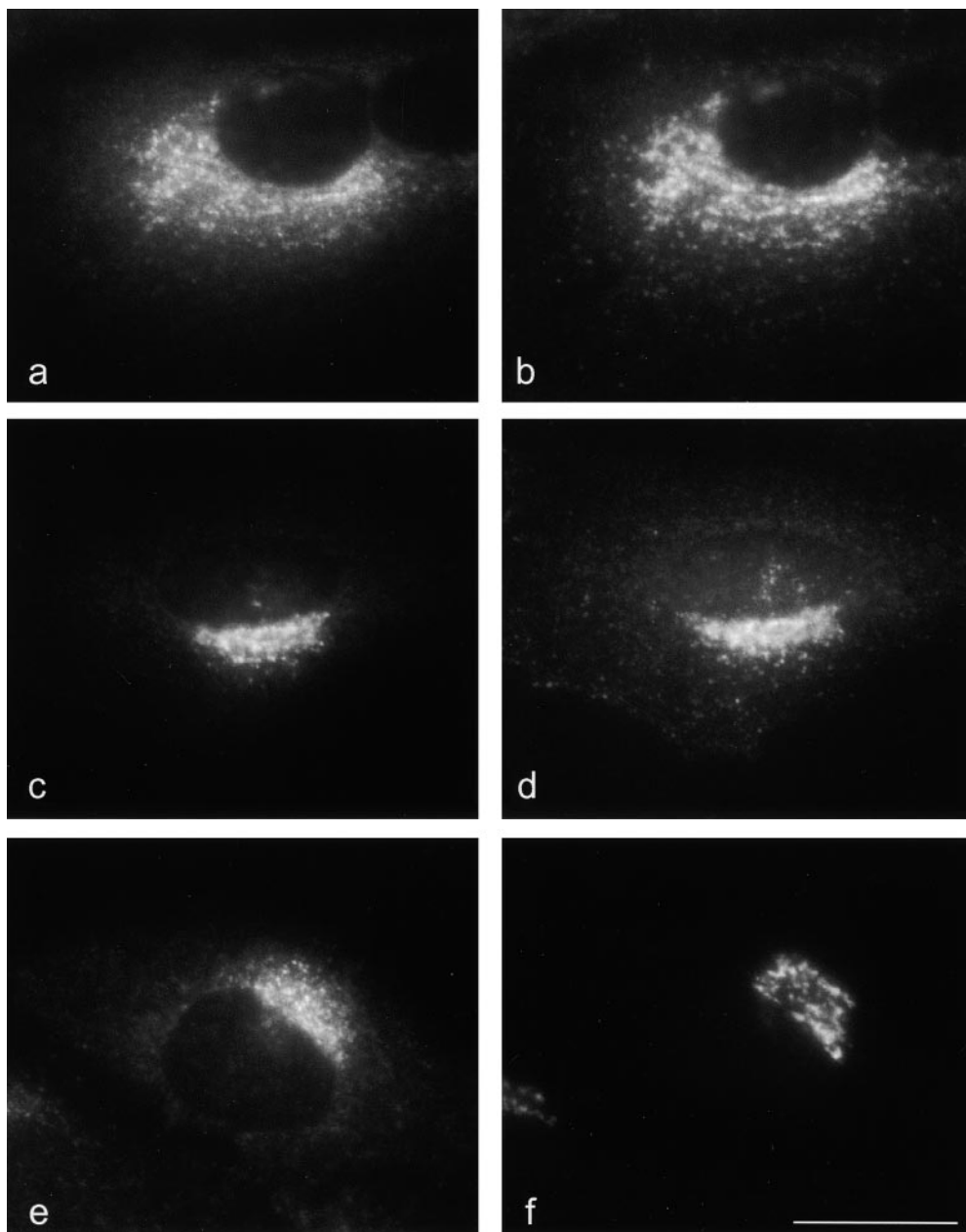


Figure 4. Distribution of GGAs compared with other TGN-associated proteins. a–d, NRK cells stably expressing FLAG-tagged GGA-2 were double-labeled with mouse anti-FLAG antibody (a and c) together with antibodies against the γ -adaptin subunit of the AP-1 adaptor complex (b) and γ -synerggin (d). The patterns are similar, but there is more peripheral labeling with the anti- γ -adaptin and anti- γ -synerggin antibodies, and the actual patterns of dots are generally distinct. e and f, NRK cells were double-labeled with an affinity-purified polyclonal antibody against GGA1 (e) and an mAb against TGN38 (f). Again, the patterns are similar, although the GGA1 labeling is more punctate than the TGN38 labeling. Bar, 20 μ m.

These observations indicate that GGA1 is associated with Golgi cisternae, particularly on the trans side, and with the TGN.

Binding Partners for γ -Adaptin, GGA1, and GGA2

What might be the functional significance of the homology between the COOH-terminal domains of γ -adaptin and the GGA family? One possibility is that they might share some of the same binding partners. To investigate this possibility, fusion proteins were constructed between GST and the COOH-terminal domains of γ -adaptin, GGA1, and GGA2. GST pulldown experiments were then performed with all three constructs, as well as with GST alone as a control, using pig brain cytosol as a source of potential binding partners. Fig. 6 shows that all three constructs

bring down bands that can be stained with Coomassie blue. Three bands can be seen in the pulldowns using GGA1 and GGA2 fusion proteins, with apparent molecular weights of \sim 200, 160, and 56 kD (Fig. 6, arrows). Bands of a similar size (labeled 2, 3, and 7), as well as a number of additional bands, can be seen in the pulldown using the γ -adaptin fusion protein.

To try to identify some of these proteins, the bands indicated with numbers were excised and subjected to MALDI mass spectrometry. Only two of the bands could be identified definitively: band 1 (MAP1A) and band 4 (rabaptin-5). Both of these proteins bound preferentially to the γ ear construct. Similarly, γ -synerggin, although it could not be identified as a Coomassie blue-stained band, was detectable by Western blotting and was found to bind preferentially to the γ ear (data not shown). Together,

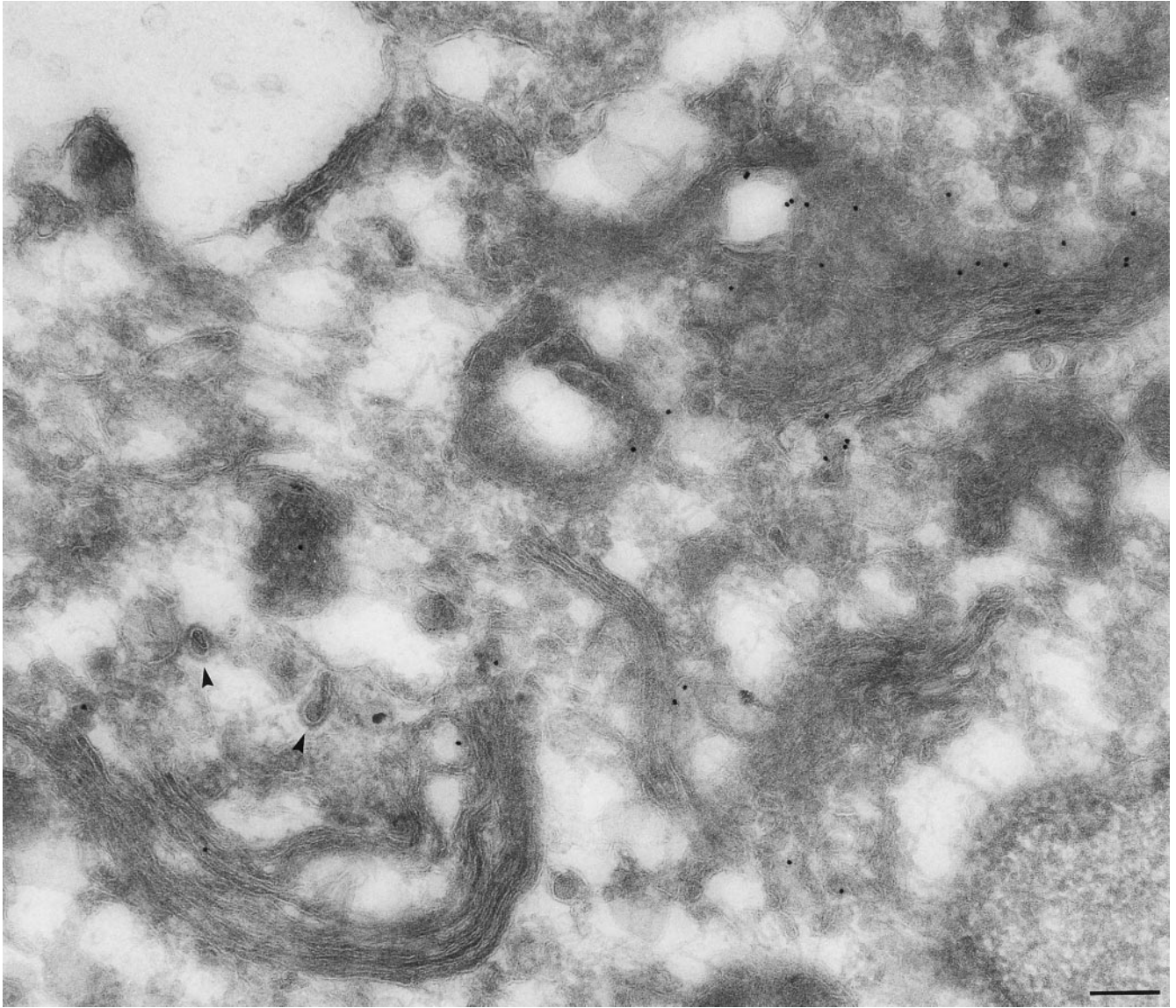


Figure 5. Localization of GGA1 at the electron microscope level. NRK cells were permeabilized by freezing and thawing, then allowed to recruit proteins from pig brain cytosol in the presence of ATP, an ATP-regenerating system, and GTP γ S. Frozen thin sections were labeled with an antibody against GGA1, followed by protein A coupled to 15-nm colloidal gold. Labeling can be seen to be associated with the Golgi stack and with tubulovesicular membranes in the Golgi region. Clathrin-coated budding profiles can be seen in the same vicinity (arrowheads), indicating that these membranes correspond to trans-Golgi cisternae and the TGN. Bar, 200 nm.

these observations indicate that the γ ear has multiple binding partners, some of which are shared by the GGA ears and some of which are not. Further details about the ear binding partners are available at <http://www.jcb.org/cgi/content/full/149/1/67/DC1> as supplemental information.

Yeast Homologues of the GGAs

A search of the *S. cerevisiae* genome revealed that there are two open reading frames that show significant homology to the mammalian GGAs, Ydr358w and Yhr108w. Fig. 7 a shows the alignment of the two yeast sequences with GGA1. The two yeast proteins are 49% identical to each

other, and each is \sim 20% identical to each of the mammalian GGAs. Like their mammalian counterparts, the yeast proteins consist of conserved NH₂-terminal and COOH-terminal domains, separated by a variable hinge-like domain. In addition, both yeast open reading frames contain VHS domains and two potential coiled coil domains, as well as a γ -adaptin homology domain; thus, the two open reading frames are likely to encode the yeast orthologues of the mammalian proteins, and we propose that the two genes be called *GGA1* (Ydr358w) and *GGA2* (Yhr108w). Schematic diagrams of the two yeast proteins are shown in Fig. 7 b.

In an attempt to determine the functions of the yeast proteins, the *GGA1* and *GGA2* genes were deleted both

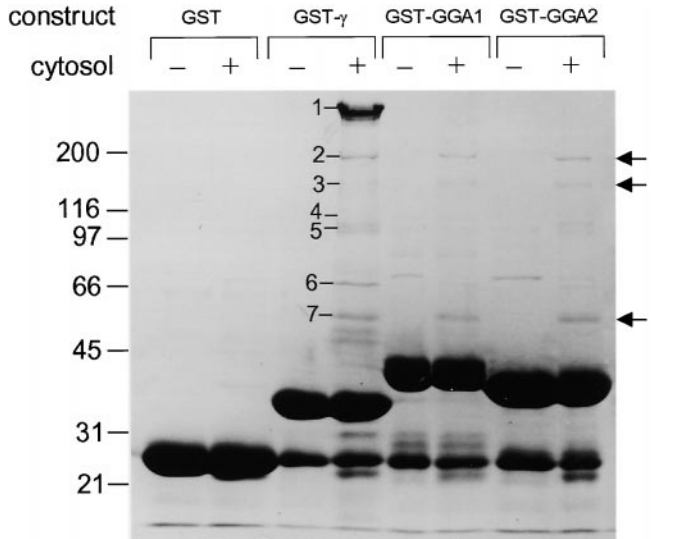


Figure 6. COOH-terminal domain binding partners. Pig brain cytosol was incubated with GST alone, with a GST fusion protein containing the γ -adapin ear domain (GST- γ), with a GST fusion protein containing the GGA1 ear domain (GST-GGA1), or with a GST fusion protein containing the GGA2 ear domain (GST-GGA2), followed by glutathione-Sepharose. The samples were subjected to SDS-PAGE and stained with Coomassie blue. The arrows indicate bands that are brought down by all three fusion proteins. The numbered bands were analyzed by MALDI mass spectrometry.

singly and together. The single knockouts of either yeast *GGA* gene were completely viable and showed no obvious phenotype. However, the double knockout strain exhibited a mild, but significant, defect in the processing of the vacuolar hydrolase, CPY. Pro-CPY is normally synthesized as a p1 precursor in the ER, undergoes processing to p2 pro-CPY in the Golgi complex, and is then transported from a late Golgi compartment to a prevacuolar compartment by its receptor, Vps10p. Here, the pro-CPY dissociates from Vps10p and is delivered to the vacuole where it is proteolytically processed to the mature (m) form. Fig. 8 a shows the result of a pulse-chase experiment to look at the processing of CPY in five different yeast strains. After a 10-min pulse with ^{35}S -methionine and a 30-min chase, in the wild-type cells, 89% of the CPY was in the mature form, while the remaining 11% was in the p2 precursor form. Similar results were obtained in the cells where only one of the *GGA* genes had been deleted (*gga2 Δ* or *gga1 Δ*). In cells where the gene encoding the protein Vps35p had been deleted (*vps35 Δ*), which results in strong CPY mis-sorting, 86% of the CPY was in the p2 precursor form and only 14% in the mature form. In the yeast cells with both *GGA* genes deleted (*gga1 Δ /gga2 Δ*), 28% of the CPY was in the p2 form and 39% in the mature form, and in addition there was a pseudomature form, running between p2 and mature CPY.

To confirm that the CPY sorting defect is a result of the double-deletion, the *gga1 Δ /gga2 Δ* strain was retransformed with either wild-type *GGA1* or wild-type *GGA2* expressed at endogenous levels. Fig. 8 b shows that *GGA1* restores CPY processing to wild-type levels, and sim-

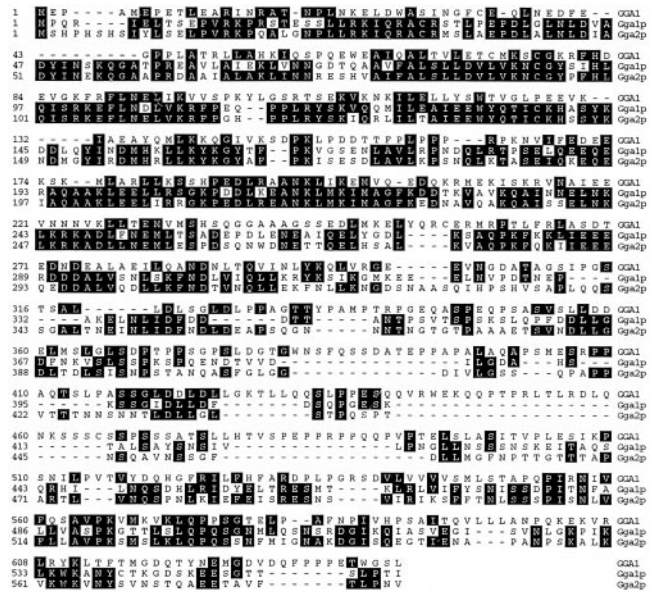


Figure 7. Sequences, alignments, and domain organization of two yeast GGAs. a, The complete protein sequences of yeast Gga1p (Ydr358w) and yeast Gga2p (Yhr108w) aligned with mammalian GGA1. The two yeast sequences are 49% identical to each other, and each is \sim 20% identical to each of the three mammalian GGAs. b, Domain organization of Gga1p and Gga2p. Like their mammalian counterparts, the yeast sequences contain a VHS domain, two coiled coil domains (CC), a central variable region, and a γ -adapin ear homology domain.

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ilar results were obtained with *GGA2* (data not shown). We also attempted to see whether we could recover a wild-type phenotype by transforming the cells with mammalian *GGA2*; however, the mammalian protein was unable to substitute for its yeast homologue (Fig. 8 b).

To examine the fate of the different forms of CPY in the *gga1 Δ /gga2 Δ* strain, the cells were spheroplasted to release proteins trapped inside the cell wall. Fig. 8 c shows that in the wild-type cells, 98% of the CPY was retained intracellularly in the mature form. In contrast, in the *vps35 Δ* cells, 86% of the CPY was secreted in the p2 form. In the *gga1 Δ /gga2 Δ* cells, 44% of the CPY was retained intracellularly, mainly in the mature form, while the rest was secreted in both the p2 and pseudomature forms. This result indicates that the *gga1 Δ /gga2 Δ* cells exhibit true missorting rather than a delay in processing and trafficking to the vacuole. Further evidence for missorting rather than delayed processing was obtained by carrying out a time course of pulse-chase experiments. Fig. 8 d shows that immediately after the pulse, the CPY was in the p1 form in all three strains. After 15 min, the p1 form had disappeared in all three strains and had been replaced either by the p2 form

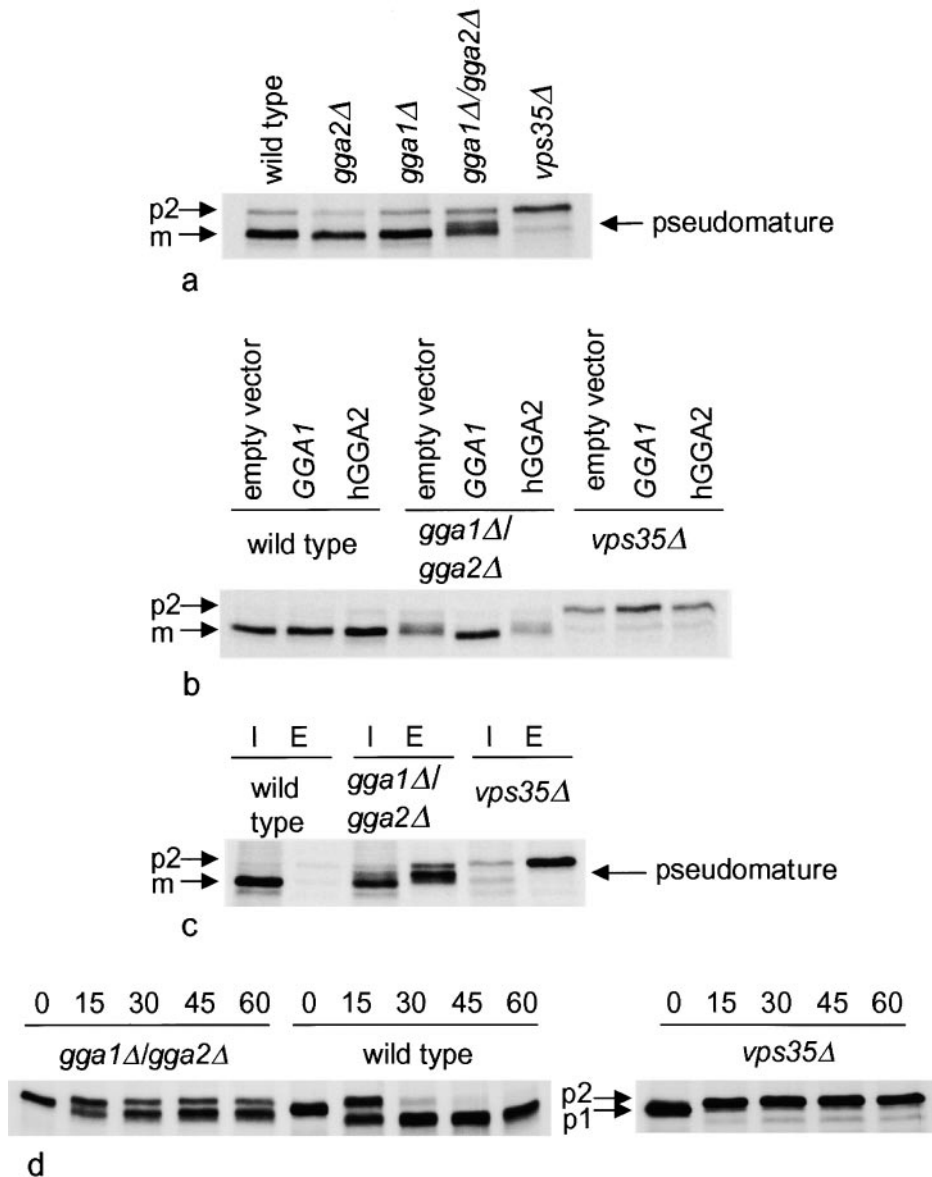


Figure 8. *GGA*-deficient yeast have a vacuolar protein sorting phenotype. **a**, Yeast cells were pulse-labeled with Promix ^{35}S for 10 min, chased for 30 min, immunoprecipitated for CPY, and subjected to SDS-PAGE. Yeast cells deleted for either *GGA* gene alone show no defect in CPY processing compared with control wild-type cells. However, cells deleted for both genes (*gga1Δ/gga2Δ*) accumulate more of the p2 relative to the m form of CPY, as well as a pseudomature form of CPY. In cells deleted for *VPS35* (*vps35Δ*), mainly the p2 form accumulates. **b**, Wild-type, *gga1Δ/gga2Δ*, and *vps35Δ* cells were transformed with either empty vector, yeast *GGA1*, or a cDNA encoding human *GGA2* (hGGA2), and were then assayed for CPY processing as above. The yeast *GGA1* gene, but not the mammalian cDNA, restores the wild-type phenotype. **c**, CPY is secreted from *gga1Δ/gga2Δ* cells. Yeast cells were pulse-labeled as above, spheroplasted, and separated into intracellular and extracellular fractions. CPY was immunoprecipitated from both fractions and subjected to SDS-PAGE. The *gga1Δ/gga2Δ* strain secretes CPY in the p2 and pseudomature forms. This is in contrast to the wild-type cells, which do not secrete CPY, and the *vps35Δ* cells, which secrete CPY in the p2 form. **d**, Kinetics of CPY processing. Yeast cells were pulse-labeled with Promix ^{35}S for 3 min, chased for 0–60 min, and then immunoprecipitated for CPY and subjected to SDS-PAGE. *gga1Δ/gga2Δ* cells lose the p1 form with wild-type kinetics, but then exhibit slow processing of the p2 form and an accumulation of the pseudomature form(s).

alone in the *vps35Δ* strain, by the p2 form together with the mature form in the wild-type strain, or by the p2, pseudomature, and mature forms in the *gga1Δ/gga2Δ* strain. After longer chase times, the protein remained in the p2 form in the *vps35Δ* strain, whereas in both the wild-type strain and *gga1Δ/gga2Δ* processing was essentially complete by 30 min, although in the *gga1Δ/gga2Δ* strain much of the protein remained in the p2 or pseudomature form.

To characterize the nature of the defect further, we looked at several criteria that have been used to group the vacuolar protein sorting mutants into different classes. The defect in the *gga1Δ/gga2Δ* strain appears to be specific for the classical vacuolar protein sorting pathway, since alkaline phosphatase, which uses a different, AP-3 mediated pathway to get to the vacuole (Cowles et al., 1997), is pro-

cessed normally and with wild-type kinetics (data not shown). We also examined the processing and localization of the CPY receptor, Vps10p. A number of *vps* mutants with a relatively mild missorting phenotype, similar to the one that we observe in the *gga1Δ/gga2Δ* strain, have been shown to accumulate Vps10p in a prevacuolar compartment, called the Class E compartment, and in these cells the Vps10p becomes proteolytically clipped and is degraded more quickly than in wild-type cells (Raymond et al., 1992; Cereghino et al., 1995). However, we find that the distribution of Vps10p is indistinguishable in the wild-type and *gga1Δ/gga2Δ* cells (Fig. 9, a and b), and the turnover time and electrophoretic mobility of Vps10p were also found to be normal (data not shown). Some of the *vps* mutants have been shown to be deficient in endocytosis, which can be observed by monitoring the uptake of the

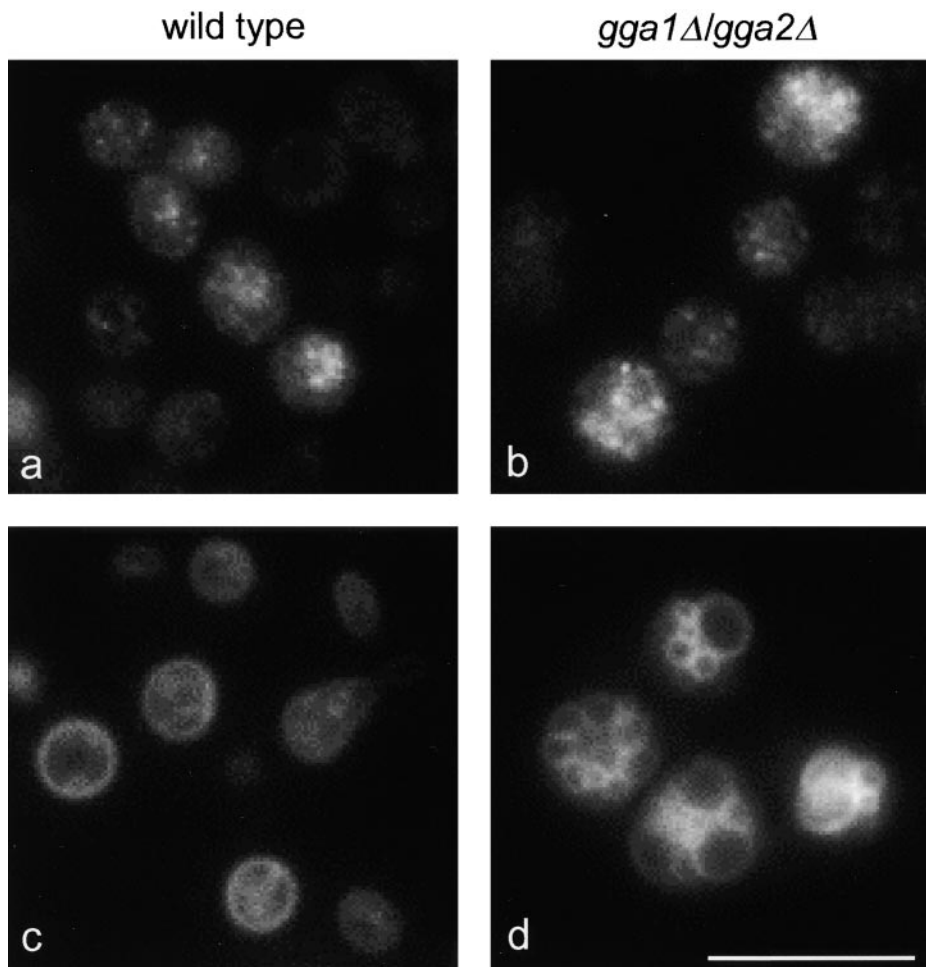


Figure 9. Localization of Vps10p and vacuolar morphology in *gga1Δ/gga2Δ* cells. a and b, Yeast cells were transformed with a myc-tagged Vps10p construct, then prepared for immunofluorescence and labeled with a mouse anti-myc antibody. The distribution of Vps10p is similar in the wild-type (a) and *gga1Δ/gga2Δ* cells (b), indicating that the *GGA* genes are not class E *VPS* genes. b, Cells were pulse-labeled with FM4-64 for 15 min, chased for 60 min at 30°C to label the vacuole, and then immobilized on a microscope slide. In the *gga1Δ/gga2Δ* cells (d) the morphology of the vacuole is significantly altered compared with wild-type cells (c), with highly fragmented vacuoles. Scale bar: 10 μm (a and b); 8 μm (c and d).

lipid-soluble styryl dye, FM4-64 (Vida and Emr, 1995). This dye then becomes concentrated in the vacuole, so it can also be used as a marker for vacuolar morphology. Fig. 9, c and d, shows that uptake of the dye appears similar in the wild-type and *gga1Δ/gga2Δ* cells. However, the appearance of the vacuoles is different in the wild-type and *gga1Δ/gga2Δ* cells. We scored 43% of the *gga1Δ/gga2Δ* cells as having fragmented vacuoles, while 31% had one large vacuole surrounded by a number of smaller vacuoles. In contrast, when we examined the wild-type strain, >99% had 1–3 vacuoles of normal appearance. Thus, in addition to their CPY sorting defect, the *gga1Δ/gga2Δ* cells also have a vacuolar morphology defect, similar to that reported for class B and class F *vps* mutants (Raymond et al., 1992).

Discussion

Here, we describe a novel family of proteins, conserved between yeast and mammals, that contain an NH₂-terminal VHS domain, one or two potential coiled coil domains, a variable hinge-like domain, and a COOH-terminal domain homologous to the COOH-terminal domain of γ -adaptin. There are at least three such proteins in mammals, GGA1, GGA2, and GGA3, and there are two in yeast, Gga1p and Gga2p.

Because of the homology between γ -adaptin and the GGAs, we set out first to determine whether the GGAs were associated with clathrin-coated vesicles or with the AP-1 complex. We found that neither GGA1 nor GGA2 was enriched in clathrin-coated vesicles, nor did they coimmunoprecipitate with any of the subunits of the AP-1 complex. Similarly, by both immunofluorescence and immunogold EM, the GGAs were found to have a distinct distribution from AP-1, although they were in close proximity on membranes of the TGN. In addition, deleting the two GGA genes in yeast gives a different phenotype from deleting AP-1 subunits or clathrin. Thus, yeast cells that are deficient in both Gga1p and Gga2p missort CPY, but endocytosis appears normal. In contrast, cells that are deficient in clathrin have reduced endocytosis, but sort CPY normally (Payne et al., 1988) (although, cells that are temperature-sensitive for clathrin missort CPY immediately after shifting them to the nonpermissive temperature, but within 3 h normal sorting is resumed; Seeger and Payne, 1992). Deleting subunits of the putative AP-1 complex has no discernible phenotype alone, although there are synthetic effects in cells that are temperature-sensitive for clathrin (Huang et al., 1999; Yeung et al., 1999). Together, these results suggest that the GGAs function independently from clathrin and AP-1.

The sequences of the GGAs provide additional clues

about their function. At the extreme NH₂-terminal end, the proteins contain a VHS domain. This is the same position where VHS domains are found in all other VHS-containing proteins so far identified. Although the function of the VHS domain is still unknown, it seems likely, based on what is known about other domains with a similar degree of conservation, that it interacts either with other proteins or with lipids. It has been proposed that the VHS domain may participate in the association of such proteins with membranes, since a construct consisting of the first 205 amino acids of EAST, including the VHS domain (amino acids 1–139), but not the coiled coil or SH3 domains, is sufficient for localization to the plasma membrane (Lohi et al., 1998). However, different VHS domain-containing proteins localize to different membranes. Thus, EAST is found on the plasma membrane, but Hrs is found on endosomes (Komada et al., 1997), and GGA1 and GGA2 are found on trans-Golgi cisternae and the TGN. One possibility is that multiple interactions participate in the localization of VHS domain-containing proteins, one of which may involve their VHS domains.

Downstream from the VHS domain, the GGAs all contain one or two predicted coiled coil domains. Although coiled coil domains are known to participate in protein-protein interactions, the native molecular weight of GGA1 and GGA2, as determined by both gel filtration (Fig. 2 d) and ultracentrifugation (Dell'Angelica et al., 2000), indicates that they are monomeric. However, both of these studies were performed on cytosol and it is possible that the proteins form coiled coils with other proteins only when they are associated with membranes. Immunoprecipitation experiments, carried out on cells extracted with NP-40 rather than on cytosol, show that GGA1 and GGA2 do not coprecipitate with each other, indicating that they do not form heterodimers; however, they might be forming homodimers, or heterodimers with some other protein. The coiled coil domains also overlap with the ARF-binding domains, as defined by Boman et al. (2000) and Dell'Angelica et al. (2000).

The third recognizable domain on the GGAs is the γ -adaptin ear homology domain, found at the COOH-terminal end. The α -adaptin ear has been shown to be the binding site for a number of accessory proteins that participate in the endocytic pathway (Owen et al., 1999), and it seems likely that the γ ear plays a similar role in the AP-1 pathway. Recently, we have identified a novel protein, γ -synergin, which binds to the γ -adaptin ear, and here we show that the γ ear binds to a number of other proteins in GST pulldown experiments. A subset of these proteins also bind to the GGA ears in GST pulldown experiments, and we are currently attempting to identify the common binding partners. At present, our working hypothesis is that the COOH-terminal domains of the GGAs, like the COOH-terminal domains of both α -adaptin and γ -adaptin, serve to recruit accessory proteins onto a particular compartment, in this case the late Golgi complex and TGN.

Are the GGAs coat proteins? Although this question has yet to be formally addressed, several lines of evidence suggest that they may be. They have a punctate distribution by immunofluorescence, and in the electron microscope GGA1 is frequently seen associated with vesicular profiles. The GGAs are sensitive to BFA, and so far, most

of the BFA-sensitive peripheral membrane proteins that have been identified are either ARFs, coat components (e.g., AP-1, AP-3, AP-4, and coatomer), or proteins associated with coat components (e.g., γ -synergin). In addition, the presence of the γ -adaptin ear homology domain suggests that, like the α -adaptin ear, this domain may recruit proteins onto the membrane that are required for vesicle budding. Finally, Dell'Angelica et al. (2000) have observed coats on GGA3-positive membranes in micrographs of transfected cells. Together, these observations suggest that the GGAs may be components of a novel type of coat, mediating the budding of vesicles from the trans-Golgi cisternae and the TGN.

What might be the fate of such vesicles? The yeast knockout experiments show that deleting the two *GGA* genes causes cells to missort the vacuolar hydrolase CPY, providing strong evidence for a role for the GGAs in the delivery of proteins to the yeast vacuole and its mammalian equivalent, the lysosome. Over 50 vacuolar protein sorting genes in yeast have now been identified and characterized by screening for CPY missorting, and the reason that the *GGA* genes have not been identified as *VPS* genes until now is presumably because they are functionally redundant, so that both of them need to be disrupted to get a *vps* phenotype. The various *VPS* genes have been grouped into different classes depending on a number of criteria, including the strength of CPY missorting, the morphology of the vacuole, the ability of the cells to sort alkaline phosphatase, and whether or not endocytosis is defective (Raymond et al., 1992). Most *vps* mutants exhibit strong CPY missorting; however, the class E mutants show ~50% missorting (Cereghino et al., 1995), which is the level that we find in the *gga1Δ/gga2Δ* cells. In addition, the gene encoding the VHS domain-containing protein Vps27p is a class E gene (Piper et al., 1995). Thus, our initial hypothesis was that *GGA1* and *GGA2* might also be class E genes. The hallmark of the class E mutants is the presence of one or two large compartments adjacent to the vacuole, called class E compartments. The class E compartment accumulates proteins that are normally resident in the vacuole or in the Golgi complex, including Vps10p, which becomes proteolytically clipped (Cereghino et al., 1995). However, we find that Vps10p has a normal size and distribution in the *gga1Δ/gga2Δ* cells, indicating that the *GGA* genes are not class E genes.

In addition to the CPY missorting phenotype, the *gga1Δ/gga2Δ* strain has a severe vacuolar morphology defect. Many of the cells were found to have fragmented vacuoles, while other cells had one large vacuole surrounded by several smaller vacuoles. Fragmented vacuoles are found in the class B mutants, while large vacuoles surrounded by smaller ones are characteristic of the class F mutants (Raymond et al., 1992). However, both class B and class F mutants have a much stronger CPY missorting phenotype than we observe in the *gga1Δ/gga2Δ* mutants. Thus, it is difficult to assign *GGA1* and *GGA2* to any of the classes of *VPS* genes. It is possible that they may be acting at a different step from any of the genes so far described. Further studies, making use of triple mutants, where not only *GGA1* and *GGA2* have been deleted, but also one of the well characterized *VPS* genes, should help to define the phenotype further. We also intend to knock out the *GGA* genes together with the genes encod-

ing clathrin and/or the γ -adaptin homologue Apl4p, to see if the phenotype is exacerbated. Since our hypothesis is that there are accessory proteins that are required for sorting to the vacuole, which can be recruited onto the membrane by binding either to γ -adaptin or to one of the Gga proteins, then by knocking out all three we may completely block CPY sorting.

Although there is still much that we do not know about the GGA family of proteins, the ability to study these proteins in both mammals and yeast has allowed us to learn much more about their function than would have been possible with either system alone. Morphological studies are much easier to perform in mammalian cells than in yeast, because of their larger size and the better defined morphology of their organelles. By localizing GGA1 and GGA2 in mammalian cells at both the light and the electron microscope level, we have demonstrated that the proteins are recruited onto trans-Golgi cisternae and the TGN. However, simply localizing the proteins does not tell us their function. By analyzing the phenotype of GGA-deficient yeast, we have shown that they are involved in vacuolar protein sorting. However, a large number of VPS genes have been described in yeast, and in many cases it is not known at what stage the proteins act: whether they are required for vesicle budding, docking, or fusion, and whether they participate in trafficking from the late Golgi to a prevacuolar endosomal compartment, from the prevacuole to the vacuole, from the prevacuole back to the Golgi, or at some other step. By combining data from both yeast and mammalian systems, we can conclude that members of the GGA family are recruited late Golgi membranes and that from there they facilitate the trafficking of proteins that are destined for the vacuole or lysosome.

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References

- Asao, H., Y. Sasaki, T. Arita, N. Tanaka, K. Endo, H. Kasai, T. Takeshita, Y. Endo, T. Fujita, and K. Sugamura. 1997. Hrs is associated with STAM, a signal-transducing adaptor molecule. Its suppressive effect on cytokine-induced cell growth. *J. Biol. Chem.* 272:32785–32791.
- Boman, A.L., C. Zhang, X. Zhu, and R.A. Kahn. 2000. A family of Arf effectors that can alter membrane transport through the trans-Golgi. *Mol. Biol. Cell.* In press.
- 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 localisation. *Mol. Biol. Cell.* 6:1089–1102.
- Cowles, C.R., G. Odorizzi, G.S. Payne, and S.D. Emr. 1997. The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell.* 91:109–118.
- Dell'Angelica, E.C., H. Ohno, C.E. Ooi, E. Rabinovich, K.W. Roche, and J.S. Bonifacino. 1997. An adaptor-like protein complex with ubiquitous expression. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:917–928.
- Dell'Angelica, E.C., C. Mullins, and J.S. Bonifacino. 1999. AP-4, a novel protein complex related to clathrin adaptors. *J. Biol. Chem.* 274:7278–7285.
- Dell'Angelica, E.C., R. Puertollano, C. Mullins, R.C. Aguilar, J.D. Vargas, L.M. Hartnell, and J.S. Bonifacino. 2000. GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. *J. Cell Biol.* 149:81–93.
- Futter, C.E., A. Gibson, E.H. Allchin, S. Maxwell, L.J. Ruddock, G. Odorizzi, D. Domingo, I.S. Trowbridge, and C.R. Hopkins. 1998. In polarized MDBK cells basolateral vesicles arise from clathrin-gamma-adaptin-coated domains on endosomal tubules. *J. Cell Biol.* 141:611–623.
- Hirst, J., and M.S. Robinson. 1998. Clathrin and adaptors. *Biochim. Biophys. Acta.* 1401:173–193.
- Hirst, J., N.A. Bright, B. Rous, and M.S. Robinson. 1999. Characterization of a fourth adaptor-related protein complex. *Mol. Biol. Cell.* 10:2787–2802.
- Huang, K.M., K. D'Hondt, H. Riezman, and S.K. Lemmon. 1999. Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:3897–3908.
- Komada, M., R. Masaki, A. Yamamoto, and N. Kitamura. 1997. Hrs, a tyrosine kinase substrate with a conserved double zinc finger domain, is localized to the cytoplasmic surface of early endosomes. *J. Biol. Chem.* 272:20538–20544.
- Lohi, O., and V.P. Lehti. 1998. VHS domain marks a group of proteins involved in endocytosis and vesicular trafficking. *FEBS Lett.* 440:255–257.
- Lohi, O., A. Poussu, J. Merilainen, S. Kellokumpu, V.M. Wasenius, and V.P. Lehto. 1998. EAST, an epidermal growth factor receptor- and Eps15-associated protein with Src homology 3 and tyrosine-based activation motif domains. *J. Biol. Chem.* 273:21408–21415.
- Manfredi, J.J., and W.L. Bazari. 1987. Purification and characterisation of two distinct complexes of assembly polypeptides from calf brain coated vesicles that differ in their polypeptide composition and kinase activities. *J. Biol. Chem.* 262:12182–12188.
- Marsh, M., and H.T. McMahon. 1999. The structural era of endocytosis. *Science.* 285:215–220.
- Nagase, T., N. Seki, A. Tanaka, K. Ishikawa, and N. Nomura. 1995. Prediction of the coding sequences of unidentified human genes. IV. The coding sequences of 40 new genes (K1AA0121–K1AA0160) deduced by analysis of cDNA clones from human cell line KG-1. *DNA Res.* 2:167–174.
- Owen, D.J., Y. Vallis, M.E. Noble, J.B. Hunter, T.R. Dafforn, P.R. Evans, and H.T. McMahon. 1999. A structural explanation for the binding of multiple ligands by the alpha-adaptin appendage domain. *Cell.* 97:805–815.
- Page, L.J., and M.S. Robinson. 1995. Targeting signals and subunit interactions in coated vesicle adaptor complexes. *J. Cell Biol.* 131:619–630.
- Page, L.J., P.J. Sowerby, W.W.Y. Lui, and M.S. Robinson. 1999. γ -Synergisin: an EH domain-containing protein that interacts with γ -adaptin. *J. Cell Biol.* 146:993–1004.
- Payne, G.S., D. Baker, E. Van Tuinen, and R. Schekman. 1988. Protein transport to the vacuole and receptor-mediated endocytosis in clathrin heavy chain-deficient yeast. *J. Cell Biol.* 106:1453–1461.
- Peyroche, A., S. Paris, and C.L. Jackson. 1996. Nucleotide exchange on ARF mediated by yeast Gea1 protein. *Nature.* 384:479–481.
- Piper, R.C., A.A. Cooper, H. Yang, and T.H. Stevens. 1995. VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in *Saccharomyces cerevisiae*. *J. Cell Biol.* 131:603–617.
- Raymond, C.K., I. Howald-Stevenson, C.A. Vater, and T.H. Stevens. 1992. Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. *Mol. Biol. Cell.* 3:1389–1402.
- 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.* 69:129–138.
- Salcini, A.E., S. Confalonieri, M. Doria, E. Santolini, E. Tassi, O. Minenkova, G. Cesareni, P.G. Pelicci, and P.P. Di Fiore. 1997. Binding specificity and in vitro targets of the EH domain, a novel protein-protein interaction module. *Genes Dev.* 11:2239–2249.
- Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Seaman, M.N.J., C.L. Ball, and M.S. Robinson. 1993. Targeting and mistargeting of plasma membrane adaptors in vitro. *J. Cell Biol.* 123:1093–1105.
- Seaman, M.N.J., E.G. Marcusson, J.-L. Cereghino, and S.D. Emr. 1997. Endosome to Golgi retrieval of the vacuolar protein sorting receptor, Vps10p, requires the function of the VPS29, VPS30, and VPS35 gene products. *J. Cell Biol.* 137:79–92.
- Seaman, M.N.J., P.J. Sowerby, and M.S. Robinson. 1996. Cytosolic and membrane-associated proteins involved in the recruitment of AP-1 adaptors onto the trans-Golgi network. *J. Biol. Chem.* 271:25446–25451.
- Seeger, M., and G.S. Payne. 1992. A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2811–2818.
- 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.
- Simpson, F., N.A. Bright, M.A. West, L.S. Newman, R.B. Darnell, and M.S. Robinson. 1996. A novel adaptor-related protein complex. *J. Cell Biol.* 133:749–760.
- Simpson, F., N.K. Hussain, B. Qualmann, R.B. Kelly, B.K. Kay, P.S. McPherson, and S.L. Schmid. 1999. SH3-domain-containing proteins function at distinct steps in clathrin-coated vesicle formation. *Nat. Cell Biol.* 1:119–124.
- Stammes, M.A., and J.E. Rothman. 1993. The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell.* 73:999–1005.
- 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.
- Yeung, B.G., H.L. Phan, and G.S. Payne. 1999. Adaptor complex-independent clathrin function in yeast. *Mol. Biol. Cell.* 10:3643–3659.