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 Set/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
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. This left Grh1, the yeast orthologue of the mammalian...
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 deletion of the genome copy of GRH1 (RBY84) one copy of GRH1 (RBY85). Representative structures containing both Bug1 and Grh1 are indicated by arrows. (B) Fluorescence micrographs of GFP-Bug1 expressed on a CEN plasmid from its own promoter in a Δgrh1 strain with the genomic copy of Bug1 tagged at the N terminus with GFP (RBY85).

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 protein obtained from a diploid strain without or with BUG1. (A) Fluorescence micrographs of Grh1-RFP expressed on a CEN plasmid from its own promoter in a Δgrh1 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 or with Bug1 expressed on a CEN plasmid from its own promoter in a Δgrh1 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. (D) Anti-Flag immunoprecipitates prepared from cells with a HA3 tag at the C terminus of genomic Bug1 and containing CEN plasmids without or with Bug1 expressed on a CEN plasmid from its own promoter in a Δgrh1 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.
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 \textit{bug1}\textsuperscript{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 \(\sim400\) 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 \textit{mak3}\textsuperscript{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.
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 grh1Δ strain, Grh1 was absent as expected, and, in the mak3Δ 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 [35S]gp of packaged into vesicles and the amount of Golgi-modified [35S]gp (Barlowe, 1997). The level of COPII-dependent budding of gp was not substantially influenced by the grh1Δ, mak3Δ, or bug1Δ 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 bug1Δ 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
cis-Golgi membranes (Sonichsen 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 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), BY4742 (MATα his3Δ3 leu2Δ2 lys2Δ2 ura3Δ0), 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 pra1Δ1 pbi1Δ1 prc1Δ1 psi1Δ1 csl3Δ1 ura3Δ5 leu2Δ3 his3Δ1) unless otherwise stated. The strains for suppression analysis were based on parental strains CBY903 (MATα trp1ΔΔ his3Δ200 ura3Δ2 leu2Δ2 ypt1Δ1Hi353 carrying pSK54) and CBY1381 (MATα his3Δ3 ura3Δ5 leu2Δ3 met15Δ3 lys2Δ2 ωα1::kanMX carrying pSK47 and pSLY1-20) and on the plasmids pSK54 (URA3 2 μm YSL1-20), pSLY1-20 (LEU2 2μm SLY1-20), pSK47 (URA3 2μm USO1), and pRB320 (URA3 2μm YPT1), which were previously described (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 MgCl2, 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 F7 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 (Scion). 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; Lu and Barlowe, 2002). Yeast strains CBY740 (MATα his3Δ3 leu2Δ2 lys2Δ2 ura3Δ0), CBY2009 (CBY740 grh1Δ::KAN), CBY2028 (CBY740 grh1Δ::KAN), and CBY2029 (CBY740 prb1Δ1::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 mak3Δ 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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