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Characterization of Grp1p, a novel *cis*-Golgi matrix protein

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Abstract

A high copy suppressor screen with *sec34-2*, a temperature-sensitive mutant defective in the late stages of ER to Golgi transport, has resulted in the identification of a novel gene called *GRP1* (also called *RUD3*). *GRP1* encodes a hydrophilic yeast protein related to the mammalian Golgi matrix protein golgin-160. A large portion of the protein is predicted to form a coiled-coil structure. Although *GRP1* is not essential for growth, the loss of Grp1p results in a growth defect at high temperature. *GRP1* genetically interacts with several genes involved in vesicle targeting/fusion stages of ER to Golgi transport. Despite these interactions, pulse chase analysis using Grp1p-depleted cells did not reveal a significant delay in the transit of the vacuolar protease carboxypeptidase Y. Grp1p-depleted cells efficiently secreted invertase which was underglycosylated, suggesting some disturbance of Golgi function. Grp1p-GFP predominantly colocalizes with the *cis*-Golgi marker Och1p. Despite lacking a signal peptide and a significant stretch of hydrophobic amino acids, Grp1p pellets with membranes. It is extracted with 1 M NaCl or 0.1 M Na_2CO_3 (pH 11.0), but is surprisingly insoluble in 1% Triton X-100. Grp1p does not recycle to the ER when forward transport is blocked and a *cis*-Golgi marker (Och1p-HA), but not a *trans*-Golgi marker (Chs5p-HA), became dispersed in *grp1*Δ cells after 1.5 h incubation at 38.5 °C. Together, these data suggest that Grp1p is a novel matrix protein that is involved in the structural organization of the *cis*-Golgi.

The Golgi apparatus is an organelle that is involved in the stepwise processing and sorting of cargo received from the endoplasmic reticulum (ER). It consists of compartmentalized and polarized stacks of membranous cisternae that reside in the juxtanuclear regions in mammalian cells whereas it comprises punctate structures scattered throughout the cytoplasm in yeast *Saccharomyces cerevisiae*. The proteins synthesized in the ER and destinated for different cellular and extracellular locations enter the Golgi complex where they are post-translationally modified and then sorted to their correct destination.

All known Golgi resident proteins so far are either integral membrane proteins or peripheral proteins located on the cytoplasmic face of the Golgi [1]. There are several types of the Golgi resident proteins: (1) modifying enzymes, (2) proteins involved in the membrane traffic, and (3) structural proteins. The modifying enzymes of the Golgi complex such as glycosylase are mainly integral membrane proteins that face the lumen of the Golgi where they carry out the glycosylation of the cargo in transit. The proteins involved in vesicular transport or the maintenance of Golgi structure are either integral membrane proteins that face the cytoplasm or peripheral Golgi proteins [2,3].

The unique structure of the Golgi apparatus is thought to depend on the cytoskeleton and cytoplasmic matrix proteins. The best characterized matrix proteins comprise the GRASP family of stacking proteins and the golgin family of fibrous, coiled-coil proteins [4]. A general feature of matrix proteins is their insolubility in Triton X-100 [2]. GRASP55 and GRASP65 are involved in the stacking of Golgi cisternae [5,6]. The golgin family has extensive coiled-coil regions and includes GM130, giantin, golgin245/p230, golgin-97, golgin-67, golgin-84, golgin-95, and golgin-160. GM130 and giantin have been shown to function in the p115-mediated docking of vesicles with Golgi cisternae [7,8]. p115 binds to the N-terminus of GM130, which itself is bound to the Golgi through a tight association with GRASP65 [5,7]. p115 also binds to giantin on COPI vesicles [8], thus serving

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as the bridge between giantin on the vesicles and GM130 on the Golgi. Golgin245/p230 and golgin-97 are peripheral Golgi proteins that are targeted to the cytoplasmic face of the Golgi apparatus. They have a Golgi localization domain (GLD, also called GRIP domain) in a non-coiled-coil portion of the C-terminus [9,10]. This domain preferentially interacts with Rab6, suggesting that this family of proteins may function in Rab6-regulated membrane-tethering events [11]. Golgin-67 and golgin-84 are integral membrane proteins of the Golgi with an N-terminal coiled-coil domain and a single C-terminal transmembrane domain [12,13]. Golgin-95 and golgin-160 are autoantigens that cross-react with the sera of patients with autoimmune diseases [14]. Golgin-95 is homologous to the C-terminus of GM130 [2] and golgin-160 is homologous to Grp1p [15]. Apart from giantin and GM130, however, the functions of these proteins are still unclear.

GRP1 (golgin-160-related protein, also called *RUD3*) has been identified as a high-copy suppressor of the *sec34-2* mutation, defective in the late stages of ER to Golgi transport [15,16]. The N-terminal 349 residues (of 484 amino acids) of Grp1p is 34% identical to the N-terminal region of golgin-160 [15]. Here, based on the homology to golgin-160, I report the characterization of Grp1p as a novel *cis*-Golgi matrix protein of yeast *S. cerevisiae* that may be involved in the structural organization of the *cis*-Golgi.

Materials and methods

Strains and growth conditions. The strains used in this study are as follows: NY2180 (*MATα ura3-52 leu2-3,112 CHS5-HA*), SFNY26-3A (*MATα ura3-52*), SFNY363 (*MATα ura3-52 leu2-3,112*), SFNY761 (*MATα/α Gal*$^+$ *leu2-3,112/leu2-3,112 ura3-52/ura3-52 GRP1/grp1Δ::URA3*), SFNY778 (*MATα ura3-52 GRP1-myc*), SFNY805 (*MATα ura3-52 leu2-3,112 sec12-4*), SFNY827 [*MATα sec35-1 ura3-52 pRS426*(*GRP1 URA3 2μm*)], SFNY828 [*MATα sec22-3 ura3-52 pRS426*(*GRP1 URA3 2μm*)], SFNY829 [*MATα bos1-1 ura3-52 pRS426*(*GRP1 URA3 2μm*)], SFNY862 (*MATα ura3-52 leu2-3,112 grp1Δ::URA3*), SFNY863 (*MATα ura3-52 leu2-3,112 sec12-4 grp1Δ::URA3*), SFNY864 (*MATα ura3-52 leu2-3,112 sec7-1 grp1Δ::URA3*), SFNY877 (*MATα ura3-52 leu2-3,112 sec7-1*), SFNY928 [*MATα GAL*$^+$ *ura3-52 leu2-3,112 grp1Δ::URA3 pRS425*(*GRP1 LEU2 2μm*)], SFNY948 [*MATα ura3-52 leu2-3,112 grp1Δ::URA3 leu2-3,112::*(*LEU2 GRP1-GFP*)], SFNY949 [*MATα sec12-4 ura3-52 leu2-3,112 grp1Δ::URA3 leu2-3,112::*(*LEU2 GRP1-GFP*), SFNY950 [*MATα sec7-1 ura3-52 leu2-3,112 grp1Δ::URA3 leu2-3,112::*(*LEU2 GRP1-GFP*)], SFNY978 [*MATα ura3-52 leu2-3,112 CHS5-HA leu2-3,112::*(*LEU2 GRP1-GFP*)], and SFNY1016 [*MATα ura3-52 leu2-3,112 pRS426*(*GRP1-GFP URA3 2μl*)]. The other strains used here were described previously [15]. In order to make *GRP1*-disrupted strains, SFNY363 (WT), SFNY805 (*sec12-4*), and SFNY877 (*sec7-1*) were transformed with a 2.6-kb *Bam*HI fragment from pBS Δ18 [15] to generate SFNY862, 863, and 864, respectively. PCR was used to confirm that *GRP1* was disrupted. To generate *GRP1-GFP* strains, the 3-kb *Bam*HI–*Bam*HI fragment from pDK206 containing the *GRP1-GFP* (see below) was inserted into the *Bam*HI site of pRS305 to yield SFNB659. SFNB659 was digested with *Bst*EII and the resulting 8.6 kb fragment was integrated into the *LEU2* locus of SFNY862, 863, and 864 to create SFNY948, 949, and 950, respectively. For colocalization experiments, SFNY363 was transformed with both *GRP1-GFP* (the 8.6-kb *Bst*EII fragment of SFNB659, Leu$^+$) and *MNN1-HA* (Ura$^+$) or *OCH1-HA* (Ura$^+$) constructs. The *GRP1-GFP* (Leu$^+$) construct was also integrated into NY2180 containing *CHS5-HA* as a late Golgi marker. To make a *GRP1-GFP* overproducing strain, SFNY363 was transformed with the pDK206 containing the *GRP1-GFP* to yield SFNY1016. A *GRP1-myc* strain (SFNY778) was constructed according to the method of Schneider et al. [17]. Briefly, a hybrid sequence containing the *URA3* gene and *myc*-epitope tag flanked by a part of the *GRP1* gene was amplified by PCR. The product was then transformed into the wild-type cells (SFNY 26-3A) to direct integration at the *GRP1* locus, and Ura+ transformants were selected. After confirming integration by PCR, the *URA* gene was popped out on plates containing 5-FOA, leaving the *myc*-epitope tag at the C-terminus of *GRP1*. Finally the colony containing *myc*-tagged Grp1p was confirmed by immunoblotting with anti-*myc* antibody. The *myc*-tagged strain showed the same growth property as cells lacking the *myc*-tag.

Construction of GFP-tagged GRP1. A *GRP1-GFP* fusion construct was made as follows: first, the promoter portion of *GRP1*, the *GFP* without a stop codon, and the N-terminus of the *GRP1* without a start codon were amplified by PCR with the following primers (5′–3′): CGG TGG CGG CCG CTC TAG AAC TAG (sense primer, #1) and TAA TTC TTC ACC TTT AGA CAT TCT AAT TAA AAA AAA TGC AAT (antisense primer, #2) for the promoter portion of *GRP1*; ATT GCA TTT TTT TTA ATT AGA ATG TCT AAA GGT GAA GAA TTA (sense primer, #3) and CTG TTT TCT TCT TAT TTT TAC CTT TGT ACA ATT CAT CCA TAC C (antisense primer, #4) for the *GFP* sequence; GGT ATG GAT GAA TTG TAC AAA GGT AAA AAT AAG AAG AAA ACA G (sense primer, #5) and TCA GAG AGA TCT TTC CCC TCA TCG (antisense primer, #6) for the N-terminus of the *GRP1*. Second, a hybrid sequence containing the promoter of *GRP1* and the *GFP* without a stop codon was then amplified with the mixture (2 μl each) of the PCR products for the promoter of *GRP1* and the *GFP* sequence as a template using #1 and #4 primers above. Likewise, a hybrid sequence containing the *GFP* and the N-terminus of the *GRP1* was amplified with the mixture (2 μl each) of the PCR products for the *GFP* and the N-terminus of the *GRP1* as a template using #3 and #6 primers. Finally, a complete hybrid sequence containing all of the three sequences (the promoter of *GRP1*, the *GFP* without a stop codon, and the N-terminus of the *GRP1* without a start codon) was amplified with the mixture (2 μl each) of two hybrid sequences as a template using #1 and #6 primers. The construct was digested with *Eag*I and *Bgl*II and inserted into the *Eag*I–*Bgl*II sites of pDK206 [15]. The *GRP1-GFP* construct was functional as pDK206 containing the cassette suppressed *sec34-2*.

Invertase assay. The glycosylation at different stages and secretion of active invertase were analyzed as follows. Cells were grown in YP medium containing 2% glucose to an OD_{599} of 2. A total of 50 OD units of cells were pelleted, washed with water, resuspended in 50 ml YP medium containing 0.1% glucose prewarmed at 37 °C for 30 min, and incubated for 1.5 h at 37 °C. The cells were washed with cold 10 mM NaF and NaN_3 and converted to spheroplasts during a 1-h incubation at 37 °C as described previously [18]. After centrifugation at 3000*g* for 5 min, the supernatant was used as an extracellular enzyme fraction. The pellet (spheroplast) was washed with spheroplast buffer without zymolase, lysed in the same volume of cold lysis buffer (20 mM Hepes/KOH, pH 7.2) as the supernatant, and homogenized six times with a Dounce tissue grinder (Wheaton Science Products, Millville, NJ). The crude lysate was centrifuged at 200*g* for 3 min at 4 °C to separate lysed (intracellular enzyme fraction) and unlysed (unbroken cells) cell fractions. Thirty microliters of each sample was analyzed in a 7.5% non-denaturing gel and invertase activity staining was performed as described previously [19].

Fluorescence microscopy. Fluorescence microscopy was described previously [20] except that mouse anti-HA monoclonal antibody (1:1000 dilution; Babco, CA, USA) and donkey anti-mouse IgG an-

tibody conjugated to Cy3 (1:1000 dilution; Jackson Immunoresearch Laboratories, PA, USA) were used as first and second antibodies, respectively, for HA epitope detection. In order to quantify the colocalization of Grp1p-GFP and Och1p-HA, 395 Grp1p-GFP positive puncta and 502 Och1p-HA positive puncta were scored in 104 cells by eye after image capture. A total of 379 puncta stained for both proteins. To quantify the colocalization of Grp1p-GFP and Mnn1p-HA, 362 Grp1p-GFP positive puncta and 420 Mnn1p-HA positive puncta were scored in 102 cells. A total of 105 puncta stained for both proteins. For the colocalization of Grp1p-GFP and Chs5p-HA, 254 Grp1p-GFP positive puncta and 297 Chs5p-HA positive puncta were scored in 93 cells. A total of 32 puncta stained for both proteins.

Fractionation and extraction studies. A strain containing tagged Grp1p-*myc* was grown in YPD medium to an OD_{599} of 2.0. A total of 150 units were washed with cold 10 mM NaN_3 and converted to spheroplasts during a 1-h incubation at 37 °C as described previously [18]. The spheroplasts were layered onto a cold sorbitol cushion (1.7 M sorbitol, 50 mM potassium phosphate, pH 7.5) and centrifuged at 3000*g* for 10 min at 4 °C. The pellet was lysed in 6 ml of cold lysis buffer (20 mM Hepes/KOH, pH 7.4) containing 1× protease inhibitor cocktail and homogenized six times with a Dounce tissue grinder (Wheaton Science Products, Millville, NJ). The crude lysate (T) was centrifuged at 200*g* for 3 min at 4 °C to generate lysed (S1) and unlysed (P1) cell fractions. The S1 fraction was centrifuged at 200,000*g* for 60 min to yield supernatant (S2) and pellet (P2) fractions. All pellet fractions were resuspended in the same volume of lysis buffer as the supernatant. For protein extractions, 0.5 ml of the S1 fraction was mixed with an equal volume of one of the following: lysis buffer, 2% Triton X-100 in lysis buffer, 2 M NaCl in lysis buffer, and 200 mM Na_2CO_3 (pH 11.0) in water. After a 45-min incubation on ice, the samples were centrifuged at 200,000*g* for 60 min and then separated into supernatant (S) and pellet (P) fractions. Equivalent amounts of the supernatant and pellet were electrophoresed on a 10% SDS–polyacrylamide gel and immunoblotted with anti-*myc* and anti-Bos1p antibodies.

Results and discussion

Grp1p-depleted strain shows a growth defect at high temperature

GRP1 encodes a highly hydrophilic protein of ~80 kDa. Tetrad analysis shows that *GRP1* is not essential for growth at 25 °C [15]. To evaluate viability of Grp1p-depleted cells at high temperature, growth rate was measured on cells grown in liquid culture at different temperatures (Table 1). At 25 °C, the growth rate of both *grp1*Δ and wild type was similar as shown by doubling time. However, the growth rate of *grp1*Δ was delayed at 30 or 37 °C and half the rate of the wild type at 38.5 °C. The diploid with one copy of *GRP1* disrupted with *URA3* was sporulated and subjected to tetrad analysis on an YPD plate that was incubated at 38.5 °C. After 3 days, each of the tetrads contained two large colonies and one tiny colony (or nothing) (data not shown). The tiny colonies were Ura^+, indicating that they contained a disrupted copy of *GRP1*. These results indicate that although *GRP1* is not an essential gene, Grp1p-depleted cells have a growth defect at temperatures over 30 °C.

Table 1
A Grp1p-depleted strain shows growth defect at high temperature

Strains	Doubling time (h)			
	Temperature:	25 °C	30 °C	37 °C	38.5 °C
*grp1*Δ		3.18	2.88	3.03	6.26
WT		3.23	2.58	2.59	3.07

Wild-type and Grp1p-depleted strains were grown overnight at 25 °C to the early exponential phase in minimal medium. To measure the growth rate, cells were pelleted, resuspended in fresh medium, and grown at 25, 30, 37, and 38.5 °C.

GRP1 genetically interacts with genes involved in vesicle targeting/fusion stages of ER to Golgi transport

In order to gain insight into the role of *GRP1,* its ability to suppress mutations that block protein trafficking at different stages was investigated (Table 2). *RUD3* (*GRP1*) was previously found to be unable to suppress mutations in ER to Golgi docking/fusion factors such as Sec35p, Ypt1p, Sec22p, Bet1p, and Bos1p although it displays genetic interactions with *SEC34* and *USO1* [16]. Sec34p forms a multiprotein complex with Sec35p [15,16] and thus a genetic interaction between Rud3p (Grp1p) and only Sec34p, but not Sec35p, seems to be counterintuitive. Also, in contrast to their results, our previous results showed that *GRP1* suppressed *sec35-1*, *sec22-3*, and *bos1-1* [15]. Thus, these conflicting results were the motivation to re-evaluate its suppression ability in more detail at different temperatures. A high copy vector (*URA3*, 2 μm) containing *GRP1* was transformed into various mutants and a suppression test was performed at various temperatures (30, 34, and 37 °C) for 2 days. As shown in Table 2, *GRP1* was found to suppress *sec22-3* (at 30 and 34 °C), *bos1-1* (at 30 and 34 °C), *uso1-1* (at 34 °C), *sec34-1* (at 37 °C), *sec34-2* (at 37 °C), and *sec35-1* (at 34 °C). Sec22p and Bos1p are v-SNAREs whereas Uso1p, Sec34p, and Sec35p are tethering factors in ER to Golgi transport [15,21–24]. However, mutations that block any other stages of the secretory pathway such as vesicle budding from the ER or post-Golgi transport were not suppressed by the overexpression of *GRP1* at any temperature. This indicates that *GRP1* shows specific genetic interactions with the genes that are involved in the late stages of ER to Golgi transport.

Grp1p resides primarily on the cis-Golgi compartment

To localize Grp1p, the protein was fused to the green fluorescent protein (GFP). The Grp1p-GFP fusion protein was functional as it suppressed *sec34-2* at 37 °C. When wild-type cells containing the Grp1p-GFP fusion protein were examined by fluorescence microscopy, bright punctate structures were found throughout the cytoplasm (Fig. 1A). The fluorescence pattern of Grp1p-GFP resembles that of the *cis*-Golgi markers Sed5p and

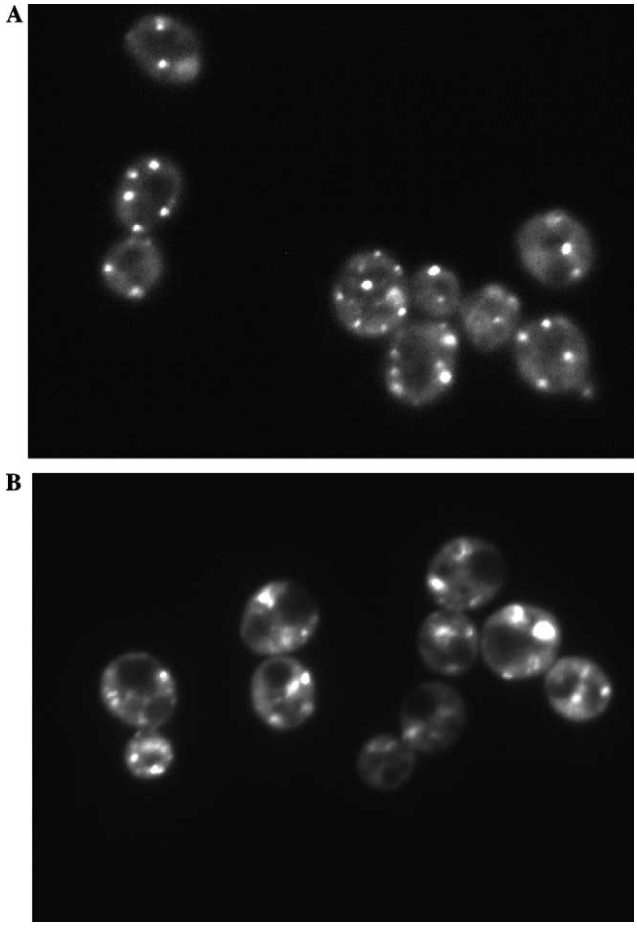

Fig. 1. Grp1p-GFP localizes to the Golgi complex. The punctate structures seen in wild-type cells (A) became larger and less numerous in the *sec7-1* mutant (B). (A) Wild-type cells containing the Grp1p-GFP fusion protein. (B) *sec7-1* mutant cells with the Grp1p-GFP fusion protein after a 2 h incubation at 37 °C in YP medium containing 0.1% glucose.

Bet3p, a component of TRAPP [25,26]. Sed5p and Bet3p were found in punctate structures throughout the cytoplasm. To confirm that Grp1p localizes to the Golgi complex, fluorescence of Grp1p-GFP in a *sec7-1* mutant was examined. The Golgi complex forms stacks in *sec7* mutant cells that have been shifted to 37 °C in low glucose-containing medium [27]. If the Grp1p-GFP fusion protein resides on the Golgi, the punctate structures observed should become larger and less numerous in the *sec7-1* mutant at 37 °C in 0.1% glucose medium. When the Grp1p-GFP fusion was localized in *sec7-1* mutant cells that have been incubated for 2 h at 37 °C in YP medium containing 0.1% glucose, punctate structures became larger and fewer (Fig. 1B). Thus, Grp1p appears to be associated with the Golgi complex. To examine intra-Golgi distribution of Grp1p, Grp1p-GFP was next localized in strains expressing the *cis*-Golgi marker Och1p-HA [28], the medial/late Golgi marker Mnn1p-HA [29], and the late Golgi marker Chs5p-HA [30] (Fig. 2). Results showed that 95.9% (379 dots/395 dots) of the Golgi puncta staining for Grp1p-GFP colocalized with puncta staining for Och1p-HA (Fig. 2A). In contrast, only 29% (105 dots/362 dots) and 12.6% (32 dots/254 dots) of the Golgi puncta staining for Grp1p-GFP co-localized with puncta staining for Mnn1p-HA (Fig. 2B) and Chs5p-HA (Fig. 2C), respectively. Looking at the reverse colocalizations, it was determined that 75.5% (379 dots/502 dots) of the Golgi puncta staining for Och1p-HA, 25% (105 dots/420 dots) of the puncta staining for Mnn1p-HA, and 10.8% (32 dots/297 dots) of the puncta staining for Och1p-HA, respectively, also stained for Grp1p-GFP. Thus, Grp1p primarily localizes to an early Golgi compartment. The fact that Grp1p predominantly localizes to the *cis*-Golgi is consistent with the suppression result whereby the suppression by Grp1p is specific for mutations that block the late stages of ER to Golgi transport (Table 2).

Secreted invertase is hypoglycosylated in Grp1p-depleted cells

In order to assess the function of Grp1p, pulse-chase analysis of carboxypeptidase Y (CPY) transport was performed in *grp1*Δ at 37 or 38.5 °C. In wild type, CPY is translocated into the ER where it is core-glycosylated (p1CPY, 67 kDa). Upon transport to the Golgi, outer chain carbohydrate is added to the core-glycosylated form (p2CPY, 69 kDa). Finally, CPY is processed to yield the mature form in the vacuole (mCPY, 61 kDa). Although *GRP1* specifically suppresses mutations that

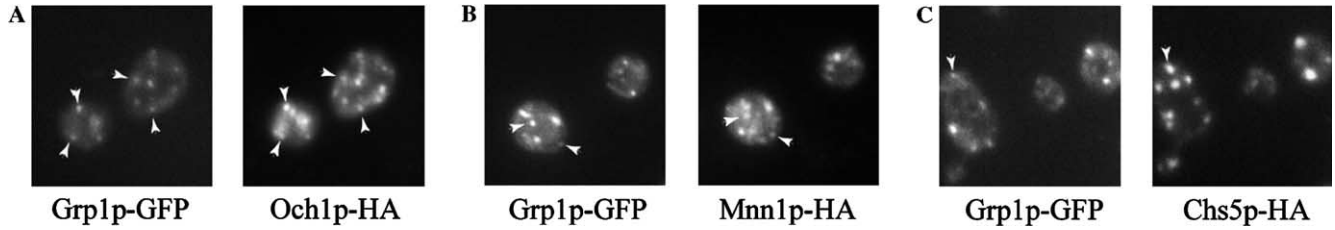

Fig. 2. The majority of Grp1p colocalizes with the *cis*-Golgi marker Och1p. Subcellular localization of Grp1p-GFP with Golgi markers: the *cis*-Golgi marker Och1p-HA (A), the medial/late Golgi marker Mnn1p-HA (B), and the late Golgi marker Chs5p-HA (C). Strains containing both Grp1p-GFP and HA-tagged Golgi markers were fixed, converted to spheroplasts, and probed with mouse anti-HA antibody. Cy3-conjugated secondary antibody was used to localize HA-tagged Golgi markers by fluorescence microscopy whereas GFP marked Grp1p-GFP through its intrinsic fluorescence. Arrowheads indicate colocalization of Grp1p with Golgi markers.

block the late stages of ER to Golgi transport (Table 2), Grp1p-depleted cells did not reveal a significant delay in the transport of CPY to the vacuole (data not shown). Thus, Grp1p does not seem to play a direct role in the secretory pathway. Next, the processing and secretion of invertase was followed in Grp1p-depleted cells. There are two forms of invertase. Secreted invertase, the larger and predominant form of the enzyme, is found outside of the cell membrane (periplasmic space) whereas cytoplasmic invertase, the smaller form, is found entirely within the cell. Secreted invertase contains carbohydrates modified in both the ER and Golgi apparatus. Cells (WT, *grp1*Δ, and *sec18-1*) were grown in YP containing 2% glucose at 25 °C and shifted to 37 °C for 1.5 h in YP medium containing 0.1% glucose for invertase induction. After separation of periplasmic (E) and intracellular (I) protein fractions, equivalent amount of samples were analyzed by activity staining in non-denaturing gels (Fig. 3). In wild-type cells, highly glycosylated invertase was transported to the periplasmic space (E) whereas the ER core-glycosylated form of invertase accumulated inside the cells in *sec18-1*. In Grp1p-depleted cells, the majority of external invertase was severely hypoglycosylated and efficiently transported to the periplasmic space (E). This may result from a general disturbance of Golgi function due to a failure to deliver modifying enzymes in the Golgi compartment. This secretion of underglycosylated invertase was previously observed in a mutant of an essential Golgi membrane protein, Yip1p [31].

Table 2
Overproduction of *GRP1* suppresses certain vesicle targeting/fusion mutants

Mutants	Vector (30 °C)	*GRP1* (30 °C)	Vector (34 °C)	*GRP1* (34 °C)	Vector (37 °C)	*GRP1* (37 °C)
ER-accumulating						
Vesicle budding						
sec12-4	++	++	–	–	–	–
sec13-1	++	++	–	–	–	–
sec16-2	+	+	–	–	–	–
sec23-1	+++	+++	–	–	–	–
Vesicle targeting/fusion						
sec17-1	++++	++++	++	++	–	–
sec18-1	++	++	–	–	–	–
sec22-3	**+**	**+++**	–	**++**	–	–
bos1-1	**+/–**	**+++**	–	**+**	–	–
uso1-1	+++	+++	–	**+**	–	–
sec34-1	+++	+++	++++	++++	**+**	**+++**
sec34-2	++	++	++	++	–	**++**
sec35-1	+++	+++	**+**	**+++**	–	–
bet1-1	++++	++++	++++	++++	–	–
bet3-1	–	–	–	–	–	–
bet5-1	+++	+++	–	–	–	–
ypt1-1	++++	++++	++++	++++	+	+
sed5-1	++	++	+	+	–	–
Golgi complex-accumulating						
sec7-1	++++	++++	++++	++++	–	–
Vesicle-accumulating (post-Golgi complex)						
sec1-1	++++	++++	–	–	–	–
sec2-41	++	++	–	–	–	–
sec3-2	+++	+++	++	++	–	–
sec4-8	+	+	–	–	–	–
sec5-24	+++	+++	–	–	–	–
sec6-4	++++	++++	–	–	–	–
sec8-9	+++	+++	++	++	–	–
sec9-4	++	++	+	+	–	–
sec10-2	+++	+++	++	++	–	–
sec15-1	+++	+++	+	+	–	–
Multiple steps						
sec19-1	+++	+++	–	–	–	–
Recycling (ER to Golgi complex)						
sec20-1	++	++	–	–	–	–
sec21-1	+++	+++	+	+	–	–

++++, colonies like wild type; +++, colonies slightly smaller than wild type; ++, colonies smaller than wild type; +, colonies much smaller than wild type; –, no growth.

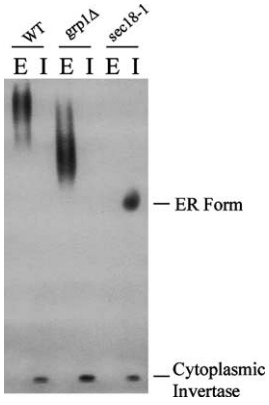

Fig. 3. Secreted invertase is underglycosylated in *grp1*Δ cells. Cells were shifted to 37 °C for 1.5 h in 0.1% glucose medium. Activity staining of invertase was performed after separation of periplasmic (E) and intracellular (I) protein fractions in 7.5% non-denaturing gels. Invertase (E in WT) secreted from wild-type cells is highly glycosylated whereas the invertase (E in *grp1*Δ) secreted from *grp1*Δ is hypoglycosylated. Accumulation of the intracellular ER core-glycosylated form (ER form in I) in *sec18-1* mutant cells is observed as a control. Cytoplasmic invertase, the smaller invertase, was only detected in intracellular fractions, indicating that samples were fractionated well.

Grp1p is a matrix protein that is not extracted by Triton X-100

Grp1p is homologous to the mammalian Golgi matrix protein golgin-160, an autoantigen [14,15]. Both Grp1p and golgin-160 proteins are predicted to have a large coiled-coil structure. Golgin-160 localizes to the Golgi and is a component of a matrix that cannot be extracted by Triton X-100 [14]. Although Grp1p has neither a signal peptide nor significant hydrophobic stretch of amino acids, it resides on the *cis*-Golgi. To determine the biochemical properties of Grp1p, fractionation and extraction studies were performed (Fig. 4). A *GRP1-myc* strain was constructed as described in the materials and methods. The *myc*-tagged strain was functional as it showed the same growth properties as the untagged cells. The *myc*-epitope in the tagged strain was confirmed by immunoblotting with anti-*myc* antibody (Fig. 4A, lane 1) whereas no signal was detected in an untagged strain (lane 2). The *myc*-tagged cells were converted to spheroplasts and lysed. A total lysate (T) was centrifuged at a low speed spin (200*g* for 3 min) to remove the unlysed cells (P1). The S1 supernatant fraction was centrifuged at high speed (200,000*g* for 1 h) to separate the soluble (S2) and the insoluble (P2) fractions. All of the Grp1p was found in the insoluble P2 fraction like the integral membrane protein Bos1p. This is a somewhat surprising result because it is a highly hydrophilic protein with no transmembrane domain, and suggests that Grp1p may be a peripheral membrane protein or part of a large, interconnected network (i.e., the matrix). To see if Grp1p behaves as a peripheral membrane protein or a matrix protein, extraction studies with 1% Triton X-100, 1 M NaCl, and 0.1 M Na_2CO_3 (pH 11.0) were performed (Fig. 4B). Peripheral membrane proteins are extracted with these reagents whereas matrix proteins are generally detergent-resistant [2]. Samples (S1 in Fig. 4A) were incubated in one of the following four reagents for 45 min on ice: buffer alone, 1% Triton X-100, 1 M NaCl, and 0.1 M Na_2CO_3 (pH 11.0) and centrifuged at 200,000*g* for 1 h to generate

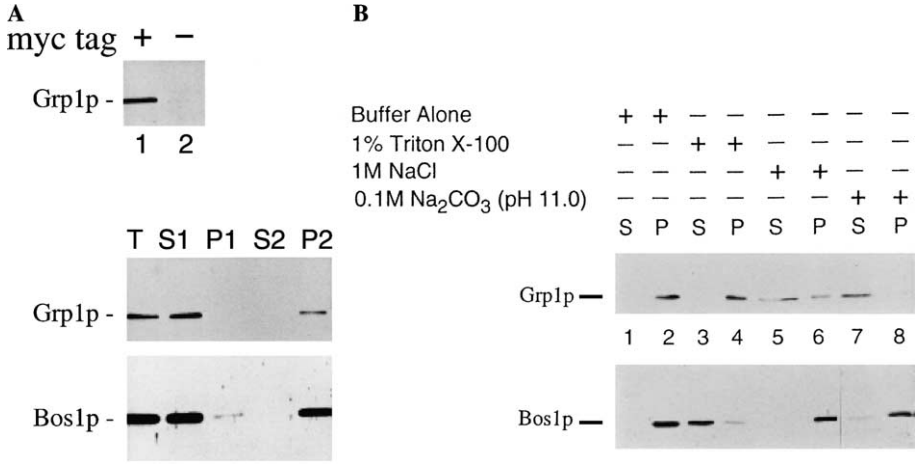

Fig. 4. Grp1p is a matrix protein which is not extracted by Triton X-100. (A) Grp1p pellets with membranes. Lysates prepared from a strain containing tagged Grp1p-*myc* (lane 1) and an untagged strain (lane 2) were subjected to Western blot analysis with the use of anti-*myc* antibody. A total lysate (T) prepared from a strain containing tagged Grp1p-*myc* was centrifuged at 200*g* for 3 min to generate the S1 supernatant and P1 pellet fractions. The S1 fraction was centrifuged at 200,000*g* for 1 h to obtain the S2 and P2 fractions. Equivalent amounts of the supernatant and pellet fractions were loaded onto the 10% SDS–polyacrylamide gel and immunoblotted with anti-*myc* and anti-Bos1p antibodies. (B) Grp1p is insoluble in Triton X-100. The S1 fraction in (A) was incubated for 45 min at 4 °C in lysis buffer alone, 1% Triton X-100 in buffer, 1 M NaCl in buffer, or 0.1 M Na_2CO_3 (pH 11.0) and centrifuged at 200,000*g* for 1 h to generate soluble (S) and insoluble (P) fractions. Equal amounts of each sample were electrophoresed on a 10% SDS–polyacrylamide gel and immunoblotted with anti-*myc* and anti-Bos1p antibodies.

soluble (S) and insoluble (P) fractions. The distribution of Grp1p and an integral membrane protein Bos1p was monitored in the S and P fractions by Western blot analysis. Most of the Bos1p was solubilized by 1% Triton X-100 as expected, but not by other reagents such as NaCl and Na_2CO_3 (pH 11.0). In contrast, these reagents efficiently released Grp1p from the pellet, but 1% Triton X-100 did not release Grp1p at all. Thus, these results suggest that Grp1p is not a peripheral membrane protein, but more likely a matrix protein.

Grp1p is a structural protein of the cis-Golgi

Several lines of evidence suggest that Grp1p may be a *cis*-Golgi structural protein. First, it displays some homology to golgin-160, a Golgi matrix protein. Second, >50% of the protein is predicted to form a long coiled-coil structure, a characteristic of structural proteins. Third, it predominantly colocalizes with the *cis*-Golgi marker Och1p. Finally, Grp1p appears to be a matrix protein that is not extracted from the pellet with the detergent Triton X-100.

In viewing the Golgi as an autonomous organelle independent of the ER, Seemann et al. [4] showed, using Brefeldin A (BFA) and a Sar1 dominant-negative mutant ($Sar1^{DN}$), that Golgi enzymes relocated to the ER whereas matrix proteins were found in well-dispersed punctate structures after treatment with BFA or in the ribbon-like Golgi structure after washout of BFA in the presence of $Sar1^{DN}$. These results suggest that the Golgi apparatus may be viewed as a meshwork of matrix proteins independent of the ER that could act as a scaffold for arranging enzyme-containing membranes.

As a way to test whether Grp1p is part of the Golgi matrix, I took advantage of the property of a *sec12* mutant. Sec12p is the guanine nucleotide exchange factor for Sar1p, a component of the COPII coat complex [32]. Vesicle budding from the ER is blocked in *sec12* mutant at the restrictive temperature, but retrograde transport from the Golgi to the ER continues during a block in forward transport. Under this condition, many proteins such as SNAREs (i.e., Sed5p, Bet1p, and Sec22p) or Emp47p, localized on the Golgi at steady-state, are redistributed to the ER where they become trapped [33]. However, some Golgi proteins like Bet3p do not relocate to the ER, but remain dispersed throughout the cytoplasm. Bet3p is a component of the TRAPP complex which is required for vesicle docking [33]. TRAPP stably associates with the Golgi and are resistant to extraction by Triton X-100 [33,34]. The localization property of Grp1p in a *sec12* mutant was investigated. As shown in Fig. 2, the Grp1p-GFP localizes primarily to the *cis*-Golgi complex. While the punctate signal of Grp1p-GFP was not affected in wild type at 37 °C (data not shown), the signal in *sec12* cells dispersed throughout the cytoplasm after 1 h incubation at 37 °C (Fig. 5A). No ER signal was detected under this condition. To make sure that no ER signal is apparent, a strain (SFNY1016) that overexpressed Grp1p-GFP was constructed. If Grp1p relocates to the ER, the ER signal would be visible in the cells that overproduced Grp1p-GFP. Approximately 200 cells were examined to see whether they have the ER signal. However, as shown in Fig. 5B, no ER signal is observed. Thus, these findings demonstrate that Grp1p does not recycle through the ER, but disperses into the cytoplasm when forward transport to the Golgi is blocked. A more likely explanation for the dispersal of the Golgi signal is that Grp1p is a matrix protein and thus functions as a Golgi structural protein unlike the Golgi enzymes or SNAREs that recycle to the ER.

To verify that Grp1p may be involved in the structural organization of the *cis*-Golgi as a matrix protein, the structure of this compartment was examined in *grp1*Δ cells. Wild-type and *grp1*Δ strains that expressed either the *cis*-Golgi marker, Och1p-HA, or the *trans*-Golgi marker, Chs5p-HA were constructed. Cells were grown in YPD medium at 25 °C and shifted to 38.5 °C for 1.5 h. Compared to that in wild type, the signal of Och1p-HA in Grp1p-depleted cells was dispersed (Fig. 6A). In contrast, the signal intensity of

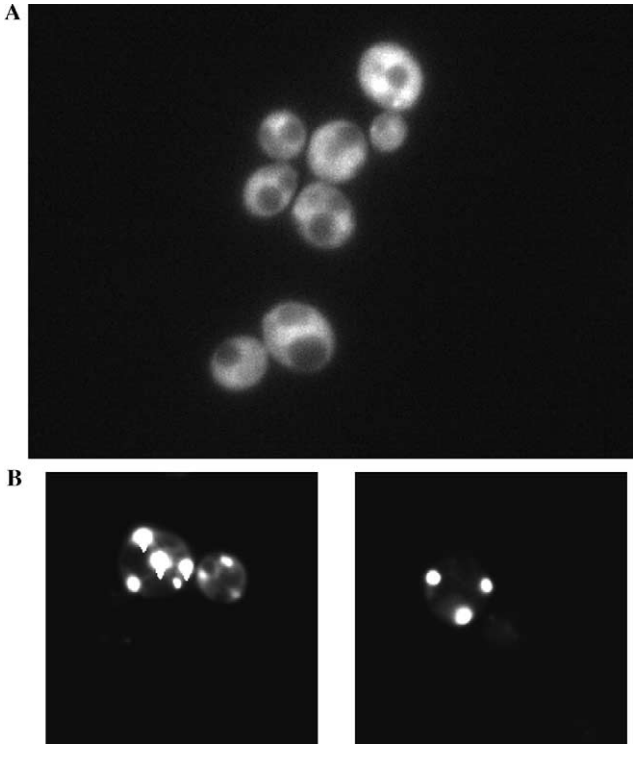

Fig. 5. Grp1p does not cycle between the Golgi and ER, but exclusively resides on the Golgi. (A) Grp1p-GFP disperses in *sec12* at 37 °C. *sec12* cells containing a Grp1p-GFP construct were grown at 25 °C and shifted to 37 °C for 1 h. (B) Overexpressed Grp1p-GFP (SFNY1016) does not show any ER staining.

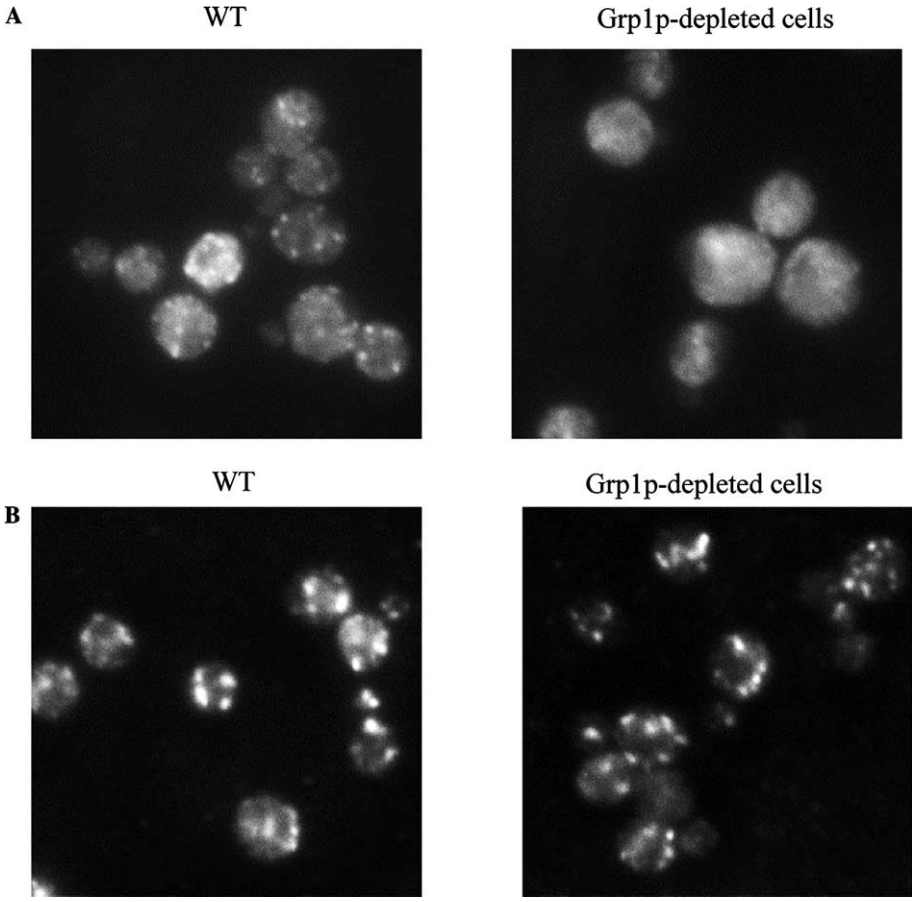

Fig. 6. Och1p-HA becomes dispersed from the *cis*-Golgi in Grp1p-depleted cells after shifting to 38.5 °C for 1.5 h. (A) Och1p-HA in WT and Grp1p-depleted cells. (B) Chs5p-HA in WT and Grp1p-depleted cells.

Chs5p-HA was not changed between wild-type and *grp1*Δ cells (Fig. 6B). This result indicates that the *cis*-Golgi, but not the *trans*-Golgi, is disrupted in *grp1*Δ cells. Taken together, Grp1p is a structural protein of the *cis*-Golgi rather than a factor which is directly involved in membrane traffic.

The Golgi apparatus in yeast *S. cerevisiae* consists of punctate structures scattered throughout the cytoplasm. The *cis*- and *trans*-Golgi do not seem to be closely connected. Thus, it is possible that the structure of the *cis*-Golgi does not influence that of the *trans*-Golgi. However, this might not be so in the more intricately connected mammalian Golgi. Interestingly, it was recently reported that caspase-2, a member of a family of cysteine proteases that function in apoptosis, is localized at the Golgi complex and cleaves golgin-160 during apoptosis [35]. Prevention of cleavage at the unique caspase-2 site delayed disintegration of the Golgi complex after delivery of a pro-apoptotic signal. Likewise, yeast *cis*-Golgi may transduce pro-apoptotic signals through any protease like caspase-2 and Grp1p may be a macromolecular substrate for the protease. Further studies are needed to elucidate the exact function of the Grp1p.

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