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Yeast *ARV1* Is Required for Efficient Delivery of an Early GPI Intermediate to the First Mannosyltransferase during GPI Assembly and Controls Lipid Flow from the Endoplasmic Reticulum

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Glycosylphosphatidylinositol (GPI), covalently attached to many eukaryotic proteins, not only acts as a membrane anchor but is also thought to be a sorting signal for GPI-anchored proteins that are associated with sphingolipid and sterol-enriched domains. GPI anchors contain a core structure conserved among all species. The core structure is synthesized in two topologically distinct stages on the leaflets of the endoplasmic reticulum (ER). Early GPI intermediates are assembled on the cytoplasmic side of the ER and then are flipped into the ER lumen where a complete GPI precursor is synthesized and transferred to protein. The flipping process is predicted to be mediated by a protein referred as flippase; however, its existence has not been proven. Here we show that yeast Arv1p is an important protein required for the delivery of an early GPI intermediate, GlcN-acylPI, to the first mannosyltransferase of GPI synthesis in the ER lumen. We also provide evidence that *ARV1* deletion and mutations in other proteins involved in GPI anchor synthesis affect inositol phosphorylceramide synthesis as well as the intracellular distribution and amounts of sterols, suggesting a role of GPI anchor synthesis in lipid flow from the ER.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) is a complex glycolipid found in all eukaryotic cells. It is covalently attached to the C-terminal end of certain secretory proteins and acts as a membrane anchor (Kinoshita and Inoue, 2000; Ikezawa, 2002; Pittet and Conzelmann, 2007). Because many proteins anchored by GPI are poorly extracted with detergents at 4°C, they have been proposed to be localized in detergent-resistant membranes (DRMs), which may be related to microdomains called lipid rafts that have been proposed to play an important role in the trafficking of GPI-anchored proteins (Simons and Ikonen, 1997; Chatterjee and Mayor, 2001; Mayor and Riezman, 2004).

Biosynthesis of GPI takes place on the membranes of the endoplasmic reticulum (ER) (Kinoshita and Inoue, 2000; Pittet and Conzelmann, 2007), and this starts with the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to the inositol of phosphatidylinositol (PI) to generate *N*-acetylglucosaminyl-PI (GlcNAc-PI), followed by de-N-acetylation to form glucosaminyl-PI (GlcN-PI). The two reactions occur on the cytoplasmic side of the ER membrane (Vidugiriene and Menon, 1993; Orlean and Menon, 2007). The GlcN-PI is then acylated on the inositol ring to form glucosaminyl-acyl-PI (GlcN-acylPI) before or after transfer of the first mannose (Smith *et al.,* 1997; Kinoshita and Inoue, 2000; Pittet and Conzelmann, 2007). Dolicholphosphomannose (Dol-P-Man) is the mannose donor for the core mannose residues (Menon *et al.,* 1990). In *Saccharomyces cerevisiae* and *Plasmodium falciparum*, inositol acylation appears to be a prerequisite for mannosylation of GPIs (Doerrler *et al.,* 1996; Gerold *et al.,* 1999), whereas, in *Trypansoma brucei* and mammalian cells, it is not essential for addition of the first mannose (Guther and Ferguson, 1995; Smith *et al.,* 1996; Murakami *et al.,* 2003), although the mammalian mannosyltransferase (GPI-MT-I), PIG-M seems to prefer GlcN-acylPI to GlcN-PI. The PIG-M has a lumenally oriented, functionally important DXD motif that is found in many glycosyltransferases (Maeda *et al.,* 2001). Based on the topological aspect of synthesis of

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Abbreviations used: AbA, aureobasidin A; CFW, calcofluor white; CP, complete GPI precursor; CPY, carboxypeptidase Y; DHS, dihydrosphingosine; Dol-P-Man, dolicholphosphomannose; EtNP, phosphorylethanolamine; GDP-Man, GDP-mannose; GlcNAc-PI, *N*-acetylglucosaminyl-phosphatidylinositol; GlcN-PI, glucosaminyl-phosphatidylinositol; GlcN-acylPI, glucosaminyl-acyl-phosphatidylinositol; GPI, glycosylphosphatidylinositol; GPI-MT, GPI mannosyltransferase; HA, hemagglutinin; IPC, inositolphosphorylceramide; LY, lucifer yellow; MIPC, mannosyl inositolphosphorylceramide; $M(IP)_2C$, mannosyl di(inositolphosphoryl)ceramide; PI, phosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; UDP-GlcNAc, UDP-N-acetylglucosamine.

GlcN-PI and subsequent mannosylation, it was suggested that the early GPI intermediates must flip from the cytoplasmic side to the ER lumen before the first mannosylation. In addition, genes coding for mammalian PIG-W and the yeast homologue *GWT1*, which are involved in inositol acylation of GPI were recently identified (Murakami *et al.*, 2003; Umemura *et al.*, 2003). They encode multi-spanning ER membrane proteins. Because the comparison of locations of predicted transmembrane domains and amino acid sequence of PIG-W homologues of various organisms showed that the location of conserved regions face the lumenal side of the ER, it was also proposed that inositol acylation occurs in the ER lumen; hence, inositol acylation is not required for flipping of GPI (Murakami *et al.*, 2003). However, it remains unclear whether the catalytic site of PIG-W is located within the conserved regions. Mutations in the temperature-sensitive (ts) mutant alleles of *GWT1*, which were randomly generated by PCR mutagenesis, were not found in the conserved regions (Umemura *et al.*, 2003). Thus, it is not yet known whether GlcN-PI or GlcN-acylPI, or both, are flipped across the ER membrane. After mannosylation and addition of phosphorylethanolamine (EtNP) residue(s), the entire GPI anchor precursor is attached to proteins by a GPI transamidase, which acts on the lumenal side of the ER (Kinoshita and Inoue, 2000; Ikezawa, 2002; Pittet and Conzelmann, 2007). Subsequently, the acyl group of the inositol is removed from the GPI-anchored proteins (Tanaka *et al.*, 2004; Fujita *et al.*, 2006a), and the GPI lipid moieties are remodeled to a more hydrophobic diacylglycerol or ceramide (Sipos *et al.*, 1997; Reggiori *et al.*, 1997). Most of the genes encoding enzymes involved in GPI biosynthetic pathway have been isolated and characterized (Kinoshita and Inoue, 2000; Pittet and Conzelmann, 2007). Recently, genes that are required for lipid remodeling of GPI-anchored proteins were also identified (Tashima *et al.*, 2006; Bosson *et al.*, 2006; Fujita *et al.*, 2006b; Ghugtyal *et al.*, 2007; Umemura *et al.*, 2007). However, despite evidence that GPI precursor flipping is a protein-mediated process (Vishwakarma and Menon, 2005; Pomorski and Menon, 2006), the putative GPI flippase has not yet been identified.

Once the inositol-linked acyl chain is eliminated by a GPI inositol deacylase, the GPI-anchored proteins are transported from the ER to the Golgi apparatus via transport vesicles (Muniz and Riezman, 2000; Ikonen, 2001; Mayor and Riezman, 2004). In *S. cerevisiae*, GPI-anchored proteins are known to exit the ER in vesicles distinct from other secretory proteins (Muniz *et al.*, 2001). Rab GTPase Ypt1p, the tethering factors Uso1p, COG complex Sec34p and Sec35p, and the ER v-SNAREs Bos1p, Bet1p, and Sec22p are necessary for the sorting of GPI-anchored proteins upon ER exit (Morsomme and Riezman, 2002; Morsomme *et al.*, 2003). The lipid composition may have a function in sorting of GPI-anchored proteins into ER-derived vesicles because GPI-anchored proteins are associated with DRMs in the ER (Bagnat *et al.*, 2000) and because ongoing ceramide synthesis is required for the efficient transport of GPI-anchored proteins (Horvath *et al.*, 1994; Sutterlin *et al.*, 1997a; David *et al.*, 1998; Barz and Walter, 1999; Watanabe *et al.*, 2002). Ceramide synthesis does not seem to be required for GPI-anchored protein transport in trypanosomes, but the anchors are not remodeled to ceramides in these cells (Sutterwala *et al.*, 2007). Because the maturation of GPI-anchored proteins is delayed when yeast cells are incubated with myriocin, an inhibitor of serine palmitoyltransferase (SPT), or when a ts *lcb1-100* mutant defective in SPT activity is incubated at nonpermissive temperature (Horvath *et al.*, 1994; Sutterlin *et al.*, 1997a), but not affected when yeast cells are treated with aureobasidin A (AbA), an inhibitor of yeast sphingolipid synthesis (Reggiori and Conzelmann, 1998), ceramide-rich domains rather than complex sphingolipid-rich domains could be important for the sorting step in the ER. This is also supported by the fact that ceramide is synthesized in the ER and then is delivered to the Golgi where it is converted to inositolphosphorylceramide (IPC), followed by mannosylation to form mannosyl IPC (MIPC) and mannosyl di(inositolphosphoryl)ceramide [$M(IP)_2C$], which are the three major yeast sphingolipids (Funato *et al.*, 2002; Dickson *et al.*, 2006). Previously, we have shown that ER-to-Golgi transport of ceramide for IPC synthesis is mediated by both vesicular and nonvesicular trafficking (Funato and Riezman, 2001).

Although the roles of the different pathways are poorly understood, the vesicular transport of ceramide could be directly coupled to transport of proteins. In addition, GPI anchor attachment to proteins and the lipid remodeling are necessary for efficient transport of GPI-anchored proteins to the Golgi (Hamburger *et al.*, 1995; Doering and Schekman, 1996; Bosson *et al.*, 2006; Fujita *et al.*, 2006b). Recent studies have shown that mutant cells defective in GPI anchor synthesis or attachment block export of some detergent-insoluble transmembrane proteins from the ER (Okamoto *et al.*, 2006), suggesting a role of GPI-anchored proteins in raft-dependent protein sorting. However, evidence indicating that GPI-anchored proteins are required for transport of raft lipid(s) per se is missing.

In this study we show that yeast *ARV1*, which has been known to be somehow involved in the regulation of sphingolipid and sterol metabolism (Tinkelenberg *et al.*, 2000; Swain *et al.*, 2002; Fores *et al.*, 2006), genetically interacts with genes involved in GPI anchor synthesis and that Arv1p is required for the efficient synthesis of GlcN-acylPI bearing one mannose, whereas *arv1*Δ cells retain both Dol-P-Man synthesis and GPI-MT activity. We propose that the primary function of Arv1p is either to deliver GlcN-acylPI from the cytoplasmic side to the luminal side of the ER or to present it to the yeast PIG-M homologue *GPI14* (Maeda *et al.*, 2001). In addition, we present evidence that GPI assembly is required not only for GPI-anchored protein transport but also for ceramide transport from the ER as well as to maintain the proper intracellular distribution and amounts of sterols.

MATERIALS AND METHODS

Strains, Media, Library Screen, Plasmids, and Reagents

The yeast strains and plasmids used in this study are listed in Supplementary Tables S1 and S2, respectively. Yeast cultures and genetic manipulations were carried out essentially as described by Sherman *et al.* (1983). To construct deletion strains, entire open reading frames were deleted and replaced with the designated genes. Deletions were confirmed by PCR. Yeast strains were grown in rich medium, YPUAD (Dulic *et al.*, 1991), semisynthetic medium, SDYE (Horvath *et al.*, 1994), or synthetic minimal medium, SD (Funato *et al.*, 2003), supplemented with the appropriate nutrients to select for plasmids. *pmi40*Δ mutant cells were supplemented with 0.5% mannose (Pitkanen *et al.*, 2004).

Temperature sensitivity was determined by spotting diluted yeast cultures on SD (−ura) plates at 25 and 37°C. To test drug sensitivity, cells were cultured on SD plates containing calcofluor white (CFW) or AbA. A genomic *LEU2*-marked library made in YEp13 (a gift from Dr. Y. Ohya, University of Tokyo, Japan) was used to isolate suppressors of the ts growth phenotype of *arv1*Δ mutant (FK137). A plasmid was isolated containing *GPI15*. *GPI15, GPI1, GPI2, GPI3,* and *ERI1* genes were amplified from *S. cerevisiae* genomic DNA by PCR. *GPI15* was cloned into BamHI/EcoRI sites of pRS426GPD, *GPI1* into BamHI/XhoI, *GPI2* into BamHI/SalI, *GPI3* into SpeI/XhoI, and *ERI1* into BamHI/XhoI of pRS426ADH vector. To construct a plasmid (pGAL-HA-GPI18) for expressing N-terminal two-hemagglutinin (HA)-tagged *GPI18*, the BamHI-SalI PCR fragment containing the open reading frame of *GPI18* was ligated into pRS316-GAL1-2xHA-BS, a pRS316-based expression vector carrying the EcoRI site, the initiation codon, two HA-epitope–encoding regions,

and multicloning sites (provided by Dr. K. Tanaka, Hokkaido University, Japan). The EcoRI-SalI fragment including the epitope-tagged *GPI18* was subcloned into pRS416GAL1. A diploid heterozygous *ARV1/arv1Δ::LEU2 GPI18/gpi18Δ::KAN^R* double deletion strain was created by deleting *ARV1* in the *GPI18/gpi18::KAN^R* diploid strain BY4743 *MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 lys2Δ0 met15Δ0 gpi18Δ::KAN^R* (generated by the *Saccharomyces* Gene Deletion Project and provided by Dr. T. Kinoshita, Osaka University, Japan) and transformed with pGAL-HA-GPI18. The transformants were sporulated and tetrads dissected to obtain haploid strains *arv1Δ::LEU2 gpi18Δ::KAN^R* (FK1015), *gpi18Δ::KAN^R* (FK1017), and wild-type (FK1019) harboring pGAL-HA-GPI18. A C-terminal triple HA-tagged *ARV1* was amplified from genomic DNA (prepared from RH6076, which carried the epitope-tagged *ARV1* gene) by PCR, and cloned into BamHI/XhoI sites of pRS426ADH. AbA and C_2-ceramide were purchased from Takara (Tokyo, Japan) and Matreya (State College, PA), respectively. YW3548 was prepared as described previously (Sutterlin *et al.*, 1997b).

Labeling of Lipids In Vivo

In vivo labeling of lipids with [^{3}H]*myo*-inositol (Perkin Elmer-Cetus Life Sciences, Boston, MA) or [^{3}H]dihydrosphingosine (DHS; American Radiolabeled Chemicals, St. Louis, MO) was performed as described previously (Zanolari *et al.*, 2000). Cells were grown overnight in SDYE, harvested and resuspended in SD without inositol for labeling with [^{3}H]*myo*-inositol, or SD medium for labeling with [^{3}H]DHS. The cells were preincubated for 15 min at 25°C and then labeled by 25 μCi of [^{3}H]*myo*-inositol or 10 μCi of [^{3}H]DHS. If present, C_2-ceramide (200 μM) was added at the start of the labeling. The incubation was stopped by the addition of 10 mM NaF and 10 mM NaN_3. The cells were then washed with cold water, and lipids were extracted with chloroform-methanol-water (10:10:3, vol/vol/vol). Half of the dried sample was treated by mild alkaline hydrolysis with 0.6 M NaOH, neutralized, and desalted by *n*-butanol partitioning. The lipids were analyzed by thin-layer chromatography (TLC) using solvent system I, chloroform-methanol-0.25% KCl (55:45:10, vol/vol/vol) for complex sphingolipid labeled with [^{3}H]*myo*-inositol, and solvent system II, chloroform-methanol-4.2 N ammonium hydroxide (9:7:2, vol/vol/vol) for sphingolipid labeled with [^{3}H]DHS. Radiolabeled lipids were visualized and quantified on a Cyclone Storage Phosphor System (Packard Instrument, Meriden, CT).

In vivo [^{3}H]mannose labeling using the *pmi40Δ* and *arv1Δ pmi40Δ* double mutant strains was performed as described (Sipos *et al.*, 1994; Sutterlin *et al.*, 1997b). In brief, the cells were grown overnight in SDCU medium (2% glucose, 1% peptone, 0.67% yeast nitrogen base, 0.5% mannose, and the required nutrients), harvested, and resuspended in SPCU medium (0.1% glucose, 2% pyruvate, 0.67% yeast nitrogen base, and the required nutrients). Wild-type and *arv1Δ* mutant strains were grown in SDCU medium without mannose. For [^{3}H]mannose labeling of strains transformed with pGAL-HA-GPI18, cells were first grown in SGYE (2% galactose, 0.67% yeast nitrogen base, 0.2% yeast extract, and the required nutrients) medium, then shifted to SDYE medium for 16 h at 25°C, and resuspended in SPCU medium. The cells were preincubated for 20 min at 25°C with 20 μg/ml tunicamycin in the presence or absence of YW3548 (10 μM) and then labeled with 25 μCi of [^{3}H]mannose (Perkin-Elmer Cetus Life Sciences) for 30 min at 25°C. The labeling was stopped by the addition of NaF and NaN_3, and lipids were extracted with chloroform-methanol-water (10:10:3, vol/vol/vol), desalted by *n*-butanol partitioning, and analyzed by TLC using solvent system III, chloroform-methanol-water (10:10:3, vol/vol/vol).

Labeling of Lipids In Vitro

Membranes for in vitro labeling experiments with [^{14}C]UDP-GlcNAc (Perkin-Elmer Cetus Life Sciences) or with [^{3}H]GDP-mannose (GDP-Man; American Radiolabeled Chemicals) were prepared as described by Schonbachler *et al.* (1995), except that for cell breakage, TDP (50 mM Tris-HCl, pH 7.4, 5 mM DTT, and 1 mM PMSF) was used. The membranes were suspended in TDP containing 20% glycerol and stored at −80°C. Samples of membranes containing equivalent amounts of protein were assayed for the early steps of GPI anchor biosynthesis using [^{14}C]UDP-GlcNAc as described (Schonbachler *et al.*, 1995) with a few modifications. Two hundred micrograms of membranes were incubated with 50 nCi [^{14}C]UDP-GlcNAc at 25°C in 50 μl of GPI buffer consisting of 100 mM Tris-HCl, pH 7.5, 1 mM EGTA, 3 mM Mg-acetate, 0.5 mM $MnCl_2$, 1 mM CoA, 1 mM ATP, 20 μg/ml tunicamycin, and 250 nM GDP-Man. Dol-P-Man synthesis was assayed in GPI buffer containing 0.5 mM UDP-GlcNAc and 0.25 μCi [^{3}H]GDP-Man (250 nM). Lipids were extracted by the addition 750 μl of chloroform-methanol (1:2, vol/vol), desalted, and analyzed by TLC using solvent system IV, chloroform-methanol-1N ammonium hydroxide (10:10:3, vol/vol/vol) for [^{14}C]UDP-GlcNAc labeled lipids (Watanabe *et al.*, 1998), and solvent system III for the synthesis of Dol-P-Man (Sutterlin *et al.*, 1997b).

For mannosylated GPI lipid synthesis assay, ER-enriched membranes were prepared as described (Funato and Riezman, 2001), and the membranes were incubated with GPI buffer, 0.5 mM UDP-GlcNAc and 0.5 μCi [^{3}H]GDP-Man in a final volume of 100 μl (Sutterlin *et al.*, 1997b). Some reactions contained 10 mM *n*-octyl β-D-glucopyranoside and 0.4 μg/ml dioctanoyl-PI [GlcN-PI(C8); a gift from Dr. M. A. Lehrman, University of Texas Southwestern Medical Center, Dallas, TX] with 6×10^{-4} % Triton X-100, which was used to add GlcN-PI(C8). After 10-min incubation at 25°C, lipids were extracted by the addition 666 μl of chloroform-methanol (1:1, vol/vol), desalted, and analyzed by TLC using solvent system III.

Pulse-Chase Experiments for Protein Maturation and GPI Anchor Attachment

Radiolabeling and immunoprecipitation to measure maturation of GPI-anchored proteins (Gas1p, Yps1p) and carboxypeptidase Y (CPY) were performed as described previously (Sutterlin *et al.*, 1997a). The samples were subjected to SDS-PAGE, analyzed, and quantified using the Cyclone Storage Phosphor System (Packard Instruments). The percentage of mature proteins was determined by taking the ratio of the mature form to the total signal (ER, Golgi, and mature forms). GPI anchor attachment was examined as described (Watanabe *et al.*, 2002).

Fluorescence Microscopy

Labeling of cells with C_6-NBD-ceramide was performed as described (Levine *et al.*, 2000). Cells were incubated with or without AbA (20 μg/ml) at 25°C for 15 min and labeled with C_6-NBD-ceramide (20 μM) at 25°C for 15 min in the presence of defatted BSA (5 mg/ml), followed by washing and back-extracting. The staining was visualized by fluorescence microscopy. For visualization of chitin (Sobering *et al.*, 2004) and sterol distribution (Beh and Rine, 2004), cells were grown in SD medium at 25°C, washed in PBS, and stained with CFW (1 mg/ml) for 5 min and filipin complex (0.1 mg/ml) for 15 min, respectively. The cells were washed and observed by fluorescence microscopy with a UV filter.

Gas Liquid Chromatography–Mass Spectrometry Analysis of Sterols

Cells were grown in SD medium at 25°C. Total sterols including free sterols and sterol esters were extracted from whole cells and analyzed by gas liquid chromatography-mass spectrometry (GLC-MS) as described previously (Munn *et al.*, 1999; Heese-Peck *et al.*, 2002, Mullner *et al.*, 2005). Cholesterol was used as an internal standard.

RESULTS

An arv1Δ Mutant Is Deficient in Ceramide Transport from the ER to the Golgi Site of IPC Synthesis

ARV1 was identified in a screen for genes that are essential in the absence of yeast sterol esterification (Tinkelenberg *et al.*, 2000). Arv1p is a multispanning integral membrane protein with six predicted transmembrane domains, and homologues have been found in a wide variety of eukaryotes. Because disruption of *ARV1* leads to not only altered levels and distribution of sterols but also reduced sphingolipid levels, it has been proposed that this protein is involved in maintenance of cellular sterol and sphingolipid homeostasis (Swain *et al.*, 2002). To obtain further evidence for the role of Arv1p in the regulation of sphingolipid homoeostasis, we re-examined sphingolipid biosynthesis in *arv1Δ* mutant cells, which were generated in our strain background. Consistent with the previous report, the *arv1Δ* mutant showed a strong reduction of sphingolipid synthesis, ~20% of IPC-C/-D/MIPC and 50% of M(IP)$_2$C levels found in wild-type cells when cells were pulse-labeled with [^{3}H]*myo*-inositol (Figure 1A). A similar reduction of IPC-C synthesis was also seen when the sphingolipid synthesis of *arv1Δ* mutant was assessed by [^{3}H]DHS labeling (Figure 1B), confirming that this mutant exhibits a sphingolipid synthesis defect. As shown by a previous experiment (Swain *et al.*, 2002), we also observed that the *arv1Δ* mutant cells made normal amounts of ceramides as determined by [^{3}H]DHS pulse-labeling for 2 h (data not shown).

Because a sphingolipid synthesis defect could result from a lack of IPC synthase activity, we next examined IPC synthase activity in *arv1Δ* mutant cells. In wild-type cells, exogenously added C_6-NBD-ceramide is efficiently converted to C_6-NBD-IPC, and the product is trapped in the Golgi compartment where Aur1p, the IPC synthase, is localized. When the fluorescent NBD probe is added to cells where IPC synthase is inhibited with AbA, an IPC synthase inhibitor,

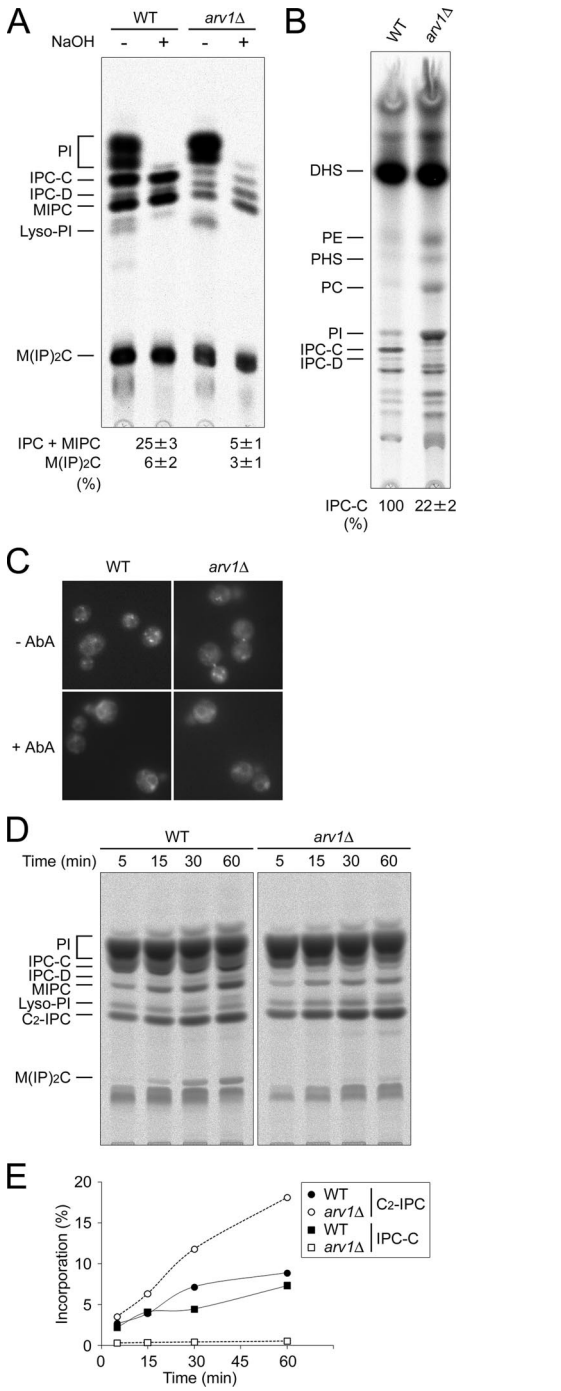

Figure 1. The *arv1*Δ mutant is defective in ceramide transport from the ER to the Golgi. (A and B) Wild-type (RH6082) and *arv1*Δ (RH6078) strains were labeled with [^{3}H]*myo*-inositol (A) or with [^{3}H]DHS (B) for 2 h at 25°C. The [^{3}H]*myo*-inositol–labeled lipids were subjected (+) or not (−) to mild alkaline hydrolysis with NaOH. (C) The same strains as in A were labeled with C_6-NBD-ceramide for 15 min at 25°C, with or without AbA as described in *Materials and Methods,* and the staining was visualized by fluorescence microscopy. (D) The same strains were also labeled with [^{3}H]*myo*-inositol for the indicated times at 25°C in the presence of C_2-ceramide. The labeled lipid products were applied to TLC plates using solvent system I (A and D) or solvent system II (B). Lipids were identified by their mobility and resistance to mild-base treatment. The band, which was identified as C_2-IPC, appeared only when the precursor C_2-ceramide was added, and the appearance was resistant to mild-base treatment and sensitive to the addition of AbA (not shown). Incorporation (%) into sphingolipids was determined as the percentage of total radioactivity into all lipids containing inositol (A and D). Incorporation of [^{3}H]DHS into IPC-C was quantified, and the relative amounts were calculated by setting the amounts in wild-type cells to 100% (B). Results of a typical experiment are shown. Data in A and B are means ± SE for six and four independent experiments, respectively, and in E are quantification of D. *PI*, phosphatidylinositol; IPC-C and -D, inositolphosphorylceramide subclasses C and D; *MIPC*, mannosyldi(inositolphosphoryl) ceramide; Lyso-PI, lyso-phosphatidylinositol; DHS, dihydrosphingosine; PE, phosphatidylethanolamine; PHS, phytosphingosine; PC, phosphatidylcholine.

the probe is preferentially incorporated into the ER membrane, indicating that localization of the NBD probe is dependent on the activity of IPC synthase (Levine *et al.*, 2000). Our data indicate that there is no apparent difference between wild-type and *arv1*Δ mutant cells in the localization of exogenous C_6-NBD-ceramide during a short labeling period, 15 min (Figure 1C), suggesting that IPC synthase in the *arv1*Δ mutant is active. For a quantitative assessment of IPC synthase activity, incorporation of exogenous C_2-ceramide into C_2-IPC in these strains was measured with [^{3}H]*myo*-inositol labeling (Figure 1D). Although the incorporation of endogenous ceramide into IPC-C in *arv1*Δ mutant cells was reduced, the incorporation of C_2-ceramide into C_2-IPC was 1.5–2-fold greater than wild-type cells after long labeling times, 30 and 60 min, respectively (Figure 1E). Increased incorporation is probably due to the low levels of competition between endogenous and exogenous ceramides. Consistently, wild-type cells treated with australifungin, an inhibitor of ceramide synthase, displayed a significant increase (1.8-fold) in C_2-IPC synthesis (data not shown). These results demonstrate that *arv1*Δ mutant cells are not compromised for IPC synthase activity and therefore suggest that sphingolipid synthesis defect of *arv1*Δ mutant cells results from a deficiency in ceramide transport.

Considering that the *arv1*Δ mutant is deficient in ceramide transport, it is reasonable to assume that this block would entail the accumulation of intermediates in ceramide synthesis. The finding that *arv1*Δ mutant cells showed an increased incorporation of [^{3}H]DHS into glycerophospholipids (Figure 1B) is consistent with this, because DHS incorporation is dependent upon its phosphorylation and subsequent cleavage by a long-chain base phosphate lyase (Funato *et al.*, 2003; Saba, 2006). Higher amounts of DHS are likely to occur when ceramide synthesis is backed up, due to its lack of transport.

ARV1 Genetically Interacts with Genes Involved in GPI Anchor Synthesis

Although the *arv1*Δ mutant can grow at 25, 30, and 35°C, it fails to grow at 37°C (Swain *et al.*, 2002, Figure 2, A and B). To gain further insight into the function of Arv1p in ceramide transport, we screened a 2 μ/*LEU2*-marked genomic yeast library for multicopy suppressors of the growth defect. A plasmid was isolated from this screen, which contained *GPI15*, and rescued the *arv1*Δ ts growth (Figure 2A). *GPI15* encodes a protein involved in the synthesis of GlcNAc-PI, the first intermediate in GPI anchor biosynthesis (Yan *et al.*, 2001). GlcNAc-PI is synthesized by the GPI-GlcNAc transferase, which is thought to act as a complex composed of six known components in yeast: Gpi1p, Gpi2p, Gpi3p, Gpi15p, Gpi19p, and Eri1p (Pittet and Conzelmann, 2007). To test whether an excess of GPI-GlcNAc transferase activity was required for the suppression, other genes were cloned into a

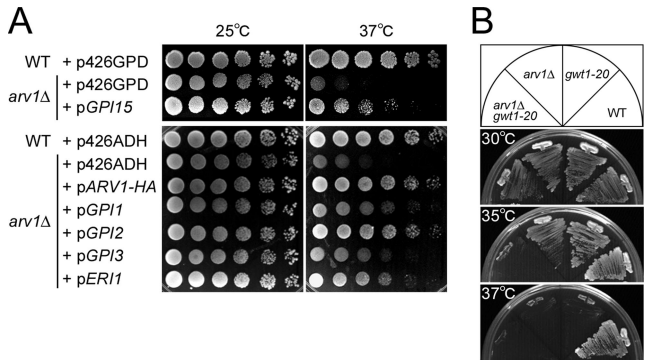

Figure 2. Overexpresssion of genes involved in GPI anchor synthesis suppresses the temperature-sensitive growth defect of *arv1*Δ, and combining *arv1*Δ and *gwt1-20* causes a synthetic growth defect. (A) Wild-type (RH6082) and *arv1*Δ (FK137) strains transformed with pRS426GPD, pRS426ADH, pGPI15, pARV1-HA, pGPI1, pGPI2, pGPI3, or pERI1 were spotted onto SD-ura plates and incubated for 4 d at 25 or 37°C. (B) Wild-type (W303-1B), *gwt1-20*, *arv1*Δ (FK245), and *arv1*Δ *gwt1-20* (FK246) were streaked onto YPUAD plates and incubated for 3 d at 30, 35, or 37°C.

2 μ vector and transformed into the *arv1*Δ mutant cells. Overexpression of Gpi2p suppressed the *arv1*Δ growth defect at 37°C almost as well as Arv1p overexpression (Figure 2A). Partial rescue of the *arv1*Δ mutant growth defect was observed by overexpressing Gpi1p, Gpi3p, and Eri1p.

Furthermore, we found that *arv1*Δ mutant causes a synthetic growth defect with the *gwt1-20* mutant allele, which shows a ts growth phenotype (Umemura *et al.,* 2003). *gwt1-20* mutant cells have a defect in inositol acylation of the GPI precursor lipid even at 24°C. Analysis of an *arv1*Δ *gwt1-20* double mutant at several temperatures revealed that although the *arv1*Δ and *gwt1-20* single mutants grow until 35°C, the *arv1*Δ *gwt1-20* double mutant is inviable at this temperature (Figure 2B), supporting a functional link between *ARV1* and GPI anchor biosynthesis.

Arv1p Is Involved in GPI Anchoring of Proteins

These genetic interactions suggest that Arv1p may have a role in GPI anchor synthesis or anchoring. To test this hypothesis, we examined the kinetics of maturation of GPI-anchored proteins in an *arv1*Δ mutant and wild-type cells, because GPI anchor synthesis and anchoring are required for efficient transport of GPI-anchored proteins from the ER to the Golgi (Schonbachler *et al.,* 1995; Hamburger *et al.,* 1995; Doering and Schekman, 1996; Sobering *et al.,* 2004). Gas1p, a GPI-anchored protein that undergoes O-linked and N-linked glycosylation, is synthesized as a 105-kDa glycoprotein in the ER, and after arrival at the Golgi compartment, fully glycosylated Gas1p has an apparent size of 125 kDa (Nuoffer *et al.,* 1993). A pulse-chase experiment revealed that the maturation of Gas1p is retarded in *arv1*Δ mutant cells (Figure 3A). A similar result was observed for the maturation of another GPI-anchored protein, Yps1p, whose ER form is found at 85 kDa, and then is modified in the Golgi and migrates as a smear of 120–180 kDa (Sievi *et al.,* 2001). By contrast, transport from the ER (P1) to the Golgi (P2) and onto the vacuole (mature form) of CPY (Stevens *et al.,* 1982) in *arv1*Δ mutant cells occurs with wild-type kinetics. Because CPY is an N-glycosylated but not a GPI-anchored protein, these results indicate that protein N-glycosylation is normal and ER-to-Golgi transport of GPI-anchored proteins is specifically delayed in *arv1*Δ mutant.

To further examine whether *arv1*Δ mutant cells have a cargo-specific defect in protein transport, we analyzed Golgi-to-ER retrograde transport, endocytosis, and endosome-to-vacuole transport. Because it has been shown that several mutants that block retrograde transport exhibit a defect in the ER-to-Golgi anterograde traffic of CPY (Duden *et al.,* 1994; Gaynor and Emr, 1997; Duden *et al.,* 1998), it is unlikely that the *arv1*Δ mutant has a retrograde defect. This is supported by our finding that, although GFP-Rer1p is mislocalized to the vacuole in a coatomer mutant, *ret1-1*, which has a defect in the Golgi-to-ER retrograde transport (Sato *et al.,* 2001), it shows normal Golgi localization in *arv1*Δ mutant cells (Figure S1A). Moreover, α-factor internalization (Figure S1B), delivery of lucifer yellow (Figure S1C) and FM4–64 (Figure S1D), fluid-phase and endocytic membrane markers were not affected in the *arv1*Δ mutant cells.

The delay in maturation of GPI-anchored proteins could result from a defect in GPI anchor synthesis (Schonbachler *et al.,* 1995; Sobering *et al.,* 2004), a defect in GPI anchoring (Hamburger *et al.,* 1995; Doering and Schekman, 1996) or a lack of stable membrane association of GPI-anchored proteins (Watanabe *et al.,* 2002). Therefore, we first examined the membrane association of Gas1p. We found that in the *arv1*Δ mutant, a significantly larger fraction of Gas1p was released from the membranes under three different conditions, buffer alone, high pH, and detergent, compared with wild-type membranes, whereas the behavior of Gap1p, an integral membrane protein, and Ypt1p, a prenylated protein, were similar in *arv1*Δ and wild-type membranes (Figure S2A), indicating that association of GPI-anchored proteins to the membranes is weakened in *arv1*Δ mutant cells.

To test whether the weakened membrane association of Gas1p was due to inefficient GPI anchoring, we examined GPI anchoring of Gas1p by pulse-chase protein labeling and Triton X-114 phase separation (Watanabe *et al.,* 2002). Anchored Gas1p partitioning into the detergent phase can only be shifted into the aqueous phase by treatment with PI-specific phospholipase C (PI-PLC), which removes the diacylglycerol moiety of GPI lipids. Unanchored Gas1p partitions into the aqueous phase and the portion remaining in the detergent phase is not affected by PI-PLC treatment (Nuoffer *et al.,* 1991). After the first partition, in *arv1*Δ mutant cells, 40% of the ER form of Gas1p was partitioned into the aqueous phase (A1) in contrast to wild-type cells (13%; Figure 3B). Furthermore, among the fraction that was partitioned into the first detergent phase (D1), most of the ER form of Gas1p in wild-type cells was shifted from the detergent phase (D2, −PI-PLC) to the aqueous phase (A2, +PI-PLC) with PI-PLC treatment, confirming that they were indeed GPI-anchored. However in *arv1*Δ mutant cells, only small fraction of the ER form of Gas1p found in D1 fraction was sensitive to PI-PLC treatment, suggesting that they were not GPI-anchored Gas1p. These results demonstrate that GPI anchoring is significantly defective in the *arv1*Δ mutant. The anchoring deficiency was confirmed by examining the incorporation of [^{3}H]*myo*-inositol or [^{3}H]DHS into proteins in *arv1*Δ mutant (Figure S2B), both of which label the glycolipid portion of GPI-anchored proteins (Reggiori *et al.,* 1997).

Because strains defective in GPI anchoring (Benghezal *et al.,* 1995) as well as GPI anchor synthesis mutants (Taron *et al.,* 2000; Umemura *et al.,* 2003; Fujita *et al.,* 2004) are hypersensitive to CFW, we reasoned that *arv1*Δ mutant cells might display the same phenotype. Indeed, the *arv1*Δ mutant cells showed hypersensitivity to CFW (Figure 3C). Hypersensitivity to CFW seen in *gpi* mutants is most likely due to elevated cell wall chitin levels that are thought to be brought about by either an up-regulated chitin synthase 3 activity in response to cell wall stress (Osmond *et al.,* 1999; Valdivieso

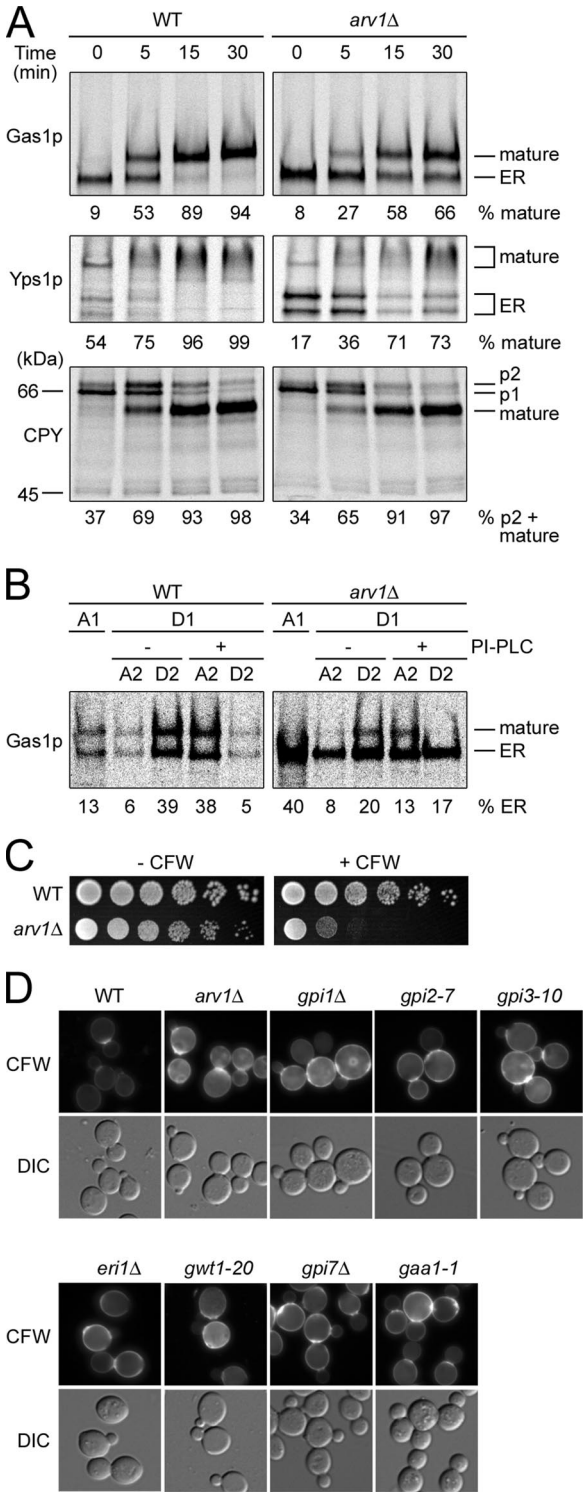

Figure 3. *arv1*Δ mutant cells show GPI-anchored protein transport and attachment defects and display the same phenotypes as *gpi* mutants. (A) ER-to-Golgi transport of protein was examined by pulse-chase experiments as described in *Materials and Methods*. Wild-type (RH6082) and *arv1*Δ (RH6078) strains were labeled with [^{35}S]methionine for 6 min at 25°C and chased for the indicated period of time. The percentage of mature Gas1p, Yps1p, or CPY is shown. (B) GPI anchor attachment was studied in wild-type (RH6082) and *arv1*Δ (RH6078) cells. The cells were labeled with [^{35}S]methionine for 6 min and chased for 5 min at 25°C. Cell extracts were prepared and solubilized with Triton X-114. After partitioning into detergent (D1) and aqueous phases (A1), the detergent phase was divided and incubated in the presence or absence of PI-PLC to remove anchors. Phases were re-extracted to yield A2 and D2 fractions and processed for Gas1p immunoprecipitation followed by SDS-PAGE. The total amount of ER form of Gas1p quantified in each partition was set to 100%. (C) Wild-type (RH6082) and *arv1*Δ (FK137) strains were spotted onto YPUAD, YPUAD supplemented with 10 μg/ml CFW, and incubated for 4 d at 25°C. (D) Wild-type (RH6082), *arv1*Δ (FK137), *gpi1*Δ (DL2831), *gpi2-7* (DL2828), *gpi3-10* (DL2829), *eri1*Δ/*eri1*Δ (DL2680), *gwt1-20*, *gpi7*Δ (FBY182) and *gaa1-1* (RH401-7C) were stained for chitin with CFW (1 mg/ml). CFW staining was visualized by fluorescence microscopy. The images were taken with equal exposures. The morphology of the cells was observed by differential interference contrast (DIC).

et al., 2000; Lesage *et al.*, 2005) or an incidental increase of UDP-GlcNAc concentration (Sobering *et al.*, 2004). Therefore, we looked for evidence of the elevated chitin levels by staining with CFW. For this, we tested six GPI anchor synthesis mutants (e.g., *gpi1*Δ, *gpi2-7*, *gpi3-10*, *eri1*Δ, *gwt1-20*, and *gpi7*Δ) and one GPI-anchoring mutant (e.g., *gaa1-1*) that are hypersensitive to CFW (data not shown). All *gpi* mutants that we tested show hyperaccumulation of chitin in the cell wall (Figure 3D), suggesting that the hyperaccumulation of chitin is a common phenotype of *gpi* mutants. Similarly, *arv1*Δ mutant cells hyperaccumulated chitin. This is consistent with our working hypothesis that Arv1p is required for GPI anchoring.

The arv1Δ Mutant Accumulates GlcN-acylPI and Has a Defect in Synthesis of Man-GlcN-acylPI

It is possible that GPI-anchoring defects result from a block in GPI anchor synthesis. Therefore, we examined whether the *arv1*Δ mutant cells have a specific defect in GPI anchor synthesis. We assayed production of GlcNAc-PI, GlcN-PI, and GlcN-acylPI by using an in vitro assay with membranes and a radiolabeled donor, [^{14}C]UDP-GlcNAc (Schonbachler *et al.*, 1995; Sobering *et al.*, 2004). All three GPI anchor intermediates were detected in membranes of *arv1*Δ mutant cells (Figure 4A), indicating that the *arv1*Δ mutant has enzyme activities for the first three steps of GPI anchor synthesis. As a control, membranes of the *gwt1-20* mutant (Umemura *et al.*, 2003) inefficiently generated GlcN-acylPI (data not shown). Remarkably, *arv1*Δ mutant membranes accumulated GlcN-acylPI when compared with wild-type membranes (Figure 4, A and B). The accumulation of GlcN-acylPI could not be detected in *arv1*Δ mutant cells by labeling with [^{3}H]*myo*-inositol (Figure 1, A and D). The reason for this discrepancy is unclear but may be due to differences between in vitro and in vivo assays (e.g., different enzymatic reaction rates) or radioactive substrates used (e.g., different labeling efficiencies). Time courses for Dol-P-Man synthase activity in the same membranes revealed that the higher accumulation of GlcN-acylPI in the *arv1*Δ membranes is not due to the lack of Dol-P-Man (Figure 4C), suggesting that later steps of GPI anchor synthesis are affected in the *arv1*Δ mutant cells.

We next examined whether deletion of *ARV1* blocks the formation of late stage GPI intermediates. Two double mutants were created by introducing the *arv1*Δ mutation into the *gaa1-1* and *gpi7*Δ mutants, which accumulate a complete GPI precursor (CP) (Hamburger *et al.*, 1995) and an abnormal GPI intermediate, Man-(EtNP)Man-Man-(EtNP)Man-GlcN-acylPI (M4; Benachour *et al.*, 1999), respectively, and tested for the accumulation of CP and M4 by pulse-labeling with [^{3}H]*myo*-inositol. The results revealed that *arv1*Δ mu-

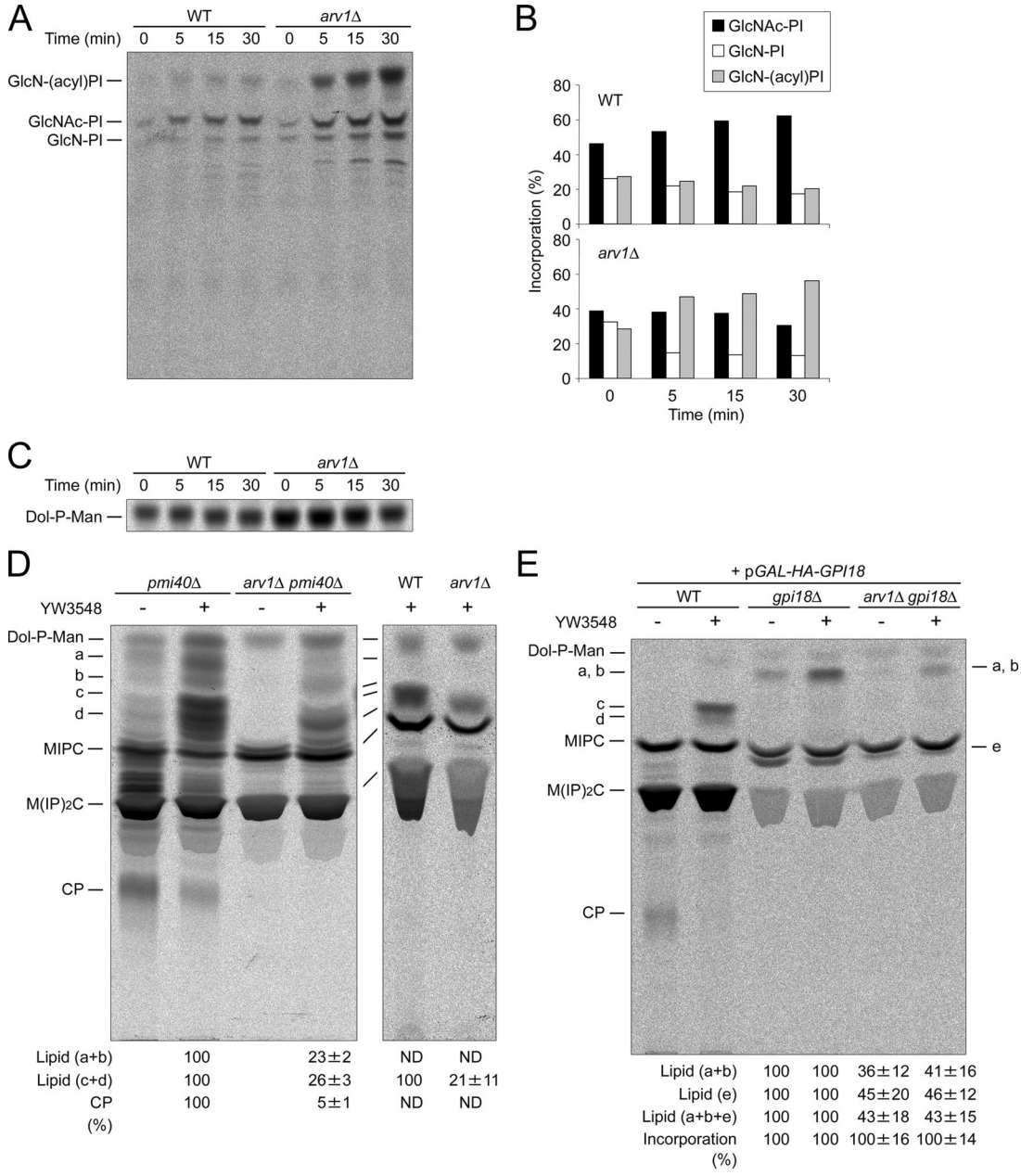

Figure 4. *arv1*Δ mutant cells accumulate GlcN-acylPI and have a strong reduction in mannosylated GlcN-acylPI synthesis. (A and B) In vitro assay for the early steps of GPI anchor biosynthesis was performed as described in *Materials and Methods*. Membranes from strains wild-type (RH6082) or *arv1*Δ (FK137) were incubated with [^{14}C]UDP-GlcNAc for the indicated times at 25°C. The lipids were extracted and analyzed by TLC plates using solvent system IV. The positions of GlcNAc-PI, GlcN-PI, and GlcN-acylPI were marked in A. Radioactivity in each GPI lipid was quantified, and incorporation (%) was determined as percentage of the total radioactivity. Data in B are quantification of A. (C) The same membranes as in A were labeled with [^{3}H]GDP-Man for the indicated times at 25°C. The lipids were extracted and analyzed by TLC plates using solvent system III. (D and E) Strains *pmi40*Δ (FK395), *arv1*Δ *pmi40*Δ (FK397), wild-type (RH6082), and *arv1*Δ (FK137) were grown as described in *Materials and Methods* (D). Wild-type [+ pGAL-HA-GPI18] (FK1019), *gpi18*Δ [+ pGAL-HA-GPI18] (FK1017), and *arv1*Δ *gpi18*Δ [+ pGAL-HA-GPI18] (FK1015) cells were grown in galactose-containing medium, and shifted to glucose-containing medium for 16 h at 25°C to repress *GPI18* expression (E). The cells were labeled with [^{3}H]mannose for 30 min at 25°C in the presence of YW3548 (10 μM) or methanol. The lipids were extracted and analyzed by TLC using solvent system III. To give better separation of Dol-P-Man and lipids a–d, some experiments were applied to a double development using the same solvent. Radioactivity in each lipid was quantified, and the relative amounts were calculated by setting the amounts in *pmi40*Δ with YW3548, wild-type with YW3548 cells (D) or in *gpi18*Δ-pGAL-HA-GPI18 without or with YW3548 (E) to 100% and were expressed as percentages of the relative controls. Data in D and E are means ± range for two independent experiments. The relative amounts of total incorporation into lipids were also shown (E). ND, not detectable; a–d, mannolipids accumulated upon YW3548 treatment; e, lipid accumulated in Gpi18-depleted cells, preferentially in the absence of YW3548 [the mobility is consistent with that of (EtNP)Man-GlcN-acylPI (M1), which has been reported to accumulate in Gpi18p-depleted cells (Fabre *et al.*, 2005)]); CP, complete GPI precursor.

tation suppresses the accumulation of CP and M4 (Figure S3), indicating that Arv1p functions upstream of Gaa1p and Gpi7p.

To further investigate which step of GPI anchor biosynthesis is defective in the *arv1*Δ mutant cells, we carried out an analogous experiment with a specific inhibitor of GPI anchor synthesis, YW3548, that inhibits the addition of EtNP onto the first mannose of the GPI core structure and leads to the accumulation of a GPI lipid, Man-Man-GlcN-acylPI (Man2; Sutterlin *et al.,* 1997b). Because a mutation in phosphomannose isomerase, *pmi40*, which causes mannose auxotrophy enhances [^{3}H]mannose labeling of mannosylated GPI intermediates (Sipos *et al.,* 1994), *pmi40*Δ and *arv1*Δ *pmi40*Δ, mutant strains were created and analyzed for synthesis of mannosylated GPIs. When YW3548 was added to the *pmi40*Δ mutant (Figure 4D) or a *pmi40* ts mutant (Figure S4A) cells, four major mannose-labeled lipids termed a, b, c, and d were accumulated, whereas synthesis of CP was significantly decreased. All a–d mannosylated lipids are acylated inositol ring-bearing GPI species because they were sensitive to GPI-phospholipase D (PLD; Figure S4, A and B), NaOH (Figure S4A), nitrous acid (Figure S4B) but resistant to PI-PLC (Figure S4, A and B). PI-PLC cleavage requires the two-position of inositol to be unacylated (Doerrler *et al.,* 1996). Because c and d migrated just above MIPC at a position expected for Man2 (Sutterlin *et al.,* 1997b) and because a and b were more hydrophobic than c and d (Figure 4D and Figure S4A), it appears that mannolipids c and d are Man2 species and that a and b species are GlcN-acylPI bearing one mannose (Man1). This was consistent with the fact that lipids (a and b) comigrated with the spot accumulated by shutting off *GPI18* expression in the presence of YW3548 (Figure 4E), which would be predicted to be a Man1 species, as Gpi18p is required for addition of the second mannose during GPI assembly (Fabre *et al.,* 2005; Pittet and Conzelmann, 2007).

GPI-PLD cleaves the linkage between the phosphate and inositol in GPI structures and generates phosphatidic acid and mannosylated GlcN-acyl-inositol. If lipid a and b species and lipid c and d species, respectively, have different acyl groups on the inositol ring, then treatment of these lipid mixtures with GPI-PLD should yield products of Man- and Man_2-GlcN-acyl-inositol consisting of at least two species. By treating the mixtures with GPI-PLD, we observed distinct subsets that migrated differently in our TLC system (Figure S4B). The GPI-PLD products derived from lipids (a and b) contained two bands that have slightly different mobilities (R_f = 0.46 and 0.44). The products from lipids c and d had multiple bands migrating at lower mobilities (R_f = 0.34–0.24). The subsets were also seen when [^{3}H]mannose-radiolabeled total lipids were tested (Figure S4A). These results suggest the existence of multiple forms of mannosylated GlcN-acylPI carrying different acyl groups on the inositol.

Importantly, deletion of *ARV1* in the *pmi40*Δ mutant strains prevented the accumulation of mannolipids (a and b) upon treatment with YW3548 (Figure 4D, left). In the absence of YW3548 a minor amount of these lipids was still observed in the *pmi40*Δ mutant, but was not detectable in the *arv1*Δ *pmi40*Δ mutant. Also, the *arv1*Δ mutation suppressed the accumulation of mannolipids (c and d) seen in the *pmi40*Δ mutant (Figure 4D, left) or wild-type (Figure 4D, right) cells in the presence of YW3548. The formation of lipids a and b, which accumulate in Gpi18p-depleted cells was reduced by disrupting *ARV1* (Figure 4E). These results show indirectly that Arv1p is required for the efficient synthesis of Man1.

Arv1p Is Required for the Delivery of GlcN-acylPI to the GPI-MT

The Man1 synthesis defect could be explained by 1) a block in production and/or translocation of Dol-P-Man across the ER membrane; 2) a defect in the activity of GPI-MT transferring mannose from Dol-P-Man to GlcN-acylPI; and 3) a defect in the delivery of GlcN-acylPI to the GPI-MT. The first possibility is unlikely because underglycosylation of CPY, a phenotype seen in the *dpm1* mutant defective in Dol-P-Man synthase (Helenius *et al.,* 2002) was not observed in the *arv1*Δ mutant cells (Figure 3A). Indeed, no defect in Dol-P-Man production was observed when *arv1*Δ mutant cells were labeled with [^{3}H]mannose (Figure 4D, right) or when *arv1*Δ mutant membranes were labeled with [^{3}H]GDP-Man (Figure 4C).

To assess GPI-MT activity, we used ER-enriched membranes from wild-type and *arv1*Δ mutant cells labeled with [^{3}H]GDP-Man. The membranes from wild-type cells generated two mannolipids comigrating with lipids b and d, respectively, whereas *arv1*Δ membranes inefficiently synthesized them (Figure 5A, lanes 1 and 3). The lipids synthesized in vitro were also sensitive to GPI-PLD but not to PI-PLC and were accumulated upon treatment with YW3548 (data not shown). These results indicate that *arv1*Δ mutant membranes have a reduced mannosylGlcN-acylPI synthesis activity, which is consistent with our in vivo results (Figure 4, D and E). Because the incorporation of [^{3}H]GDP-Man into these lipids (b and d) was linear over 15 min and that of [^{3}H]GDP-Man into Dol-P-Man was constant (Figure S5), all subsequent assays were performed at the incubation time of 10 min.

To definitively estimate GPI-MT activity, we measured the incorporation of [^{3}H]GDP-Man into the lipids with the membrane topology being destroyed by detergent. *n*-octyl β-D-glucopyranoside was chosen for this assay because it has been shown to be the most suitable detergent for preserving GPI-MT activity in *T. brucei* (Smith *et al.,* 1996). In the presence of 10 mM detergent, wild-type membranes could still generate the lipids b and d, but lost the activities to generate more hydrophilic GPI intermediates (Figure S5). As expected from the previous results with *T. brucei*, addition of higher concentrations of the detergent (e.g., 25 and 50 mM) resulted in an almost complete block of synthesis of all GPI intermediates including lipids b and d. Also, 25 mM *n*-octyl β-D-glucopyranoside inhibited the synthesis of all mannolipids generated with the *arv1*Δ mutant membranes (Figure S5). Therefore, we assayed the enzymatic activity of mannolipid synthase at the concentration of 10 mM *n*-octyl β-D-glucopyranoside. In comparison with wild-type membranes (Figure 5A, lanes 1 and 2), the *arv1*Δ mutant membranes showed an increased amount of labeled mannolipids (b and d) in the presence of the detergent (lanes 3 and 4). Because an increase in Dol-P-Man production was also observed with the *arv1*Δ mutant membranes, we normalized the amounts of mannolipid synthesis with those of Dol-P-Man synthesis. Normalized mannolipid synthesis was identical in wild-type and *arv1*Δ membranes (Figure 5B), suggesting that *arv1*Δ mutant membranes have full GPI-MT activity. Under the conditions where the membranes were not intact, a sufficient level of GPI-MT activity in *arv1*Δ membranes was also detected using a synthetic substrate, GlcN-PI with dioctanoyl-PI [GlcN-PI(C8); Figure 5B], which is an efficient substrate for GPI-MT-I (Doerrler *et al.,* 1996) and is converted into Man-GlcN-acylPI(C8) (Figure 5A, lipid f). Therefore, Man-GlcN-acylPI synthesis defect of *arv1*Δ mutant is neither due to the defect in Dol-P-Man synthesis nor

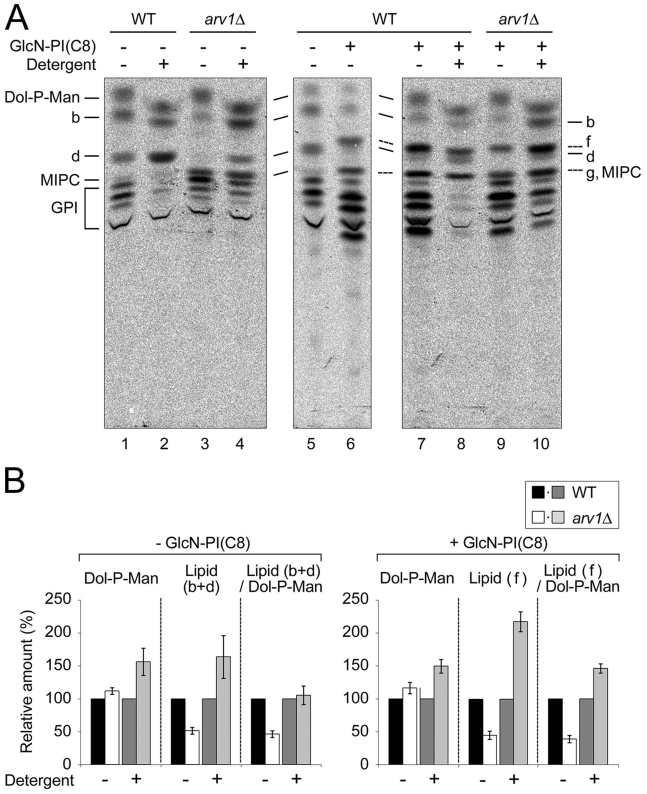

Figure 5. *arv1*Δ mutant cells have full Dol-P-Man synthesis and GPI-MT activity. (A) To measure GPI-MT activity, the ER-enriched membranes from wild-type (RH6082) and *arv1*Δ (FK137) were incubated with [^{3}H]GDP-Man for 10 min at 25°C in the presence (lanes 2, 4, 8, and 10) or absence (lanes 1, 3, 5–7, and 9) of 10 mM *n*-octyl β-D-glucopyranoside. The experiments were also done in the presence (lanes 6–10) or absence (lanes 1–5) of 0.4 μg/ml GlcN-PI(C8). The lipids were extracted and analyzed by TLC using solvent system III. The detergent causes a slightly altered migration of Dol-P-Man and lipid b. (B) Radioactivity in each lipid was quantified, and the relative amounts were calculated by setting the amounts in wild-type membranes in the presence or absence of detergent to 100%. For the estimation of GPI-MT activity, the amounts of lipids (b and d) or lipid (f) were normalized by dividing by the amounts of Dol-P-Man. Because lipid g comigrated with MIPC, we could not obtain the proper amounts of lipid g for quantification. The results represent the means ± SD from four independent experiments. Lipids f and g represent Man-GlcN-acylPI(C8) and Man_2-GlcN-acylPI(C8), respectively, and GPI designates more hydrophilic GPI intermediates than lipid d. Addition of 10 mM *n*-octyl β-D-glucopyranoside inhibited the incorporation into GPI to 24 ± 9% (wild-type) and 35 ± 19% (*arv1*Δ) of the amounts without detergent (lanes 1–4; see also Figure S5).

GPI-MT activity. Thus, we conclude that Arv1p is required for the delivery of GlcN-acylPI to the GPI-MT.

gpi Mutants Affect Sphingolipid Synthesis and Sterol Amounts and Distribution

If the primary function of Arv1p protein was to transport ceramides out of the ER, the defect of Man1 synthesis seen in the *arv1*Δ mutant would result from the accumulation of ceramides in the ER. To investigate this possibility, we assayed mannosylGlcN-acylPI synthesis in cells treated with AbA and in *sec12* mutant cells, which are defective in ER-to-Golgi protein transport at nonpermissive temperature of 37°C. When wild-type cells were labeled with [^{3}H]mannose, lipids a and b were not detectable at 25°C (Figure 4D, right), but they were detected at 37°C (Figure S6). Neither AbA treatment nor the *sec12* allele affected lipid a and b synthesis nor lipid c and d synthesis at 37°C. This result suggests that the accumulation of ceramides in the ER cannot be the reason for the defect of mannosylated GlcN-acylPI synthesis, thereby suggesting that Arv1p functions primarily in the delivery of GlcN-acylPI to the GPI-MT.

On the other hand, we consistently, found that other *gpi* mutants affect sphingolipid synthesis. When wild-type and *gaa1* mutant cells were labeled with [^{3}H]*myo*-inositol, the *gaa1-1* mutant cells showed ~30% of the level of IPC-C synthesis as found in wild-type cells (Figure 6A). The extent of reduction was similar to *arv1*Δ mutant cells. Moreover, *gpi1*Δ, *gpi2-7,* and *gpi3-10* mutant cells made ~50–60% of the amount of IPC-C synthesized in the wild-type cells. These results underscore that a GPI anchor synthesis or anchoring defect causes a defect in sphingolipid biosynthesis.

Changes in ceramide levels could be monitored using AbA. The fact that wild-type strains are sensitive to AbA, whereas *lac1*Δ *lag1*Δ double and *lip1*Δ single mutant strains defective in ceramide synthesis are resistant to AbA (Schorling *et al.*, 2001; Vallee and Riezman, 2005) implies that AbA sensitivity is associated with an increased level of ceramides rather than a decreased level of sphingolipids. Thus one would predict that *arv1*Δ mutant cells should be hypersensitive to AbA, because the *arv1*Δ mutant strain accumulates ceramides (Swain *et al.*, 2002), and we found that the ceramide synthesis pathway appears to be overloaded in the *arv1*Δ mutant (Figure 1B). Wild-type and *arv1*Δ mutant strains were sensitive to 1 μg/ml AbA, whereas the *lac1*Δ *lag1*Δ mutant was resistant (Figure 6B). This is consistent with our finding that *arv1*Δ mutant cells had normal ceramide synthase activity (data not shown). At a lower concentration (0.1 μg/ml) of AbA, wild-type cells grew, but *arv1*Δ mutant cells could not grow (Figure 6C), indicating that the *arv1*Δ mutant is hypersensitive to AbA. Other *gpi* mutants (e.g., *gpi1*Δ, *gpi2-7*, *gpi3-10,* and *gaa1-1*) were also hypersensitive to the inhibitor. These results imply that a GPI anchor synthesis or anchoring defect causes an accumulation of ceramides, most likely as a consequence of the ceramide transport defect.

Because the *ARV1* deletion has been shown to accumulate sterols in internal membrane fractions (Tinkelenberg *et al.*, 2000; Beh and Rine, 2004), we asked whether *gpi* mutants affect intracellular sterol distribution. As detected by filipin staining, the majority of sterols in *arv1*Δ and *gpi* mutants were found in internal membranes of cells. Wild-type cells displayed much less internal filipin levels than the mutants (Figure 7A). In the mutant cells, internal filipin fluorescence was observed in ring-like perinuclear structures of the ER (Figure 7A, arrows). Fluorescence was also seen in the lipid particles, which can be easily visualized with differential interference contrast (DIC; Figure 7A, arrowheads). Although the number and size of lipid particles per cell varies among cells and experiments, *gpi* mutants seem to have an increased number of lipid particles.

Moreover, we measured total sterols in these mutants by GLC/MS analysis. Compared with the wild-type cells, *gpi* mutants showed a 2–4-fold increase in total cellular sterols (Figure 7B). TLC analysis further indicated that the free sterol level was 50–200% increased in *gpi* mutants and *arv1*Δ cells compared with wild-type (data not shown). Thus, GPI assembly not only affects sphingolipid metabolism and transport but also the intracellular distribution and level of sterols.

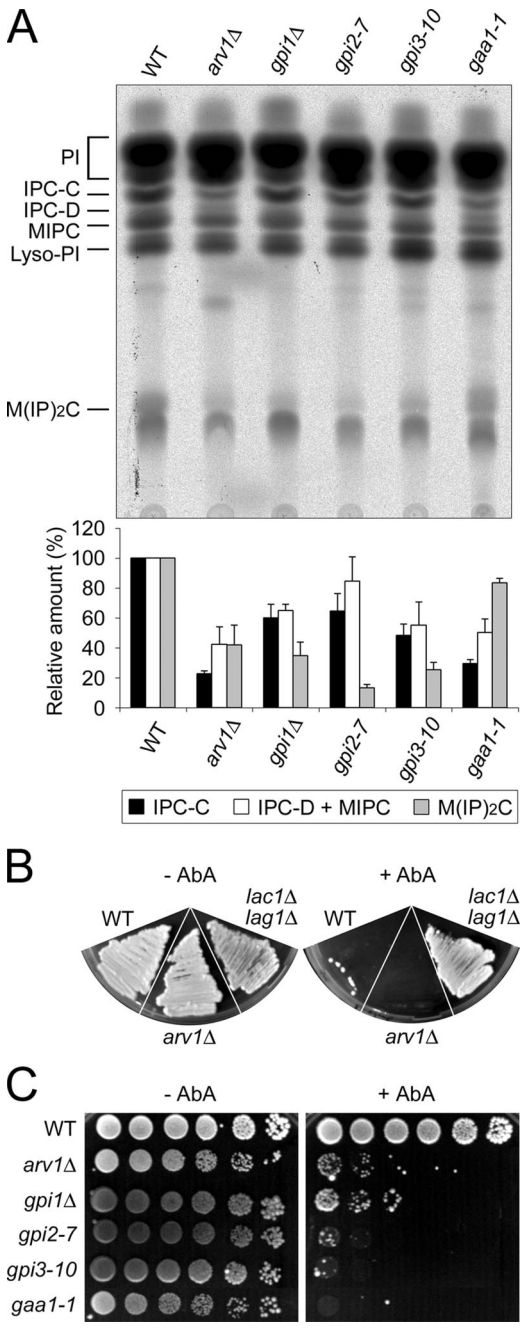

Figure 6. *gpi* mutants have a reduced level of sphingolipids and are hypersensitive to aureobasidin A. (A) Wild-type (RH6082), *arv1*Δ (FK137), *gpi1*Δ (DL2831), *gpi2-7* (DL2828), *gpi3-10* (DL2829), and *gaa1-1* (RH401-7C) were labeled with [^{3}H]*myo*-inositol for 1 h at 25°C. The lipids were extracted and analyzed by TLC plates using solvent system I. Incorporation (%) into sphingolipids was determined as the percentage of total radioactivity into all lipids containing inositol, and the relative amounts of each species were calculated by setting the amount in wild-type cells to 100%. Data in A are means ± SE for three independent experiments. (B) AbA (final concentration ≈1 μg/ml, right) or ethanol (left) was spread onto YPUAD plates. Wild-type (RH6082), *lac1*Δ *lag1*Δ (RH5308), and *arv1*Δ (RH6078) were streaked on the plates and incubated for 3 d at 25°C. (C) The same strains as in A were spotted onto YPUAD, YPUAD supplemented with 0.1 μg/ml AbA or ethanol and incubated for 4 d at 25°C.

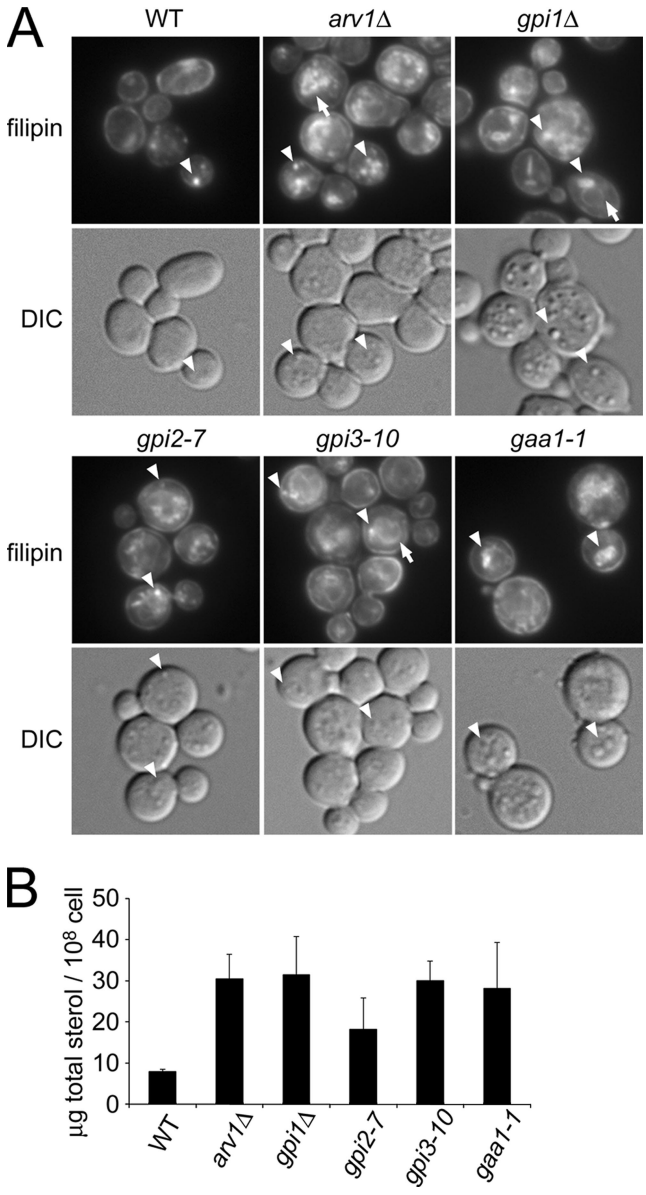

Figure 7. *gpi* mutants affect intracellular distribution and level of total sterols. (A) Wild-type (RH6082), *arv1*Δ (FK137), *gpi1*Δ (DL2831), *gpi2-7* (DL2828), *gpi3-10* (DL2829), and *gaa1-1* (RH401–7C) were grown to similar densities (7–10 × 10^6 cells/ml) and then stained for sterols with filipin complex (0.1 mg/ml). The staining was visualized by fluorescence microscopy, and the images were taken with equal exposures. The morphology of the cells and lipid particles were observed by DIC. Arrows and arrowheads indicate ring-like perinuclear ER and lipid particles, respectively. (B) The same strains as in A were grown likewise, and total sterol levels were analyzed by GLC/MS using cholesterol as an internal standard. The amounts of total sterols were determined in two independent experiments analyzed in duplicate, and data are shown as means; error bars, ± range.

DISCUSSION

In this study, we have shown that yeast deleted for *ARV1* accumulates GlcN-acylPI and is impaired for GlcN-acylPI mannosylation without having defects in Dol-P-Man synthesis or GPI-MT activity. Because N-glycosylation of Gas1p, Yps1p and CPY was not affected in *arv1*Δ mutant, the delivery of Dol-P-Man required for synthesis of N-linked oligo-

saccharides to the mannosyltransferases appears to be normal. Therefore, our results suggest a specific role of Arv1p in the delivery of GlcN-acylPI to the GPI-MT. Furthermore, we provide evidence that GPI anchor synthesis and attachment to proteins are not only required for GPI-anchored protein transport but also regulate ceramide transport from the ER and intracellular sterol distribution.

When we labeled *pmi40*Δ or *pmi40* ts mutant cells with [^{3}H]mannose, we detected four mannolipids, a–d, that were accumulated upon treatment with YW3548. On the basis of labeling experiments (Figure 4, D and E; Figure S4, A and B), we suggest that the lipids a and b are Man-GlcN-acylPI and c and d are Man_2-GlcN-acylPI species. The mannosylGlcN-acylPI species might contain different types of acyl groups on the inositol ring, which to our knowledge has not been documented in yeast GPI anchor synthesis (Sipos *et al.,* 1997; Reggiori *et al.,* 1997; Bosson *et al.,* 2006; Fujita *et al.,* 2006b). Several studies demonstrated that acyl chains linked to inositol of GPI are heterogeneous in trypanosomes (Guther *et al.,* 1996) and mammalian cells (Houjou *et al.,* 2007). At present, we cannot exclude that the appearance of multiple forms of mannosylGlcN-acylPI might be a secondary effect, because it was neither found in wild-type cells without YW3548 (data not shown) nor with membranes in our in vitro system (Figure 5A and Figure S5). However, it has been shown that yeast membranes can utilize acyl-CoAs containing different lengths of fatty acid (C14–C20) as exogenous substrates for inositol acylation (Costello and Orlean, 1992; Doerrler *et al.,* 1996; Umemura *et al.,* 2003), suggesting that the various species of mannosylated GlcN-acylPI bearing different acyl chain lengths can be generated in yeast.

Murakami *et al.* (2003) demonstrated that in mammalian cells, the first mannosylation occurs without inositol acylation, similar to the *T. brucei* GPI biosynthetic pathway (Guther and Ferguson, 1995; Smith *et al.,* 1996). This indicates that mammalian GPI-MT-I can accept GlcN-PI as a substrate. On the other hand, yeast GPI-MT does not seem to act on GlcN-PI. Previous studies have shown that all mannosylated GPI lipids accumulating in yeast mutants, *gaa1* (Hamburger *et al.,* 1995), *gpi11* (Taron *et al.,* 2000), *gpi7* (Benachour *et al.,* 1999), *smp3* (Grimme *et al.,* 2001), *gpi10* (Sutterlin *et al.,* 1998), and *gpi18* (Fabre *et al.,* 2005) were acylated GPI species. Also, we never detected any mannosylated GPI species that was not acylated. Only two acylated species, Man-GlcN-acylPI and (EtNP)Man-GlcN-acylPI, were detected in Gpi18p-depleted cells (Figure 4E). These observations strongly support the idea that the substrate specificity of yeast GPI-MT is restricted to GlcN-acylPI.

Three possibilities could explain how the GPI precursor lipid flips across the ER membrane. First, only GlcN-acylPI synthesized on the cytoplasmic side of the ER may flip into the ER lumen. Second, both GlcN-PI and GlcN-acylPI can flip but only GlcN-acylPI can be mannosylated. Finally, GlcN-PI flipped into the ER lumen can be acylated to yield GlcN-acylPI. In the last model, inositol acylation would occur on the luminal side of the ER, and fatty acyl-CoAs must be present in the ER lumen. Consistent with this notion, it is known that secretory proteins such as Hedgehog and Wnt are acylated in the ER lumen by the related acyl-CoA–dependent acyltransferases (Hofmann, 2000; Orlean and Menon, 2007). However, fatty acyl-CoAs are synthesized in the cytosol or the cytoplasmic side of organelle membranes (Black and Dirusso, 2007), and it has been reported that they do not normally penetrate into microsomal membranes (Gooding *et al.,* 2004). In addition, there is evidence that the yeast ER does not contain enzymes involved in carnitine-dependent acyl-CoA transport across membranes (Tehlivets *et al.,* 2007). These findings lead to the proposal that fatty acyl-CoAs may not reside in the ER lumen. Thus, it remains to be determined if inositol acylation can occur on the luminal side of the ER at all.

What might be the biochemical function of Arv1p? If GlcN-acylPI is synthesized on the cytoplasmic side of the ER, it is possible that Arv1p functions as a GPI flippase or as its regulator. Based on the biochemical analysis using proteoliposomes from a detergent extract of ER with fluorescent GPI analogues (Vishwakarma and Menon, 2005), it was recently proposed that flipping of early GPI intermediates occurs via an ATP-independent process mediated by specific proteins. Such energy-independent flipping processes are often observed with lipids across the ER. For example, flip-flop of phospholipids across the ER membrane does not require ATP (Pomorski *et al.,* 2004; Gummadi and Kumar, 2005; Pohl *et al.,* 2005). In addition, the translocation of lipid-linked oligosaccharides from the cytosolic leaflet to the luminal leaflet of the ER, which is mediated by a putative flippase Rft1p, also seems to be independent of metabolic energy. *RFT1* encodes a multimembrane-spanning protein but does not have ATP-binding domains (Helenius *et al.,* 2002; Helenius and Aebi, 2002). Similarly, Arv1p contains six predicted transmembrane domains and lacks any ATP-binding site motifs. Instead, this protein contains an N-terminal domain highly conserved among Arv1 proteins from different organisms, defined as the Arv1 homology domain (AHD) that consists of the N-terminal subdomain with a putative zinc-binding motif and the C-terminal subdomain with a transmembrane domain. Although the role of the N-terminal subdomain remains unclear, it was recently suggested that the N-terminal subdomain of the AHD is not required for Arv1p function, whereas the C-terminal subdomain plays an essential role (Fores *et al.,* 2006). This may imply an important role of the membrane-spanning region within the C-terminal subdomain in Arv1p function. Furthermore, our analysis of hydrophobicity profiles and ClustalW algorithms suggested that well-conserved regions among Arv1 homologues were mainly located close to or in the membrane and that they were found in both leaflets of the membranes (data not shown). Interestingly, it was also predicted by intragenic complementation analysis that Arv1p functions as a multimer, presumably a dimer, resulting in an arrangement of 6 + 6 transmembrane domains in the membrane (Tinkelenberg *et al.,* 2000). This appears to be a common characteristic of flippases and transporters such as ATP-binding cassette transporters (Pohl *et al.,* 2005; Tusnady *et al.,* 2006). Although GPI flipping activity of Arv1p still has to be proven directly, these observations make it likely that Arv1p is either a GPI flippase or an accessory protein that facilitates the flipping of GPI. In this case, the remaining activity for mannosylGlcN-acylPI synthesis in the absence of Arv1p may be due to the spontaneous flipping of GlcN-acylPI or the flipping mediated by other proteins that are not yet identified. As an attractive possibility, it has been proposed that GPI flippase may be the same as the putative glycerophospholipid flippase (Vishwakarma and Menon, 2005). It would be interesting to see if Arv1p is involved in flip-flop of glycerophospholipids. However, because no direct evidence is provided yet for the site of GlcN-acylPI synthesis, we cannot rule out that Arv1p plays a role in the lateral movement of GlcN-acylPI in the ER lumen, as in the case of SL15/Lec35 for the utilization of Dol-P-Man (Anand *et al.,* 2001). Alternatively, Arv1p might be involved in specific delivery of Dol-P-Man to the GPI-MT, although we consider this unlikely.

We have previously reported that ceramides synthesized in the ER are transported to the Golgi compartment by both vesicular and nonvesicular pathways in yeast (Funato and Riezman, 2001). The vesicular transport of ceramide occurs via COPII-coated vesicles and requires ATP, whereas nonvesicular transport does not require ATP. In this study, we observed that *ARV1* deletion and *GAA1* mutation result in strong reductions of IPC synthesis by ~75–80 and 70%, respectively, which are similar to the effect on IPC synthesis (−80%) seen in *sec18* mutant cells (Funato and Riezman, 2001). In addition, our analysis showed that ATP depletion using NaN_3 and NaF inhibited IPC synthesis of wild type by ~50%, but had no effect on IPC synthesis of *arv1*Δ mutant cells (data not shown). This suggests that IPC synthesis via an ATP-requiring vesicular pathway is almost completely abolished in the absence of Arv1p. Collectively, these results imply that GPI anchor synthesis regulates sphingolipid synthesis mainly by vesicular ceramide transport, which is consistent with the model previously proposed that GPI-anchored proteins and ceramides are cotransported from the ER in yeast.

Filipin staining revealed that sterols probably hyperaccumulate in *gpi* mutant cells in lipid particles as well as in the ER. Ergosterol, the major sterol of yeast is not required for ER-to-Golgi transport of GPI-anchored proteins (Heese-Peck *et al.*, 2002), suggesting that the sterol structural requirements for ER-to-Golgi transport may be not strict or that sterols might not be cotransported with GPI-anchored proteins and ceramides. Because sterol transport to the plasma membrane is not blocked in mutants affecting the secretory pathway, such as *sec18*, *sec12* (Baumann *et al.*, 2005) and *sec23* (Schnabl *et al.*, 2005), we propose that the sterol distribution defect in the *arv1*Δ mutant is an indirect effect stemming from its sphingolipid synthesis and transport defect. Without the proper amount of sphingolipids in the plasma membrane, sterols would no longer properly equilibrate between the ER and the plasma membrane, as proposed previously (Baumann *et al.*, 2005). Lipid particles are thought to be storage compartments for steryl esters and triacyglycerols, but they also may play a role in sterol synthesis and/or transport in yeast, because some enzymes involved in sterol synthesis are localized to the lipid particles (Daum *et al.*, 1998; Sorger *et al.*, 2004). It is plausible that if sterol transport from the ER to the plasma membrane is defective, the excess of sterols found in the ER could be packaged into lipid particles. Lipid particles are thought to be formed from the ER membrane. Consistent with this idea, we observed that the total level of sterols as well as the free sterol pool was increased in *gpi* mutants. A similar observation has been reported in a GPI-deficient lymphocyte lacking caveolins, which has more cholesterol than control cells (Abrami *et al.*, 2001). We also found that *ARE1* and *ARE2* genes, which encode yeast acyl-CoA: sterol acyltransferases, interact genetically with *GAA1* (Figure S7) as they do with *ARV1* (Tinkelenberg *et al.*, 2000). These observations, together with the fact that sphingolipid-deficient *lcb1-100* mutant cells cause a weak membrane association of GPI-anchored proteins with the ER membrane (Watanabe *et al.*, 2002), accumulate sterols in lipid particle structures similar to those seen in *gpi* mutant, and increase the level of sterols (Baumann *et al.*, 2005), suggests the existence of a coordinated regulation for the trafficking of GPI-anchored proteins and raft lipids.

Finally, the results presented here support the model that the primary function of Arv1p is to deliver GlcN-acylPI to GPI-MT and that GPI anchor synthesis is required for efficient ceramide transport from the ER as well as intracellular distribution of sterols. How these events are connected in detail remains to be elaborated. It is possible that GPI anchor synthesis might be required for the formation and/or maintenance of functional lipid domains in the yeast ER.

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