The yeast orthologue of GRASP65 forms a complex with a coiled-coil protein that contributes to ER to Golgi traffic

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The mammalian Golgi protein GRASP65 is required in assays that reconstitute cisternal stacking and vesicle tethering. Attached to membranes by an N-terminal myristoyl group, it recruits the coiled-coil protein GM130. The relevance of this system to budding yeasts has been unclear, as they lack an obvious orthologue of GM130, and their only GRASP65 relative (Grh1) lacks a myristoylation site and has even been suggested to act in a mitotic checkpoint. In this study, we show that Grh1 has an N-terminal amphipathic helix that is N-terminally acetylated and mediates association with the cis-Golgi. We find that Grh1 forms a complex with a previously uncharacterized coiled-coil protein, Ydl099w (Bug1). In addition, Grh1 interacts with the Sec23/24 component of the COPII coat. Neither Grh1 nor Bug1 are essential for growth, but biochemical assays and genetic interactions with known mediators of vesicle tethering (Uso1 and Ypt1) suggest that the Grh1–Bug1 complex contributes to a redundant network of interactions that mediates consumption of COPII vesicles and formation of the cis-Golgi.

Introduction

Formation of the first compartment of the Golgi apparatus involves recognition events between several different membranes. These include tethering of both anterograde COPII vesicles and retrograde COPI vesicles to the cis-Golgi. In addition, intermediate compartment structures that formed in the cell periphery fuse with the cis-Golgi membranes after movement to the cell center, and Golgi stacks undergo homotypic fusion to form the elongated ribbon characteristic of vertebrate cells. Finally, the cis-Golgi is attached to an adjacent medial compartment in the stack. Several proteins have been proposed to guide these membrane recognition events, including the coiled-coil proteins GM130 and p115, the multisubunit complexes transport protein particle (TRAPP) and conserved oligomeric Golgi (COG), and the PDZ-like protein GRASP65 (Whyte and Munro, 2002; Barr and Short, 2003). This latter protein was identified using an in vitro assay for the postmitotic reassembly of mammalian Golgi stacks (Barr et al., 1997). It consists of two PDZ-like domains flanked by a C-terminal Ser/Pro-rich domain and an N-terminal myristoylation site, which is required for its association with the cis-Golgi (Barr et al., 1998; Kondylis et al., 2005). GRASP65 recruits the coiled-coil protein GM130 to the cis-Golgi by binding to the latter's C terminus (Barr et al., 1997). Golgi association of the two proteins appears mutually interdependent, as mutation of the interfacial residues in either protein causes a loss of localization (Barr et al., 1998). GRASP55, a GRASP65 paralogue unique to vertebrates, is found on the medial Golgi but may also contribute to GM130 recruitment (Shorter et al., 1999; Vasile et al., 2003). GM130 also interacts with the coiled-coil protein p115 and the small GTPase Rab1, and it has been proposed that the GRASP65–GM130 complex acts in a variety of tethering interactions at the cis-Golgi (Nakamura et al., 1997; Weide et al., 2001; Kondylis et al., 2005; Puthenveedu et al., 2006).

Despite these compelling physical interactions, analysis of the importance of GRASP65 in vivo has not yet reached a clear consensus. Removal of the protein by RNAi has been reported to affect the formation of Golgi ribbons, the number of cisternae, the structure of the cisternae themselves, and even the formation of the mitotic spindle (Kondylis et al., 2005; Sutterlin et al., 2005; Puthenveedu et al., 2006). However, in all cases, transport through the Golgi appeared relatively normal, and a similar result has been reported for the loss of GM130 (Vasile et al., 2003). These rather variable and perhaps surprisingly mild phenotypes may reflect a degree of redundancy in membrane traffic steps at the cis-Golgi. Not only are there other large

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coiled-coil proteins and tethering factors that could compensate for the removal of GRASP65–GM130, but there may also be redundancy among the multiple membrane fusion events that generate the cis-Golgi (Whyte and Munro, 2002; Barr and Short, 2003; Gillingham et al., 2004).

The understanding of membrane traffic in mammalian cells has been helped by studies of model organisms and, in particular, of the budding yeast *Saccharomyces cerevisiae*. However, the GRASP65–GM130 complex has not been investigated by this route, in part because an *S. cerevisiae* orthologue of GM130 is not detectable by similarity searches. Moreover, the one protein in yeast that is related to GRASP65, Grh1, does not have a myristoylation site at its N terminus and is not essential for growth. Indeed, one study suggested that it could act in a mitotic checkpoint, although this has not been subsequently investigated (Norman et al., 1999). We have investigated Grh1 in more detail, as we noticed that it has an N-terminal amphipathic helix that is a likely target for N-terminal acetylation by the NatC N-terminal acetyltransferase. We had previously found that a similar helix on the Golgi-localized GTPase Arl3 is responsible for targeting it to Golgi membranes (Behnia et al., 2004; Setty et al., 2004). We report here that Grh1 is on the cis-Golgi, and its acetylated N-terminal amphipathic helix appears to replace the membrane-associating role provided by the N-terminal myristoyl group for GRASP65. We find that Grh1 forms a complex with a previously uncharacterized coiled-coil protein, which, although not related in primary sequence, shares several structural features with GM130. Thus, it appears that the role of GRASP65 is better conserved in evolution than previously thought, and our data suggest that this role is in membrane traffic even if it is not essential for secretion in either yeast or mammalian cells.

Results and discussion

Grh1, the yeast orthologue of GRASP65, is a putative substrate for N-terminal acetylation by the NatC complex

To identify proteins whose membrane targeting might be dependent on N-terminal acetylation by the NatC complex, we searched for yeast proteins that had a NatC consensus (F, Y, I, L, or W at the second position; Polevoda and Sherman, 2003) and also an N-terminal amphipathic helix. We then determined whether both the hydrophobic residue at position 2 and the amphipathic helix were conserved in other yeasts and filamentous fungi. This left Grh1, the yeast orthologue of the mammalian

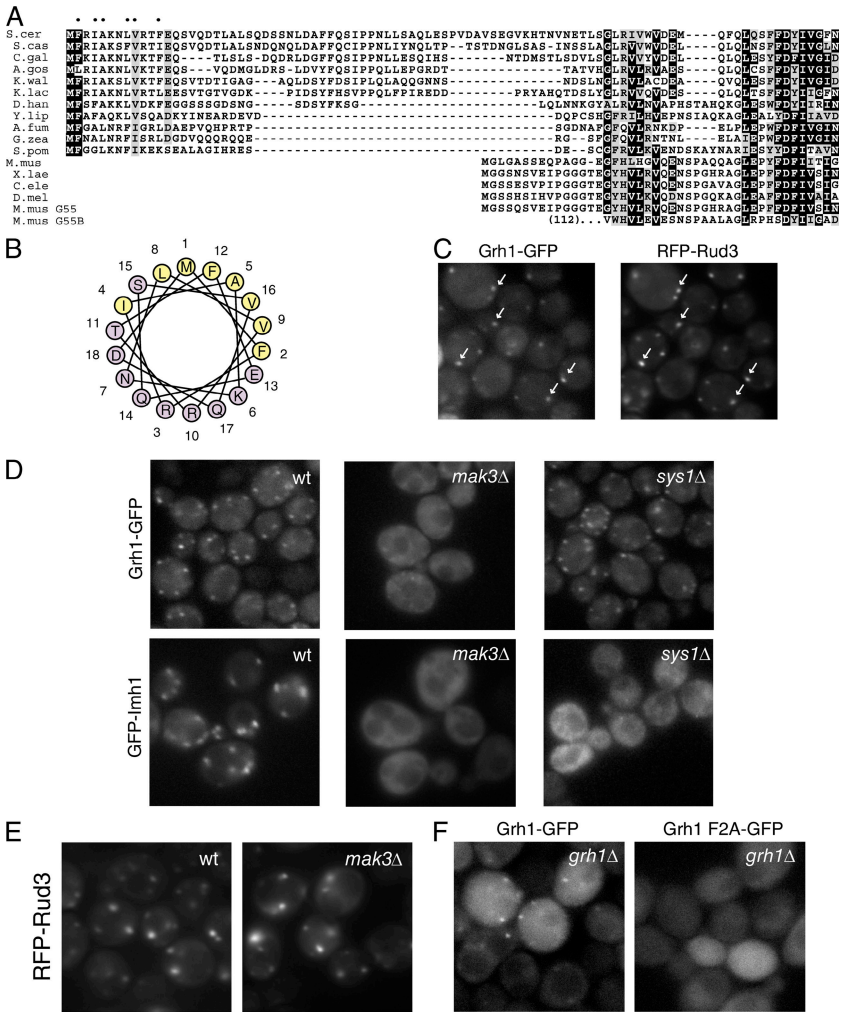

Figure 1. **The N-terminal amphipathic helix of Grh1 is conserved in other fungal homologues.** (A) Alignment of the N termini of *S. cerevisiae* Grh1 and its relatives in *Saccharomyces castellii*, *Candida glabrata*, *Ashbya gossypii*, *Kluyveromyces waltii*, *Kluyveromyces lactis*, *Aspergillus fumigatus*, *Gibberella zeae*, *Debaryomyces hansenii*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe*. The N termini of *Mus musculus* GRASP65 and GRASP55, *Xenopus laevis* GRASP55, and the single orthologues in *Caenorhabditis elegans* (Y42H9AR.1) and *Drosophila melanogaster* (CG7809) are also shown. The second PDZ-like repeat from *M. musculus* GRASP55 (G55B) is meant to indicate where the first repeat starts. The hydrophobic residues that form an amphipathic helix are conserved (dots). Residues are shaded where half or more are identical (black) or similar (gray). (B) Helical wheel representation of the N terminus of Grh1 and the hydrophobic (yellow) and hydrophilic (purple) amino acids. (C) Fluorescence micrographs of live yeast expressing RFP-Rud3 from a constitutive form of the *PHO5* promoter on a CEN plasmid (Gillingham et al., 2004). Cells are BY4741 with genomic *GRH1* tagged with C-terminal GFP, and these and all other images in the paper were obtained on a wide-field microscope as described in Materials and methods. Representative structures containing both Rud3 and Grh1 are indicated by arrows. (D) Fluorescence micrographs of Grh1-GFP and GFP-Imh1 in live cells lacking a genomic copy of *MAK3* or *SYS1* and in the corresponding wild-type (wt) BY4741 strain. Imh1 targeting is dependent on Arl3, which binds to Sys1 in a Mak3-dependent manner (Behnia et al., 2004; Setty et al., 2004). (E) Fluorescence micrographs of BY4741 cells (wt) or the same lacking MAK3, expressing RFP-Rud3 as in C. (F) Fluorescence micrographs of BY4741 cells lacking *GRH1* and expressing wild-type or F2A Grh1-GFP from CEN plasmids as in C.

protein GRASP65 (Fig. 1, A and B). The putative N-terminal amphipathic helix is followed by a region of poorly conserved length and sequence, which could act as a flexible linker. The metazoan orthologues lack this N-terminal extension and instead have a short region with a glycine in position 2, which forms part of the consensus sequence for N-terminal myristoylation.

The NatC complex is required for recruitment of Grh1 to early Golgi membranes

To determine whether NatC action is required for the membrane association of Grh1, we initially examined the subcellular localization of Grh1. When *GRH1* was tagged in the genome with GFP at the C terminus, Grh1-GFP was found to be on punctate structures, which colocalized with the early Golgi marker RFP-Rud3 (Fig. 1 C). When the gene encoding the Mak3 catalytic subunit of the NatC complex was deleted, Grh1-GFP was much more diffuse, with only a few faint dots still visible (Fig. 1 D). This altered distribution of Grh1-GFP did not reflect a general perturbation of the early Golgi, as other markers of this compartment, such as Rud3 and Sed5, were apparently unaffected by the deletion of *MAK3* (Fig. 1 E and not depicted). The use of fractionation to examine membrane association was precluded by the membrane association of Grh1 being readily reversible (>95% being in the soluble fraction after cell lysis and centrifugation at 10^5 *g* for 30 min). However, photobleaching of the diffuse pool of Grh1-GFP in the *mak3*Δ strain showed that it diffuses much more rapidly than a GFP-labeled membrane protein in a strain known to accumulate Golgi-derived vesicles (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb200607151/DC1). This indicates that the loss of Mak3 results in a reduction in the membrane association of Grh1 rather than a vesiculation of Grh1-positive membrane structures. In addition, when Phe2 of Grh1 was mutated into an alanine, which precludes the generation of an acetylated N terminus by NatC, the mutant protein was substantially mislocalized to the cytosol, indicating that the precise structure of the N terminus is important for Grh1 localization (Fig. 1 F).

To examine the modification state of the N terminus of Grh1, Grh1-Flag3 was purified from both wild-type and *mak3*Δ strains. Mass spectrometry of proteolytic products from the protein obtained from the wild-type strain revealed a peptide corresponding to an acetylated N terminus, with this being replaced in the *mak3*Δ-derived protein with a nonacetylated peptide beginning with Met1 (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200607151/DC1). A similar NatC-dependent N-terminal modification is found on the Golgi GTPase Arl3, and, in this case, the modification is required for recognition by the Golgi membrane protein Sys1 (Behnia et al., 2004; Setty et al., 2004). However, deletion of the gene encoding Sys1 did not affect the localization of Grh1 (Fig. 1 D). Thus, the localization of Grh1 to the cytosolic face of the early Golgi membranes is Mak3 dependent but Sys1 independent.

Grh1 binds to a second protein, Bug1, and the two are interdependent for their localization to the early Golgi

Mammalian GRASP65 interacts with the coiled-coil protein GM130, but there is no obvious homologue of GM130 outside of metazoans. Thus, binding partners for Grh1 were sought using the protein as bait in a yeast two-hybrid screen. Of 25 positives, two were Grh1 itself, and the remainder was an uncharacterized open reading frame YDL099w that we named *BUG1* (bound to Grh1). Bug1 is a 342-residue protein with an N-terminal basic region and a potential coiled-coil domain (see Fig. 3 A), which is a structure reminiscent of that of GM130.

When *BUG1* was tagged in the genome with GFP at either the C or N terminus, the resulting fusion proteins localized to punctate structures (Fig. 2 A and not depicted), but the N-terminally tagged version gave less cytosolic background

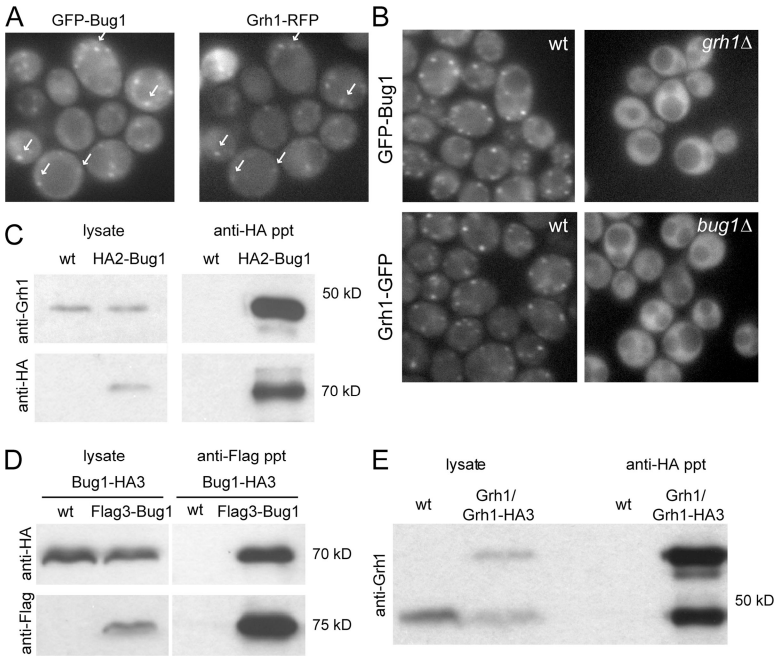

Figure 2. **Grh1 and Bug1 colocalize on the cis-Golgi and form a heterooligomeric complex.** (A) Fluorescence micrographs of Grh1-RFP expressed on a CEN plasmid from its own promoter in a Δ*grh1* strain with the genomic copy of *BUG1* tagged at the N terminus with GFP (RBY85). Representative structures containing both Bug1 and Grh1 are indicated by arrows. (B) Fluorescence micrographs of GFP-Bug1 in strains with or without *GRH1* and of Grh1-GFP in strains without or without *BUG1*. (C) Anti-HA immunoprecipitates from a protease-deficient strain with or without (wt) *BUG1* tagged with HA2 in the genome and then probed with the indicated antibodies. (D) Anti-Flag immunoprecipitates prepared from cells with a HA3 tag at the C terminus of genomic *BUG1* and containing CEN plasmids without (wt) or with Flag3-Bug1 expressed as in Fig. 1 C. Samples were blotted with the indicated antibodies. (E) Anti-HA immunoprecipitates prepared from a diploid strain without (BY4743; wt) or with (RBY84) one copy of *GRH1* tagged C terminally with HA3 and probed for Grh1.

and was used for the rest of the experiments. GFP-Bug1 colocalizes with Grh1-RFP but is completely mislocalized to the cytosol in the absence of Grh1 (Fig. 2, A and B) and is diffuse, with only a few dots remaining in the absence of Mak3 (not depicted). Interestingly, Grh1-GFP, in turn, is mislocalized in a *bug1*Δ strain (Fig. 2 B). Thus, it appears that Grh1 and Bug1 are interdependent for their localization to the Golgi.

To confirm the interaction between Grh1 and Bug1, HA2-Bug1 was immunoprecipitated, and endogenous Grh1 was found to be enriched in the precipitate (Fig. 2 C). Consistent with this result, recent high throughput screens of yeast protein–protein or genetic interactions have indicated that Bug1 coprecipitates with Grh1-TAP (Schuldiner et al., 2005; Gavin et al., 2006; Krogan et al., 2006). We also found that Bug1-HA3 could be immunoprecipitated with Flag3-Bug1 and that untagged Grh1 could be coprecipitated with Grh1-HA3 (Fig. 2, D and E). Thus, both of the proteins form at least dimers, and, therefore, Bug1–Grh1 is a heterooligomer composed of at least a dimer of Grh1 and a dimer of Bug1. Indeed, by gel filtration, Grh1 runs at ∼400 kD, which corresponds to the mass of two molecules each of Bug1 and Grh1 (unpublished data).

Grh1 binds to the C terminus of Bug1

We next generated truncated forms of Bug1 and compared their ability to interact with Grh1 with their effect on the latter's localization. Removal from Bug1 of the N-terminal basic region either alone or with the adjacent poorly conserved region (amino acids 44–340 and 185–340) had no effect on Grh1 binding or localization (Fig. 3). A construct also lacking the coiled-coiled region (i.e., just the last 65 amino acids of Bug1 [275–340]) could only be detected after immunoprecipitation but was clearly able to bind to Grh1. Moreover, a construct lacking the last 30 amino acids of Bug1 (1–310) was no longer able to bind Grh1. Thus, the binding site for Grh1 is located at the well-conserved C terminus of Bug1. However, recruitment of Grh1 to membranes in vivo appears to require not only this region but also the adjacent putative coiled coil (Fig. 3 B). Therefore, the C terminus of Bug1 interacts with Grh1, but its coiled-coil region also contributes to Golgi membrane association. Together, this results in a robustly localized complex.

Grh1 binds to the components of the inner COPII coat but is not required for budding of COPII-coated vesicles

During the purification of Grh1-Flag3 to examine the N terminus, we noticed several proteins of 80–100 kD in the precipitate from wild type that were absent from that prepared from the *mak3*Δ strain (Fig. 4 A). Mass spectrometry of tryptic peptides identified these proteins as the components of the COPII coat, Sec23 and Sec24, along with the two relatives of the latter, Sfb2 and Sfb3. Probing with anti-Sec23 antibodies confirmed this identification (Fig. 4 B), and this interaction was also detected in recent high throughput screens (Schuldiner et al., 2005; Gavin et al., 2006; Krogan et al., 2006). Sec23 forms stable dimers with Sec24 or its relatives even when the COPII coat is disassembled (Peng et al., 2000). These results suggest that Grh1 can interact with this dimer and that this interaction requires N-terminal acetylation.

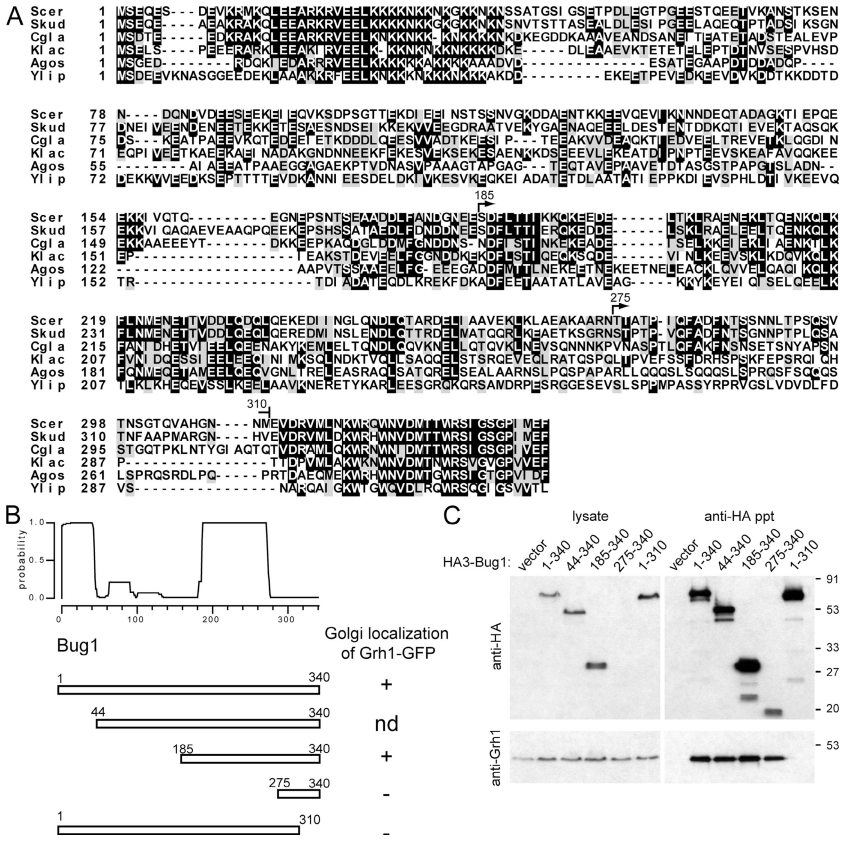

Figure 3. **The binding site for Grh1 is located at the C terminus of Bug1.** (A) Alignment of Bug1 with its homologues in other yeasts. The locations of the truncations are indicated. Residues are shaded where half or more are identical (black) or similar (gray). (B) Coiled-coil prediction for Bug1 (MacStripe) along with Bug1 truncations that were tagged at the N terminus with HA3 and expressed as in Fig. 1 C in a Δ*bug1* strain (RBY46) to examine the effect on the localization of Grh1-GFP. (C) Anti-HA immunoprecipitates from a *bug1*Δ strain expressing HA3-tagged Bug1 truncations as in B. Lysates and precipitates were probed with anti-Grh1 or -HA antibodies.

To investigate the role of Grh1 and Bug1 in ER to Golgi transport, we examined COPII-dependent budding and transport reactions in an in vitro assay based on semi-intact cells (Barlowe, 1997). ER-derived transport vesicles were produced from washed membranes after incubation with purified COPII proteins (Sar1, the Sec23/24 complex, and the Sec13/31 complex). Grh1 was detected on COPII vesicles produced from wild-type membranes (Fig. 4 C). The level of Grh1 associated with vesicles relative to total membranes was less than Sec22 (a vesicle SNARE protein) but above the level of an ER-resident protein, Sec12. In the *grh1*Δ strain, Grh1 was absent as expected, and, in the *mak3*Δ strain, the amount of Grh1 that associated with vesicles was reduced.

To quantify COPII-dependent budding and transport to the Golgi complex in the reconstituted assays, we measured the amount of [^{35}S]gpαf packaged into vesicles and the amount of Golgi-modified [^{35}S]gpαf (Barlowe, 1997). The level of COPII-dependent budding of gpαf was not substantially influenced by the *grh1*Δ, *mak3*Δ, or *bug1*Δ deletions (Fig. 4 D). In the additional presence of Uso1 (the yeast homologue of the coiled-coil tethering protein p115), Sec18, and LMA1, such vesicles fuse with Golgi membranes, as detected by the acquisition of Golgi-dependent carbohydrate modifications (Barlowe, 1997), and this showed a small but reproducible decrease for all three deletions (Fig. 4 E). The *bug1*Δ deletion produced the strongest decrease and reduced overall transport by ~60%. When just Uso1 is present, the vesicles are known to become tethered without fusing, as assayed by a reduction in the population of freely diffusible vesicles (Cao et al., 1998). Strikingly, this Uso1-dependent tethering was greatly diminished for all three deletions (Fig. 4 E). These results indicate that semi-intact cell membranes lacking Grh1 or Bug1 produce COPII vesicles normally but are partially compromised in the fusion stage of ER-derived vesicles with Golgi membranes. This appears to reflect, at least in part, Uso1-dependent tethering being reduced to the point where it is, at most, no longer sufficient to maintain a tethered state through the centrifugation step used to separate tethered from diffusible vesicles.

Deletions of *GRH1* and *BUG1* show genetic interactions with components of ER to Golgi traffic

The lethality of yeast strains lacking the GTPase Ypt1 or the tether Uso1 can be suppressed by *SLY1-20*, a dominant mutation in Sly1, which is a member of the Sec1/Munc18 family of SNARE regulators (Dascher et al., 1991; Sapperstein et al., 1996). This suggests that tethering of COPII vesicles before consumption can occur by Uso1-independent processes, and we hypothesized that these processes might also depend on the Grh1–Bug1 complex. Fig. 5 shows that yeast cells lacking *USO1* or *YPT1* cannot be rescued by *SLY1-20* if either *BUG1* or *GRH1* is absent. The deletion of *MAK3* also affected the ability of *SLY1-20* to rescue the loss of *USO1*, although growth was not completely impaired with a few larger colonies appearing after

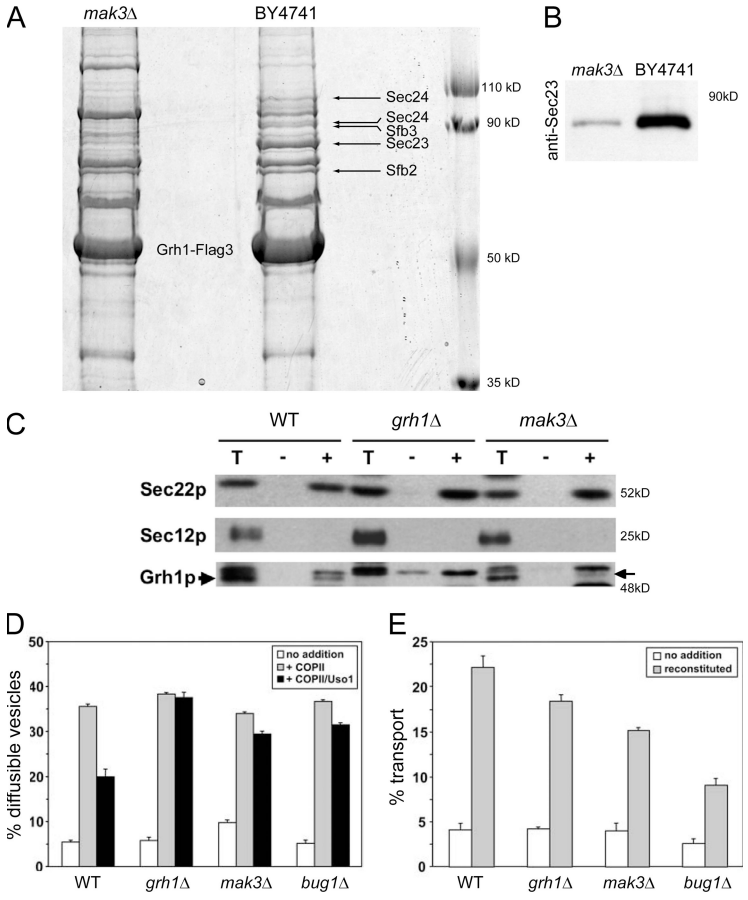

Figure 4. **Grh1 binds to the Sec23/24 complex and to reconstituted COPII-coated vesicles.** (A) Coomassie-stained gel of anti-Flag precipitates from 1 g of yeast lacking *MAK3* or the corresponding wild-type strain, both expressing Grh1-Flag3 from a CEN plasmid as in Fig. 1 C. The indicated bands were identified by mass spectrometry. The bottom Sec24 band presumably corresponds to a degradation product, and Sfb2 was in a mixture with the heat-shock protein Ssb1. (B) 10% of the samples used in A were blotted with an anti-Sec23 antiserum. (C) Reconstituted COPII budding assays with indicated membranes and purified COPII proteins. One tenth of a total reaction (T) and budded vesicles produced in the absence (−) or presence (+) of COPII proteins were immunoblotted for the indicated ER and vesicle marker proteins. Grh1 (arrowhead) migrated just below a major cross-reactive species that was present in all three strains. Grh1 (arrow) migrated just ahead of a prominent background band. WT, wild type. (D) Level of [^{35}S]gpαf packaged into freely diffusible COPII vesicles in an in vitro budding reaction. The reaction was performed with no addition (white bars), with the addition of purified COPII proteins (gray bars), or the same with the tethering factor Uso1 (black bars). The latter tethers COPII vesicles to the Golgi and reduces the diffusible pool. (E) Overall ER/Golgi transport of [^{35}S]gpαf with washed membranes and no addition (white bars) or with the addition of purified factors (COPII, Uso1, and LMA1) to reconstitute transport (gray bars). Error bars represent SD.

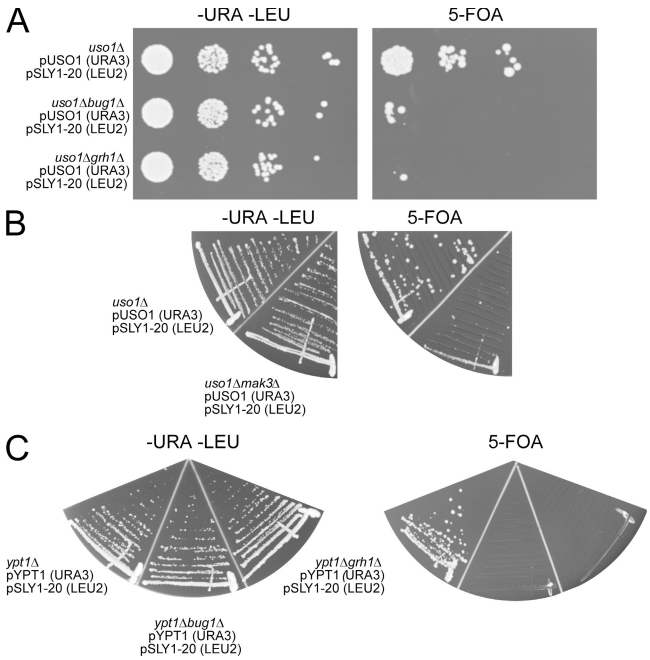

Figure 5. **Grh1, Bug1, and Mak3 are required to allow suppression of loss of the early Golgi transport components Uso1 or Ypt1.** (A) Serial dilutions of *uso1*Δ/pUSO1 pSLY1-20, *uso1*Δ*bug1*Δ/pUSO1 pSLY1-20, or *uso1*Δ*grh1*Δ/pUSO1 pSLY1-20 strains spotted on −URA −LEU or 5-fluoorotic acid (5-FOA) plates and incubated at 30°C. 5-fluoorotic acid is toxic to cells expressing Ura3. (B) *uso1*Δ/pUSO1 pSLY1-20 and *uso1*Δ*mak3*Δ/pUSO1 pSLY1-20 were streaked on −URA −LEU or 5-fluoorotic acid plates and incubated at 30°C. (C) *ypt1*Δ/pYPT1 pSLY1-20, *ypt1*Δ*bug1*Δ/pYPT1 pSLY1-20, or *ypt1*Δ*grh1*Δ/pYPT1 pSLY1-20 strains were streaked on −URA −LEU or 5-fluoorotic acid plates and incubated at 25°C because *SLY1-20* does not rescue the *ypt1*Δ phenotype at 30°C.

several days, perhaps corresponding to suppressor mutations. This partial effect is consistent with Grh1-GFP not being completely mislocalized in a *mak3*Δ strain. Thus, the rescue of strains lacking Uso1 or Ypt1 by *SLY1-20* relies on the Grh1–Bug1 complex.

The Grh1–Bug1 complex appears to contribute to membrane traffic at the cis-Golgi, and biochemical and genetic interactions suggest a role in ER to Golgi transport. Recruitment of the complex to membranes is dependent on an N-terminally acetylated amphipathic helix on Grh1, indicating that N-terminal acetylation has a relevance to membrane targeting beyond the two Arf-like GTPases for which it has been previously shown to be important (Behnia et al., 2004; Hofmann and Munro, 2006). The role of Grh1 and Bug1 may be to improve the efficiency of both Uso1-dependent and independent tethering events. Formation of the cis-Golgi is likely to involve several heterotypic and homotypic fusion events between nascent cis-cisternae, COPI vesicles, and COPII vesicles, and such multiplicity could allow cell growth even when one or more fusion pathways are compromised. Alternatively, it may be that multiple interactions are used to increase the accuracy of recognition of a specific fusion target such as the cis-Golgi and that a partial reduction in accuracy does not prevent viability. Interestingly, in higher eukaryotes, p115 but not GM130 or GRASP65 are required for secretion in cultured cells even though the latter clearly interact with p115 in assays that reconstitute fusion with cis-Golgi membranes (Sonnichsen et al., 1998; Puthenveedu and Linstedt, 2001; Kondylis et al., 2005; Puthenveedu et al., 2006). This suggests that the GM130–GRASP65 complex serves to optimize rather than allow membrane traffic in the early Golgi and that the constraints of the in vitro systems highlight a role for components whose loss can normally be bypassed in cells growing in optimal conditions. Given that this mammalian machinery now appears to have yeast analogues, it seems likely that further examination of Grh1 and Bug1 and other putative cis-Golgi tethers will shed light on the precise function of the mammalian proteins.

Materials and methods

Yeast strains, plasmids, and antibodies

Unless otherwise stated, yeast strains were based on BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), BY4742 (MATα *his3 leu2 lys2 ura3*), or disruptions in this background (Open Biosystems), with further genes disrupted or epitope tagged by PCR-based homologous recombination (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200607151/DC1). Immunoprecipitations were performed from the protease-deficient strain c13-ABYS-86 (MATα *pra1-1 prb1-1 prc1-1 cps1-3 ura3Δ5 leu2-3 his*$^-$) unless otherwise stated. The strains for suppression analysis were based on parental strains CBY903 (MATa *trp1Δ63 his3Δ200 ura3-52 leu2 Δypt1::HIS3* carrying pSK54) and CBY1381 (MATα *his3Δ ura3Δ leu2Δ met15Δ lys2Δ Δuso1::kanMX* carrying *pSK47* and pSLY1-20) and on the plasmids pSK54 (*URA3 2μm SLY1-20*), pSLY1-20 (*LEU2 2μm SLY1-20*), pSK47 (*URA3 2μm USO1*), and pRB320 (*URA3 2μm YPT1*), which were described previously (Ballew et al., 2005). An anti-Grh1 antiserum was generated in sheep, and anti-Sec23 antibodies were described previously (Peng et al., 2000).

Immunoprecipitations and fluorescence microscopy

For small-scale immunoprecipitations, 50–100 mg of yeast pellets were lysed by the addition of 200 μl of glass beads (425–600 μm; Sigma-Aldrich) and 200–400 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, and 1% Triton X-100) containing protease inhibitors (Roche), and the tubes were vortexed twice in a RiboLyser (Thermo-Hybaid) at speed setting six at 4°C. After centrifugation for 15 min at 12,000 *g*, the supernatants were incubated with 20 μl anti-HA F-7 agarose beads (Santa Cruz Biotechnology, Inc.) or anti-Flag M2 agarose beads (Sigma-Aldrich) for 2 h at 4°C. The beads were washed in lysis buffer and eluted with SDS sample buffer.

Grh1-Flag3 was expressed on a CEN *URA3* plasmid under the control of a constitutive *PHO5* promoter in the wild-type strain BY4741 or the same lacking *MAK3*, and protein was precipitated from 1 g of cells as described previously (Behnia et al., 2004). For reasons that are not clear, coprecipitation of COPII coat components with Grh1-Flag3 was more efficient from 1 liter rather than 100-ml cultures. For mass spectrometry, the gel was stained with Coomassie blue, and bands were excised, digested with trypsin or Lys-S, and peptides were subjected to matrix-assisted laser desorption ionization mass spectrometry.

Fluorescence micrographs were obtained at room temperature with a 100× 1.3 NA plan Neofluor objective on a microscope (Axioplan2; Carl Zeiss MicroImaging, Inc.) and with a camera (CCD-1300; Princeton Instruments) using 1–2-s exposures controlled with IPLab software (Scanalytics). Image processing was restricted to adjusting levels in Adobe Photoshop but maintaining a γ value of 1.0.

In vitro assays for COPII-dependent transport

Yeast semi-intact cells were prepared and analyzed in reconstituted cell-free budding and transport assays as previously described (Barlowe, 1997; Liu and Barlowe, 2002). Yeast strains CBY740 (*MATα his3 leu2 lys2 ura3*), CBY2009 (CBY740 *grh1Δ::KAN*), CBY2028 (CBY740 *mak3Δ::KAN*), and CBY2029 (CBY740 *bug1Δ::KAN*) were purchased from Invitrogen and are isogenic with BY4742.

Online supplemental material

Fig. S1 shows that Grh1-GFP is displaced from membranes by the loss of Mak3. Fig. S2 shows that the N terminus of Grh1 is acetylated in wild-type but not in *mak3*Δ cells. Table S1 provides information about the yeast

strains generated during this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200607151/DC1.

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