Deficiencies in the Endoplasmic Reticulum (ER)-Membrane Protein Gab1p Perturb Transfer of Glycosylphosphatidylinositol to Proteins and Cause Perinuclear ER-associated Actin Bar Formation

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Submitted January 15, 2004; Revised March 5, 2004; Accepted March 26, 2004

Monitoring Editor: Reid Gilmore

The essential *GAB1* gene, which encodes an endoplasmic reticulum (ER)-membrane protein, was identified in a screen for mutants defective in cellular morphogenesis. A temperature-sensitive *gab1* mutant accumulates complete glycosylphosphatidylinositol (GPI) precursors, and its temperature sensitivity is suppressed differentially by overexpression of different subunits of the GPI transamidase, from strong suppression by Gpi8p and Gpi17p, to weak suppression by Gaa1p, and to no suppression by Gpi16p. In addition, both Gab1p and Gpi17p localize to the ER and are in the same protein complex in vivo. These findings suggest that Gab1p is a subunit of the GPI transamidase with distinct relationships to other subunits in the same complex. We also show that depletion of Gab1p or Gpi8p, but not Gpi17p, Gpi16p, or Gaa1p causes accumulation of cofilin-decorated actin bars that are closely associated with the perinuclear ER, which highlights a functional interaction between the ER network and the actin cytoskeleton.

INTRODUCTION

Morphogenesis in the budding yeast *Saccharomyces cerevisiae* is the result of coordinated actions of the actin cytoskeleton, the secretory pathway, and cell wall biosynthetic and remodeling enzymes. The actin cytoskeleton, including actin patches and cables, is polarized at or toward the bud site at the beginning of the cell cycle (Adams and Pringle, 1984). Actin patches are believed to be required for endocytosis, whereas actin cables may be the molecular tracks along which secretory vesicles are transported by a type V myosin to the budding site to deliver wall synthetic enzymes and wall glycoproteins for bud growth (Pruyne *et al.*, 1998; Pruyn and Bretscher, 2000). The shape of the forming bud is maintained by the newly synthesized wall. Because many enzymes of cell wall biosynthesis, all cell wall glycoproteins, and glycosylphosphatidylinositol (GPI)-linked plasma-membrane proteins enter the secretory pathway in the endoplasmic reticulum (ER) and become covalently modified

in that organelle, the ER plays a critical role in wall biogenesis (Orlean, 1997).

About 0.5–1.0% of the proteins encoded in eukaryotic genomes are predicted to receive a COOH-terminal GPI (Eisenhaber *et al.*, 2001) that anchors the protein in the extracellular face of the plasma membrane. In fungi, a subsequent transglycosylation reaction can cross-link the protein to cell wall polysaccharide via the GPI glycan (Lipke and Ovalle, 1998). In mammals and protozoa, GPI-linked proteins are implicated in cell–cell and cell–environment communications (McConville and Ferguson, 1993; Kinoshita *et al.*, 1995; Nosjean *et al.*, 1997). In *S. cerevisiae*, there are some 60 potential GPI-linked proteins, some of which seem to remain predominantly plasma-membrane anchored, whereas others become mainly cell wall-linked (Caro *et al.*, 1997; Hamada *et al.*, 1999). Functional studies on yeast GPI-linked proteins suggest that they are mainly involved in cell wall integrity and also in cell–cell communication during mating (Lipke *et al.*, 1989; Popolo and Vai, 1999; Rodriguez-Pena *et al.*, 2000).

GPIs are preassembled in the membrane of the ER and transferred to protein in the lumen of that organelle by the multiprotein GPI transamidase complex. This complex seems to be conserved between yeast and humans. In humans, five subunits (GAA1, GPI8, PIG-S, PIG-T, and PIG-U) have been identified (Ohishi *et al.*, 2001; Hong *et al.*, 2003), whereas in *S. cerevisiae*, four of the subunits (Gaa1p, Gpi8p, and the PIG-S and PIG-T homologues Gpi17p and Gpi16p)

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04-01-0035. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-01-0035.

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have been shown to be involved in GPI transfer (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996; Fraering *et al.*, 2001; Ohishi *et al.*, 2001). A PIG-U-like protein from *S. cerevisiae* can partially rescue a human PIG-U-deficient cell line (Hong *et al.*, 2003), but its roles in the GPI transfer reaction and perhaps other cellular functions have not been analyzed in yeast.

In *S. cerevisiae*, interactions between the actin cytoskeleton (or its motors) and different organelles such as mitochondria, vacuoles, and nucleus are required for the inheritance of these organelles into the buds (Catlett *et al.*, 2000; Yin *et al.*, 2000; Boldogh *et al.*, 2001). The mechanisms underlying ER inheritance are less clear. *S. cerevisiae* has an elaborate ER network consisting of perinuclear ER (nuclear envelope), cortical ER, and ER tubules that connect the first two structures (Preuss *et al.*, 1991; Prinz *et al.*, 2000). A part of the ER network is enriched near the tips of tiny and small buds, ahead of the movement of vacuoles and mitochondria (Preuss *et al.*, 1991). Video microscopy of cells carrying Sec63p-GFP, an ER reporter protein, suggests that the ER network displays F-actin-dependent dynamic behavior (Prinz *et al.*, 2000) and that a branch of ER extends from the perinuclear ER to the presumptive bud site and is anchored to the bud tip in an F-actin-dependent manner (Fehrenbacher *et al.*, 2002; Estrada *et al.*, 2003). This anchorage may be important for coupling the polarity machinery at the bud tip to ER partitioning into the daughter cell. However, a direct ER-actin association has not been visualized in yeast. ER or ER-derived vesicles are transported by myosin V in squid neurons (Tabb *et al.*, 1998) and in mitotic *Xenopus* egg extracts (Wollert *et al.*, 2002). In addition, ER seems to be linked directly to F-actin through the adaptor protein spectrin in honey bee photoreceptor cells (Baumann, 1998) and interaptin, a member of α -actinin superfamily, in *Dictyostelium* (Rivero *et al.*, 1998). These findings suggest that part of the ER has the capacity to interact with the actin cytoskeleton, which seems to be a feature conserved between yeast and animal cells.

We screened for mutants defective in cellular morphogenesis and identified an essential gene, *GAB1*, which encodes an ER-membrane protein. Functional studies indicate that Gab1p likely defines the fifth subunit of the yeast GPI transamidase. These studies also led to the visualization of a direct association between the perinuclear ER and actin.

MATERIALS AND METHODS

Strains, Growth Conditions, and Genetic Methods

Yeast strains used in this study are listed in Table 1. Standard media and growth conditions were used (Sherman, 1991). Where noted, a buffered rich medium, YM-P (Lillie and Pringle, 1980), was used. In some experiments, 2% galactose plus 2% glucose was used in the growth medium to keep the expression of *pGAL1*-controlled genes at a moderate level. Media and growth conditions used in radiolabeling experiments were as described previously (Grimme *et al.*, 2001). *Escherichia coli* strain DH12S (Invitrogen, Carlsbad, CA) was used routinely as a plasmid host.

Isolation of Ochre Mutants Defective in Cell Morphogenesis

The screen for ochre mutants developed by Riles and Olson (1988) was used. Strain AB1377 [*ade2-1(ochre) can1-100(ochre) trp1* containing plasmid pC689_{UAA} (*CEN TRP1 SUP4-o*)] was mutagenized by UV, and colonies were screened for nonselecting mutants. From ~82,000 UV-mutagenized colonies, 532 independent mutants harboring ochre mutations in essential genes were obtained. These mutants, plus 115 isolated by Riles and Olson (1988), were screened by phase contrast microscopy to identify those containing cells that had become morphologically aberrant after plasmid loss.

Cloning of the Gene ComPLEMENTING KC62BD1-3D

KC62BD1-3D was transformed with an YCp50-LEU2 genomic library (provided by F. Spencer and P. Hieter, University of British Columbia, Canada) (Bi and Pringle, 1996), and selection was imposed for colonies of red *Trp1*⁻ cells

that had lost the suppressor plasmid. Plasmids YCp50-LEU2-GAB1#1 and YCp50-LEU2-GAB1#2 were recovered from two such colonies, and the complementing gene was localized on their genomic DNA insert by subcloning and insertional mutagenesis, shown to be tightly linked to the ochre mutation, and sequenced.

Plasmids

The following key plasmids were used in this study. Details of these, of intermediates in their construction, and of plasmids generated during subcloning are available upon request. Plasmids pRS316-GAL1-GPI17 (*CEN URA3*) and YEp24-GPI8SU (2 μ m *URA3*) were isolated in screens for overexpression and multicopy suppressors of the temperature sensitivity of *gab1-1* cells from a pRS316-GAL1-based cDNA library (Liu *et al.*, 1992) and from a YEp24-based genomic library (Carlson and Botstein, 1982), respectively. Plasmids pRS315-GAL1-GAB1, pRS316-GAL1-GAB1, pRS316-GAL1-GAA1, pRS316-GAL1-GPI16, and pRS316-GAL1-GPI8 contain *GAB1*, *GAA1*, *GPI16*, and *GPI8*, respectively, under the *GAL1* promoter from pRS316-GAL1/10 (Bi and Pringle, 1996). Plasmid YEp351-GAB1-BS contains an ~3.8-kb *BamHI-Sall* fragment carrying *GAB1* in vector YEp351 (2 μ m *LEU2*) (Hill *et al.*, 1986). Plasmid YEp181-GPI8 contains an ~1.7-kb *HindIII-PstI* fragment carrying *GPI8* in vector YEplac181 (2 μ m *LEU2*) (Gietz and Sugino, 1988). Plasmid pEGKT-GAB1 expressed an NH₂-terminal GST-GAB1 fusion under the control of the *GAL1* promoter of pEGKT (2 μ m *URA3*) (Mitchell *et al.*, 1993). Plasmids pRS315-GAB1-C-GST (*CEN LEU2*) (Sikorski and Hieter, 1989) and YEp351-GAB1-C-GST carry the same DNA insert expressing Gab1p with an in-frame glutathione S-transferase (GST) tag inserted between amino acids 386 and 387. Integrative plasmid Ylp211-GAB1-GFP (Gietz and Sugino, 1988) expresses Gab1p into which green fluorescent protein (GFP) has been inserted between amino acids 386 and 387. Plasmid pSEC63-GFP expresses Sec63p-GFP (Prinz *et al.*, 2000).

Isolation of Temperature-Sensitive Mutations in GAB1

A polymerase chain reaction (PCR)-based method (Cadwell and Joyce, 1992) was used to mutagenize randomly the entire *GAB1* gene, which had been subcloned into pBluescript KS(+) (Stratagene, La Jolla, CA) as a 3.8-kb *BamHI-Sall* fragment excised from YCp50-LEU2-GAB1#1. A pair of oligonucleotides (forward primer, CACTCCTTAGGATCCTAGATTGCTGTATCTGTGTCCATAT, and reverse primer, TCGTATTTAGTCTGCTGTGTCGCAATCAAGATACGCTTCAA) was used to amplify the *GAB1*-containing fragment under mutagenic conditions (Caviston *et al.*, 2002). Plasmid pRS315-GAB1-SS, which contained an ~5.4-kb *SmaI-Sall* fragment of genomic DNA containing *GAB1* from YCp50-LEU2-GAB1#2, was digested with *AatII* (153 base pairs upstream of the start codon of *GAB1*) and *NdeI* (1003 base pairs downstream of the stop codon of *GAB1*) to remove the entire *GAB1* open reading frame (ORF). Mutagenized PCR products were then mixed with equal amount of digested plasmid DNA and transformed into YEF1228 (*gab1 Δ :HIS3*, pRS316-GAL1-GAB1) to allow gap repair between the PCR fragments and the linearized plasmid. Transformants were selected on SC-Leu plates at 23°C and then replicated onto SC-Leu plates containing 5-fluoroorotic acid to select for cells that had lost pRS316-GAL1-GAB1. The Leu⁺ Ura⁻ transformants were replicated onto two sets of SC-Leu plates that were incubated at 24 and 37°C, respectively, to allow the identification of temperature-sensitive (Ts) mutants. From ~24,000 transformants screened, nine Ts mutants of *GAB1* were identified. One of the Ts alleles, *gab1-1*, was used to replace the chromosomal *GAB1* gene, generating the strain YEF1792.

GST Pull-Down Assay

Strains YEF3734 (*gab1 Δ HIS3 GPI17:HA-TRP1*, pRS315-GAB1-C-GST) and YEF3735 (*gab1 Δ HIS3 GPI17:HA-TRP1*, YEp351-GAB1-C-GST) were grown each in 1 liter of SC-Leu medium until OD₆₀₀ ~1.0. Cells were harvested by centrifugation, washed with sorbitol buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.4), and then resuspended in 20 ml of the sorbitol buffer containing a cocktail of protease inhibitors and 1% NP-40. Cells were then broken by vortexing in the presence of glass beads at 4°C. Cellular debris was removed by centrifugation at 10,000 $\times$ g for 10 min. Supernatant was incubated with 1.0 ml of 75% (wt/vol) slurry of glutathione-Sepharose 4B beads at 4°C with gentle shaking for 1 h. The beads were washed five times with the sorbitol buffer containing 0.2% NP-40, and proteins bound to the beads were eluted with SDS sample loading buffer. Samples were then analyzed by 7.5% SDS-PAGE and standard Western blotting by using enhanced chemiluminescence reagents.

Microscopy

Differential interference contrast and fluorescence microscopy were performed with a Nikon microscope (E800; Nikon, Tokyo, Japan) and a digital camera (model 4742-95; Hamamatsu, Bridgewater, NJ). Indirect immunofluorescence was performed as described by Pringle *et al.* (1991). Primary antibodies include goat anti-actin antibodies (Karpova *et al.*, 1993), the rat monoclonal anti-tubulin antibody YOL1/34 (Accurate Chemical & Scientific, Westbury, NY), affinity-pure rabbit anti-GST polyclonal antibodies

Table 1. Yeast strains used in this study

Strain	Genotype	Reference or source
AB1377	α <i>ade2-1(o) can1-100(o) lys2-1(o) leu2_{UGA} his5_{UGA} trp1</i> [pC689 _{UAA} (CEN TRP1 SUP4-o)]	(Riles and Olson, 1988)
KC62BD1-3D	a <i>ade2-1(o) can1-100(o) leu2 trp1 ura3 gab1(o)</i> [pC689 _{UAA}]	See text
YPH500	α <i>ade2-101(o) his3-Δ200 leu2-Δ1 lys2-801(a) trp1-Δ63 ura3-52</i>	(Sikorski and Hieter, 1989)
YPH501	<i>a/α ade2-101(o)/ade2-101(o) his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801(a)/lys2-801(a) trp1-Δ63/trp1-Δ63 ura3-52/ura3-52</i>	(Sikorski and Hieter, 1989)
YEF165	as YPH501 except <i>GAB1/gab1Δ::URA3</i>	See text
YEF473	a/α his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3	(Bi and Pringle, 1996)
YEF473A	a his3 leu2 lys2 trp1 ura3	(Bi and Pringle, 1996)
YEF604	YEF165 [pRS315-GAL1-GAB1]	This study
YEF614	α <i>ade2-101(o) his3-Δ200 leu2-Δ1 lys2-801(a) trp1-Δ63 gab1Δ::URA3</i> [pRS315-GAL1-GAB1]	Segregant of YEF604
YEF624	a/α ade2-1(o)/ade2-101(o) CAN1/can1-100(o) HIS3/his3-Δ200 leu2/leu2-Δ1 LYS2/lys2-801(a) trp1/trp1-Δ63 ura3/ura3-52 GAB1/gab1(o) [pRS315-GAL1-GAB1]	This study ^a
YEF625	as YEF624 except <i>gab1(o)/gab1Δ::URA3</i>	This study ^b
YEF626	KC62BD1-3D [pEGKT-GAB1]	This study
YEF920	as YEF473 except <i>GAB1/gab1Δ::HIS3</i>	See text
YEF1224	a his3 leu2 lys2 trp1 ura3 gab1Δ::HIS3 [pRS315-GAL1-GAB1]	This study ^c
YEF1226	a his3 leu2 lys2 trp1 ura3 [pRS315-GAL1-GAB1]	This study ^c
YEF1228	a his3 leu2 lys2 trp1 ura3 gab1Δ::HIS3 [pRS316-GAL1-GAB1]	This study ^a
YEF1789	a his3 leu2 lys2 trp1 ura3 GAB1:GFP-URA3	See text
YEF1792	a his3 leu2 lys2 trp1 ura3 gab1-1	See text
YEF2684	As YEF473 except <i>GPI17/GPI17:GFP-Kan</i>	See text
YEF2791	α <i>his3 leu2 lys2 trp1 ura3 GPI17:GFP-kan</i>	Segregant of YEF2684
YEF2828	as YEF473 except <i>GPI17:GFP-kan/GPI17:GFP-kan</i>	This study ^d
YEF2882	a his3 leu2 lys2 trp1 ura3 gab1-1 GPI17:GFP-Kan	This study ^e
SDP24270	a/α his3Δ1/his3Δ1 leu2Δ/leu2Δ met15Δ/MET15 LYS2/lys2Δ ura3Δ GPI17/gpi17Δ::Kan	This study ^f
YEF3437	α <i>his3Δ1 leu2Δ lys2Δ ura3Δ</i> [pRS316-GAL1-GPI17]	This study ^g
YEF3438	a his3Δ1 leu2Δ met15Δ ura3Δ gpi17Δ::Kan [pRS316-GAL1-GPI17]	This study ^g
YEF3712	a his3Δ1 leu2Δ ura3Δ gaa1Δ::Kan [pRS316-GAL1-GAA1]	This study ^g
YEF3716	a his3Δ1 leu2Δ lys2Δ ura3Δ gpi16Δ::Kan [pRS316-GAL1-GPI16]	This study ^g
YEF3720	a his3Δ1 leu2Δ ura3Δ gp18Δ::Kan [pRS316-GAL1-GPI8]	This study ^g
YEF3734	a his3 leu2 lys2 trp1 ura3 gab1Δ::HIS3 GPI17:HA-TRP1 [pRS315-GAB1-C-GST]	This study ^h
YEF3735	a his3 leu2 lys2 trp1 ura3 gab1Δ::HIS3 GPI17:HA-TRP1 [YEp351-GAB1-C-GST]	This study ^h
POY133	α <i>ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 gaa1-1</i>	(Grimme <i>et al.</i> , 2001)
POY134	α <i>ade2-1 ade3Δ22 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 gpi8</i>	This study ⁱ
BWY197	a gaa1 leu2 ura3 his3 trp1 ade2 ade3	P. Orlean
22AB Δ 1	a/α PFY1/pfy1Δ::LEU2 leu2-3,112/leu2-3,112 ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-1(am)/trp1-1(am) lys2-80(am)/lys2-80(am)	(Haarer <i>et al.</i> , 1993)
JGY519	a pfy1Δ::LEU2 leu2-3,112 ura3-52 his3-Δ200 trp1-1(am) lys2-80(am)	Segregant of 22AB Δ 1

^a YEF624 was constructed by crossing KC62BD1-3D to YPH500 and curing the plasmid pC689_{UAA}.^b YEF625 was constructed by crossing KC62BD1-3D to YEF614 and curing the plasmid pC689_{UAA}.^c Segregants of YEF920 carrying pRS315-GAL1-GAB1 or pRS316-GAL1-GAB1.^d Two segregants of YEF2684 were crossed to generate YEF2828.^e Segregant from the cross between YEF1792 and YEF2791.^f Generated by *Saccharomyces* Gene Deletion Project and obtained from Research Genetics (Huntsville, AL).^g Segregants from SGD24720 carrying pRS316-GAL1-GPI17; YEF3680 (**a/ α gaa1 Δ ::Kan/GAA1**) carrying pRS316-GAL1-GAA1; YEF3681 (**a/ α gpi16 Δ ::Kan/GPI16**) carrying pRS316-GAL1-GPI16; and YEF3682 (**a/ α gp18 Δ ::Kan/GPI8**) carrying pRS316-GAL1-GPI8. YEF3680-3682 were purchased from Research Genetics.^h YEF1228 carrying *GPI17:HA-TRP1* was constructed through a PCR-based method (Longtine *et al.*, 1998). The resulting strain was transformed with pRS315-GAB1-C-GST and YEp351-GAB1-C-GST, respectively. Transformants were streaked out onto an SC-Leu+5-fluoroorotic acid plate to cure them of the *URA3*-containing plasmid, generating strains YEF3734 and YEF3735, respectively.ⁱ Segregant from two successive backcrosses of FBY11 (Benghezal *et al.*, 1996) to YMW1, a W303 derivative.

(Bi and Pringle, 1996), mouse monoclonal anti-GST antibody (Covance, Richmond, CA), mouse monoclonal anti-hemagglutinin (HA) antibody (Covance), affinity-pure rabbit anti-Cofilin (cofilin) polyclonal antibodies (from D. Drubin, University of California at Berkeley, Berkeley, CA), and rabbit anti-Kar2p polyclonal antibodies (from M. Rose, Princeton University, Princeton, NJ). Secondary antibodies include rhodamine-conjugated donkey anti-goat IgG, fluorescein isothiocyanate-conjugated goat anti-rat IgG (both from Jackson ImmunoResearch Laboratories, West Grove, PA), BODIPY-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR), Cy2-conjugated donkey anti-rabbit IgG, and Cy3-conjugated donkey anti-mouse IgG. Alexa 568-phalloidin (10 U/ml; Molecular Probes) was used to stain F-actin. Chitin was visualized by staining with Calcofluor (Sigma-Aldrich, St. Louis, MO) and DNA by staining with 1 μ g/ml bisBenzimide (Sigma-Aldrich).

Radiolabeling of Lipids

Procedures for [³H]inositol labeling of lipids were essentially as described previously (Grimme *et al.*, 2001), except that radiolabeling was carried out for 180 min, and the solvent used for developing thin layer chromatograms was chloroform:methanol:water (4:4:1 by volume).

RESULTS

Mutations in *GAB1* Cause a Loss-of-Polarity Phenotype

To identify mutations in novel genes involved in cell morphogenesis, we used the strategy of Riles and Olson (1988)

Figure 1. Morphological defect of a *gab1(o)* mutant. Cells of strains AB1377 (wild-type) and KC62BD1-3D [*gab1(o)*] were streaked onto YPD plates and incubated for 12–16 h at 24 and 37°C, respectively, before being observed by differential interference contrast microscopy. Bar, 10 μ m. For all figures, cells in the same panel have the same magnification.

to isolate ochre mutations that result in grossly abnormal cell morphology in the absence of the *SUP4* ochre suppressor. Aberrant morphologies included cell chains with multiple nuclei and elongated buds, cells with a single nucleus and multiple elongated buds, and large, round cells with one or more nuclei (mutants defective in polarity establishment and/or maintenance). Sixteen mutants harboring single recessive ochre mutations were isolated and divided into 11 complementation groups. Further analyses revealed that six complementation groups had mutations in the *CDC24*, *CDC53*, *TSM1*, *CDC28*, *CDC5*, and *GRR1* genes. One of the remaining five complementation groups, represented by mutant KC62BD1-3D, was analyzed further, because in the absence of the suppressor plasmid, this strain produced a typical loss-of-polarity phenotype characterized as large, round cells, particularly at 37°C (Figure 1). The gene defined by the ochre mutation was cloned, sequenced, and deposited in databases under the tentative name *CDC91* (YLR459W); as described below, we have now renamed it GPI and Actin Bars (*GAB1*).

***GAB1* Is an Essential Gene and Encodes a Conserved Protein with Multiple Transmembrane Domains**

To determine whether *GAB1* is required for cell growth, we carried out deletion analysis on *GAB1* in two yeast strains with different genetic backgrounds. In one case, the *EcoRI*-*NsiI* fragment (an internal 750-base pair fragment, from 103 base pairs downstream of the start codon to 334 base pairs upstream of the stop codon) of *GAB1* was replaced by *URA3* (strain YEF165). In the other, the entire *GAB1* sequence was replaced by *HIS3* (strain YEF920). Dissection of 20 tetrads for YEF165 and 48 for YEF920 showed that *GAB1* was required for cell viability in both backgrounds (our unpublished data), indicating that *GAB1* is an essential gene. *GAB1* encodes a protein of 394 amino acids and likely defines the counterpart of the PIG-U subunit of the mammalian GPI transamidase (Hong *et al.*, 2003). Sequences with significant homologies to Gab1p are encoded in the genomes of other

yeasts, mammals, and plants (Figure 2A). Pairwise comparisons indicate that most proteins in this family share 20–30% sequence identity over the entire length of the proteins with the exception of the virtually identical Chinese hamster and human proteins. Like Gab1p, all members of this family are highly hydrophobic (Figure 2B), being predicted to have eight to 10 transmembrane domains, depending on the analytical software used.

***Gab1p* Localizes to the ER**

We used two approaches to determine the cellular location of Gab1p. First, we integrated a single copy of a functional *GAB1*-GFP at the *GAB1* locus, under the control of the *GAB1* promoter. Gab1p showed the localization of a typical ER protein, being localized to the cell cortex (cortical ER) and surrounding the nuclear DNA (perinuclear ER) (Figure 3A, cells 1–3). This was confirmed by the demonstration of co-localization of Gab1p and the ER marker Kar2p (Figure 3B) (Rose *et al.*, 1989). Furthermore, when a GST-Gab1p fusion was overexpressed from a galactose-inducible promoter, the fusion protein fully complemented the *gab1(o)* mutant and was clearly observed to decorate ER tubules that connect the perinuclear ER to the cortical ER (Figure 3C, cells 4 and 5). In both cases, especially with GST-Gab1p, the Gab1p cortical signal was not in the form of a continuous line with uniform intensity (Figure 3C, cells 4–6), suggesting that Gab1p does not localize to the plasma membrane.

Differential Suppression of a gab1-Ts Allele by Overexpression of Different Subunits of the GPI Transamidase, with the Strongest Suppression Displayed by GPI8 and GPI17

To investigate the cellular function of Gab1p, we isolated a temperature-sensitive allele of *GAB1*, *gab1-1*, by random PCR mutagenesis. A strain harboring *gab1-1* showed temperature sensitivity for growth, and this phenotype was corrected upon introduction of *GAB1* on a centromeric plasmid (our unpublished data). Like the original ochre mutant, *gab1-1* cells were larger and rounder than the corresponding wild-type cells at 37°C, and chitin in the mutant cells was more diffused over the entire cell surface, but it still had a relatively higher concentration at the neck region (Figure 4A).

Next, we performed two genetic screens to identify genes that, when overexpressed, were able to suppress the lethality of *gab1-1* cells at the restrictive temperature. In one screen, a *pGAL1*-controlled cDNA library was used, and among 2.1×10^6 transformants screened, we found 50 overexpression suppressors. Southern blotting and DNA sequencing showed that 36 were *GAB1* and 14 were *GPI17* (Figure 4B), a gene whose product is involved in transferring GPI anchors to proteins (Ohishi *et al.*, 2001). In the second screen, a 2μ -based, high-copy plasmid library was used. In this case, all yeast genes were under the control of their own promoters and should be expressed at moderately elevated levels. Of 62,900 transformants screened, 18 harbored plasmids with suppressor activity. Seventeen of these contained *GAB1*, and the other contained five full ORFs and two truncated ones. One of the full ORFs encodes Gpi8p, the catalytic subunit of the GPI transamidase complex (Meyer *et al.*, 2000). Subcloning and deletion analysis showed that *GPI8* was solely responsible for the suppression (Figure 4C).

Because the overexpression suppressors from the genetic screens encode the subunits of the GPI transamidase, we decided to compare the suppression of the *gab1* mutant by overexpression of each known subunit of the GPI transamidase in the same genetic context. Each of the subunit genes was cloned into the same expression vector and transformed into the

A

Figure 2. (A) Alignment of Gab1p-homologous sequences from different organisms by using MacVector software. Gab1p(Sc) (accession no. L31649; full-length protein, 394 amino acids) from *S. cerevisiae*; CA0739 (Ca) (accession no. CA0739; 482 amino acids) from *Candida albicans*; SPAC1952.01 (Sp) (accession no. CAB52565; 408 amino acids) from *Schizosaccharomyces pombe*; CG13089 (Dm) (accession no. AAL13897; 426 amino acids) from *Drosophila melanogaster*; PIG-U (Cg) (accession no. BAC53627; 435 amino acids) from *Cricetulus griseus* (Chinese hamster); PIG-U (Hs) (accession no. BAC53626; 435 amino acids) from *H. sapiens*; ATLG12730 (At) (accession no. AAL34242; 476 amino acids) from *Arabidopsis thaliana*. Residues conserved in more than one-half of the aligned sequences are highlighted in bold letters in gray boxes. (B) Hydrophobicity plot for Gab1p generated with MacVector software.

B

gab1-1 mutant for the suppression test. Interestingly, different subunits displayed consistent and differential suppression of the *gab1* mutant with the suppression length in the descending order of Gpi8p→Gpi17p→Gaa1p (weak suppression)→Gpi16p (no obvious suppression) (Figure 4B). We also tested for suppression of a *gaa1*-Ts mutant by overexpression of different subunits of the GPI transamidase, by using the same set of plasmids used in the *gab1* tests. None of the subunits except

Gaa1p suppressed the *gaa1* mutant (Figure 4D). These data suggest that Gab1p has distinct functional relationships with different subunits of the GPI transamidase.

Gab1p Forms a Complex with Gpi17p In Vivo

Like Gab1p, a functional Gpi17p-GFP localized to the ER throughout the cell cycle (Figure 5A). The genetic suppression and the localization results suggested that Gab1p and

Figure 3. Gab1p localizes to the endoplasmic reticulum. (A) Cells of strain YEF1789 (*GAB1:GFP*) were scraped from a SC-Ura plate after growth for 12–16 h at 24°C. DNA was visualized by staining the cells with bisBenzimide. (B) Cells of strain KC6BD1-3D [*gab1(o)*] carrying plasmid pEGKT-GAB1 were grown in liquid SC-Ura medium containing 1% raffinose plus 2% galactose for 6 h at 30°C and then processed for immunofluorescence with mouse monoclonal anti-GST antibody and rabbit anti-Kar2p antibody. (C) Cells from B were processed for immunofluorescence with rabbit anti-GST polyclonal antibodies.

Gpi17p may interact with each other in the ER. Because Gab1p has eight to 10 transmembrane domains and was virtually undetectable at its endogenous level, we determined whether Gab1p and Gpi17p form a complex in vivo by growing up 1-liter cultures of each of the strains YEF3734 (*gab1Δ* *GPI17:HA-TRP1*, *GAB1:GST*-single copy under the *GAB1* promoter) and YEF3735 (*gab1Δ* *GPI17:HA-TRP1*, *GAB1:GST*-multiple copies under the *GAB1* promoter) for a single GST pull-down experiment. From the strain expressing the endogenous level of Gab1p, even after being concentrated by adsorption on glutathione-Sepharose beads,

Figure 4. Suppression of the temperature sensitivity of *gab1-1* cells by overexpression of *GPI17* and *GPI8*. (A) Temperature-sensitive *gab1-1* mutant shows defects in chitin deposition and cellular morphogenesis. Cells of YEF473A (wild-type) and YEF1792 (*gab1-1*) were grown to exponential phase in YM-P medium at 24°C and then shifted to 37°C for 3 h before staining with Calcofluor to visualize chitin. Bar, 10 μ m. (B) Differential suppression of the temperature sensitivity of *gab1-1* cells by overexpression of different subunits of the GPI transamidase. Cells of YEF1792 (*gab1-1*) carrying pRS316-GAL1 (Vector), pRS316-GAL1-GAB1, pRS316-GAL1-GAA1, pRS316-GAL1-GPI8, pRS316-GAL1-GPI16, and pRS316-GAL1-GPI17 were streaked onto SC-Ura plates containing 1% raffinose plus 2% galactose and incubated at 24 and 37°C, respectively, for 4 d. (C) Suppression of *gab1-1* cells by multicopy *GPI8*. Cells of YEF1792 (*gab1-1*) carrying YEp1ac181 (vector), YEp351-GAB1-BS (*GAB1*), and YEp181-GPI8 (*GPI8*) were streaked onto two SC-Leu plates and incubated at 24 and 35.5°C, respectively for 3 d. (D) Overexpression of different subunits of the GPI transamidase did not suppress the temperature sensitivity of a *gaa1* mutant. The same suppression assay as in B was performed here, except the strain used for transformation was BWY197 (*gaa1*).

Gab1p was still difficult to visualize (Figure 5B, lane 1). However, GST-Gab1p was clearly detected in the pull-down precipitate from the strain expressing a higher level of Gab1p (Figure 5B, lane 2). GST-Gab1p ran as a smear of bands in the range of 135–200 kDa, much larger than the expected size of ~72 kDa. This is not too surprising, because the human homologue PIG-U behaves similarly by SDS-PAGE analysis (Hong *et al.*, 2003). It was also clear from Figure 5B that GST-Gab1p quantitatively pulled down

Figure 5. Gpi17p localizes to the ER and coimmunoprecipitates with Gab1p. (A) Cells of YEF2828 (*GPI17::GFP-Kan/GPI17::GFP-Kan*) were streaked onto a YPD plate and incubated at 24°C for 12–16 h before visualization of GFP and DNA. (B) To pull down GST-Gab1p, cell lysates made from strains YEF3734 (*gab1ΔHIS3 GPI17::HA-TRP1*, pRS315-GAB1-C-GST) and YEF3735 (*gab1ΔHIS3 GPI17::HA-TRP1*, YEp351-GAB1-C-GST) were mixed with equal amounts of a suspension of glutathione-Sepharose 4B beads. GST-Gab1p and Gpi17p-HA in the precipitates, as well as Gpi17p-HA in the input cell lysates, were detected by Western blotting with mouse anti-GST or anti-HA monoclonal antibody.

Gpi17p-HA from the two cell lysates, even though Gpi17p-HA was expressed from its native promoter with similar levels in both strains. These results suggest that Gab1p and Gpi17p are able to form a complex *in vivo*.

[³H]Inositol Lipid Accumulation Phenotype of *gab1-Ts* Cells Indicates a Defect in GPI Precursor Transfer to Protein

To explore whether Gab1p also plays a role in the GPI transamidase reaction that requires Gpi8p and Gpi17p, we examined the *gab1-1* mutant for accumulation of GPI precursor(s) at the restrictive temperature. The *gab1-1* mutant accumulated a range of [³H]inositol-labeled lipids (Figure 6, lanes 2 and 3), whose thin layer chromatographic mobilities were the same as those of the GPIs accumulated by GPI transamidase mutants *gaa1* and *gpi8* (Figure 6, lanes 6 and 7) (Hamburger *et al.*, 1995; Benghezal *et al.*, 1996; Grimme *et al.*, 2001), and by a Gpi17p-depleted strain (Ohishi *et al.*, 2001) (Figure 6, lanes 4 and 5). The two most polar lipids correspond to “complete precursors” 1 and 2 defined by Sipos *et al.* (1994). These results are strong evidence that *gab1-1* mutant is indeed defective in transfer of GPI precursors to proteins. Introduction of a 2 μ plasmid containing *GAB1* under its native promoter relieved the lipid accumulation phenotype (our unpublished data).

Figure 6. The *gab1-1* mutant accumulates [³H]inositol-labeled GPIs characteristic of GPI transamidase mutants. Cells were pulse-labeled with [³H]inositol at 25 or 37°C, and radiolabeled lipids were extracted, separated by thin layer chromatography by using chloroform:methanol:water (4:4:1 by volume) as solvent, and detected by fluorography. Lane 1, lipids from wild-type (WT) control strain (YEF473A) labeled in glucose-containing medium; lanes 2 and 3, lipids from *gab1-1* cells (YEF1792) labeled in glucose at 25 and 37°C, respectively; lanes 4 and 5, lipids from *gpi17Δ::Kan*, pRS316-GAL1-GPI17 cells (YEF3438), labeled in medium containing glucose and galactose, respectively; lanes 6 and 7, *gaa1* (POY133) and *gpi8* cells (POY134), respectively, labeled in glucose at 37°C. CPs, complete precursors defined by Sipos *et al.* (1994); o, origin.

Depletion of Gab1p Alters ER Organization and Causes Actin Bar Formation

Because Gab1p localizes to the ER and has multiple transmembrane domains, we asked whether Gab1p is normally involved in the organization of the ER structure. By using Sec63p-GFP as an ER marker (Prinz *et al.*, 2000; Fehrenbacher *et al.*, 2002), we observed that most of the Gab1p-depleted cells lacked detectable ER tubules and contained many fluorescent spots of differing brightness (Figure 7A). The perinuclear ER in these cells looked relatively normal, although portions of it became brighter. Similar alterations in the distribution of two other ER markers, ss-GFP-HDEL (Prinz *et al.*, 2000) and Kar2p (Rose *et al.*, 1989) confirmed

Figure 7. Depletion of Gab1p alters ER organization and causes actin bar formation. (A) ER organization in Gab1p-depleted cells. Cells of YEF1226 (*GAB1*, pRS315-GAL1-GAB1) (control) and YEF1224 (*gab1Δ*, pRS315-GAL1-GAB1) carrying plasmid pSEC63-GFP were grown in SC-Ura liquid medium for 22 h at 30°C to deplete Gab1p and then examined for Sec63p-GFP localization. (B) Strains YEF1226 and YEF1224 were inoculated from SC-Leu plates containing 2% galactose and 2% glucose into YP medium containing 2% galactose and 2% glucose and grown to exponential phase. Cells from both cultures were then washed with YPD and diluted into fresh YPD for 23 h at 30°C to deplete Gab1p. Cells were then fixed with formaldehyde before staining with Alexa 568-phalloidin. The Gab1p-depleted cells stained by phalloidin were left slightly out of focus to visualize the actin cables. (C) As in B, except that cell wall was removed before these cells were stained with anti-actin antibodies. (D) Depletion of Gpi17p does not cause actin bar formation. Cells of strains YEF3437 (*GPI17*, pRS316-GAL1-GPI17) and YEF3438 (*gpi17Δ::Kan*, pRS316-GAL1-GPI17) were grown exponentially in liquid YP medium containing 2% galactose plus 2% glucose and then washed with YPD medium and released into fresh YPD to deplete Gpi17p for 23 h before the cells were processed for immunofluorescence with anti-actin antibodies. (E) Depletion of different subunits of the GPI transamidase displays a differential effect on actin bar formation. Different subunits of the GPI transamidase in strains YEF1224 (*gab1Δ::HIS3*, pRS315-GAL1-GAB1), YEF3720 (*gpi8Δ::Kan*, pRS316-GAL1-GPI8), YEF3438 (*gpi17Δ::Kan*, pRS316-GAL1-GPI17), YEF3716 (*gpi16Δ::Kan*, pRS316-GAL1-GPI16), and YEF3712 (*gaa1Δ::Kan*, pRS316-GAL1-GAA1) were depleted by growing the strains in YPD medium for 28.5 h at 30°C, after which the cells were processed for immunofluorescence with anti-actin antibodies. At least 100 cells of each strain were scored for the actin bar phenotype. Bar, 10 μ m.

that ER structure was perturbed in the Gab1p-depleted cells (our unpublished data).

Examination of the original *gab1(o)* mutant suggested that Gab1p might be involved in cellular morphogenesis. To explore this further, we characterized the phenotypes of Gab1p-depleted cells in more details. These cells were generally larger than the isogenic control cells and have a mild defect in cell separation (Figure 7B, top). Moreover, the actin cytoskeleton, including actin cables and patches as revealed

by Alexa-phalloidin staining, was largely polarized in the Gab1p-depleted cells (Figure 7B, bottom).

Strikingly, when the actin cytoskeleton was stained with anti-actin antibodies, ~25% of Gab1p-depleted cells ($n = 102$) contained actin bars (Figure 7C, right), whereas similar structures were not observed in the control strain ($n = 300$) (Figure 7C, left), indicating a defect in actin organization in Gab1p-depleted cells. The majority of the cells contained a single actin bar and very few cells contained two bars. We

also examined whether depletion of other subunits of the GPI transamidase would cause actin bar formation. Like Gab1p, ~20% of Gpi8p-depleted cells formed actin bars (Figure 7E). In contrast, very few or no bars were observed in Gpi17p-, Gpi16p-, and Gaa1p-depleted cells (Figure 7, D and E). In addition to the actin bar phenotype, the percentage of small-budded cells with polarized actin patches seemed to decrease more in Gab1p-, Gpi8p-, and Gaa1p-depleted cells (Figure 7E). Because GPI transfer reaction was affected to the same degree by depletion of each subunit of the GPI transamidase (Fraering *et al.*, 2001; Ohishi *et al.*, 2001) (Figure 6; this study), these results suggest that the actin bar phenotype is unlikely caused by a deficiency in the GPI transamidase activity per se and that Gab1p and Gpi8p somehow play an additional role in actin organization.

Actin bar formation was not a general consequence of a GPI assembly defect. The GPI assembly mutants *gpi1Δ* (Leidich and Orlean, 1996), *gpi3Δ* (Leidich *et al.*, 1995), *smp3-2* (Grimme *et al.*, 2001), *mcd4-174* (Gaynor *et al.*, 1999), and *gpi7Δ* (Benachour *et al.*, 1999) formed 10, 1, 0, 0, and 1% actin bars, respectively (at least 100 cells were scored for each strain; our unpublished data). Furthermore, neither the *alg1* nor *alg2* mutants (Huffaker and Robbins, 1983), which are defective in essential, early steps in the assembly of the dolichol-linked precursor in asparagine-linked glycosylation exhibited detectable actin bar formation. However, the secretion (*sec*) mutants examined formed actin bars to varying degrees after shifting the mutants to 37°C for 3.5 h. The early *sec* mutants defective in ER-to-Golgi trafficking formed the most bars, including *sec18-1* (bars formed in 94% of the cells) and *sec20-1* (93%). The late-stage *sec* mutants defective in Golgi-to-plasma membrane trafficking also displayed actin bar formation, including *sec1-1* (52%), *sec2-41* (40%), *sec3-2* (45%), *sec4-8* (25%), *sec5-24* (32%), *sec6-4* (48%), *sec8-9* (31%), *sec9-4* (22%), *sec10-2* (52%), and *sec15-1* (34%). In addition, actin bars in all *sec* mutants were near the nucleus (our unpublished data).

None of the actin bars in Gab1p-depleted cells contained detectable tropomyosins, which normally decorate actin cables only (Liu and Bretscher, 1989) (Figure 8A). In contrast, all the actin bars were decorated by the actin-binding protein Cof1p (cofilin) (Figure 8A), which normally decorate actin patches, but not the cables (Figure 8A, arrowheads) (Moon *et al.*, 1993). Actin bars formed in cells at different stages of the cell cycle and did not colocalize with the mitotic spindle in most, if not, all cells (Figure 8B, cells 1–5). Moreover, in all cells examined (*n* = 50), actin bars were closely associated with the nucleus as indicated by DNA staining (Figure 8B, middle). Even in cells with two actin bars (Figure 8B, cell 2), the nucleus was located at the point of convergence of the bars. In addition, many actin bars exhibited an uneven distribution of fluorescence along their entire length, with their dimmer region always intersecting with the nucleus (100%, *n* = 30 bars) (Figure 8C, cell 3). Double labeling of the actin bars and the ER network with anti-actin and anti-Kar2p antibodies revealed that a portion of the actin bar was invariably associated with the perinuclear ER (Figure 8C), strongly suggesting that the actin bars are outside the nucleus, but in close association with the perinuclear ER. We also characterized the actin bars in cells deleted for *PFY1*, which encodes profilin, an actin monomer-binding protein, in *S. cerevisiae* (Haarer *et al.*, 1993). Like Gab1p-depleted cells, ~24% of the *pfy1Δ* cells (*n* = 204) displayed actin bars, all of which were decorated with cofilin and were closely associated with the perinuclear ER (Figure 8D; our unpublished data). Together, these data suggest that Gab1p somehow affects actin organization and that there might be a

Figure 8. Characterization of actin bars formed in Gab1p-depleted cells and profilin-deficient cells. (A) Actin bars in Gab1p-depleted cells are decorated with cofilin, but not tropomyosin. Gab1p-depleted cells (YEF625) and the wild-type control cells (YEF624), after being processed as described in Figure 7(B), were double-stained for actin and tropomyosin with anti-actin and anti-Tpm1p antibodies (left and middle) or double-stained for actin and cofilin with anti-actin and anti-cofilin (Cof1p) antibodies (right). (B) Cells from A were triple stained for actin, DNA, and tubulin with anti-actin antibodies, bisBenzimide, and an anti-tubulin antibody. (C) Cells from A were triple stained for actin, ER, and DNA with anti-actin, anti-Kar2p antibodies, and bisBenzimide. (D) Actin bars in profilin-deficient cells are also decorated with cofilin and are closely associated with DNA. Cells of JGY519 (*pfy1Δ::LEU2*) were grown to exponential phase in YPD medium at 24°C and then processed for immunofluorescence. Cells were then triple-stained with anti-actin, anti-Cof1p antibodies, and bisBenzimide.

Figure 9. Model for the possible relationships between the subunits of the GPI transamidase. The core components (Gpi8p, Gpi16p, and Gaa1p) of the GPI transamidase are highlighted in pink with the disulfide bond between Gpi8p and Gpi16p being indicated by the black box. Other components (Gab1p and Gpi17p) are highlighted in blue. The distance between Gab1p and each subunit reflects the relative strength in suppression of the *gab1-1* mutation by the specific subunit.

common mechanism underlying actin bar formation in Gab1p-depleted and other mutant cells.

DISCUSSION

Gab1p and Its Relationships with Other Subunits of the GPI Transamidase in Yeast

Our ochre-suppressor screen for morphogenesis mutants led to the identification of a previously uncharacterized protein, Gab1p. Functional studies presented here indicate that Gab1p is the fifth subunit of the yeast GPI transamidase. First, a *gab1-Ts* mutant accumulates the same GPI precursors as those accumulated by three GPI transamidase mutants. Second, overexpression of *GPI8* or *GPI17*, genes encoding two GPI transamidase subunits, strongly suppresses the temperature sensitivity of *gab1-Ts* cells. Third, Gab1p coimmunoprecipitates with Gpi17p. Finally, the human Gab1p homologue PIG-U has recently been identified as the fifth subunit of the human GPI transamidase, and yeast Gab1p can partially complement a PIG-U-deficient cell line (Hong *et al.*, 2003). Previous efforts to identify additional subunits of the yeast GPI transamidase complex by coimmunoprecipitation with known transamidase subunits failed to identify Gab1p (Fraering *et al.*, 2001). This could be due to the great hydrophobicity (8–10 transmembrane domains) of Gab1p, its low endogenous level, and the smeared bands in the higher molecular weight range on SDS-polyacrylamide gels.

The functions of individual subunits in the GPI transamidase complex and the relationships among these subunits are still being uncovered. Our functional studies on yeast Gab1p, together with previous biochemical and structural studies on the GPI transamidase in several organisms, has led us to propose a possible arrangement of the subunits in the complex (Figure 9). Gpi8p, the catalytic subunit (Meyer *et al.*, 2000), is the centerpiece of the complex with four other

subunits, Gpi16p, Gaa1p, Gpi17p, and Gab1p, surrounding it in the clockwise direction. Moreover, this five-component complex may be further divided into two subcomplexes: one containing Gpi8p, Gpi16p, and Gaa1p; and the other containing Gab1p and Gpi17p. This arrangement is supported by the following facts. First, it reflects the differential suppression of a *gab1* mutant by overexpression of other subunits, with strong suppression displayed by Gpi8p and Gpi17p, weak suppression by Gaa1p, and no suppression by Gpi16p (Figure 4). Second, affinity purification by using endogenous level of GST-Gpi8p as the biochemical bait led to the identification of the three-component subcomplex (Fraering *et al.*, 2001). Third, all known GPI transamidases have five subunits and all subunits are conserved between yeast and human. However, only the three-component subcomplex is conserved between humans and the protozoan *Trypanosoma brucei*, whose other two components seem unique to this organism (Nagamune *et al.*, 2003). Fourth, Gpi16p is linked to Gpi8p via a disulfide bond (Ohishi *et al.*, 2003). Structural modeling (Eisenhaber *et al.*, 2003) led to the notion that Gpi16p may regulate the access of substrate protein to Gpi8p and that Gaa1p has been suggested to interact with the substrate GPI (Vainauskas and Menon, 2004) and maybe involved in presenting the free GPI anchor to Gpi8p (Eisenhaber *et al.*, 2003). Thus, theoretically, Gpi8p, Gpi16p, and Gaa1p could form a minimal functional complex. Finally, we have shown that Gab1p is in the same complex as Gpi17p (Figure 5). The functions of these two subunits in the GPI transfer reaction are not known, although Gpi17p has been speculated to stabilize the transamidase complex or play a role in substrate selection (Eisenhaber *et al.*, 2003). The great hydrophobicity of Gab1p, and the presence of a stretch of amino acids shared with fatty acid elongases (Hong *et al.*, 2003), are consistent with the possibility that Gab1p is involved in presenting free GPI to the catalytic subunit Gpi8p.

The Perinuclear ER-Actin Bar Association in Gab1p-depleted Cells and Its Implications for ER Inheritance

In addition to the GPI transfer defect, Gab1p-depleted cells had a second, dramatic phenotype: the formation of actin bars. These structures can be detected with anti-actin bodies, but not with Alexa-phalloidin, suggesting that the bars could be either aggregates of G-actin or actin filaments that are decorated with actin-binding proteins (ABPs) that would prevent the binding of phalloidin. The fact that all actin bars in Gab1p-depleted cells were decorated by cofilin, an ABP that is known to prevent phalloidin from binding to actin filaments (McGough *et al.*, 1997; Bamburg *et al.*, 1999) suggests that actin bars may in fact contain bundles of actin filaments. Interestingly, only a single bar was formed in each cell, and the bar was always associated with the perinuclear ER. Because Gab1p is a subunit of the GPI transamidase, we also examined the role of other subunits in actin bar formation. Except Gpi8p, depletion of the other three subunits caused little or no bar formation. These data suggest that a general defect in the activity of the GPI transamidase is unlikely to be responsible for the actin bar formation. Furthermore, because actin bar formation was not a characteristic of GPI assembly mutants, this phenotype is not a general consequence of a deficiency in GPI anchoring and GPI-dependent protein processing and transport in yeast.

An actin bar-like structure, "actin rods" containing actin depolymerization factor ADF/cofilin, has also been observed in the neurites of Alzheimer's patients and in neurons stimulated with neurodegeneration signal, although the significance of the actin rod formation is not known

(Minamide *et al.*, 2000; Ashworth *et al.*, 2003). In yeast mutants deficient in ABPs such as profilin (Haarer *et al.*, 1990, 1993), in a strain overexpressing an ABP (Drubin *et al.*, 1988), or in a mutant carrying a specific actin mutation (*act1-2*) (Novick and Botstein, 1985), actin bars are frequently observed and found to be near the nucleus. In this study, we report that actin bars in a profilin deletion strain are also decorated with cofilin and are associated with the perinuclear ER (Figure 8D). In addition to the actin-related mutants, we also observed that all the *sec* mutants examined so far formed actin bars to varying extents, with mutants blocked in ER-to-Golgi transport forming them more extensively than mutants affected in Golgi-to-plasma membrane trafficking. The bars formed in *sec* mutants were all near the nucleus. Because all these *sec* mutants are known to block secretion to a similar degree yet display a significant variation in their ability to form actin bars, a general defect in secretion seems unlikely to be responsible for the actin bar phenotype.

In summary, the actin bar phenotypes in all mutants examined so far are very similar: a single bar per cell that is often positioned near the nucleus. These analyses suggest that there might be a common mechanism underlying the actin bar phenotype. What triggers actin bar formation? Is the actin bar a pathological structure or does it represent an adaptive strategy for the cell to cope with internal and/or external stress? Does an actin bar consist of G-actin aggregates or does it represent specialized F-actin? Is an actin bar dynamic? Why do the majority cells form only one bar and why it is always associated with the nucleus? Currently, we cannot answer all these questions, but we have some clues to the last one.

The close association between an actin bar and the perinuclear ER in mutant cells may reflect a normal interaction between the ER and the actin cytoskeleton in wild-type cells. ER-actin interactions have been observed in other organisms. In animal cells, ER is directly linked to actin filaments through ABPs such as spectrin, interaptin, and myosin V (Baumann, 1998; Rivero *et al.*, 1998; Tabb *et al.*, 1998; Wollert *et al.*, 2002). In *S. cerevisiae*, the anchorage of an ER tubule to the bud tip and the maintenance of ER in the cell cortex both involve F-actin (Fehrenbacher *et al.*, 2002; Estrada *et al.*, 2003). One possible explanation for the position of the actin bar in different mutants is presented here. In wild-type cells, part of the ER network (the tip of an ER tubule emanating from the perinuclear ER and/or the ER in the bud cortex) might be attached to the plasma membrane via a chain of molecular interactions, from ABPs associated with the ER surface, to actin, and then to an ER-anchorage complex, and finally to plasma membrane. The ER-associated ABPs might include the type V myosin Myo4p, which is involved in the transport of several mRNAs to the daughter cell and also in the cortical ER inheritance (Kruse *et al.*, 2002; Estrada *et al.*, 2003). At least, a fraction of Myo4p is associated with the ER membrane (Estrada *et al.*, 2003), and at a steady state Myo4p seems to be concentrated at the bud tip and/or the bud cortex (Kruse *et al.*, 2002). The ER-anchorage complex might contain polarity proteins and Sec3p, a component of the exocyst involved in spatial regulation of exocytosis (Finger *et al.*, 1998). This model couples ER inheritance to polarity establishment and/or maintenance and also explains why actin bar is always associated with the perinuclear ER in mutants defective in diverse cellular processes. With this model, any event that breaks the ER anchorage to the bud tip or the bud cortex, including ER disorganization, defects in ABPs or actin itself, or defects in ER-anchorage complex could lead to the collapse of part of the ER onto or near the

nuclear envelope. The ABP on the ER surface binds to the actin bar, thus explaining the position of the bar in different mutants. Depletion of Gab1p, and the early and late *sec* mutants all affect ER organization (this study) (Kaiser and Schekman, 1990; Wiederkehr *et al.*, 2003). A particularly interesting finding is that a *sec3* mutant displays an ER inheritance defect (Wiederkehr *et al.*, 2003). Electron microscopy study of this mutant shows that only 33% of the cells had cortical ER in the bud in comparison with 94% for the wild-type cells. Remarkably, 27% of the *sec3* mutant cells contain a cluster of ER tubules near the nucleus, lending strong support to our model. The nature of the ABP-ER interaction is unknown, but our findings with selected GPI transamidase subunits are consistent with the notion that one or more resident proteins of the ER membrane are directly or indirectly involved. A relationship between components of the GPI transamidase, and the cytoskeleton also has been noted in mammalian cells. The cytoplasmically oriented NH₂ terminus of human Gaa1 interacts with tubulins and that removal of that NH₂-terminal sequence affects its interaction with tubulins and consequently the ER structure and the morphology of cultured mammalian cells (Vainauskas *et al.*, 2002). Furthermore, the NH₂ terminally deleted forms of human Gaa1 showed a dramatically altered distribution that was suggested to reflect a collapse of the ER around the nucleus of cultured cells (Vainauskas *et al.*, 2002).

In this study, we have defined Gab1p as the fifth subunit of the yeast GPI transamidase and identified some functional relationships among its different subunits of the complex, which will inform future mechanistic studies of this enzymatic complex. We have also found that Gab1p-depleted cells form actin bars that are always associated with the perinuclear ER. Although it is not clear whether Gab1p is directly involved in the ER-actin interaction, our findings highlight a close relationship between the ER and the actin cytoskeleton, which has broad implications for ER inheritance and/or ER dynamics in *S. cerevisiae*.

ACKNOWLEDGMENTS

We thank J.R. Pringle for encouragement and discussions throughout this work; Herman Chen for excellent technical assistance; D. Drubin for pointing out actin bar formation in secretory mutants; M. Longtine for sharing unpublished data; L. Riles, M.V. Olson, W. Guo, C. Burd, and P. Brennwald for strains; J. Cooper, S. DiNardo, D. Drubin, P. Melloy, L. Pon, and M. Rose for plasmids and antibodies; and P. Tran for help with confocal microscopy. This work was supported by National Institutes of Health grants GM-59216 (to E.B.), GM-46220 (to P.O.), and GM-31006 (to J.R. Pringle), and by grant RSG-02-039-01-CSM from the American Cancer Society (to E.B.).

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