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The ADP Ribosylation Factor-Nucleotide Exchange Factors Gea1p and Gea2p Have Overlapping, but Not Redundant Functions in Retrograde Transport from the Golgi to the Endoplasmic Reticulum

Anne Spang,[*†‡] Johannes M. Herrmann,[†] Susan Hamamoto,[†] and Randy Schekman[†]

*Friedrich Miescher Laboratorium, Max Planck Gesellschaft, D-72076 Tübingen, Germany; and †Department of Molecular and Cell Biology and Howard Hughes Medical Institute, University of California, Berkeley, California 94720

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The activation of the small ras-like GTPase Arf1p requires the action of guanine nucleotide exchange factors. Four Arf1p guanine nucleotide exchange factors have been identified in yeast: Sec7p, Syt1p, Gea1p, and its homologue Gea2p. We identified *GEA2* as a multicopy suppressor of a *sec21-3* temperature-sensitive mutant. *SEC21* encodes the γ-subunit of coatomer, a heptameric protein complex that together with Arf1p forms the COPI coat. *GEA1* and *GEA2* have at least partially overlapping functions, because deletion of either gene results in no obvious phenotype, whereas the double null mutant is inviable. Conditional mutants defective in both *GEA1* and *GEA2* accumulate endoplasmic reticulum and Golgi membranes under restrictive conditions. The two genes do not serve completely overlapping functions because a Δ*gea1* Δ*arf1* mutant is not more sickly than a Δ*arf1* strain, whereas Δ*gea2* Δ*arf1* is inviable. Biochemical experiments revealed similar distributions and activities for the two proteins. Gea1p and Gea2p exist both in membrane-bound and in soluble forms. The membrane-bound forms, at least one of which, Gea2p, can be visualized on Golgi structures, are both required for vesicle budding and protein transport from the Golgi to the endoplasmic reticulum. In contrast, Sec7p, which is required for protein transport within the Golgi, is not required for retrograde protein trafficking.

INTRODUCTION

Retrograde transport from the Golgi to the endoplasmic reticulum (ER) is an essential process. The most important functions of retrograde transport may be the retrieval of proteins that have escaped the ER and the recycling of transport factors, such as SNAP receptors (SNAREs), for subsequent rounds of anterograde transport. This transport step is mediated by the heptameric protein complex, coatomer, and the small ras-like GTPase Arf1p, which together form the COPI coat. The recruitment of the COPI coat to the *cis*-Golgi is thought to be initiated by the binding of Arf1p to the membrane. Arf1p is myristoylated at its N-terminus. In the GDP-bound (inactive) state, the fatty acid modification is buried within the molecule, and thus Arf1p is soluble in the cytoplasm. Upon exchange of GDP for GTP on Arf1p, the N-terminus is exposed and anchors Arf1p into the membrane. The activation of Arf1p is mediated by guanine nucleotide exchange factors (GEFs), which have been identified in yeast, plants, protozoa, worms, flies, and mammals (Jackson and Casanova, 2000). After the association of Arf1p with the membrane, coatomer and cargo can be recruited into budding complexes, resulting in the formation of COPI-coated vesicles that pinch from the donor membrane (Springer *et al.*, 1999).

In the yeast *Saccharomyces cerevisiae*, four GEFs that can act on Arf1p have been identified: Sec7p, Syt1p, Gea1p, and Gea2p (for review, see Roth, 1999; Jackson and Casanova, 2000). Each contains a conserved *SEC7* domain, which is the catalytic site of the GEFs. In higher eukaryotes, ADP ribosylation factor (ARF)-GEFs can be distinguished by their sensitivity to the fungal metabolite brefeldin A, which is determined by the sequence of the *SEC7* domain. In yeast, the GEFs are sensitive to brefeldin A (Peyroche *et al.*, 1999).

‡ Corresponding author. E-mail: anne.spang@tuebingen.mpg.de.
Abbreviations used: ARF, ADP ribosylation factor; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; SNARE, SNAP-receptor; ts, temperature sensitive; YPD, yeast extract, peptone, and dextrose growth medium.

Table 1. Strains used in this paper

Strain	Genotype	Source
YPH499	MATa *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801 ade2-101*	Sikorski and Hieter (1989)
YPH500	MATα *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801 ade2-101*	Sikorski and Hieter (1989)
EGY021a	MATα *trp1 leu2 his3 lys2 suc2Δ9 sec21::HIS3 pRS416-SEC21*	Gaynor and Emr (1997)
EGY021c	MATα *trp1 leu2 his3 lys2 suc2Δ9 sec21::HIS3 pRS416-sec21-3*	Gaynor and Emr (1997)
CJY049-3-4	MATα *ura3-52 leu2-3,112 his3-Δ200 lys2-801 ade2-101*	C.L. Jackson
CJY062-10-3	MATα *ura3-52 leu2-3,112 his3-Δ200 lys2-801 ade2-101 gea1-4 gea2::HIS3*	C.L. Jackson
CJY049-11-4	MATα *ura3-52 leu2-3,112 his3-Δ200 lys2-801 ade2-101 gea2::HIS3*	C.L. Jackson
CJY049-2-2	MATa *ura3-52 leu2-3,112 his3-Δ200 lys2-801 gea1::URA3*	C.L. Jackson
EHY154	MATa *ura3 trp1 arf3::hisG-URA3-hisG*	E. Harsay
EHY255	MATa *ura3 trp1 his3 arl1::HIS3*	E. Harsay
RSY299	MATα *ura3-1 leu2-3,112 trp1-Δ1 his3-11,15 ade2-1 sec7-1*	Schekman strain collection
RSY301	MATa *ura3-1 leu2-3,112 trp1-Δ1 his3-11,15 sec7-4*	Schekman strain collection
RSY445	MATα *ura3-52 leu2-3,112 trp1-289 pep4::URA3 prb1 his4-579*	Schekman strain collection
RSY913	MATα *ura3 leu2 his3 trp1 lys2 arf2::LEU2*	Schekman strain collection
RSY1163	MATa *leu2-3,112 ura3-53 ade2-101 kar2-133*	Schekman strain collection
RSY1169	MATa *leu2-3,112 ura3-53 pep4::URA3 gls1-1*	Schekman strain collection
YAS31	MATα *ura3-52 leu2-3,112 trp1-289 pep4::URA3 prb1 his4-579 LEU2-EMP47-MYC TRP1-OCH1-HA*	Spang and Schekman (1998)
YAS61	MATa *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801 ade2-101 GEA2-mychis$_6$-URA3*	This study
YAS64	MATa *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801 ade2-101 HIS3-GAL-GEA2-mychis$_6$-URA3*	This study
YAS65	MATa *ura3-52 leu2-3,112 trp1-Δ1 his3-Δ200 arf1::HIS3*	This study
YAS78	MATα *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801 ade2-101* p92	This study
YAS79	MATα *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801 ade2-101* pAFB1	This study
YAS88	MATα *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801 ade2-101 GEA1-myc-KAN*	This study
YAS89	MATα *ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 lys2-801 ade2-101 GEA1-HA-KAN*	This study
Y05512	MATa *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 YPR095c::kanMX4*	Eurofan

Whereas GEFs in yeast are fairly large proteins (160–230 kDa), in higher eukaryotes smaller GEFs exist, such as ARNO, which is ~45 kDa (Chardin *et al.*, 1996). The role of the extra domains on the large GEFs is not understood. One domain may be required for homodimerization as was shown for the *Arabidopsis* ARF-GEF GNOM (Grebe *et al.*, 2000). Other segments may help to localize a GEF by promoting contact with a protein anchored more directly to a membrane target.

Sec7p, Gea1p, and Gea2p have been shown to be required for transport from the ER through the Golgi apparatus (Franzusoff and Schekman, 1989; Peyroche *et al.*, 1996; Wolf *et al.*, 1998). However, the details of their individual roles are not established. We show that Gea1p and Gea2p have overlapping functions, specifically in retrograde transport from the Golgi to the ER, whereas Sec7p is not required for this transport step.

MATERIALS AND METHODS

Yeast Strains, Media, and Yeast Transformation

Yeast strains used in this study are summarized in Table 1. Cells were grown in either yeast extract, peptone, and dextrose growth medium (YPD) or synthetic complete medium lacking specific nutrients to maintain or select for plasmids. Yeast strains were transformed as described by Schiestl and Gietz (1989).

sec21-3 Suppressor Screen

The *sec21-3* mutant strain was provided by E. Gaynor and S. Emr (University of California, San Diego, San Diego, CA) and transformed with a YEp24 (*URA3* 2μ)-based yeast library (Carlson and Botstein, 1982). Transformants (40,000) were grown at 24°C, replica plated, and screened for growth at 35°C. From growing colonies, plasmids were isolated and retransformed into the *sec21-3* mutant. From transformants that remained temperature resistant, plasmid DNA was isolated and sequenced.

Preparation of Perforated Yeast Spheroplasts and Cytosol

Perforated yeast spheroplasts (semi-intact cells) were prepared as described by Rexach *et al.* (1994).

For cytosol, we grew yeast cells to early to mid log phase in YPD. Wild-type strains were grown at 30°C, and temperature-sensitive (ts) mutant strains at 23°C. The cells were harvested by centrifugation and washed twice with water. The cell pellet was resuspended in a minimal volume of buffer B88 (20 mM HEPES, pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM Mg(OAc) acetate) and pipetted into liquid nitrogen. The cell beads were ground up under liquid nitrogen in a blender (Worthington Biochemical, Lakewood, NJ) for large-scale preparations or in a mortar for small-scale preparations. The cell powder was thawed in an ice-water bath, and 1 mM dithiothreitol and protease inhibitors were added. The lysate was centrifuged (5 min at 3000 × *g*, 15 min at 20,000 × *g*, 1 h at 100,000 × *g*), and the 100,000 × *g* supernatant was collected, carefully avoiding the pellet and the lipids which floated to the top.

Antibodies

Antibodies directed against Sec21p (Hosobuchi *et al.*, 1992), Sec22p (Bednarek *et al.*, 1995), Sec23p (Barlowe *et al.*, 1994), Bet1p, Bos1p, and coatomer (Rexach *et al.*, 1994), Sec7p (Franzusoff *et al.*, 1991), Arf1p (Spang and Schekman, 1998), and α-1,6–linked mannose (Baker *et al.*, 1988) have been described. Anti-Anp1p, anti-Gea1p, and anti-Emp47p antibodies were generous gifts from S. Munro (Cambridge, UK), C.L. Jackson (Saclay, France), and S. Schröder-Köhne (Göttingen, Germany), respectively.

Monoclonal anti-HA (12CA5), anti-myc (9E10), anti-PGK antibodies were purchased from Roche (Mannheim, Germany) and Molecular Probes (Leiden, Netherlands), respectively. Donkey anti-rabbit and sheep anti-mouse secondary antibodies coupled to horseradish peroxidase were obtained from Amersham (Freiburg, Germany). Secondary antibodies coupled to fluorescein isothiocyanate or CY3 were purchased from Dianova (Hamburg, Germany).

Purification of Coatomer, COPII, Uso1p, Sec18p, and N*-Myristoylated Yeast Arf1p*

Coatomer was purified according to the method of Hosobuchi and Schekman (1992). The purification of Sar1p, the Sec23/24 complex, and the Sec13/31 complex was performed as described by Barlowe *et al.* (1994) and Salama *et al.* (1993). A myc-tagged Uso1p was purified from yeast cytosol according to the method of Barlowe (1997). Bacterially expressed Sec18p-His_6 and *N*-myr-yArf1p were purified as previously described (Kahn *et al.*, 1995). The Lma1p complex was a generous gift of Zuoyu Xu (Dartmouth Medical School, Hanover, NH) and was prepared as described previously (Xu *et al.*, 1997).

In Vitro Round-Trip Retrieval Assay and Golgi Budding Assay

Both in vitro assays were performed as described before (Spang and Schekman, 1998). The retrieval assay was performed in the presence of either wild-type cytosol (2 mg/ml) or coatomer (37.5 μg/ml) and Arf1p (12 μg/ml) in stage IV (retrieval from the Golgi to the ER) of the transport reaction. In brief, the assay consists of four stages: stage I, translocation. The translocation reaction using [^{35}S]ppα-factor-myc-HDEL (Dean and Pelham, 1990) and *gls1-1* mutant donor membranes was performed as described; stage II, budding. To the membranes of the stage I reaction, we added 25 μg/ml Sar1p, 25 μg/ml Sec23/24 complex, 75 μg/ml Sec13/31 complex, 50 μM GTP, and an ATP regeneration system (without GDP-mannose; Baker *et al.*, 1988). The reaction mixture was incubated for 30 min at 20°C, chilled for 5 min on ice, and subjected to a medium speed centrifugation (12,000 × *g* for 30 s), which retained COPII vesicles in the supernatant fraction; stage III, fusion. The medium speed supernatant from stage III was supplemented with an ATP-regenerating system (without GDP-mannose), 50 μM GTP, 1 μg/ml Lma1p complex, 1 μg/ml Sec18p, 1.5 μg/ml Uso1p, and 600 μg/ml perforated spheroplast membranes from a *GLS1* strain and were washed twice with B88 or washed with 2.5 M urea and B88 before addition. Fusion was allowed to take place for 15 min at 20°C; stage IV, retrieval. Arf1p (12 μg/ml) or Arf1Q71Lp (12 μg/ml) and coatomer (37.5 μg/ml) were added. Coatomer and Arf1p could be replaced by cytosol at a final concentration of 2 mg/ml. Reactions (100 μl) were incubated for 30 min at 30°C. The reaction mixture was chilled on ice for 5 min, and the acceptor ER was sedimented by centrifugation at 12,000 × *g* for 30 s. The pellet was washed once with 2.5 M urea in B88 for 10 min on ice and once with B88. Membranes in the pellet were resuspended to 100 μl with B88. Fusion with the acceptor ER was measured by precipitation of protease-protected [^{35}S]gpαF-HDEL with concanavalin A-Sepharose.

Nondenatured Yeast Extracts

Yeast cells were grown in YPD to early log phase (OD_{600} 0.5–1.0). The equivalent of 10 OD_{600} was resuspended in 150 μl of B88* (20 mM HEPES, pH 6.8, 150 mM KOAc, 5 mM $Mg(OAc)_2$ in the presence of 1 mM dithiothreitol and protease inhibitors. The cells were lysed with glass beads, and unlysed cells were removed by centrifugation. The cell lysates were adjusted to 1 M NaCl or 0.2% Triton X-100 and incubated for 5 min on ice. For fractionation purposes, we subjected the extracts to a differential centrifugation regimen (5 min at 1000 × *g*, 10 min at 10,000 × *g*, 30 min at 100,000 × *g*) before SDS-PAGE and immunoblot analysis.

Immunofluorescence and Electron Microscopy

Cells were grown in rich medium to early log phase. Immunofluorescence was performed as described by Chuang and Schekman (1996). The staining was observed on an Axioplan microscope (Zeiss, Oberkochen, Germany), and pictures were captured with a charge-coupled device camera. For electron microscopy, we fixed and prepared the cells according to the method of Wuestehube *et al.* (1996). Thin sections were examined under a CM10 electron microscope (Philips Electron Instruments, Eindhoven, Netherlands).

Subcellular Fractionation

The fractionation was performed as described by Newman *et al.* (1992) with the modification described by Sacher *et al.* (1998). In brief, cells of strains YAS61 or YAS88 were grown to early log phase. Cells (600 U at OD_{600}) were harvested, converted into spheroplasts, and lysed in 8 ml of lysis buffer (20 mM HEPES, pH 7.2, 1 mM EDTA, 0.8 M sorbitol). Unbroken cells were removed by a centrifugation for 3 min at 450 × *g*, and membranes were harvested by centrifugation for 10 min at 10,000 × *g*. Particulate material was resuspended and homogenized in 1.5 ml of 55% (wt/vol) sucrose buffered with 20 mM HEPES, pH 7.2, and placed on the bottom of a ultracentrifuge tube. The homogenate was overlaid with 1 ml of 50%, 1 ml of 47.5%, 1.5 ml of 45%, 1.5 ml of 42%, 1.5 ml of 40%, 1 ml of 37.5%, 1 ml of 35%, and 1 ml of 30% sucrose (wt/vol) in 20 mM HEPES, pH 7.2. Gradients were centrifuged for 16 h at 170,000 × *g* in an SW41 rotor (Beckman Instruments, Fullerton, CA). Fractions (800 μl) were collected from the top and sampled for GDPase activity and immunoblot analysis.

GDPase Enzyme Assay

The GDPase assay was performed as described by Yanagisawa *et al.* (1990). Samples (5 μl) were mixed with 50 μl of 20 mM imidazole-HCl, pH 7.4, 2 mM $CaCl_2$, 0.1% Triton X-100, and 5 mM GDP or CDP as a control and incubated for 20 min at 30°C. Reactions were terminated by the addition of 10 μl of 6.7% SDS. P_i was detected by adding 200 μl of 0.35% ammonium molybdate, 0.15% ascorbic acid in 0.86 N H_2SO_4. The assay was allowed to develop for 20 min at 42°C and quantified by reading the absorbance at 700 nm in a plate reader (Tecan Sunrise, Tecan, Crailsheim, Germany).

RESULTS

GEA2 Is a Multicopy Suppressor of sec21-3

To isolate components that regulate vesicle transport between the ER and the Golgi apparatus, we performed a multicopy suppressor screen using a ts COPI mutant. The overexpression of activators or stimulators of the COPI-dependent retrograde transport should mitigate the transport defect and lead to an increased temperature tolerance. We chose the γ-COP mutant *sec21-3* isolated by Gaynor and Emr (1997), because this allele exhibits a very rapid and complete transport block and is therefore suitable for both genetic and biochemical experiments.

A YEp24 (*URA3*, 2μ)-based yeast library (Carlson and Botstein, 1982) was transformed into the *sec21-3* strain, and

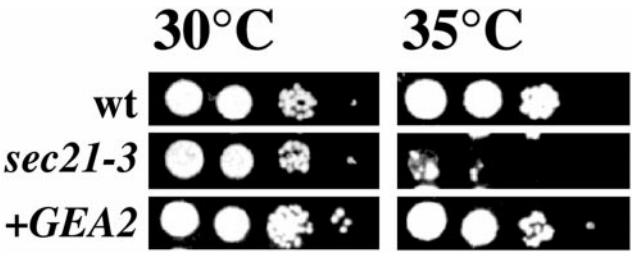

Figure 1. GEA2 is a multicopy suppressor of *sec21-3*. Strains were grown overnight at the permissive temperature in synthetic complete medium-URA to select for the plasmids. Cells were diluted to a final concentration of 10^6 cells/ml, and 10 μl of 10-fold dilutions were spotted on YPD plates. The plates were incubated at the indicated temperatures. *GEA2* overexpression from a 2μ plasmid restored viability of *sec21-3* at 35°C. wt, wild type.

colonies were grown at the permissive temperature (24°C) for 2 d, replica plated, and incubated at 35°C. Of ~40,000 transformants, 26 colonies exhibited a temperature resistance phenotype. From these, the YEp24 plasmids were isolated and retransformed into *sec21-3*. Of these strains, 23 again grew at 35°C, 20 of which contained *SEC21*, one a piece of chromosome VII from bp 399,250–406,876 (according to the Stanford Genome Database), and two a stretch of chromosome V (Figure 1).

The stretch on chromosome V carried one complete open reading frame, which codes for *GEA2. GEA2* is 51% identical to *GEA1*, which was shown to be a GEF for Arf1p (Peyroche *et al.*, 1996). The disruption of the *GEA1* or the *GEA2* gene did not lead to any obvious phenotype. However, the deletion of *GEA1* in a Δ*gea2* background causes lethality (Peyroche *et al.*, 1996; Spang, unpublished results). This suggests that Gea1p and Gea2p have at least partly overlapping functions. *GEA1* overexpression did not rescue the ts defect of a *sec21-3* mutant. Next, we examined the effect of overexpression of *GEA1* and *GEA2* on other *sec21* alleles and different coatomer mutants. Whereas *GEA2* overexpression did not significantly alter the viability of other *sec21* alleles or coatomer mutants, *GEA1* on a 2μ plasmid exaggerated the ts phenotype of the *sec21-2* and *sec33-1* (*ret1-1*) mutants and suppressed a *sec27-1* allele at 33°C (Figure 2). These results suggest that both *GEA1* and *GEA2* genetically interact with coatomer components. However, the mode of interaction of the GEFs with coatomer may be different.

Because yeast has two ARFs that together make up an essential pair, *GEA1* and *GEA2* may operate interchangeably or with overlapping specificity on the two ARFs. To distinguish the contributions of *GEA1* and *GEA2*, we combined the null alleles of *GEA* with a null allele of *ARF1* or *ARF2*. The results are summarized in Table 2. A Δ*gea1*Δ*arf1* double mutant was viable, whereas a Δ*gea2* Δ*arf1* double was not. These results suggest that Gea2p may exchange GTP on Arf2p, which can functionally substitute for Arf1p, whereas Gea1p may be unable to perform this function. Alternatively, Gea2p may have additional functions. An obvious alternative, that *GEA2* is more highly expressed than *GEA1*,

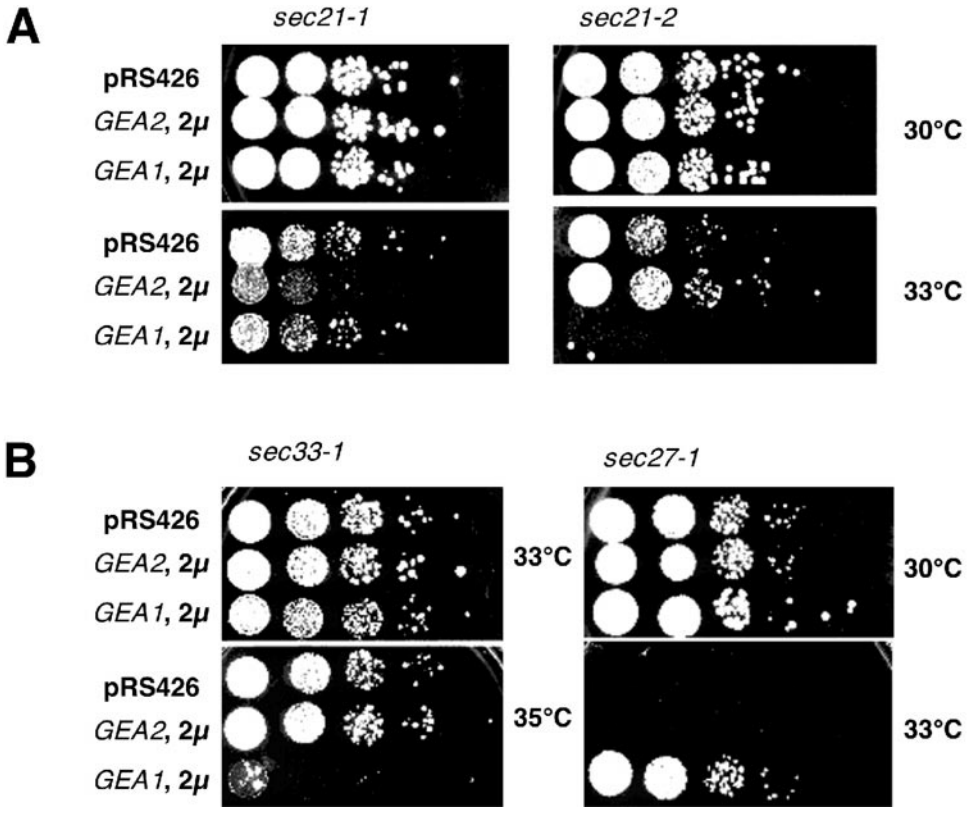

Figure 2. *GEA1* and *GEA2* genetically interact with coatomer. Mutant strains were transformed with a 2μ plasmid (pRS426) either empty or bearing *GEA1* or *GEA2*. Strains were grown and treated as described in Figure 1. The temperatures indicated were chosen because considerable growth of the mutant strains with the empty vector was observed. (A) *GEA1* and *GEA2* do not suppress *sec21-1* and *sec21-2*. *GEA2* suppressed specifically the *sec21-3* allele and did not significantly alter the growth of *sec21-1* and *sec21-2*. In contrast, *GEA1* overexpression enhanced the ts phenotype of the *sec21-2* mutant, whereas *sec21-1* was unaffected. (B) *GEA1* genetically interacts with *sec33-1* and *sec27-1*. Similar to the effect observed in the *sec21-2* mutant, *GEA1* overexpression enhanced the ts sensitivity. In contrast, multicopy *GEA1* suppressed *sec27-1* at 33°C.

Table 2. Genetic interaction between ***GEAs* and *ARFs***

Genotype	Survival	ts
Δ*arf1* Δ*gea1*	Yes	No
Δ*arf1* Δ*gea2*	No	
Δ*arf2* Δ*gea1*	Yes	No
Δ*arf2* Δ*gea2*	Yes	No
Δ*arf3* Δ*gea1*	Yes	No
Δ*arf3* Δ*gea2*	Yes	No

is not indicated by relative mRNA abundance (Holstege *et al.*, 1998). Furthermore, immunoblot analysis, where *GEA1* or *GEA2* were appended with a myc-epitope tag, revealed very similar protein levels in cell lysates (Figure 6A). Thus, we conclude that Gea1p and Gea2p are not fully redundant.

SEC21 and other coatomer genes are essential. The *GEA2*-containing plasmid did not suppress a null allele of *SEC21.* Thus, overexpression of *GEA2* does not bypass the need for Sec21p function.

The gea1-4 Δgea2 *Mutant Accumulates Intracellular Membranes at the Nonpermissive Temperature*

Although the growth rate of Δ*gea1* or Δ*gea2* strains was normal, morphological changes may not be reflected in a growth phenotype. We examined the morphology of single and double mutants grown at a permissive temperature (23°C) or transferred to a restrictive temperature (37°C) for 1 h. Cells were fixed and prepared for electron microscopy as described by Wuestehube *et al.* (1996). The single mutant strains showed normal morphology at either temperature compared with wild type. However, the double mutant was severely affected. ER membranes proliferated extensively at 37°C, and even at 23°C, Golgi membranes and associated vesicles were more abundant than normally seen in wild-type cells (Figure 3, compare A and B to C and D). The defect seen in this strain was consistent with that observed in other retrograde transport mutants, such as *ufe1-1* (Lewis and Pelham, 1996) or in strains depleted of Sec20p (Sweet and Pelham, 1992).

Gea1p and Gea2p Have Overlapping Functions in Retrieval from the Golgi to the ER

Peyroche *et al.* (1996) found that conditional double mutants of *GEA1* and *GEA2* are defective in transport of the vacuolar carboxypeptidase Y. Although GEFs could be involved in anterograde transport, it is more likely that they activate ARFs required to retrieve essential proteins from the Golgi to the ER. To test this hypothesis, we performed a retrieval assay (Spang and Schekman, 1998). Our in vitro assay recapitulates a complete round of transport from the donor ER to the Golgi and back to an acceptor ER compartment. The detection is based on a differential glycosylation pattern in the *N*-glycans of the reporter molecule (core-glycosylated proα-factor to which an HDEL sequence has been fused). The donor membrane is defective in glucosidase I, which is responsible, together with glucosidase II, for the trimming the *N*-glycans of glucosylated proteins before they leave the ER. Therefore, the reporter leaving the ER and arriving in the Golgi apparatus remains untrimmed. The HDEL sequence of the reporter is recognized by Erd2p, the HDEL receptor, and recycled back to the ER. When an acceptor ER that contains functional glucosidase I is used, trimming of the *N*-glycan can occur only when a retrograde vesicle fuses with the acceptor ER membrane. The untrimmed reporter is resolved from the trimmed reporter by SDS-PAGE. The assay starts with donor membranes incubated with COPII proteins to generate COPII-coated vesicles. The vesicles are then allowed to fuse to the acceptor Golgi membranes provided by wild-type or mutant strains. The acceptor Golgi and the acceptor ER are derived from a common source of membranes.

Retrograde transport was detected with wild-type acceptor membranes (Figure 4A). However, acceptor membranes from either Δ*gea1* or Δ*gea2* strains were partially defective in the production of trimmed reporter (Spang, unpublished results; Figure 4A). This defect was more pronounced when the conditional double mutant was used. The addition of wild-type cytosol instead of purified COPI components did not rescue the transport defect. This result indicates that the GEFs were not recruited from the cytosol to the Golgi membrane during the time course of the experiment. Although Gea1p and Gea2p were both capable of sustaining retrograde transport, Sec7p and Syt1p, the two other Arf1-GEF appear not to be involved in this transport step, because *sec7* mutant and Δ*syt1* acceptor membranes were not defective in retrieval from the Golgi to the ER (Figure 4B; Spang, unpublished results). Thus, Gea1p and Gea2p are required at a different step than Sec7p and Syt1p. This conclusion was supported by the observation that overexpression of *SEC7* did not rescue a Δ*gea1* Δ*gea2* strain and did not complement the ts phenotype of the *gea1-4* Δ*gea2* mutant. The defect in retrograde transport was specific because the *gea1-4* Δ*gea2* mutant sustained wild-type levels of gpαF transport from the ER to the Golgi apparatus as assessed by addition of α-1,6-linked mannose residues to the *N*-glycan of the gpαF.

The results above indicate a specific defect in retrieval. However, the defect could occur either at the Golgi membrane in the generation of COPI-coated vesicles or in the consumption of the retrograde transport vesicles by the ER. To distinguish between these possibilities, we enriched Golgi membranes from wild-type and *gea1-4* Δ*gea2* strains and subjected them to a Golgi vesicle budding assay as described by Spang and Schekman (1998). These enriched Golgi membranes were devoid of ER. The formation of the vesicles was examined by immunoblot detection of cargo molecules, such as Emp47p, or v-SNAREs, such as Sec22p and Bos1p (Figure 5). Wild-type Golgi-enriched membranes produced vesicles containing cargo molecules and SNAREs (fractions 6 and 7); however, membranes from the *gea1-4* Δ*gea2* mutant strain were unable to produce COPI-coated vesicles. This result indicates that the *gea1-4* Δ*gea2* mutant strain is specifically defective in the generation of COPI-coated vesicles from the Golgi, consistent with a participation of Gea1p and Gea2p in nucleotide exchange by Arf1p localized to the *cis*-Golgi membrane.

Gea1p and Gea2p Have Partially Overlapping Localization

To follow the Gea1p and Gea2p distribution in the cell, *GEA1* was chromosomally tagged with a myc- or HA-epitope (Knop *et al.*, 1999), and *GEA2* was appended with a

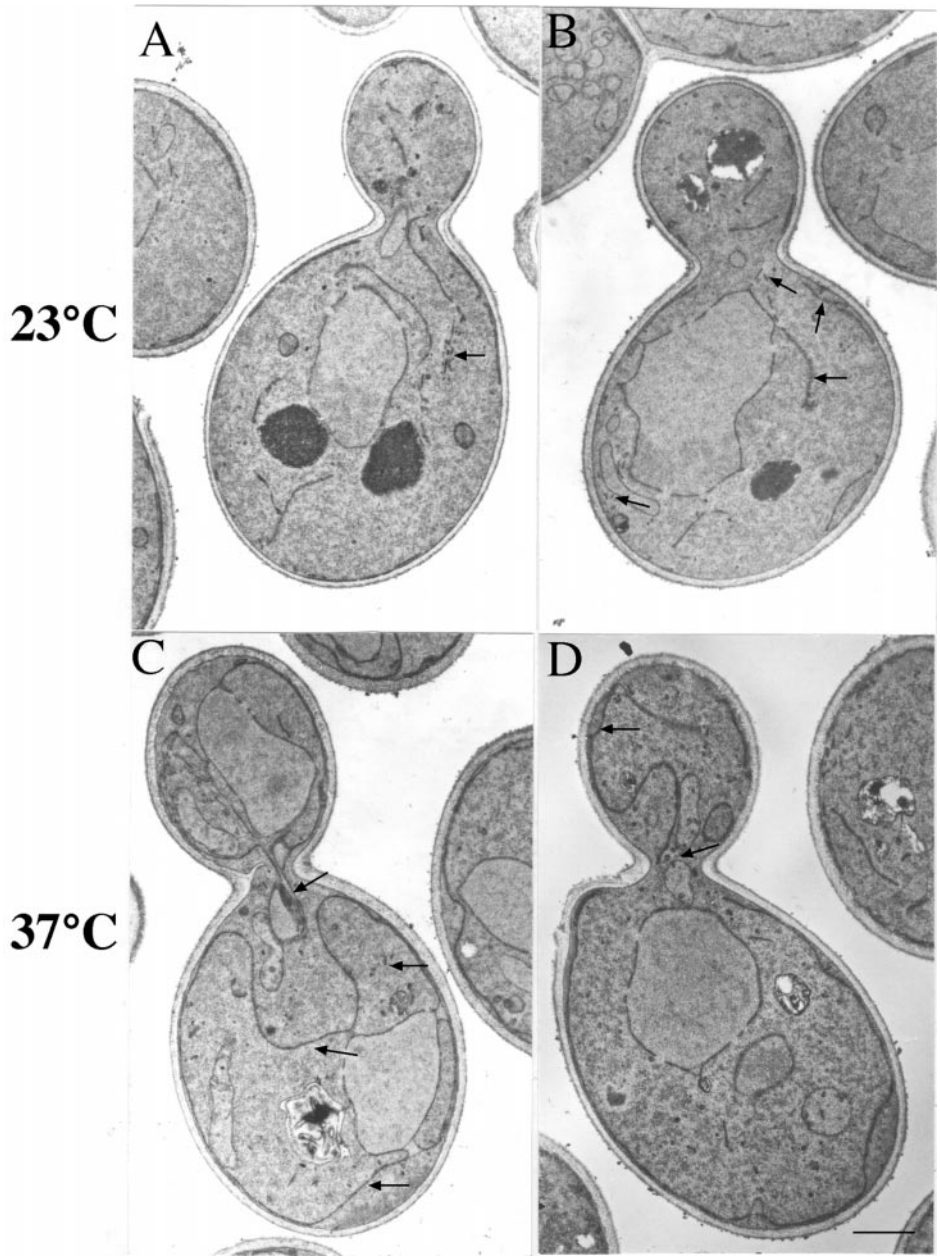

Figure 3. *GEA1 GEA2* conditional double mutants accumulate membranes at the nonpermissive temperature. *gea1-4* Δ*gea2* cells were grown to early log phase in YPD at 23°C (permissive temperature). The culture was split in half. One-half remained at 23°C, and the other half was transferred to 37°C (restrictive temperature) for 1 h. The cells were prepared for electron microscopy and ultrathin sections were stained. (A and B) Cells grown at 23°C. (C and D) Cells that were shifted to 37°C for 1 h. Note the membrane accumulation, especially ER membranes at 37°C and Golgi structures even at 23°C. Some examples are highlighted with arrows. Pictures were taken at the same magnification. Bar, 0.9 μm.

myc-his$_6$-tag to the 3′-coding region. *GEA1:HA* or *GEA1:myc* did not lead to a detectable phenotype in a Δ*gea2* strain background. We showed that the Gea2p fusion protein complemented the ts phenotype of the *gea1-4* Δ*gea2* strain. To determine the subcellular localization of Gea1p and Gea2p, we performed differential centrifugation of gently lysed yeast cells (Figure 6). Most of both GEFs (50–80% depending on the experiment) were soluble. The remaining pool of GEFs was associated with membranes, which sedimented at 10,000 and 100,000 × *g*. Gea1p and Gea2p were solubilized from membranes incubated with 1 M NaCl or 0.2% Triton X-100, whereas the distribution of the Golgi-resident transmembrane protein, Anp1p, or the cytoplasmic protein, Pgk1p, behaved as expected by their domain structure (Figure 6B). Thus, both GEAs are peripherally associated with membranes.

To further characterize the localization of Gea2p, we performed indirect immunofluorescence. Gea2p-myc-his$_6$ was localized to numerous spots within the cytoplasm, which is indicative of Golgi or endosomal compartments in yeast (Figure 7). This staining pattern was comparable to that of two Golgi marker proteins: Och1p, which is an α-1,6-mannosyl transferase, and Emp47p, a Golgi protein known to cycle between the Golgi and the ER.

Gea1p, however, did not show the same distribution (Figure 8). Gea1p appeared to localize in a large number of small

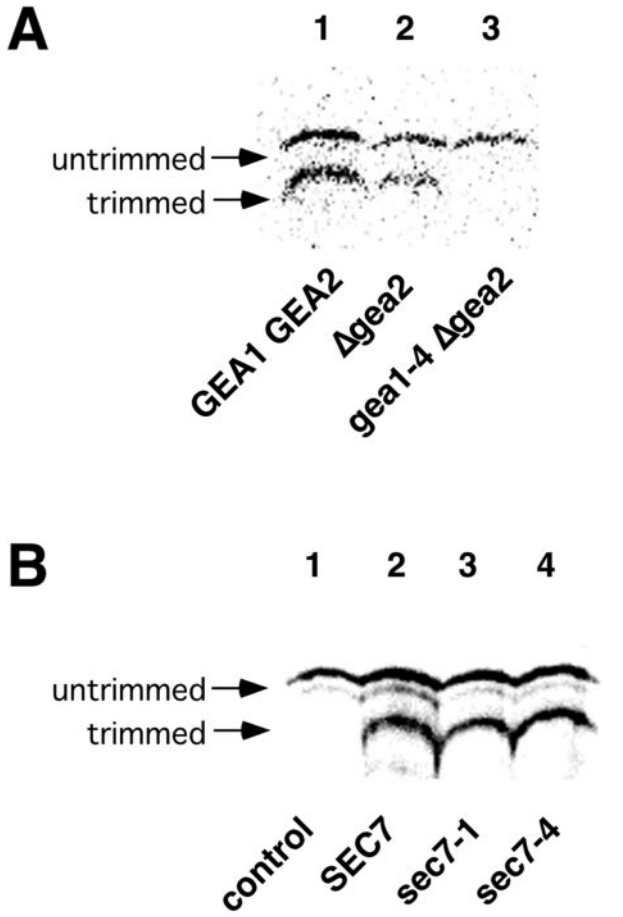

Figure 4. ARF-GEF requirement for retrograde transport from the Golgi to the ER. Membranes of mutant and wild-type strains were prepared from cells grown at a permissive temperature. The membranes were added to stage III of the retrieval assay (for details see MATERIALS AND METHODS). After incubation at 20°C, the samples were complemented with coatomer and Arf1p. Retrieval reactions were incubated at 30°C. At the end of the reaction, samples were subjected to trypsin digestion, concanavalin A-Sepharose precipitation, and SDS-PAGE. The species migrating at the position of trimmed gpαF represents the reporter that has been transported back to the ER. Representative gels are shown. (A) Gea1p and Gea2p support retrograde transport. (B). Sec7p is not required for retrieval of reporter molecules back to the ER. Two different ts mutants were compared with wild-type membranes. In the control, the stage IV (retrieval from the Golgi to the ER) was performed at 4°C.

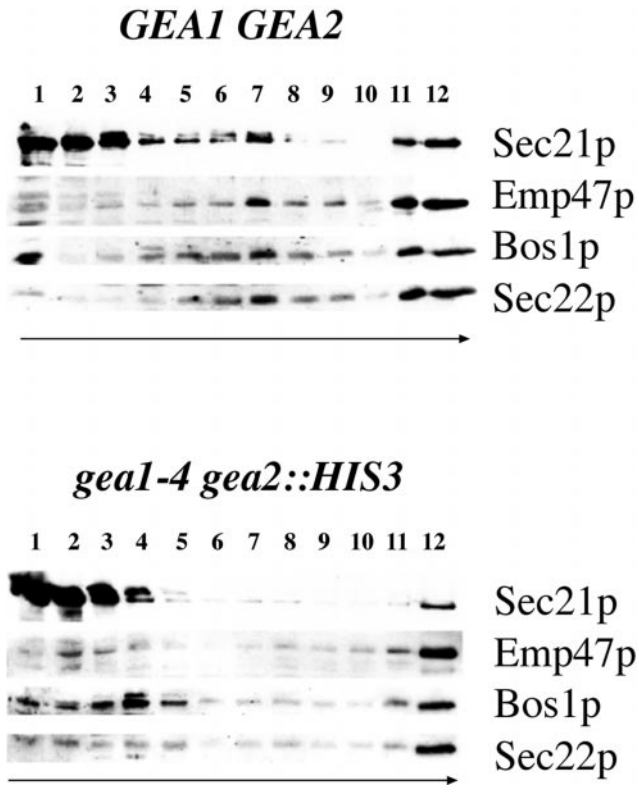

Figure 5. Gea1p and Gea2p are required for the formation of retrograde transport vesicles. Golgi-enriched membranes from either wild-type or *gea1-4 gea2:: HIS3* cells were prepared from cells grown at a permissive temperature. A Golgi budding assay was performed in the presence of Arf1p, guanosine 5′-[γ-thio]triphosphate, and coatomer at 30°C. The reaction sample was loaded on a Ficoll/sucrose gradient and centrifuged for 2 h at 100,000 × *g*. Fractions were collected from the top and concentrated by trichloroacetic acid precipitation, and proteins were separated by SDS-PAGE and analyzed by immunoblot with antibodies directed against Sec21p, Emp47p, Bos1p, and Sec22p. The arrows indicate the direction of the gradient from the top to the bottom. Golgi membranes sedimented to the bottom of the gradient and soluble proteins remained at the top. Coated vesicles peaked in fractions 6 and 7.

foci distributed throughout the cell. No bright foci as in the case of Gea2p were observed. This does not exclude the possibility that Gea1p and Gea2p localization partially overlaps. The localization of Gea1p was unaffected by the use of an epitope-tagged strain using a monoclonal antibody for detection or the preabsorbed anti-Gea1p antibody on a wild-type strain. Overexpression of Gea1p from a 2μ plasmid resulted in an increase of the cytoplasmic pool, and no changes were observed for the localization in the foci. The pattern of Gea1p localization remained unchanged in a Δ*gea2* mutant strain.

To extend these results, we used a fractionation scheme based on membrane buoyant density that allows the separation of different Golgi compartments (Sacher *et al.*, 1998). A membrane pellet of a 10,000 × *g* centrifugation was separated on a sucrose gradient. The location of *cis*-Golgi and medial-Golgi subcompartments was followed by immunoblot and GDPase assays (Yanagisawa *et al.*, 1990). The *cis*-Golgi markers, the KKXX-containing protein Emp47p, the SNAREs Sed5p and Bos1p, and the small GTPase Arf1p peaked in fractions 5 through 9 (Figure 9A). In contrast, the markers for a later Golgi compartment, Anp1p and GDPase activity, were found predominantly in the first five fractions of the gradient (Figure 9). The same distribution was found when a 100,000 × *g* pellet was subjected to fractionation (Spang, unpublished results). Both Gea1p and Gea2p comigrated with *cis*-Golgi markers on this gradient (Figure 9A). In the experiment shown in Figure 9A, we used antibodies

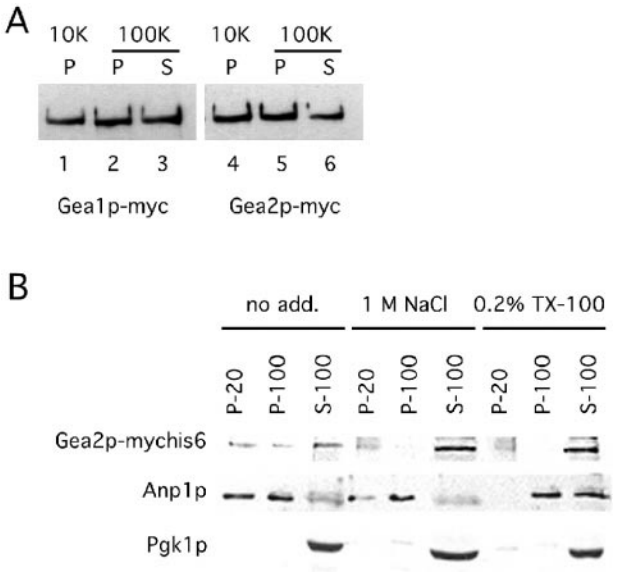

Figure 6. Gea1p and Gea2p are peripheral membrane proteins. (A) Strains that contained either a chromosomal C-terminal myc-tagged Gea1p or Gea2p were grown in YPD to early to mid log phase. Cells were harvested, gently lysed, and subjected to differential centrifugation. To enrich for Gea1p and Gea2p, we subjected the supernatant (S) and the solubilized pellets (P) to immunoprecipitation with anti-myc antibodies coupled to proteinA-Sepharose. The precipitates were analyzed by immunoblot decorated with anti-myc-antibodies. (B) Gea2p is peripherally associated with membranes. YAS61, which contains a chromosomal Gea2p-myc-his$_6$, was grown to early log phase. Cells were harvested and lysed with glass beads under native conditions. Aliquots of the lysate were supplemented with either 1 M NaCl or 0.2% Triton X-100 (TX-100) before differential centrifugation. Samples were analyzed by immunoblot with antibodies directed against the myc-epitope, Anp1p and Pgk1p.

against Gea1p, which gave a very weak signal. However, by this means we were able to visualize Gea1p and Gea2p in the same strain background. An epitope-tagged strain of Gea1p was used, and some Gea1p coincided also in the fractions containing Anp1p and GDPase. This analysis suggested that Gea1p and Gea2p colocalize to some extent consistently with their partially overlapping functions.

DISCUSSION

We identified *GEA2* as a multicopy suppressor of a *sec21-3* mutant. *GEA2* overexpression did not rescue the *SEC21* deletion, indicating that it is not part of the coatomer complex or it can function in place of coatomer. Gea2p is part of the *SEC7* domain-containing family of GEFs for ARFs (Jackson and Casanova, 2000). Peyroche *et al.* (1996) have shown that the Gea1p/Gea2p pair is required in the early secretory pathway: a mutant deleted in *GEA2* and mutated in *GEA1* was not able to process the vacuolar carboxypeptidase Y correctly. We extended these results and show that Gea1p and Gea2p cooperate to sustain retrograde transport from the Golgi to the ER. Because the single deletions of the *GEAs* had no obvious effect, Peyroche *et al.* (1996) concluded that Gea1p and Gea2p are functionally redundant.

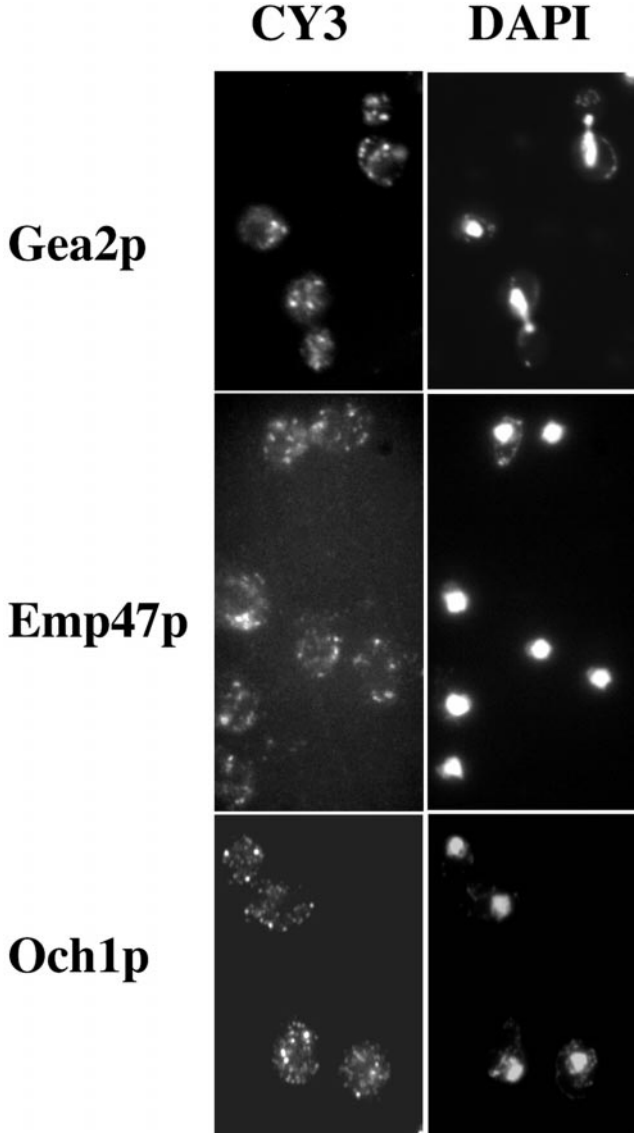

Figure 7. Gea2p localizes to the Golgi apparatus. Cells that bear chromosomal tagged forms of *GEA2*, *EMP47*, and *OCH1* were grown to early log phase and prepared for immunofluorescence. The proteins were visualized by incubation with a monoclonal anti-myc (Gea2p-myc-his$_6$ and Emp47p-myc) or anti-HA (Och1p-HA) antibody followed by a secondary anti-mouse antibody coupled to CY3. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI).

Alternatively, Gea1p and Gea2p may share common functions but also have distinct nonessential roles in the cell such as shown for the another pair of proteins regulating the ARF-cycle: GTPase-activating proteins (GAPs). Gcs1p and Glo3p are ARF-GAPs with overlapping functions in retrograde transport from the Golgi to the ER (Poon *et al.*, 1999). Recently, Dogic *et al.* (1999) reported that *GLO3* is identical to *RET4*, mutant alleles of which display a defect in retrograde transport. Because *ret4-1* cells retain the other ARF-GAP, *GCS1*, Glo3p must be principally responsible for early

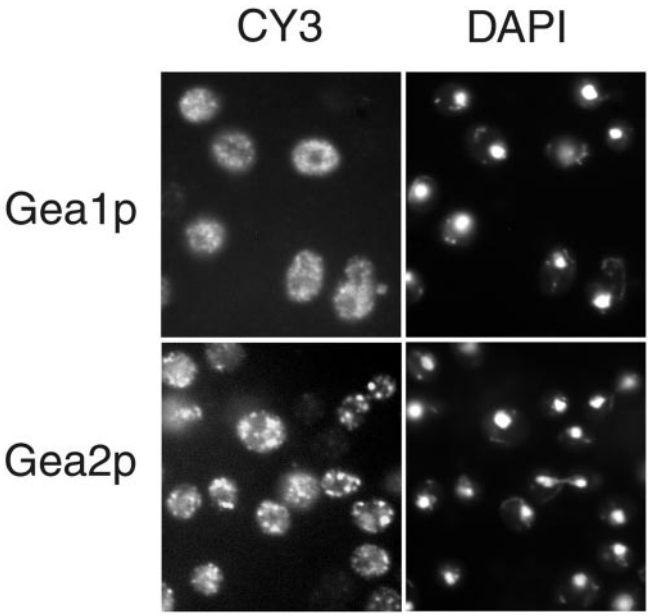

Figure 8. Gea1p and Gea2p show different localization patterns. Strains YAS61 (Gea2p-myc-his$_6$) and YAS88 (Gea1p-myc) were grown in YPD to early to mid log phase and prepared for immunofluorescence. The GEFs were stained with anti-myc antibodies followed by anti-mouse coupled to CY3 antibodies. The DNA was visualized with 4′,6-diamidino-2-phenylindole.

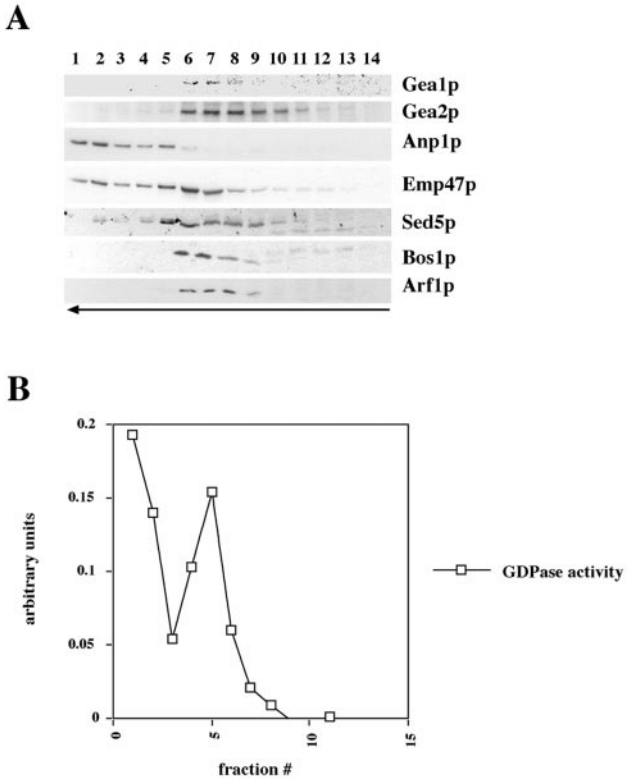

Figure 9. Gea1p and Gea2p cofractionate with *cis*-Golgi proteins. Strain YAS61 (Gea2p-myc-his$_6$) was grown to early log phase. A membrane pellet of a 10,000 × *g* spin was floated to equilibrium on a sucrose gradient. Fractions were collected from the top. The fractions were either separated by SDS-PAGE, and analyzed by immunoblot with antibodies directed against Gea1p, the myc-epitope (Gea2p-myc-his$_6$), Anp1p, Emp47p, Sed5p, Bos1p, and Arf1p (A) or sampled for GDPase activity assays (B). The arrow in A indicates the direction of movement of membranes within the gradient.

retrograde transport. In contrast, Gcs1p has been implicated in normal actin cytoskeletal organization in vivo and stimulates actin polymerization in vitro (Blader *et al.*, 1999). Thus, GEFs normally exert their function at different places and perhaps at different times during the cell cycle. However, three GEFs, Sec7p, Gea1p, and Gea2p, have been implicated in ER to Golgi transport (Franzusoff *et al.*, 1992; Peyroche *et al.*, 1996; Wolf *et al.*, 1998). Although it is possible that the three GEFs have distinct roles in traffic between the ER and Golgi, it is more likely that one or more of these GEFs plays a role later in the pathway, such as in vesicle traffic within or from the Golgi. Mutations in such a GEF may exert a rapid but indirect effect on an early step in the pathway. Sec7p is the GEF least likely to be involved in traffic between the ER and Golgi because *sec7* mutants show no defect in retrograde transport from the Golgi to the ER and *SEC7* overexpression does not suppress a *gea1-4* Δ*gea2* mutant. Furthermore, cargo containing COPI-coated vesicles are produced normally from Golgi membranes derived from a *sec7* mutant strain (A.S., unpublished result). At the *trans*-Golgi, Sec7p could recruit Arf1p to sites for clathrin-coated vesicle formation. Alternatively, Sec7p may be the ARF-GEF for anterograde or retrograde vesicular intra-Golgi transport. A role in the *trans*-Golgi is indicated by the localization of Sec7p to late Golgi cisternae (Franzusoff *et al.*, 1991; Preuss *et al.*, 1992; Seron *et al.*, 1998). In addition, *sec7* ts mutants are suppressed by overexpression of Ypt-GTPases (*YPT 31*, *YTP32*, and *SEC4*) that act at or after the *trans*-Golgi (Jones *et al.*, 1999). Furthermore, mouse *msec7-1* has been reported to be involved in neurotransmitter release (Ashery *et al.*, 1999; Neeb *et al.*, 1999). Thus, Sec7p and Syt1p, which has been identified as a multicopy suppressor of *YPT31* and *YPT32*, may have partially overlapping functions late in secretion.

Gea1p and Gea2p are very similar at first glance: they are 51% identical, they exist in soluble and membrane-bound pools, they both interact at least genetically with coatomer subunits, and each can sustain retrograde vesicular transport from the Golgi to the ER. Nevertheless, there are differences between them: *GEA2* is synthetically lethal with *ARF1*, overexpression of *GEA1* or *GEA2* leads to opposite effects on some coatomer mutants, and the subcellular localizations are not completely coincident. The localization of Gea2p and its interaction with Arf1p makes it the more likely ARF-GEF for early retrograde transport. If so, what is the role of Gea1p? Perhaps the different ARF-GEFs act preferentially at each of the COPI-mediated transport steps: Gea2p for retrograde transport from the Golgi to the ER, Gea1p for intra-Golgi transport, and Sec7p for *trans*-Golgi vesicle budding or later steps. If so, one must explain how GEFs are recruited to their place of action. Is there a "receptor" or does the lipid composition of the different compartments dictate localization? Wolf *et al.* (1996) have reported the existence of a 90-kDa membrane protein that may be the

receptor for Sec7p. However, the identity of this 90-kDa protein has not been reported. In addition, distinct localization could be achieved by being part of a complex with other proteins. The yeast ARF-GEFs are fairly large proteins, and only a small segment (~200 amino acids), the *SEC7* domain, is sufficient for enzymatic activity (Goldberg, 1998; Peyroche *et al.*, 1999). The rest of each protein contains sequences that are much less conserved, which could be involved in achieving the right localization in the cell. ARNO, the little brother of the ARF-GEF in higher eukaryotes, carries a PH domain helping to localize it to phosphoinositide-rich membranes. The yeast ARF-GEFs lack this motif or any other known membrane-localizing motif such as the GRIP domain (Munro and Nichols, 1999). Further analysis, e.g., by use of domain swaps among the yeast GEFs, may illuminate sequences involved in localization.

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