Characterizing the Sphingolipid Signaling Pathway That Remediates Defects Associated with Loss of the Yeast Amphiphysin-like Orthologs, Rvs161p and Rvs167p*

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Loss of function of either the RVS161 or RVS167 Saccharomyces cerevisiae amphiphysin-like gene confers similar growth phenotypes that can be suppressed by mutations in sphingolipid biosynthesis. We performed a yeast two-hybrid screen using Rvs161p as bait to uncover proteins involved in this sphingolipid-dependent suppressor pathway. In the process, we have demonstrated a direct physical interaction between Rvs167p and the two-hybrid interacting proteins, Acf2p, Gdh3p, and Ybr108wp, while also elucidating the Rvs167p amino acid domains to which these proteins bind. By using subcellular fractionation, we demonstrate that Rvs167p, Ybr108wp, Gdh3p, and Acf2p all localize to Rvs161p-containing lipid rafts, thus placing them within a single compartment that should facilitate their interactions. Moreover, our results suggest that Acf2p and Gdh3p functions are needed for suppressor pathway activity. To determine pathway mechanisms further, we examined the localization of Rvs167p in suppressor mutants. These studies reveal roles for Rvs161p and the very long chain fatty acid elongase, Sur4p, in the localization and/or stability of Rvs167p. Previous yeast studies showed that rvs defects could be suppressed by changes in sphingolipid metabolism brought about by deleting SUR4 (Desforges, L., Durrens, P., Juguelin, H., Cassagne, C., Bonneu, M., and Aigle, M. (1993) Yeast 9, 267–277). Using rvs167 sur4 and rvs167 sur4 double null cells as models to study suppressor pathway activity, we demonstrate that loss of SUR4 does not remediate the steady-state actin cytoskeletal defects of rvs167 or rvs161 cells. Moreover, suppressor activity does not require the function of the actin-binding protein, Abp1p, or Slp1p, a protein that is thought to regulate assembly of the cortical actin cytoskeleton. Based on our results, we suggest that sphingolipid-dependent suppression of rvs defects may not work entirely through regulating changes in actin organization.

Human amphiphysins, along with human BIN1, BIN2, and BIN3, the Schizosaccharomyces pombe hob1+ and hob3+ genes, the murine Apl1 and the Saccharomyces cerevisiae amphiphysin orthologs, RVS161 and RVS167, are all members of the BAR family of proteins (1, 2). Initially, BAR family function was thought to be involved only in regulating early endocytosis and the actin cytoskeleton (3, 4), but with the discovery of the amphiphysin II isoform, BIN1, functions have expanded to include signaling from the plasma membrane to the nucleus (5, 6). In addition, it is now believed that BAR family proteins may function to regulate critical membrane topological/morphological changes. Work has demonstrated that Drosophila amphiphysin regulates the organization of specialized membrane domains that are required for cortical protein localization (7). Moreover, studies have shown that the BAR domain (Fig. 1) itself binds directly to membranes and may initiate membrane curvature events that facilitate the development of a tubulovesicular membrane system required for clathrin-mediated endocytosis, regulation of membrane dynamics, and depolarization-contraction coupling in striated muscle (1, 2, 8–10). Although the exact functions of BAR proteins in yeast are not known, they do appear to regulate similar events, such as endocytosis (11), actin cytoskeletal structure (3, 4, 11–13), and certain nuclear events (14, 15). Thus, understanding how BAR proteins function within various yeast pathways should increase our understanding of their roles in higher eukaryotes, as well. All members of the BAR family have an N-terminal conserved BAR domain (Fig. 1). All but Rvs161p have a central domain enriched in proline and a C-terminal SH3 domain. These two regions bind each other and interact with other actin-binding proteins (see below) (16, 17). The central domain of Rvs167p is not as well conserved and is enriched in proline, glycine, and alanine (14). Bin1, an isoform of the human amphiphysin II, has additional domains that either bind the Myc oncoprotein or function in nuclear localization (5). Bin1 interacts with the actin cytoskeleton and functions in endocytosis, actin function, and signal transduction (5, 6). Amphiphysin I is localized at presynaptic terminals (18, 19). Its central region interacts with α-adaptin and clathrin, whereas the SH3 domain binds dynamin and synaptotagmin, and all regulate endocytosis (20–22). Interestingly, amphiphysin I is a dominant autostabilized in paraneoplastic stiff man syndrome (23).

The S. cerevisiae RVS161 and RVS167 genes were isolated by selecting for recessive mutations causing reduced viability upon nutrient starvation (3). Neither RVS gene is essential for

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1 The abbreviations used are: RVS161, wild type gene designation (uppercase italics); rvs161, gene deletion (lower case italics); Abp, actin-binding protein; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; BAR, BIN/amphiphysin/Rvs; Cdk, cyclin-dependent kinase; DIGs, detergent-insoluble glycolipid-enriched membrane fraction; Gdh, mammalian glutamate dehydrogenase; GFP, green fluorescent protein; GPA, glycine-alanine-proline-rich; HA, hemagglutinin; LCB, long chain sphingoid base; Rvap, Rvs proteins; SH3, SRC homology 3.
Sur4p/Elo3p and Elo2p are very long chain fatty acid elongases whereas Sur2p is a LCB hydroxylglyceramide synthase (32), whereas Sur2p is a LCB hydroxylglyceramide synthase (32). It was shown that depending on the gene deleted, either Rvs161p function was rendered nonessential for repolarization of actin under high salt stress, or depolarization in rvs161 cells was decreased under the same conditions (38). These results suggest a link among sphingolipid metabolism, Rvs161p, and the regulation of actin repolarization.

In the present study, we used a two-hybrid screen to isolate proteins interacting with Rvs161p/Rvs167p, and we determined their role in the sphingolipid-dependent suppression of rvs defects brought about by loss of function of the SUR4 gene (Table I). We have uncovered a novel two-hybrid interaction that may link RVS function, sphingolipid metabolism, and nitrogen assimilation. Moreover, we present evidence that our Rvs167p two-hybrid interactions exist in vivo, possibly within sphingolipid/sterol rafts. Additional studies aimed at determining how altering sphingolipid metabolism suppresses rvs defects show that rvs167 sur4 and rvs161 sur4 double mutant cells harbor rus null steady-state actin cytoskeletal defects. Moreover, we demonstrate that rvs161 sur4 abp1 and rvs161 sur4 sla1 triple mutant cells are viable and grow under wild-type conditions. Thus, the sur4-dependent suppression of rvs defects does not require certain factors that are thought to be involved in regulating actin organization (14, 41, 42). Overall, our results hint at the possibility that sphingolipid-dependent suppression of rus defects may not function entirely through remediating the actin cytoskeletal defects of mutant cells.

Sphingolipids are essential membrane lipids that are conserved structurally throughout evolution (29–31). Eukaryotic sphingolipids, including those of the budding yeast, S. cerevisiae, are composed of a LCB sphingobind that is C2 amide linked to a very long chain fatty acid. Ceramide serves as the backbone for all complex sphingolipids (29–31). S. cerevisiae and mammalian sphingolipid biosyntheses are very similar up to ceramide production (Fig. 2). Various polar head groups are attached to the C1 position of ceramide of complex sphingolipids, and it is at this point in sphingolipid bio synthesis where yeast and mammalian cells diverge. In higher eukaryotes, polar head groups include phosphocholine for sphingomyelin, carbohydrates for glycosphingolipids, and sialic acid residues (31). Yeast sphingolipid head groups are all inositol-based, with S. cerevisiae producing only three complex sphingolipids: inositolphosphorylceramide, mannose inositolphosphorylceramide, and mannose diinositolphosphorylceramide (29, 30).

Recessive mutations that suppress rvs defects have been isolated and reside within the sphingolipid biosynthetic pathway in S. cerevisiae. Loss of function mutations in the SUR1/CSG1, SUR2, SUR4/ELO3, ELO2, and IPT1 genes alter sphingolipid biosynthesis in yeast and suppress rvs defects (24) (Fig. 2). Sur1p/Csg1p is a subunit of the mannose inositolphosphorylceramide synthase (32), whereas Sur2p is a LCB hydroxylase that converts dihydrophosphogamine to phytosphingosine (33). Sur4p/Elo3p and Elo2p are very long chain fatty acid elongases required for the production of the C26 fatty acids found in all yeast complex sphingolipids (34). Ip3p is a mannose diinositolphosphorylceramide synthase that catalyzes the terminal step in yeast sphingolipid biosynthesis (35). SUR7, isolated as a multicopy suppressor of rvs defects, including those associated with the actin cytoskeleton, regulates sphingolipid levels but by a mechanism that has yet to be elucidated (36, 37).

How rus defects are suppressed by altering sphingolipid metabolism is not known but may be mediated through regulating changes in the actin cytoskeleton (38). Studies have demonstrated that deleting the individual sphingolipid genes described above suppresses the actin depolarization defects of rvs161 cells; the process of actin depolarization/repolarization is important for many cell events including cell stability during stress and for maintaining proper polarized cell growth (39, 40). Yeast viability, and rvs161 rus167 mutants can grow. rus161 and rus167 null cells have shared phenotypes that include an inability to thrive under nutrient starvation and actin cytoskeletal and endocytosis defects (4, 11, 13, 24). However, Rvs161p and Rvs167p seem not to have totally redundant functions because BAR domain exchange between the two proteins does not yield functionally redundant chimeras (16), and Rvs161p functions independently of Rvs167p in nuclear fusion during the yeast conjugation process (25). Studies have shown that Rvs161p and Rvs167p form a complex in vivo (26) which likely associates with factors regulating the actin cytoskeleton, endocytosis, and the cell cycle, such as actin itself (27), the actin-binding protein, Abp1p (12, 14), Sla2p, which is involved in membrane cytoskeleton assembly and cell polarization (28), and the Pho85p Cdk cyclin, Pcl2p (15) (Table I).

Sphingolipid-dependent Suppression of rvs Defects (Fig. 1). The BAR family of proteins. Comparative structures of a subset of the proteins in the BAR family are shown. P, proline-rich region; A, alanine-rich region; N, nuclear localization domain; MBD, Myc binding domain; aa, amino acids. Adapted from Refs. 23 and 62.

**Materials and Methods**

**Yeast Strains, Miscellaneous Microbial Techniques, and Growth Conditions**—The yeast strain P369-4A (YJN737) was used for all two-hybrid studies and was obtained from Phil James (43). All other strains were constructed in the W303-1A (MATa ure3-1 leu2-3,112 his3-11,15 trp1-1 ade2 can1-100) or MY2792 (YJN146) (MATa ura3-52 leu2-1 his3-200) backgrounds (25). The plasmids Yip-rvs161::LEU2, Yip-rvs167::HIS3, and Yip-sur4::HIS3 were used to generate rvs161, rus167, and sur4 deletion alleles. ace2, abp1, sla1, gdh3, and ydr108w deletion alleles were constructed using the plasmid pFA6a-hanMX and the PCR method described previously (14) using the plasmid pAS2-1 (BD Biosciences). Long-tail pGAL4-YIp-rvs161::HIS3, pGAL4-YIp-rvs167::HIS3, and pGAL4-Yip-sur4::HIS3 were used to construct the pGAL4-HIS3 integrants YIP167HIS3, YIP167HIS3, and YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3. A functional pGAL4-HIS3 integrant was constructed using the plasmid pGAL4-HIS3 integrant YIP167HIS3.
Sphingolipid-dependent Suppression of *rus* Defects

**Table 1**

| Gene     | Gene translation* | Biological process* | Function* |
|----------|-------------------|---------------------|-----------|
| ACF2     | Assembly-Complementing Factor | Actin cytoskeleton organization and biogenesis | Glucan 1,3-β-glucosidase activity |
| ELO2     | ELOgation-defective | Fatty acid elongation | Fatty acid elongase activity |
| FUS2     | Cell FUSion-defective | Plasma membrane fusion | Unknown |
| GA51     | Glycophospholipid-Anchored Surface protein | Cell wall organization and biogenesis | 1,3-β-Glucanoyltransferase activity |
| GDH3     | Glutamate DeHydrogenase | Glutamate dehydrogenase activity | Glutamate dehydrogenase activity |
| IPT1     | InositolPhosphoTransferase | Mannose diphosphorylinositolceramide metabolism | Inositolphosphotransferase activity |
| LAS17    | | Actin filament organization, actin polymerization, and/or depolymerization endocytosis | Cytoskeletal protein binding |
| PCL2     | Pho85p CycLin | Cell cycle | Cyclin-dependent protein kinase regulator activity |
| PHO85    | PHOosphate metabolism | Cell cycle; phosphate metabolism | Cytoskeletal protein binding |
| RVS161   | Reduced Viability upon nutrient Starvation | Endocytosis; polar budding | Cytoskeletal protein binding |
| RVS167   | Reduced Viability upon nutrient Starvation | Response to osmotic stress mating | Cytoskeletal protein binding |
| SAC6     | Suppressor of Actin mutations | Actin filament organization endocytosis; polar budding | Actin filament binding |
| SRA1     | Syntethically Lethal with ABP1 | Actin cortical patch assembly | Protein binding, bridging |
| SRA2     | Syntethically Lethal with ABP1 | Actin filament organization cell wall organization and biogenesis; endocytosis exocytosis | Protein binding, bridging |
| SUR1/CSCG1 | SuPPressor of Rvs | Mannose inositolphosphoceramide metabolism | Mannosyltransferase activity |
| SUR2     | SuPPressor of Rvs | Sphingolipid biosynthesis | Sphinganine hydroxylase activity |
| SUR4/ELO3 | SuPPressor of Rvs | Fatty acid elongation | Fatty acid elongase activity |
| SUR7     | SuPPressor of Rvs | Sphingolipid biosynthesis | Unknown |
| VPS20    | Vacuolar Protein Sorting | Late endosome to vacuole transport | Unknown |
| VPS21    | Vacuolar Protein Sorting | Endocytosis | GTPase activity |
| YBR108w  | Open reading frame | Unknown | Unknown |

* Obtained from the *S. cerevisiae* genome data base; www.yeastgenome.org.

scale two-hybrid screen was performed using YJN737 containing the pBD-GAL4Cam-RVS161 plasmid and a yeast activation domain genomic library (Y2H-C1) (a gift from Phil James) (43). Approximately 2.7 × 10^6 independent transformants were initially screened on His - Leu - Trp - agar containing 2.5 mM 3-aminitriazole. Transformed cells were grown at 30 °C for 10 days at which time randomly selected His + colonies were transferred to Leu - Trp - master plates. After 2 days at 30 °C, these colonies were replicated to Leu - Trp - Ade - plates and grown for 1 week at 30 °C. Ade + colonies were subsequently assayed for β-galactosidase activity (LacZ') using a filter assay.

His + , Ade + , LacZ + yeast clones were randomly chosen for further analysis. After overnight growth in SD-Leu broth at 30 °C, plasmid DNA was precipitated from cells and propagated in XLIBlue cells, which were plated on LB medium supplemented with 200 μg/ml ampicillin. All plasmids isolated were retested for interaction with Rvs161p. The nucleotide sequence of the DNA inserts from these reactive clones was determined using a primer specific for the activation domain vector.

**Liquid β-Galactosidase Assays—LacZ assays were performed as described in the Yeast Protocols Handbook (BD Biosciences) using the substrate chlorophenolred-β-D-galactopyranoside. Briefly, three independent colonies from a single positive clone were inoculated into Leu - Trp - medium and grown to exponential phase (A400 ~ 0.5–0.8). Cells were pelleted from 1.5 ml of growing culture and resuspended in 300 μl of buffer 1 (100 mM HEPES, pH 7.3, containing 150 mM NaCl, 4.5 mM l-aspartate (hemimagnesium salt), 2.0 g of bovine serum albumin, and 100 μl of Tween 20). 100 μl of cells in buffer 1 was then applied to three chambers of freeze/thawing using a dry ice/ethanol water bath. 700 μl of buffer 2 (20 μl of buffer 1 containing 27.1 μg of chlorophenolred-β-D-galactopyranoside) was added, and the reaction was allowed to proceed until color development became visible. The reaction was terminated by the addition of 500 μl of 3.0 mM ZnCl2. Cellular debris was pelleted by centrifugation, and β-galactosidase activity was measured at A600 using the resulting supernatant. Miller units were calculated as described previously (46). The Miller units represented are the average values of five independent experiments.

**Fluorescence Microscopy Procedures—**YJN1554 harbors a functional *rus167*:RVS167-GFP::TRP1 allele that was constructed using the plasmid pFA6a-GFP(S65T)-TRP1 (44). Rvs167p-GFP was functional, as *rus161* cells expressing this protein did not show any of the pleiotropic phenotypes associated with cells lacking Rvs167p function (data not shown). YJN1829, YJN1830, and YJN1831 were generated by mating YJN1554 to individual *rus161*, *sur4*, and *rus161 sur4* haploid strains, sporulation of the resulting diploid, and subsequent selection of the appropriate haploid. Rhodamine phallolidin (Molecular Probes, Eugene, OR) staining of actin was performed as described by Bringle et al. (47). All fluorescence microscopy studies were performed using a Leica DRME fluorescence microscope, and either FITC (GFP) or RITC (rhodamine phallolidin) optics and a PlanAPO 100× objective. Data were collected using a Hamamatsu DIG-15 CCD digital camera and Open Labs software (version 2.1). Final fluorescence images were generated using Adobe Photoshop (version 4.0).

**Immunoprecipitation and Western Analysis—**YJN1556 carries a functional *rus167*:RVS167-HA::TRP1 allele that was constructed using the plasmid pFA6a-HA-TRP1 (44). YJN1657 (Ybr108wp-Myc), YJN1658 (Acf2p-Myc), and YJN1722 (Gdh3p-Myc) were constructed using YJN1556 and the plasmid pFA6a-13Myc-kanMX6 (44). 20-ml cultures of exponentially growing cells (YPD; 30 °C) were used to obtain a total cell protein extract for immunoprecipitation. Briefly, cells were pelleted, washed once with distilled H2O, and subsequently lysed in buffer A (200 mM Tris-HCl, pH 7.9, containing 390 mM (NH4)2SO4, 10
mm MgSO4, 20% v/v glycerol. 1 mM EDTA, 1 mM AEBS, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 10 μl of Sigma protease inhibitor solution (Sigma). 1 mg of total cell protein extract was resuspended in 500 μl of buffer A and incubated with 1 μl of anti-c-Myc antibodies (mouse monoclonal antibody clone 9E10; Hoffman-LaRoche Ltd., Basel, Switzerland) or 10 μl of anti-HA mouse monoclonal antibodies (12CA5) l of anti-HA mouse monoclonal antibodies (12CA5) l of anti-c-Myc antibodies (mouse monoclonal antibody clone 9E10; Hoffman-LaRoche Ltd., Basel, Switzerland) or 10 μl of anti-HA mouse monoclonal antibodies (12CA5) l of Sigma protease inhibitor solution (Sigma). 1 mg of total cell protein extract was resuspended in 500 μl of buffer A and incubated with 1 μl of anti-c-Myc antibodies (mouse monoclonal antibody clone 9E10; Hoffman-LaRoche Ltd., Basel, Switzerland) or 10 μl of anti-HA mouse monoclonal antibodies (12CA5) l of anti-HA mouse monoclonal antibodies (12CA5) l of Sigma protease inhibitor solution (Sigma). 1 mg of total cell protein extract was resuspended in 500 μl of buffer A and incubated with 1 μl of anti-c-Myc antibodies (mouse monoclonal antibody clone 9E10; Hoffman-LaRoche Ltd., Basel, Switzerland) or 10 μl of anti-HA mouse monoclonal antibodies (12CA5) l of anti-HA mouse monoclonal antibodies (12CA5) for 2 h at 4°C. Incubations with primary and secondary antibodies were performed for 1 h in buffer C. Membranes were washed six times after antibody incubations with buffer C containing 0.05% Tween 20. Anti-c-Myc and anti-HA antibodies were used at 1:1,000 and 1:2,500, respectively. Horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (Amersham Biosciences) was used at a 1:2,000 dilution. Proteins were visualized using the ECL system (Amersham Biosciences).

When total cell extracts from strains expressing epitope-tagged proteins were analyzed by Western analysis, they all expressed tagged proteins of the appropriate size either for Rvs167p-HA, Gdh3p-Myc, Ybr108wp-Myc, or Acf2p-Myc. Monoclonal antibodies that were directed against the HA tag immunoprecipitated Rvs167p-HA alone, whereas those directed against Myc pulled down only Gdh3p-Myc, Ybr108wp-Myc, or Acf2p-Myc. Under our assay conditions, neither monoclonal antibody preparation immunoprecipitated any nonspecific proteins, and protein-A did not nonspecifically bind and pull down any tagged fusion proteins.

**RESULTS**

**Rvs161p Two-hybrid Screen Reveals Novel Rvs167p Interactions**—We were interested in understanding how altering sphingolipid metabolism suppresses rvs161 null phenotypes. Our approach was to perform a two-hybrid screen using Rvs161p as bait, with the intention of isolating proteins having a unique interaction with this protein alone. 54 independent clones showing positive interactions with Rvs161p were isolated. 6 genes were recovered based on restriction digest analyses. They were ACF2, FUS2, GDH3, LAS17, RVS167, and the open reading frame, YBR108w (Fig. 3; RVS161 RVS167 pBD-

![Diagram](http://www.cell.org)
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Fig. 3. Rvs161p two-hybrid screen reveals a novel interaction between Rvs167p and Gdh3p. Various Pj69-4A strains harboring pBD-GAL4Cam-RVS161 or pBD-GAL4Cam-RVS167 and the indicated pACT2 vectors (denoted by gene designation) were patched onto Leu-Trp medium and grown for 1 day at 30 °C. Cells were then replica-plated onto Leu-Trp Ade medium and grown for 2 days at 30 °C. Liquid β-galactosidase assays also were performed on individual strains, and Miller units were determined as described under “Materials and Methods.”

Las17p, and is known to form a complex with Rvs167p (26). However, Rvs161p (Table I). Rvs161p has been coprecipitated with Fus2p (25) and is known to form a complex with Rvs167p (26). Previous studies demonstrated that Rvs161p itself was unable to interact with Acf2p, Gdh3p, and Ybr108wp. We found that Acf2p, Gdh3p, and Ybr108wp had a two-hybrid interaction with one or more domains of Rvs167p.

Rvs167p Coprecipitates with Acf2p, Gdh3p, or Ybr108wp—Our two-hybrid studies suggested that Rvs167p might interact physically with Acf2p, Gdh3p, and/or Ybr108wp in vivo. To determine whether any direct physical interactions occur, we asked whether Rvs167p and/or the opposite were also true (Fig. 4). Strains were constructed which expressed epitope-tagged chimeras of all proteins of interest, and coimmunoprecipitation experiments were performed using total cell extracts to uncover any interactions. We found that Rvs167p-HA coprecipitated Ybr108wp-Myc, Gdh3p-Myc, or Acf2p-Myc from total cell extracts (Fig. 4, lane 1). Moreover, we found that the inverse was also true. Ybr108wp-Myc (lane 2), Gdh3p-Myc (lane 3), and Acf2p-Myc (lane 4) all were able to coprecipitate full-length Rvs167p-HA.

Rvs167p, Ybr108wp, Gdh3p, and Acf2p Localize within Detergent-insoluble Glycolipid-enriched Membranes—Rvs161p and Rvs167p colocalize to actin patches where they are thought to interact (17, 38). Rvs161p also localizes to DIGs that contain lipid rafts composed of sphingolipid and most likely sterol (38). Thus, we tested whether Rvs167p-HA coprecipitated Ybr108wp-Myc, Gdh3p-Myc, or Acf2p-Myc from total cell extracts (Fig. 4, lane 1). Moreover, we found that the inverse was also true.

TABLE III

|               | pAS2-1a pACT-YBR108w pACT-GDH3 pACT-ACF2 |
|---------------|-------------------------------------------|
| BAR           | 40.7 18.1 15.5 15.5                       |
| BARGPA        | 27.0 12.0 10.3 10.3                       |
| SH3           | 6.8 9.0 1.0 1.0                           |
| GPASH3        | 1.0 12.5 31.0 25.3                       |
|               | 0.7 8.3 20.6 20.6                       |

* pAS2-2 vector expressing the indicated Rvs167p domains.
* Miller units.
* Fold activation over pACT vector alone.

Sphingolipid-dependent Suppression of rvs Defects—Certain pleiotropic growth defects associated with the loss of RVS161 or RVS167, such as nutrient deprivation and high salt stress intolerance, are suppressed by altering sphingolipid metabolism (24). Based on the fact that our results pointed to Ybr108wp, Gdh3p, and Acf2p associating with and functioning within an Rvs161p-Rvs167p complex in vivo, we explored
Sphingolipid-dependent Suppression of rvs Defects

Approximately 5 x 10^5 cells were in the first well. WT, wild type.

Typically, we observed four types of localization patterns for Rvs167p-GFP in asynchronous wild type cell cultures using fluorescence microscopy. In mother cells at a point just prior to daughter bud emergence, Rvs167p-GFP was found clustered at the newly formed bud site (Fig. 7A). In the majority of small budded cells, it localized to both mother and daughter cells (Fig. 7B), although in most large budded cells, it was found either at the mother/daughter bud neck (Fig. 7C) or localized to the daughter cell alone (Fig. 7D). Thus, Rvs167p movement and localization may be cell cycle-regulated. These localization patterns for Rvs167p have been observed previously (17).

We found that in rvs161, sur4, or rvs161 sur4 null cells either the intensity of the Rvs167p-GFP signal was dramatically weakened, or Rvs167p-GFP was mislocalized in the cell. First, we discovered that in both rvs161 (Fig. 7, E–I) and rvs161 sur4 (Fig. 7, M–P) cells, Rvs167p-GFP fluorescence was almost completely absent. Second, the localization of Rvs167p-GFP in sur4 cells was more diffuse, and at no point during cell growth did we observe localization patterns similar to wild type cells (Fig. 7, I–L). Rvs167p-GFP was unable to localize to the bud site of the ACF2 gene in rvs167 sur4 cells (rvs167 sur4 versus rvs167 sur4 acf2) resulted in growth phenotypes similar to those seen for rvs161 and rvs167 null cells, respectively. Similar results were obtained when we examined the role of a second glutamate dehydrogenase, GDH1. These growth defects were not observed for sur4 gdh3, sur4 gdh1, or sur4 acf2 null cells. The overexpression of YBR108w, ACF2, or GDH1 alone in rvs161 or rvs167 cells did not remediate any rvs defects tested. Thus, Gdh3p and Acf2p functions play roles in the sphingolipid-dependent suppression of rvs defects.

Rvs161p and Sur4p Are Required for Proper Localization and Stability of Rvs167p—To understand further how loss of SUR4 suppresses rvs growth defects, we determined the localization of Rvs167p-GFP in wild type, rvs161, sur4, and rvs161 sur4 cells.

FIG. 4. Rvs167p coprecipitates with Gdh3p, Ybr108wp, and Acf2p. Strains harboring various tagged alleles were grown to exponential phase at 30 °C in YEPD. Cell extracts from these strains were obtained and incubated with anti-HA monoclonal antibodies to immunoprecipitate Rvs167-HA, or anti-Myc monoclonal antibodies to immunoprecipitate Gdh3p-Myc, Ybr108wp-Myc, or Acf2p-Myc. Coprecipitated fusion proteins were resolved by SDS-PAGE and visualized by Western analysis using the appropriate antibody preparation. The top row (IP) shows the concentration of immunoprecipitated protein (input); the remaining rows show the concentration of the coprecipitated protein (output).

FIG. 5. Rvs167p, Ybr108wp, Gdh3p, and Acf2p all colocalize within lipid rafts. Lipid rafts were isolated using the Optiprep density gradient ultracentrifugation method described under “Materials and Methods.” Proteins were resolved using SDS-PAGE, and their localization within lipid rafts was determined using Western analysis. The localization of Rvs161p-HA, Rvs167p-HA, Ybr108wp-Myc, Gdh3p-Myc, and Acf2p-Myc was determined using either anti-HA or anti-Myc monoclonal antibodies. Dpm1p and Gas1p were localized using anti-Dpm1p and anti-Gas1p polyclonal antibodies, respectively. R, detergent-resistant membranes containing lipid rafts; S, detergent-solubilized membranes.

whether the proper functions of these proteins were required for the suppression of rvs growth defects by loss of SUR4. Thus, we examined how deleting YBR108w, GDH1, or ACF2 in either rvs161 sur4 or rvs167 sur4 null cells altered their growth under conditions of glucose starvation or high salt concentration.

Using conventional plate assays, we found that rvs161 null cells showed robust growth on YEPD medium but were completely inviable under conditions of nutrient starvation (YEPD). These growth defects were not observed for sur4 gdh3, sur4 gdh1, or sur4 acf2 null cells. The overexpression of YBR108w, ACF2, or GDH1 alone in rvs161 or rvs167 cells did not remediate any rvs defects tested. Thus, Gdh3p and Acf2p functions play roles in the sphingolipid-dependent suppression of rvs defects.

RVs167p coprecipitates with Gdh3p, Ybr108wp, and Acf2p. Strains harboring various tagged alleles were grown to exponential phase at 30 °C in YEPD. Cell extracts from these strains were obtained and incubated with anti-HA monoclonal antibodies to immunoprecipitate Rvs167-HA, or anti-Myc monoclonal antibodies to immunoprecipitate Gdh3p-Myc, Ybr108wp-Myc, or Acf2p-Myc. Coprecipitated fusion proteins were resolved by SDS-PAGE and visualized by Western analysis using the appropriate antibody preparation. The top row (IP) shows the concentration of immunoprecipitated protein (input); the remaining rows show the concentration of the coprecipitated protein (output).

FIG. 6. Gdh3p and Acf2p functions are required for sphingolipid-dependent suppression of rvs defects. Various W303-1A strains were grown to exponential phase at 30 °C in YEPD. 10-fold serial dilutions of cells were aliquoted onto the indicated media plates and grown for 3 (YEPD and YEPDLG) or 4 days (YEPDHS) at 30 °C. Approximately 5 x 10^5 cells were in the first well. WT, wild type.

Typically, we observed four types of localization patterns for Rvs167p-GFP in asynchronous wild type cell cultures using fluorescence microscopy. In mother cells at a point just prior to daughter bud emergence, Rvs167p-GFP was found clustered at the newly formed bud site (Fig. 7A). In the majority of small budded cells, it localized to both mother and daughter cells (Fig. 7B), although in most large budded cells, it was found either at the mother/daughter bud neck (Fig. 7C) or localized to the daughter cell alone (Fig. 7D). Thus, Rvs167p movement and localization may be cell cycle-regulated. These localization patterns for Rvs167p have been observed previously (17).

We found that in rvs161, sur4, or rvs161 sur4 null cells either the intensity of the Rvs167p-GFP signal was dramatically weakened, or Rvs167p-GFP was mislocalized in the cell. First, we discovered that in both rvs161 (Fig. 7, E–I) and rvs161 sur4 (Fig. 7, M–P) cells, Rvs167p-GFP fluorescence was almost completely absent. Second, the localization of Rvs167p-GFP in sur4 cells was more diffuse, and at no point during cell growth did we observe localization patterns similar to wild type cells (Fig. 7, I–L). Rvs167p-GFP was unable to localize to the bud site of...
mother cell having an emerging bud growth (Fig. 7I) or to the mother/daughter bud neck (Fig. 7K) of large budded cells in sur4 cells, but in the majority of cells examined was localized throughout mother and daughter. Thus, Rvs167p-GFP localization required Sur4p function.

Next, we determined whether the weakened Rvs167p-GFP signal correlated with a reduced level of Rvs167p. Western analyses using anti-GFP monoclonal antibodies showed that Rvs167p levels were significantly reduced in rvs161 cells and almost completely absent in rvs161 sur4 cells (Fig. 7; Western). Moreover, sur4 cells showed a modest reduction in the amount of full-length Rvs167p, although they accumulated a lower molecular weight form of the protein. The reduction in Rvs167p levels was not the result of C-terminal proteolysis of the GFP moiety from full-length protein. Pulse-chase experiments using the galactose-inducible pGAL-GFP vector demonstrated that GFP is highly stable protein, thus a poor proteolytic substrate in cells.

\textit{rvs167} sur4 and \textit{rvs161} sur4 Cells Have Actin Cytoskeletal Defects Similar to Those Seen in \textit{rvs167} and \textit{rvs161} Cells—\textit{rvs161} and \textit{rvs167} cells harbor actin cytoskeleton defects (4, 13, 38). To determine further how deletion of \textit{SUR4} suppresses \textit{rus} defects, we asked whether its loss causes changes in the steady-state actin cytoskeletal structure of \textit{rvs167} or \textit{rvs161} cells. Actin was visualized by fluorescence microscopy using rhodamine-conjugated phalloidin.

In wild type (Fig. 8A) and sur4 (Fig. 8C) cells grown in YEPDGL, we observed that the majority of actin was localized to the growing daughter bud, although in rvs167 and rvs161 (not shown) cells, the characteristic \textit{rus} defect was seen, revealing actin patches that were distributed evenly between mother and daughter (Fig. 8B). More importantly, rvs167 sur4 and rvs161 sur4 (not shown) cells both mislocalized actin in a similar manner (Fig. 8D). Similar results were obtained using YEPDHS medium. Thus steady-state actin cytoskeletal defects remain in rvs167 and rvs161 cells lacking Sur4p function.

Suppression of \textit{rus} Defects by Deletion of \textit{SUR4} Does Not Require the Function of Ahp1p or Sla1p—Synthetic lethality occurs when two genes deleted together in the same strain cause inviability, while either gene deletion alone does not. Synthetic lethality is used to explore whether certain gene products function within a single pathway or function together to regulate a single event. The loss of \textit{RVS167} is synthetic lethal in combination with the loss of several genes regulating the actin cytoskeleton, including \textit{SLA1}, \textit{SLA2}, and \textit{SAC6}, and the \textit{VPS21} gene required for late endosome function (12) (Table I). In contrast, \textit{rvs167} abp1 cells are viable (12). We obtained similar results when these synthetic interactions were examined in our W303-1A strain background (not shown). We also discovered that \textit{RVS167} was synthetically lethal with another late endosome gene, \textit{VPS20} (50), further linking Rvs167p function to late endosomal activity.

We then tested whether loss of \textit{RVS161} gave rise to these same synthetic interactions. We did this for two reasons. First, we wanted to examine in more detail the redundant nature of Rvs161p and Rvs167p functions. Second, we also wanted to see whether we could generate viable mutant strains that would allow us to examine whether the loss of factors associated with actin cytoskeletal regulation affects the suppression of \textit{rus} defects by deletion of \textit{SUR4}. We discovered that loss of \textit{RVS161} gene function was synthetically lethal in combination with deletions in \textit{SLA2}, \textit{SAC6}, \textit{VPS20}, and \textit{VPS21}, but we did recover \textit{rvs161} abp1 and \textit{rvs161} sla1 mutant strains (Fig. 9A) (Table II). We constructed rvs161 sur4 abp1 and rvs161 sur4 sla1 triple mutants and tested whether Ahp1p or Sla1p functions were needed for suppression of \textit{rus} defects by deletion of \textit{SUR4}. rvs161 sur4 abp1 cells grew as well as rvs161 sur4 and sur4 abp1 cells under conditions of nutrient starvation (YEPDGL) or high salt stress (YEPDHS) (Fig. 9B). Similar results were obtained when we examined rvs161 sur4 sla1 and rvs167 sur4 abp1 cells (not shown). We could not test rvs167 sla1 cells...
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**Fig. 9.** Abp1p or Slalp functions are dispensable for sur4 suppression of rvs defects. A, growth on YE PD of meiotic segregants of a representative tetraplicate ascus from strains heterozygous for rvs161 and the indicated genes. Leu’ (rvs161:LEU2) and kan’ (gene x:kan’) were used to score for the presence of specific deletions and infer the genotype of nonviable spores. B, various deletion strains were grown on the indicated plates for 3 days at 30 °C.

because they were inviable. The loss of ABP1 (Fig. 9B) or SLA1 (not shown) alone in rvs161 cells did not suppress the glucose starvation or salt intolerance phenotypes. Thus, deletion of SUR4 and resulting suppression of rvs defects did not require the functions of Abp1p or Sl1p.

**DISCUSSION**

We were interested in understanding how defects in sphingolipid biosynthesis suppress rvs defects. Our approach was to perform a yeast two-hybrid screen as a means to isolate proteins interacting with Rvs161p. Unexpectedly, we isolated several proteins having known two-hybrid interactions with Rvs167p. Using rvs161 and rvs167 deletion strains, we discovered that our putative Rvs161p two-hybrid interactions were actually Rvs167p-dependent. These results further emphasize the need to use deletion strains when using the two-hybrid to understand the direct/indirect interactions of a protein complex.

Gdh3p and Acf2p were found to interact with Rvs167p and play roles in the suppression of rvs defects by deletion of the SUR4 gene. The NADP-glutamate dehydrogenase, Gdh3p, along with Gdh1p, is involved in ammonium utilization in yeast through catalyzing the reductive amination of α-ketoglutarate to form glutamate (52). gdh3 null cells have starvation defects and rapidly lose viability (53). Gdh3p activity is minimal under normal growth conditions, but growth in ethanol results in a severalfold stimulation (52). Thus, Gdh3p regulation seems important for growth under environmental stress conditions. Interestingly, mammalian Gdh exhibits complex allosteric regulation (54), whereas the yeast enzymes do not. The allosteric regulation of mammalian Gdh plays a role in insulin homeostasis, linking amino acid catabolism to insulin secretion (54). Rather than possessing allosteric regulation, control of yeast Gdh3p activity may occur through protein-protein binding, possibly with Rvs167p. Alternatively, a Rvs167p-Gdh3p complex and its association/dissociation may link actin cytoskeletal reorganization to nitrogen deprivation stress, which may be important for cell survival under these conditions. Endothelial cells subjected to oxidative stress re-model the actin cytoskeleton, characterized by increased actin polymerization and reorganization into stress fibers (55). Whether BAR proteins from higher eukaryotes function to regulate nutrient sensing signaling pathways, such as glucose sensing-insulin signaling (56), murine target of rapamycin (mTOR) signaling (57) or cAMP-protein kinase A signaling (58) is not known.

Acf2p/Eng2p has endo-1,3-β-glucanase activity (59) and is required for cortical actin assembly in vitro (60). Why might Acf2p activity be important for suppression of rvs defects? Some insight comes from the characterization of the ELO2 gene. ELO2 has been isolated as a recessive mutation causing reduced glucan synthase activity, which is required for cell wall biosynthesis, and resistance to the cell wall biosynthesis inhibitor, pneumocandin B0 (61). However, Elo2p is a very long chain fatty acid elongase that is required to synthesize C26 fatty acid for sphingolipid biosynthesis, and elo2 mutants have an altered sphingolipid composition (34). These results together suggest that sphingolipids may regulate cell wall biosynthesis in some way. They may do so by regulating Acf2p activity, with regulation and subsequent cell wall changes being necessary for rvs suppression by deletion of SUR4. It is also possible that Rvsp, whether it be a Rvs1617p-Rvs161p complex or higher eukaryote forms of amphiphasin, acts as a “dockinng station” for proteins regulating diverse processes, all of which require modulation of the actin cytoskeleton and topological changes to membranes, or both, to function properly. Bon *et al.* (62) performed a yeast two-hybrid screen using both Rvs161p and Rvs167p and isolated 34 open reading frames, suggesting that specific protein networks involved in diverse cellular events converge on Rvs161p/Rvs167p.

Although cells deleted for the SUR4 gene have normal cytoskeletal structure, rvs161 sur4 null cells harbor the rvs actin phenotype. Yet these double mutant cells lack most other rvs phenotypes; they still do harbor the conjugation defect seen in rvs161 cells. Moreover, suppression of rvs defects by deletion of SUR4 does not require the functions of Abp1p or Sl1p; both play roles in actin cytoskeletal assembly and organization (12, 14, 41). Studies have suggested that the BAR domain of the BAR family of proteins may be involved in regulating membrane curvature (1, 2, 8–10). If Rvsp in yeast also functions to regulate membrane topology, rvs defects may be caused by the loss of proper membrane morphology. Loss of SUR4 and subsequent changes in sphingolipid metabolism may restore to some extent proper membrane topology and viability. sur4 null cells accumulate the LCB, phytosphingosine (34), and this LCB has been shown to reinitiate endocytosis in yeast cells defective in sphingolipid biosynthesis (63). rvs null cells harbor defects in endocytosis (11). Thus, deletion of SUR4 could possibly suppress rvs defects by mechanisms that are independent of any effects on the actin cytoskeleton (24). However, it is also possible that yeast Rvsp BAR works directly through the actin cytoskeleton to mediate changes in membrane topology indirectly, and loss of SUR4 results in actin remodeling that is sufficient enough to suppress rvs defects. Additional studies are necessary to resolve these issues completely.

It is also possible that suppression of rvs defects through changes in sphingolipid biosynthesis may proceed by an altogether different mechanism. Here, the accumulation of a specific sphingolipid intermediate in mutant cells acts to initiate a signaling pathway that is required to remediate rvs defects. elo2, sur4 elo3, and most likely sur2 null cells all accumulate
LCB (34, 64). LCBS in mammalian cells have long been recognized as highly bioactive signaling lipids, playing important roles in modulating the pathways regulating cell cycle, differentiation, and apoptosis (65–72). Recently, Zhang et al. (73) demonstrated that LCB accumulation in yeast activates the Pck1p-Map1p cell wall integrity pathway. Birchwood et al. (74) have shown that accumulation of the mammalian LCB, sphingosine 1-phosphate, in yeast stimulates Ca2+

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example. Alternatively, each individual sphingolipid metabolite accumulated because of a specific mutation may affect independent and nonoverlapping cell events. Neither of these hypotheses can be ruled out at this time.

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Characterizing the Sphingolipid Signaling Pathway That Remediates Defects Associated with Loss of the Yeast Amphiphysin-like Orthologs, Rvs161p and Rvs167p

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