Characterization of the Yeast Amphiphysins Rvs161p and Rvs167p Reveals Roles for the Rvs Heterodimer In Vivo

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We have used comprehensive synthetic lethal screens and biochemical assays to examine the biological role of the yeast amphiphysin homologues Rvs161p and Rvs167p, two proteins that play a role in regulation of the actin cytoskeleton, endocytosis, and sporulation. We found that unlike some forms of amphiphysin, Rvs161p-Rvs167p acts as an obligate heterodimer during vegetative growth and neither Rvs161p nor Rvs167p forms a homodimer in vivo. *RVS161* and *RVS167* have an identical set of 49 synthetic lethal interactions, revealing functions for the Rvs proteins in cell polarity, cell wall synthesis, and vesicle trafficking as well as a shared role in mating. Consistent with these roles, we show that the Rvs167p-Rvs161p heterodimer, like its amphiphysin homologues, can bind to phospholipid membranes in vitro, suggesting a role in vesicle formation and/or fusion. Our genetic screens also reveal that the interaction between Abp1p and the Rvs167p Src homology 3 (SH3) domain may be important under certain conditions, providing the first genetic evidence for a role for the SH3 domain of Rvs167p. Our studies implicate heterodimerization of amphiphysin family proteins in various functions related to cell polarity, cell integrity, and vesicle trafficking during vegetative growth and the mating response.

INTRODUCTION

RVS161 and *RVS167*, which encode closely related proteins in *Saccharomyces cerevisiae*, were first identified in a screen for mutants that exhibited *r*educed *v*iability upon *s*tarvation (Bauer *et al.*, 1993). Mutation of *RVS161* or *RVS167* causes a phenotype consistent with a role for the Rvs proteins in cortical actin cytoskeleton organization and endocytosis: loss of viability and unusual cell morphology in poor growth medium or salt-containing medium, delocalized actin distribution under suboptimal growth conditions, abnormal (random) budding in diploids, and defects in endocytosis and sporulation (Bauer *et al.*, 1993). Ultrastructural studies have revealed that *rvs* mutants accumulate late secretory vesicles at sites of membrane and cell wall construction (Breton *et al.*, 2001), suggesting an additional role for Rvs161p and Rvs167p in vesicle trafficking.

Rvs161p and Rvs167p are members of the BAR-domain family of proteins, which includes *Bin1*, *A*mphiphysin, and *R*vs proteins (Sivadon *et al.*, 1997). Amphiphysins are enriched in the mammalian brain and seem to function in synaptic vesicle endocytosis (for review, see Zhang and Zelhof, 2002). Bin1 is a splice isoform of amphiphysin 2 that

has features of a tumor suppressor (Sakamuro *et al.*, 1996). Proteins in this family are characterized by the presence of a conserved BAR domain, and it is through their BAR domains that Rvs161p interacts with Rvs167p (Navarro *et al.*, 1997; Sivadon *et al.*, 1997; Colwill *et al.*, 1999). The crystal structure of the BAR domain of *Drosophila* amphiphysin has recently been solved (Peter *et al.*, 2004). It is a crescent-shaped dimer, in which each monomer forms a coiled coil. The curved shape is only revealed upon dimerization of the two slightly kinked monomers, suggesting that the domain functions only as a dimer. The BAR domain binds preferentially to highly curved negatively charged membranes in vitro, and this property is thought to promote membrane deformation, leading to vesicle formation (Lee and Schekman, 2004; Peter *et al.*, 2004). The central portion of Rvs167p consists of a region rich in glycine, proline, and alanine (the GPA region) and may play a role in Rvs regulation because it is phosphorylated in vivo (Friesen *et al.*, 2003). At its carboxy terminus, Rvs167p, like the amphiphysins, has a Src homology 3 (SH3) domain (Figure 1A), a protein module well defined for binding proline-rich sequences (Pawson and Scott, 1997). Large-scale two-hybrid and phage-display screens have identified a number of proteins that bind to the SH3 domain of Rvs167p (Bon *et al.*, 2000; Uetz *et al.*, 2000; Drees *et al.*, 2001; Ito *et al.*, 2001; Tong *et al.*, 2002); however, few of these interactions have been confirmed. Domain mapping of Rvs167p has revealed that the GPA region and the SH3 domain are largely dispensable for all Rvs167p functions tested (Navarro *et al.*, 1997; Colwill *et al.*, 1999). Thus, although the SH3 domain is conserved among amphiphysins, and several biologically important ligands have been seen to bind to the SH3 domain, its biological function remains unknown.

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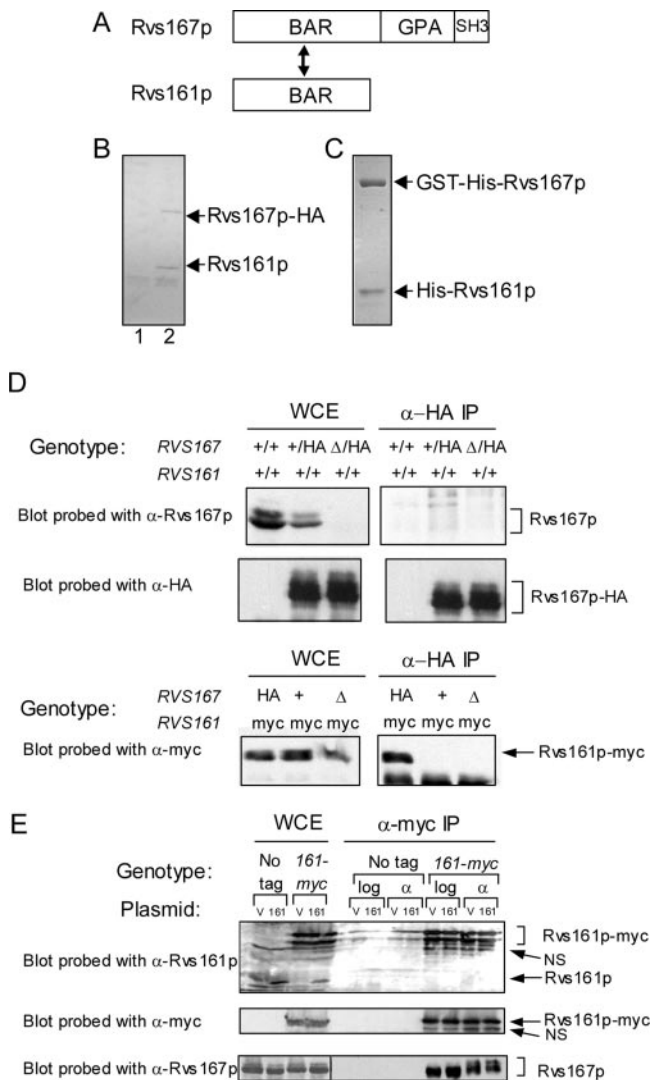Address correspondence to: Brenda Andrews (brenda.andrews@utoronto.ca).

Figure 1. Interaction of Rvs167p and Rvs161p. (A) Schematic illustration of the domain structures of Rvs167p and Rvs161p. The relative positions of the N-terminal BAR domain, the GPA region, and the C-terminal SH3 domain in Rvs167p and the BAR domain of Rvs161p are indicated. Rvs167p and Rvs161p interact with each other through their BAR domains. (B) Coimmunoprecipitation of Rvs167p-HA and Rvs161p from yeast extracts. Coomassie blue-stained gel showing α-HA immunoprecipitates from an untagged yeast strain, BY263 (lane 1), and from a yeast strain expressing *RVS167-HA*, BY1034 (lane 2). The lower molecular weight band was cut out of the gel, processed for mass spectrometry, and identified as Rvs161p (see *Materials and Methods*). The positions of migration of Rvs167p-HA and Rvs161p are indicated on the right. (C) Copurification of GST-His-Rvs167p and His-Rvs161p proteins coexpressed in insect cells. GST-His-Rvs167p and associated His-Rvs161p were purified using glutathione-Sepharose beads and analyzed by SDS-PAGE followed by staining with Coomassie blue. (D) Assay for homodimerization of Rvs167p. Lysates from diploid strains with either two untagged copies of *RVS167*, BY264 (lane 1), one untagged and one tagged copy of *RVS167*, BY1910 (lane 2), or one tagged copy of *RVS167* and one deleted copy of *RVS167*, BY2591 (lane 3) were incubated with anti-HA antibodies. Immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting with antibodies against Rvs167p (top) and anti-HA antibodies (middle). The anti-Rvs167p antibody does not detect HA-tagged Rvs167p. Lysates from haploid strains expressing *RVS167-HA* and *RVS161-MYC*, BY2592 (lane 4), *RVS167-HA* only, BY1034 (lane 5), or deletion of *RVS167* and *RVS161-MYC*, BY2502 (lane 6) were incubated with

Several lines of evidence support a model in which Rvs161p and Rvs167p function together. First, Rvs161p and Rvs167p interact with each other through their respective BAR domains in two-hybrid assays, and Rvs161p can be immunoprecipitated from cells overproducing tagged Rvs167p (Navarro *et al.*, 1997; Colwill *et al.*, 1999). Second, each *RVS* gene is required for the stability of its Rvs protein partner during vegetative growth in vivo (Lombardi and Riezman, 2001). Third, the phenotype of an *rvs161Δ* mutant seems identical to that of an *rvs167Δ* mutant in vegetative cells (Crouzet *et al.*, 1991; Bauer *et al.*, 1993; Sivadon *et al.*, 1995), and the extragenic suppressors *sur1Δ*, *sur2Δ*, and *sur4Δ* (Desfarges *et al.*, 1993), *fen1Δ*, and *ipt1Δ* (Balguerie *et al.*, 2002) and the multicopy suppressor *SUR7* (Sivadon *et al.*, 1997) are able to bypass the defects of either an *rvs161Δ* or *rvs167Δ* strain (Desfarges *et al.*, 1993; Sivadon *et al.*, 1997).

Nonetheless, evidence for distinct roles for the Rvs proteins has also been reported. The BAR domains of Rvs161p and Rvs167p cannot be functionally exchanged with each other, showing that, despite being structurally similar, this domain confers specificity to the activity of each Rvs protein (Sivadon *et al.*, 1997). Rvs167p homodimerization has also been reported (Colwill *et al.*, 1999; Bon *et al.*, 2000; Lombardi and Riezman, 2001). Furthermore, the localization patterns of Rvs161p and Rvs167p do not completely overlap: Rvs167p is localized to cortical actin patches (Balguerie *et al.*, 1999), whereas Rvs161p is reportedly mainly cytoplasmic with small dots distributed randomly within the cell cortex in unbudded cells and at the mother-bud neck during bud emergence and cytokinesis (Brizzio *et al.*, 1998), although Rvs161p-green fluorescent protein has also been localized to small cortical patches during G$_1$ (Balguerie *et al.*, 2002). Finally, synthetic mating defects in combination with *fus1Δ* are seen with *rvs161Δ* but not *rvs167Δ*, revealing a specific role for Rvs161p in cell fusion during mating (Brizzio *et al.*, 1998).

In this study, we have used genetic analyses of *RVS161* and *RVS167* to ask whether Rvs161p and Rvs167p have separate functions in vivo and to characterize the roles of the Rvs proteins. Comprehensive synthetic lethal screens and biochemical studies have identified shared roles for Rvs161p and Rvs167p in cell polarity, cell integrity, cell wall synthesis, and vesicle trafficking, all processes that require proper formation and fusion of vesicles. Consistent with these biological roles, we found that purified Rvs161p-Rvs167p is able to bind to phospholipid vesicles, as is seen with its *Drosophila* and mammalian homologues. We have also devised specific genetic screens that uncovered a previously unappreciated role for Rvs167p in mating and an essential role for the Rvs167p SH3 domain in some strain backgrounds. We conclude that Rvs161p and Rvs167p function as an obligate heterodimer in vegetative cells and have partially overlapping roles during mating.

antibodies against HA. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting with anti-myc antibodies (*bottom*). (E) Assay for homodimerization of Rvs161p. Lysates from haploid yeast strains (either untagged, BY263, or with *RVS161-myc*, BY1182) bearing a plasmid encoding untagged Rvs161p were analyzed by coimmunoprecipitation and Western blot. Cells were grown to mid-log phase and then treated or mock treated with 5 μM α factor for 1 h. The positions of migration of relevant proteins is marked on the right; NS, nonspecific band.

Table 1. Yeast strains used in this study

Strain		Reference
BY263[a]	*MAT**a** trp1Δ63 GAL2+ ura3-52 lys2-801[am] ade2-107° his3Δ200 leu2-Δ1*	Measday *et al.* (1994)
BY1034[a]	BY263 *RVS167-HA::kan*	This study
BY264[a]	*MAT**a**/α trp1Δ63/trp1Δ63 GAL2/gal2 ura3-52/ura3-52 lys2-801/lys2-801 ade2-107°/ade2-107° his3Δ200/his3Δ200*	Measday *et al.* (1994)
BY1910[a]	BY264 *RVS167/RVS167-HA::kan*	This study
BY2591[a]	BY264 *rvs167Δ::TRP1/RVS167-HA::kan*	This study
BY2592[a]	BY263 *RVS167-HA::kan RVS161-myc::kan*	This study
BY2502[a]	BY263 *rvs167Δ::TRP1 RVS161-myc::kan*	This study
BY1182[a]	BY263 *RVS161-MYC::kan*	This study
BY4741a[b]	*MAT**a** his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met15Δ0*	Brachmann *et al.* (1998)
BY1404[b]	*MATα mfa1ΔMFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 lys2Δ0 rvs167Δ::Nat*	Friesen *et al.* (2003)
BY1796[b]	*MATα mfa1ΔMFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 lys2Δ0 rvs161Δ::Nat*	Tong *et al.* (2004)
[b]	BY4741a *rvs167Δ::kan*	Deletion consortium
[b]	BY4741a *rvs161Δ::kan*	Deletion consortium
BY2504[c]	*MATα mfa1ΔMFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 lys2Δ0 rvs167Δ::URA3*	This study
BY1690[c]	*MATα mfa1ΔMFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 lys2Δ0 rvs161Δ::URA3*	This study
BY2001[c]	*MATα mfa1ΔMFA1pr-HIS3 can1Δ his3Δ1 leu2Δ0 lys2Δ0 sla1Δ::Nat*	This study
BY2102[b]	BY4741 *MATα end3Δ::kan*	This study
BY1891[b]	*MAT**a** slt2Δ::kan*	Deletion consortium
BY2506[b]	*MAT**a** rvs167Δ::Nat slt2Δ::kan*	This study
BY2805[b]	*MAT**a** rvs167Δ::Nat sla1Δ::kan*	This study
BY2806[b]	*MAT**a** abp1Δ::Nat sla1Δ::kan*	This study

[a] BY263-based; BY263 is an *ssd1-d* strain derived from S288C.

[b] BY4741-based; BY4741 is an *SSD1-V* strain derived from S288C.

[c] BY4741-based query strain for SGA.

MATERIALS AND METHODS

Strains and Media

S. cerevisiae strains used in this study are listed in Table 1. Strains were obtained from the yeast gene-deletion mutant collection constructed by the deletion consortium (Winzeler *et al.*, 1999) or were constructed using standard yeast genetic techniques (Sherman, 1991). Deletions of open reading frames (ORFs) were constructed by integrative transformation (Longtine *et al.*, 1998). Standard methods and media were used for yeast growth and transformation (Guthrie and Fink, 1991). Supplemented minimal medium containing 2% dextrose (SD) or 2% galactose (SG) with appropriate amino acid supplements was used to select for plasmids. Salt-containing medium was made by adding NaCl to synthetic dextrose medium or YPD to a final concentration (wt/vol) as indicated. Sorbitol was added to minimal synthetic medium containing glucose or galactose to a final concentration of 1 M to maintain *rvs167Δ slt2Δ* mutants. Where indicated, α-factor (obtained from the Louisiana State University Health Sciences Center Core Laboratory, New Orleans, LA) was added to log-phase cultures in YPD to a final concentration of 5 μM, and cells were incubated as indicated before harvesting. For spot assays, cultures grown to mid-log phase in SD were serially diluted 10-fold and spotted onto the appropriate plates.

Plasmids

Plasmids are described in Table 2. Details of construction of all plasmids are available upon request. The *ABP1ΔPXXP* (BA1732) plasmid was constructed with the use of PCR with Platinum Pfx (Invitrogen, Carlsbad, CA). Two PCR products were synthesized, using a plasmid containing *ABP1* under the control of its own promoter as template. Each PCR product introduced an *Nhe*I site within the *ABP1* coding sequence (the sequence of the primers is available upon request), one at each end of the proline-rich region. The 5′-PCR product was digested with *Nhe*I and *Bam*HI and the 3′-PCR product was digested with *Nhe*I and *Apa*I. These were ligated into pRS315 (Sikorski and Hieter, 1989) that had been digested with *Bam*HI and *Apa*I, giving pRS315-ABP1ΔPXXP, which contained an in-frame deletion of amino acids 448–525. The integrity of all PCR products was confirmed by sequencing.

Protein Immunoprecipitation and Western Blotting

Yeast strains were grown at 30°C to log phase, harvested, and lysed by vortexing with glass beads in lysis buffer as described previously (Tennyson *et al.*, 1998). Where specified, α-factor was added to 5 μM and the cultures treated as described in figure legends. For large-scale immunoprecipitation of Rvs167p-hemagglutinin (HA), log-phase cells were harvested in immunoprecipitation-lysis buffer (50 mM Tris-Cl, pH 7.4, 250 mM NaCl, 50 mM NaF,

0.1% NP-40, 40 mM β-glycerophosphate, 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.1 mg/ml insulin, and protease inhibitor tablet without EDTA; Boehringer Mannheim, Laval, Quebec, Canada). Cells were lysed by vortexing in the presence of glass beads. One hundred milligrams of lysate was precleared with 300 μl of protein A-Sepharose for 1 h and then incubated with 200 μl of α-HA antibodies coupled to protein A-Sepharose for 1 h. The beads were then washed in 3 × 5 ml lysis buffer and boiled in SDS sample buffer. For Western blotting of whole cell extract, the protein concentration of the lysates was measured using the Bradford assay (Bio-Rad, Mississauga, Ontario, Canada). Equivalent amounts of each sample were analyzed by SDS-PAGE and transferred to nitrocellulose using a dry transfer apparatus. Coimmunoprecipitations were done as described previously (Friesen *et al.*, 2005). Immunoblotting was done with antibodies against Rvs161p (gift from H. Riezman, University of Geneva, Geneva, Switzerland), Rvs167p (Lee *et al.*, 1998) and HA (12CA5). As a control, antibodies against Crn1p were used (Humphries *et al.*, 2002). The protein signal was revealed using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom).

Slt2p-HA was immunoprecipitated with monoclonal antibody 12CA5 (Wilson *et al.*, 1984) from 100-ml cultures either grown at 24°C or grown at 24°C and transferred to 39°C for 2 h. Cells were collected by centrifugation, and lysates were prepared as described previously (Tennyson *et al.*, 1998). Five milligrams of protein from the lysates were incubated with 1 μl of 12CA5 ascitic fluid for 1 h on ice and then rocked in the presence of protein A-Sepharose for 1 h at 4°C. Beads were collected by centrifugation and washed in lysis buffer four times.

Expression and Purification of Rvs167p and Rvs161p from Insect Cells

A *Bam*HI fragment containing full-length *RVS161* was subcloned into pAC-HLT (BD Biosciences PharMingen, San Diego, CA) that had been digested with *Bgl*II to generate a His-tagged Rvs161p fusion product. A *Bam*HI fragment containing full-length *RVS167* was subcloned into pAC-GHLT (BD Biosciences PharMingen) that had been digested with *Bgl*II to generate a glutathione *S*-transferase (GST)-His-tagged Rvs167p fusion product as described previously (Friesen *et al.*, 2003). Recombinant baculoviruses containing *RVS161* and *RVS167* were isolated from Sf9 insect cells and coinfected into Hi5 cells as recommended by the manufacturer (Invitrogen). Approximately 48 h after infection, cells were harvested, washed, and lysed in insect cell lysis buffer (50 mM Tris-HCl, 20% glycerol, 50 mM NaCl, 0.5 mM EDTA, and 10 mM β-mercaptoethanol). Cell extracts were incubated with glutathione-Sepharose beads (Amersham Biosciences) for 1 h at 4°C. Beads were washed 4 times in 10 volumes of lysis buffer, and GST-His-Rvs167p and associated His-Rvs161p were eluted with lysis buffer containing 100 mM glutathione.

Table 2. Plasmids used in this study

	Plasmid	Description	Source
BA1533	pHis-RVS161	*RVS161* ORF with an N-terminal 6-His-tag subcloned in pAcHLT-C for baculoviral expression	This study
BA1534	pRVS167-HIS-GST	*RVS167* ORF subcloned in pAcGHLT-C for baculoviral expression	Friesen *et al.* (2003)
BA1766	pRS316-RVS161	Genomic fragment of *RVS161* in low copy plasmid	This study
BA1387	p416MET-RVS167	*RVS167* ORF under MET25 promoter in low copy plasmid	Friesen *et al.* (2003)
BA1840	pRS426-RVS161	Genomic fragment of *RVS161* in high copy plasmid	This study
BA1658	p416MET-RVS167-P473L	*RVS167* ORF with P473L substitution in SH3 domain under *MET25* promoter in low copy plasmid	This study
BA1437	p416MET-RVS167–4A	*RVS167* ORF with S299A, S321A, T323A, S379A substitutions under *MET25* promoter in low copy plasmid	Friesen *et al.* (2003)
BA1019	pSLT2-HA	*SLT2–3HA* fusion on high copy plasmid	Madden *et al.* (1997)
BA1422	pGAL-GST-MNN9	*MNN9* ORF fused to *GST-His* at N-terminus under *GAL1* promoter on high copy plasmid	Zhu *et al.* (2000)
BA1577	pGAL-CCW12	*CCW12* ORF under *GAL* promoter on high copy plasmid	This study
BA1695	pRS316-ABP1ΔSal	Genomic fragment of *ABP1* on low copy plasmid	This study
BA1732	pRS315-ABP1ΔPXXP	Genomic fragment of *ABP1* encoding a protein with a deletion of the PXXP-containing region (codons 448–525) on low copy plasmid	This study
BA1697	pRS315-ABP1	Genomic fragment of *ABP1* on low copy plasmid	This study

Synthetic Lethality Screens and Complementation Assays

We used the synthetic genetic array (SGA) method to systematically identify mutant backgrounds in which *RVS167* or *RVS161* is required for viability or wild-type growth at 30°C (Tong *et al.*, 2001, 2004). All interactions were confirmed by tetrad dissection. Nonoverlapping interactions between the *RVS161* and *RVS167* screens were tested against both *rvs* deletions. Because the frequency of false negatives in SGA analysis can be as high as 40% (Tong *et al.*, 2004), we also tested candidate genes encoding members of a protein complex for interactions with our query genes if genes encoding two or more members of the protein complex were identified to have synthetic interactions (e.g., members of the prefoldin complex). The data for the *RVS161* and *RVS167* screens have been published previously (Tong *et al.*, 2004).

Complementation of the *sla1Δ abp1Δ* strain was assayed using the plasmid shuffle method. The *sla1Δ abp1Δ* strain containing the *URA3*-based plasmid pRS316-ABP1 was transformed with the *LEU2*-based plasmid pRS315-ABP1 or pRS315-ABP1-ΔPXXP. Double transformants were pregrown on SD-Leu, and then cells that had lost the *URA3* plasmid were selected on 5-fluoroorotic acid (5-FOA) and assayed by spot dilution on YPD at 37°C and YPD + 3 mM caffeine at 35°C.

Synthetic Mating Defects Screen and Mating Assays

SGA screens were done to construct *MAT**a*** double mutants that had *rvs161Δ* marked with *URA3* (*rvs161Δ::URA3 xxxΔ::kan*) (see above; Tong *et al.*, 2001). To identify genes that were important for mating in the absence of *RVS161*, the ~5000 viable *MAT**a*** *rvs161Δ::URA3 xxxΔ::kan* mutants were crossed to a *MATα* strain in which the *RVS161* ORF had been replaced with the gene encoding nourseothricin resistance (*rvs161Δ::Nat*) by replica pinning onto YPD plates. The cells were allowed to mate for 6 h at 30°C and then replica pinned onto YPD plates containing nourseothricin (Nat) and G418 to select for diploid cells. Strains that had apparent synthetic mating defects in combination with *rvs161Δ* were retested in limited plate matings with *rvs161Δ* essentially as described previously (Brizzio *et al.*, 1998). In brief, cells of each mating type were patched on top of each other on YPD plates. The plates were incubated at 30°C and replica plated after 2, 3, and 4 h to YPD + G418 + Nat to select for diploids. Likewise, limited mating assays of other strains were done by crossing a *MATα rvs167Δ::Nat* strain with *MAT**a*** *rvs167Δ::URA3 fus1Δ::kan*, *rvs167Δ::URA3 fus2Δ::kan*, *rvs167Δ::URA3 prm9Δ::kan*, and *rvs167Δ::URA3 fig2Δ::kan* strains; a *MATα sla1Δ::Nat* strain with *MAT**a*** *sla1Δ::URA3 fus1Δ::kan*, *sla1Δ::URA3 fus2Δ::kan*, *sla1Δ::URA3 prm9Δ::kan*, and *sla1Δ::URA3 fig2Δ::kan* strains; and a *MATα end3Δ::Nat* strain with *MAT**a*** *end3Δ::URA3 fus1Δ::kan*, *end3Δ::URA3 fus2Δ::kan*, *end3Δ::URA3 prm9Δ::kan*, and *end3Δ::URA3 fig2Δ::kan* strains.

Fluorescence Microscopy

Ten milliliters of cells were grown to mid-log phase in YPD, fixed in 4% formaldehyde for 1.5 h with shaking, and then washed two times with phosphate-buffered saline (PBS), resuspended in 100 μl of PBS, and stored at 4°C. Thirty-six microliters of fixed cells were incubated with 4 μl of rhoda-mine-phalloidin (Invitrogen), 0.5 μl of 10% Triton X-100, and 4 μl of 1 mg/ml Fluorescent Brightener (also known as Calcofluor White; Sigma-Aldrich, St. Louis, MO) for 1.5 h in the dark with occasional gentle vortexing. Cells were washed three times in PBS, visualized using VectaShield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA), and viewed through fluorescein isothiocyanate and rhodamine filters. Photographs were taken with a Micromax 1300y high-speed digital camera (Princeton Instruments, Trenton, NJ) mounted on a Leica DM-LB microscope. Images from the camera were analyzed with MetaVue software (Universal Imaging, Media, PA).

Overexpression of MNN9 and CCW12 in an rvs167Δ slt2Δ Strain

Strains BY1891 (*slt2Δ*) and BY2506 (*rvs167Δ slt2Δ*) were transformed with pGAL-ORFs or empty vector, and BY2506 was kept alive on selective synthetic medium plus glucose and 1 M sorbitol at 30°C. Cells were then replica plated onto synthetic selective media plus 2% galactose and 1 M sorbitol, to induce expression of the pGAL-ORF. Ten-fold serial dilutions were made onto SD and SG containing 1 M sorbitol, and plates were incubated at 30°C to assess the growth of the cells.

Actin Binding Assay

Actin filament binding assays were performed as described previously (Goode *et al.*, 1999). Purified Rvs167-GPA-SH3p-His (2.5 μM; Friesen *et al.*, 2003) or Crn1p (2.5 μM; Humphries *et al.*, 2002) was added to preassembled yeast actin filaments (5 μM) and incubated for 15 min at 25°C. The reactions were centrifuged at 90,000 rpm for 15 min in a TLA100.3 rotor, and the supernatants and pellets were analyzed by SDS-PAGE and Coomassie blue staining.

Liposome Sedimentation Assays

Purified GST-Rvs167p-Rvs161p was subjected to centrifugation at 140,000 $\times$ *g* for 30 min to remove insoluble material. Soluble Rvs167p-Rvs161p heterodimer (7 nM) was incubated with 0.8 mg/ml liposomes from total bovine brain lipids (Avanti Polar Lipids, Alabaster, AL; prepared as described by Peter *et al.*, 2004) or buffer for 30 min at 25°C and then centrifuged at 140,000 $\times$ *g* for 20 min as described previously (Peter *et al.*, 2004). After centrifugation, supernatants were removed immediately and pellets were resuspended in an equal volume of buffer. Samples of pellet and supernatant were analyzed by PAGE and Western blot with α-Rvs167p antibodies.

RESULTS

Rvs167p and Rvs161p Interact as a Heterodimer In Vivo and In Vitro

The structure of the BAR domain of the *Drosophila* am-phiphysin protein suggests that BAR domain proteins are

likely active only as dimers (Peter *et al.*, 2004). *Drosophila* amphiphysin and other mammalian amphiphysins form homodimers; however, heterodimers have been observed for some members of the BAR domain family, for example, rat amphiphysin 1 and 2 (Wigge *et al.*, 1997). A search of the SMART database (Letunic *et al.*, 2004) reveals that Rvs161p and Rvs167p are the only BAR domain-containing proteins in *S. cerevisiae*. Thus, if dimerization is required for BAR-domain activity, the Rvs proteins must act as either an obligate heterodimer or they must homodimerize. Several laboratories have detected a two-hybrid interaction between the BAR domains of Rvs161p and Rvs167p (Navarro *et al.*, 1997; Colwill *et al.*, 1999; Lombardi and Riezman, 2001; Figure 1A), and Rvs161p has been found to coimmunoprecipitate with overproduced Rvs167p (Navarro *et al.*, 1997). To test whether Rvs proteins expressed from their endogenous loci interact and to assay for other proteins that interact with Rvs167p at a stoichiometric level, we used an immunoaffinity purification procedure to isolate Rvs167p–HA complexes from yeast extracts (Figure 1B). By Coomassie blue staining, the only protein that immunoprecipitated with Rvs167p-HA was a protein of ~30 kDa. Mass spectrometric analysis of peptides derived from the 30-kDa band indicated that the protein was Rvs161p (our unpublished data). The similar recovery of Rvs161p and Rvs167p in the Rvs167p-HA immune complexes suggests that Rvs161p-167p may form a stoichiometric complex in vivo.

In both *rvs161Δ* and *rvs167Δ* mutants, the Rvs protein levels are reduced because of reduced stability of one Rvs protein in the absence of the other (Lombardi and Riezman, 2001). This suggests that the interaction between Rvs161p and Rvs167p is required for their stability. Based on our finding that Rvs161p and Rvs167p interact in vivo, we thought that their interaction might function to stabilize each component of the complex in a heterologous system. Consistent with the requirement for an Rvs161p–167p interaction, we found that proteins encoded by either *His-RVS161* or *GST-His-RVS167* was largely insoluble when expressed (separately) in insect cells (Friesen *et al.*, 2003; our unpublished data), and His-Rvs161p and GST-Rvs167p were insoluble when produced (separately) in *Escherichia coli* (our unpublished data), suggesting that the protein products may not fold properly in the absence of their partner (Colwill *et al.*, 1999; Maxwell *et al.*, 1999). To test whether the interaction between the Rvs proteins might allow production of a stable protein complex, we coinfected insect cells with baculoviral vectors that expressed both *GST-His-RVS167* and *His-RVS161*. Cell lysates from coinfected cells were bound to glutathione-Sepharose columns, and both Rvs167p and Rvs161p were detected in the column eluates (Figure 1C). Thus, when coexpressed, Rvs167p and Rvs161p were able to form a soluble protein complex. These biochemical data are consistent with the hypothesis that Rvs167p and Rvs161p must function together in vivo.

Several laboratories have reported a two-hybrid interaction between the BAR domain of Rvs167p and full-length Rvs167p (Colwill *et al.*, 1999; Bon *et al.*, 2000; Lombardi and Riezman, 2001). However, a two-hybrid assay does not address whether homodimerization of Rvs167p occurs in vivo. We tested for Rvs167p homodimerization in a coimmunoprecipitation assay using a diploid strain expressing two alleles of *RVS167*, one encoding an HA-tagged version of Rvs167p and the other encoding an untagged Rvs167p protein. We found that Rvs167p did not coimmunoprecipitate with Rvs167p-HA, showing that homodimerization of Rvs167p does not occur in log-phase cells (Figure 1D, top). To confirm that the epitope-tagged Rvs167p was functional

(i.e., able to bind Rvs161p), we showed that Rvs167p could immunoprecipitate an Rvs161p-myc fusion protein (Figure 1D, bottom). These data suggest that previous two-hybrid interactions between the BAR domain of Rvs167p and full-length Rvs167p may have been indirect or an artifact of BAR domain overproduction. In support of this, Bon *et al.* (2000) saw a two-hybrid interaction between Rvs167p and Rvs167p when both genes were on high-copy plasmids but not when they were on low-copy plasmids.

We also tested whether Rvs161p could homodimerize. Because Rvs161p and Rvs167p are the only BAR-domain-containing proteins in yeast and because Rvs161p is required for fusion during mating and Rvs167p is not (Brizzio *et al.*, 1998), we were especially interested in whether Rvs161p would form homodimers in cells during the mating response. To test for Rvs161p homodimerization, we constructed a strain expressing *RVS161-myc* from its endogenous locus and transformed it with a plasmid containing an untagged version of *RVS161*. As a negative control, we used an untagged strain containing the *RVS161* plasmid. We immunoprecipitated Rvs161p-myc with anti-myc antibodies and assayed for the presence of Rvs161p. As a positive control, we assayed for coimmunoprecipitation of Rvs167p. Although Rvs167p efficiently coimmunoprecipitated with Rvs161p-myc, Rvs161p did not immunoprecipitate with Rvs161p-myc, either in log-phase cells or in cells treated with α factor (Figure 1E). Together, these data show that Rvs161p is the predominant protein partner for Rvs167p in vivo and that these proteins do not form homodimers. These data strongly suggest that the Rvs161p-Rvs167p heterodimer is the functional unit of the Rvs proteins in vivo.

RVS161 and RVS167 Are Genetically Identical as Revealed by Analysis of Synthetic Lethality

To test whether the heterodimer of Rvs161p and Rvs167p accounts for all the cellular functions of the Rvs proteins, we looked at whether one Rvs protein had a function in the absence of its Rvs partner. We predicted that if Rvs161p and Rvs167p function only as a heterodimer, deletion of one *RVS* gene would be genetically identical to deletion of the other. To test this, and to explore the biological roles of Rvs161p and Rvs167p, we screened for genes that had synthetic genetic interactions with *RVS161* and *RVS167* by using SGA analysis. Double mutant strains that failed to grow, or that grew more slowly than wild type, identified candidate genetic backgrounds in which *RVS* was required. All the interactions with either *RVS167* or *RVS161* were confirmed by tetrad analysis in crosses with both *rvs161Δ* and with *rvs167Δ* mutants. The complete data set from these screens has been published previously (Tong *et al.*, 2004).

We found 49 strains whose deletion caused death or synthetic growth defects in combination with deletion of *RVS167* (Figure 2A). All of these mutant strains (and *no* others) also showed synthetic lethal or synthetic growth defects with a deletion of *RVS161*. Genes that are required in the absence of *RVS161* and *RVS167* were organized into categories according to their roles as defined by the Yeast Proteome Database (https://proteome.incyte.com/control/tools/proteome), the Saccharomyces Genome Database (http://www.yeastgenome.org), and published reports (Figure 2A). As expected from the phenotypes of *rvs* deletion mutants (Crouzet *et al.*, 1991; Sivadon *et al.*, 1995, 1997; Lee *et al.*, 1998; Breton *et al.*, 2001; Lombardi *et al.*, 2001; Balguerie *et al.*, 2002), the *RVS* interactions were enriched for genes with roles in cell polarity, cell wall synthesis, cell wall integrity, and vesicle trafficking.
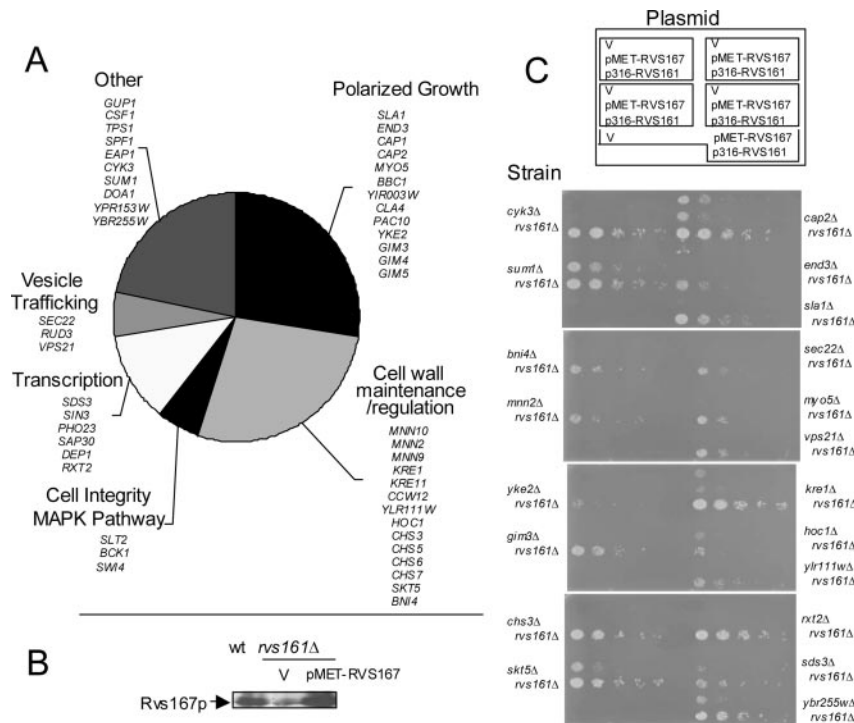
Figure 2. *RVS161* and *RVS167* have identical synthetic genetic interactions. (A) Pie chart summarizing the results of the *rvs167Δ* and *rvs161Δ* synthetic lethality screens with query strains BY1404 and BY1796. Genes were categorized according to their annotations in the Saccharomyces Genome Database (SGD), the Yeast Proteome Database (YPD), and/or on the basis of review of published studies concerning the genes. (B) Western blot showing levels of Rvs167 protein in wild-type and *rvs161Δ* cells containing either vector or p416-MET-RVS167. (C) Assay for suppression of the synthetic growth defect of slow-growing *rvs161Δ xxxΔ* mutant strains by overproduction of Rvs167p. Twenty slow-growing viable *rvs161Δ xxxΔ* mutant strains were transformed with vector (p416-MET; V), p416-MET-RVS167, and pRS316-RVS161 and assayed by serial dilution onto SD-Ura and SD-Ura + 3% NaCl. Only the SD-Ura + 3% NaCl plates are shown. Strains were arrayed as shown in the diagram at the top with five yeast strains transformed with three plasmids on each plate. For many of the double mutant strains, cells containing vector or p416-RVS167 were unable to grow on 3% NaCl so that only growth of cells containing p316-RVS161 is apparent.

The Pattern of Identical Synthetic Genetic Interactions with RVS161 and RVS167 Is Not Because of Destabilization of the Rvs Partner

Because each Rvs protein is required for the solubility and stability of its Rvs partner in vivo (Lombardi and Riezman, 2001; Figure 1C), it was possible that the identical SGA profiles for *RVS161* and *RVS167* reflected the decreased protein levels of the remaining Rvs protein. We next asked whether increasing the cellular concentration of Rvs167p to wild-type levels in the *rvs161Δ* double mutants (which we refer to as *rvs161Δ xxxΔ* strains) could suppress their growth defect. Expression of *RVS167* from the *MET25* promoter restores Rvs167p protein to wild-type levels in the *rvs161Δ RVS167⁺* strain (Figure 2B). We predicted that if the *rvs161Δ xxxΔ* mutant strains were slow growing because Rvs167p was destabilized, heterologous expression of *RVS167* should rescue the growth defects of the double mutants. We compared the ability of empty vector, p416-MET-RVS167 and p316-RVS161 (*RVS161* under the control of its own promoter) to complement the growth defects of 20 slow-growing *rvs161Δ xxxΔ* mutants grown on medium containing 3% salt, which provides more stringent conditions to detect the subtle growth defects of some of the double mutants. We found that restoring levels of Rvs167p by expressing *RVS167* failed to rescue the growth defects of these strains (Figure 2C). Weak suppression of the *rvs161Δ xxxΔ* slow growth phenotype by *RVS167* was detected in the *sum1Δ*, *skt5Δ*, and *ybr255wΔ* double mutants, but in all cases the *RVS161* plasmid complemented the growth defect of the *rvs161Δ xxxΔ*

strains much more efficiently than the *RVS167* plasmid (Figure 2C; our unpublished data). These data show that restoring Rvs167p protein to wild-type levels is not sufficient to restore wild-type growth in strains in which *RVS161* is needed. Thus, the decreased protein levels of the Rvs partner are not sufficient to explain the overlap of the genetic profiles of strains deleted for *RVS167* or *RVS161*.

Reduced Stability of Each Rvs Protein in the Absence of its Partner in Pheromone-treated Cells

As noted above, previous studies showed that the levels of Rvs167p protein are reduced in an *rvs161Δ* mutant during log phase, and, similarly, the levels of Rvs161p are strongly reduced in an *rvs167Δ* mutant (Lombardi *et al.*, 2001). The instability of the Rvs proteins in the absence of their partner is consistent with the model that they function together during vegetative growth. However, distinct phenotypes for *rvs* mutants have been reported during the mating response, and it is unclear whether the Rvs proteins function as a heterodimer during mating (Brizzio *et al.*, 1998). We hypothesized that if Rvs167p has a role in mating, then Rvs161p protein levels might remain low in an *rvs167Δ* strain exposed to mating pheromone, despite the rapid induction of *RVS161* expression in response to α factor (Brizzio *et al.*, 1998). To test this, we used Western blots to look at the protein levels of Rvs161p and Rvs167p from extracts of cells treated with pheromone. Rvs161p protein levels were greatly decreased in extracts from *rvs167Δ* mutants in log phase compared with levels in wild-type cells (Figure 3A,
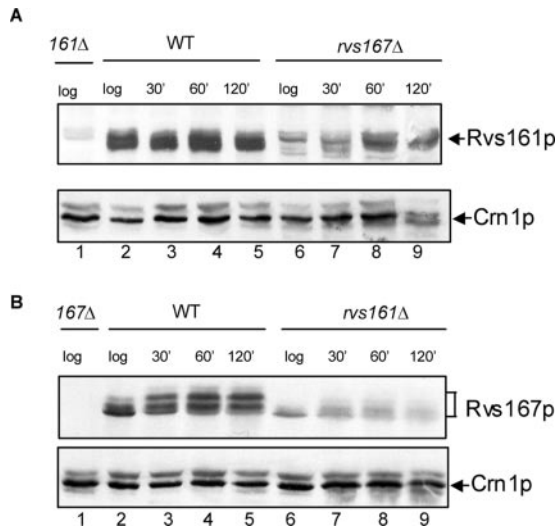
Figure 3. Reduced stability of Rvs161p and Rvs167p protein in the absence of its partner in log phase and during treatment with mating pheromone. (A) Western blot analysis of protein extracts from wild-type (BY4741a) and *rvs167Δ* cells in log phase and after 30-, 60-, and 120-min exposure to 5 μM α factor as indicated. Extracts were analyzed by SDS-PAGE and immunoblotted with anti-Rvs161p antibody (gift from H. Riezman). (B) Western blot analysis of protein extracts from wild-type and *rvs161Δ* cells in log phase and after 30-, 60-, and 120-min exposure to mating pheromone as indicated. Extracts were analyzed by SDS-PAGE and immunoblotted with anti-Rvs167p antibody. The same extracts were blotted with antibodies against Crn1p as a loading control (bottom). The position of migration of Rvs167p and Rvs167p phosphoforms, Rvs161p and Crn1p are indicated.

compare lanes 2 and 6). On treatment with α factor, Rvs161p was detected at much lower levels in the *rvs167Δ* strain than in wild-type cells, even after incubation for 2 h in phero-mone-containing medium (Figure 3A, lanes 7–9). In an analogous assay, we examined the protein levels of Rvs167p in *rvs161Δ* extracts after exposure to pheromone. Rvs167p protein levels were strongly reduced in log-phase *rvs161Δ* cells compared with wild-type cells and remained reduced during treatment with α factor (Figure 3B). These data reveal a requirement for the Rvs partner for the stability of the Rvs proteins during pheromone exposure, suggesting that the Rvs161p-Rvs167p heterodimer may function during mating.

Identification of Mutations That Lead to Synthetic Mating Defects in Combination with Deletion of RVS161 and RVS167

Our finding that stability of Rvs161p depends on Rvs167p during pheromone response suggests a function for both Rvs161p and Rvs167p during mating. However, only *RVS161*, but not *RVS167*, has been shown to be important for mating (Brizzio *et al.*, 1998). The mating defects of a strain deleted for *RVS161* alone are subtle but are exacerbated in combination with a deletion of *FUS1* (Brizzio *et al.*, 1998). We were therefore interested in identifying additional genetic backgrounds in which the mating defect of the *rvs161Δ* strain was exacerbated, to test whether a role for *RVS167* in mating could be uncovered in these backgrounds. We used SGA to create double mutants of *rvs161Δ* in combination with the ~5000 yeast gene deletions. These *rvs161Δ xxxΔ* mutants were then screened in limited mating assays for synthetic mating defects in crosses with an *rvs161Δ* mating
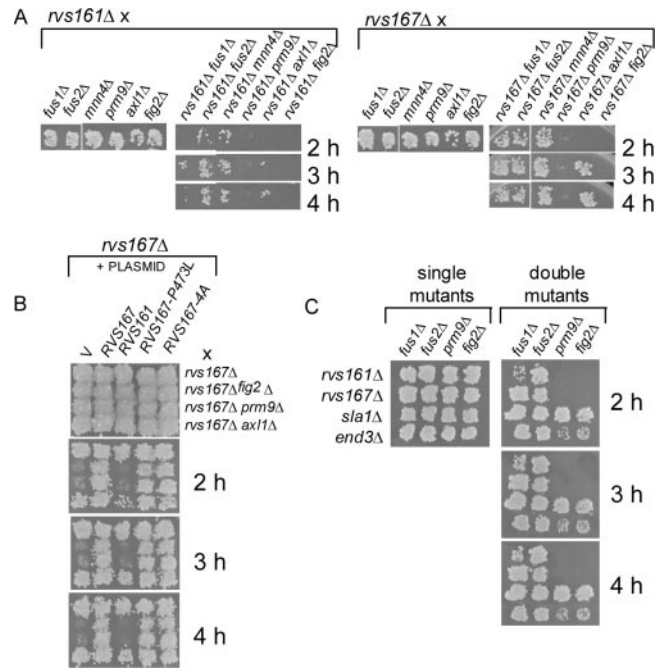

Figure 4. Diploid selection plates from limited mating assays for *rvs161Δ* and *rvs167Δ* double mutants. (A) *MAT*a *rvs161Δ::URA3 xxxΔ::kan* strains were mixed with a toothpick on a YPD plate with a *MAT*α *rvs161Δ::Nat* strain, incubated at 30°C for 2, 3, and 4 h and replica plated to YPD + G418 + Nat to select for diploids. Likewise, *MAT*a *rvs167Δ::URA3 xxxΔ::kan* strains were mated with a *MAT*α *rvs167Δ::Nat* strain. On the left, for each strain, is shown a control plate of single mutant strains (e.g., *fus1Δ::kan*) crossed to *rvs161Δ::Nat* or *rvs167Δ::Nat* in which diploids were selected 2 h after mating. (B) *MAT*a *rvs167Δ::URA3 xxxΔ::kan* strains transformed with p416-MET, p416-MET-RVS167, p426-RVS161, p416-MET-RVS167-P473L, or p416-MET-RVS167–4A were crossed with a *MAT*α *rvs167Δ::Nat* strain as described above. (C) Limited-mating assay for mutants defective in endocytosis. *MAT*a double mutants of *rvs161Δ::URA3*, *rvs167Δ::URA3*, *sla1Δ::URA3*, and *end3Δ::URA3* in combination with *fus1Δ::kan*, *fus2Δ::kan*, *prm9Δ::kan*, and *fig2Δ::kan* were crossed to *MAT*α *rvs161Δ::Nat*, *rvs167Δ::Nat*, *sla1Δ::Nat*, or *end3Δ::Nat*, incubated at 30°C for 2, 3, and 4 h and replica plated to YPD + G418 + Nat.

partner using SGA (see *Materials and Methods*). We identified a number of genes that seemed to be required for mating in the absence of *RVS161* (our unpublished data). We arbitrarily chose three genes, each of which has a role in cell fusion during mating: *PRM9*, *AXL1*, and *FIG2*. *PRM9* encodes a pheromone-regulated membrane protein (Heiman and Walter, 2000), and its mutant phenotype has not yet been characterized. *AXL1* encodes a protease required for cell fusion during mating (Elia and Marsh, 1998) and is required for axial budding (Fujita *et al.*, 1994). *FIG2* is involved in agglutination during mating (Zhang *et al.*, 2002). We next tested whether some of these genes were required for mating in the absence of *RVS167* using limited mating assays. We also included crosses with *fus1Δ* and *fus2Δ* mutants as positive and negative controls, because a strain with a deletion of *RVS161* has mating defects in combination with *fus1Δ* but not *fus2Δ* (Brizzio *et al.*, 1998), and *mnn4Δ*, another positive control, which had no defect in combination with *rvs161Δ* or *rvs167Δ*. All of these mutants showed no mating defect in an *RVS*[+] background when crossed to *rvs161Δ* or *rvs167Δ* strains (Figure 4A, left). In an *rvs161Δ* background, a synthetic mating defect was seen in *fus1Δ*, *prm9Δ*, *axl1Δ*,

and *fig2Δ*, but not in *fus2Δ* or in *mnn4Δ* (Figure 4A, right). In the *rvs167Δ* background, no mating defect was seen in *fus1Δ*, *fus2Δ*, *axl1Δ*, or in *mnn4Δ* strains, although the *axl1Δ* strain seemed to be delayed in mating (Figure 4A, right). But *rvs167Δ prm9Δ* and *rvs167Δ fig2Δ* strains had a mating defect similar to that seen in an *rvs161Δ* background. All these results were confirmed in quantitative mating assays (our unpublished data). We also found that the *rvs161Δ* and *rvs167Δ* double mutants (with *fus1Δ*, *fus2Δ*, *prm9Δ*, and *fig2Δ*) were able to form shmoos with >50% wild-type efficiency, indicating that their pheromone signaling pathway was functional, consistent with the defect in cell fusion previously reported in *rvs161Δ* strains. In summary, we found that *rvs161Δ* has synthetic mating defects with *fus1Δ* and *axl1Δ* that are not seen with *rvs167Δ*. Both *rvs161Δ* and *rvs167Δ* have defects in combination with deletions in *prm9Δ* and *fig2Δ*.

Because we had found that Rvs161p levels were significantly decreased during exposure to mating pheromone in an *rvs167Δ* strain (Figure 3A), one possibility was that the mating defect was because of destabilized Rvs161p and not absence of Rvs167p. To test this possibility, we assayed *rvs167Δ* cells transformed with a high copy plasmid containing *RVS161* under its own promoter in a limited mating assay. As a positive control, we also tested *rvs167Δ* cells with a plasmid containing *RVS167*. In this experiment, *RVS167* but not high copy *RVS161* was able to complement the mating defect of the *rvs167Δ xxxΔ* mutants (Figure 4B). This suggests that the mating defect in *rvs167Δ* cells is not because of reduced stability of Rvs161p.

The BAR domain of Rvs167p is largely able to complement all the defects of an *rvs167Δ* strain during log-phase growth, but we were interested in whether the SH3 domain or the phosphorylation sites within the GPA region of Rvs167p were needed for mating. Rvs167p is hyperphosphorylated during the mating response (Lee *et al.*, 1998; Friesen *et al.*, 2003). We tested the *rvs167Δ xxxΔ* mutants for complementation using a plasmid that encodes Rvs167p with a mutant SH3 domain (Rvs167p-P473L). This Rvs167p SH3 mutant protein does not interact with the proline-rich region of Abp1 in a two-hybrid assay, but it does bind other proteins that interact with Rvs167p outside the SH3 domain, indicating that this mutant specifically disrupts the function of the SH3 domain (Colwill *et al.*, 1999). We also tested for complementation using a plasmid with *RVS167* encoding a protein with alanine substitutions at all four of the residues that are phosphorylated in vivo (Rvs167p-4A; Friesen *et al.*, 2003). The *rvs167Δ xxxΔ* mutants were complemented equally efficiently by wild-type *RVS167* and by *RVS167-P473L* and by *RVS167-4A* (Figure 4B). This indicates that neither an intact SH3 domain nor phosphorylation of Rvs167p is required for Rvs167p function during mating.

We were interested in whether the mating defects in *rvs167Δ* cells were characteristic of all endocytosis mutants. Brizzio *et al.* (1998) assayed several mutants defective in endocytosis for defects in mating and found that *end3-1*, *end4-1*, *end6-1*, and *end7-1* behaved like wild type, suggesting that endocytosis did not play a role in cell fusion. However, it was possible that these defects could only be uncovered in certain specific genetic backgrounds, as we had seen for *rvs167Δ*. We tested two well-characterized endocytosis mutants, *end3Δ* (Raths *et al.*, 1993) and *sla1Δ* (Warren *et al.*, 2002), along with *rvs161Δ* and *rvs167Δ*, for mating in the absence of *FUS1*, *FUS2*, *PRM9*, and *FIG2* (Figure 4C). As described above (Figure 4, A and B), *rvs161Δ* had a mating defect in the absence of *FUS1*, and *rvs161Δ* and *rvs167Δ* had mating defects in the absence of *PRM9* and *FIG2* (Figure 4C).

In contrast, the *sla1Δ* strain and the *end3Δ* strain were able to mate in the absence of *PRM9* and *FIG2* (Figure 4C). Mating of the *end3Δ* strain was not as efficient as mating in the other strains, possibly because the *end3Δ* strain is slow growing by itself. Thus, in these assays, *RVS167* has a role in mating that is partially distinct from that of *RVS161* but is not found in other endocytosis mutants.

Synthetic Defects in Actin Organization and Chitin Distribution in Double Mutants

Identification of synthetic lethal interactions or synergistic growth defects provides support for a functional interaction between gene products (Bender and Pringle, 1991, Tong *et al.*, 2004). Furthermore, the subtle defects in the organization and polarity of cortical actin structures of null mutations in cytoskeleton protein genes, and the large number of synthetic lethal interactions among these genes reveal their multiple overlapping functions (Ayscough and Drubin, 1996; Goode and Rodal, 2001). However, the cellular basis for the synthetic lethality is not always clear, and we decided to characterize the phenotypes of a subset of the mutants identified in our SGA analysis to determine whether polarity was defective in the double mutant strains. We first decided to examine the actin cytoskeleton. The *rvs161Δ* and *rvs167Δ* strains have partial defects in the polarization of their cortical actin cytoskeleton, suggesting their function in this process must be partially redundant with other cytoskeleton proteins (Sivadon *et al.*, 1995; Lee *et al.*, 1998; Balguerie *et al.*, 1999). In a subset of the viable *rvs167Δ xxxΔ* mutants with synthetic growth defects and in the corresponding single mutants, we visualized filamentous actin using rhodamine-phalloidin staining (Figure 5A). Quantification of cells showing a defect in actin polarization revealed that *rvs167Δ* cells had a partially depolarized actin cytoskeleton (Figure 5A, left), as reported previously. In some of the *rvs167Δ xxxΔ* mutants, the defects in actin organization were no worse than in the single mutants. However, in the *myo5Δ rvs167Δ* and *yke2Δ rvs167Δ* double mutants cortical actin patches were seen randomly distributed over the periphery of both mother and daughter cell at all stages of the cell cycle (Figure 5A). This suggests that in these mutants the synergistic growth defect may be because of a depolarized actin cytoskeleton.

The *rvs167Δ xxxΔ* mutants were also examined for another phenotype that is often associated with perturbation of the actin cytoskeleton: cell wall defects. Mutations affecting actin can cause cell wall defects as indicated by depolarized chitin deposition (Novick and Botstein, 1985; Yang *et al.*, 1997). We looked at cells stained with Calcofluor White to visualize the chitin deposited in the cell wall in bud scars and with DAPI to monitor position in the cell cycle. As reported previously (Bauer *et al.*, 1993), the *rvs167Δ* mutant contained slightly diffuse chitin distributed over the entire mother cell surface (Figure 5B). Of the six *rvs167Δ xxxΔ* strains tested, *rvs167Δ myo5Δ*, *rvs167Δ sla1Δ*, *rvs167Δ vps21Δ*, and *rvs167Δ yke2Δ* showed a more diffuse chitin localization and weaker staining of bud scars than either of the single mutants (Figure 5B, quantification shown on the left). Interestingly, deletion of the genes with roles in endocytosis, *MYO5* (Geli and Riezman, 1996), *SLA1* (Warren *et al.*, 2002), and *VPS21* (Singer-Krüger *et al.*, 1994), in combination with deletion of *RVS167*, resulted in cell wall defects. Thus, although *rvs167Δ* mutants have only partial defects in their actin cytoskeleton and cell wall, one or both of these defects were made more severe in several of the double mutants tested. Together, these data show that the growth defects of *rvs167Δ* double mutants may be caused by synergistic de-
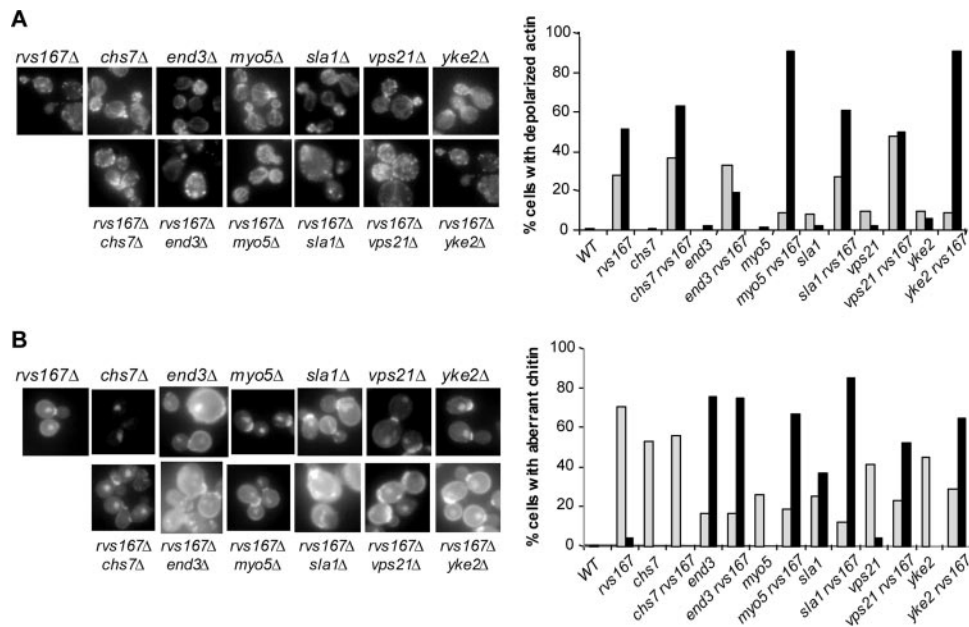
Figure 5. Actin and chitin staining of single and double mutant cells of *chs7Δ*, *end3Δ*, *myo5Δ*, *sla1Δ*, *vps21Δ*, and *yke2Δ* in combination with *rvs167Δ*, as indicated above and below each panel. (A) Cells were grown in YPD to log phase, fixed and their actin stained using rhodamine-conjugated phalloidin as described in *Materials and Methods*. On the left are shown representative cells from each strain. On the right is shown a graph depicting percentage of cells with depolarized actin. Approximately 100 small- and medium-budded cells were scored as having: >90% of their actin in the bud (not graphed); 50–90% of their actin in the bud (gray); or <50% of their actin in the bud (black). (B) Cells were stained with Calcofluor White and DAPI, washed, and observed for chitin distribution. On the left are shown representative cells from each strain. On the right is shown a graph depicting percentage of cells with aberrant chitin distribution. Approximately 100 cells were scored for each strain as having: staining localized almost completely to the bud scars (not graphed), some diffuse chitin with easily discernible bud scars (gray), or completely diffuse chitin (black).

fects in cell wall structure and in the organization of the actin cytoskeleton.

The Cell Integrity Pathway Is Activated in rvs Mutants

The Slt2p kinase becomes phosphorylated on T190 and Y192 upon activation of the Slt2p MAP kinase pathway during periods in which yeast cells are undergoing polarized cell growth, namely, during bud formation and in response to mating pheromone and in response to environmental conditions such as cell wall damage and heat stress (Zarzov *et al.*, 1996; de Nobel *et al.*, 2000). Based on the observations that *rvs161Δ* and *rvs167Δ* mutants have defects in their cell wall (Figure 5B) and synthetic lethal interactions with genes that encode components of the Slt2p pathway (Figure 2A; Breton *et al.*, 2001), we hypothesized that *rvs* mutants may have a constitutively activated Slt2p pathway. We used anti-phospho-p42/p44 MAP kinase antibody, which specifically recognizes the phosphorylated (activated) form of Slt2p (Verna *et al.*, 1997; de Nobel *et al.*, 2000), to examine activation of the Slt2p pathway in wild-type and *rvs161Δ* and *rvs167Δ* cells. Because we were unable to detect endogenous Slt2p using this antibody (our unpublished data), we transformed cells with a high-copy plasmid encoding HA-tagged Slt2p and immunoprecipitated Slt2p-HA from these cells using anti-HA antibodies, for detection with the anti-phospho-p42/p44 MAP kinase antibody (Madden *et al.*, 1997). We immunoprecipitated Slt2p-HA using anti-HA antiserum from wild-type, *rvs161Δ*, and *rvs167Δ* cells grown at 24°C. As a positive control for Slt2p activation, we also activated the Slt2p pathway in a wild-type strain by incubating the cells at 39°C for 2 h (Kamada *et al.*, 1995). No proteins were recognized by the anti-phospho-p42/44 antibody in immu-

noprecipitates from a wild-type strain with untagged Slt2p (Figure 6A, top). In wild-type cells grown at 24°C, only a small amount of phosphorylated Slt2-HA was detected by the anti-phospho-p42/p44, whereas incubation of cells at 39°C resulted in the accumulation of a larger amount of phosphorylated Slt2p (Figure 6A, top, lanes 2 and 3). Consistent with the hypothesis that Slt2p is constitutively activated in the absence of Rvs161p and Rvs167p, *rvs161Δ* and *rvs167Δ* mutants grown at 24°C displayed a higher level of phosphorylated Slt2p than the wild-type strain grown at 24°C (Figure 6A, lanes 4 and 5 respectively). Immunoblot analysis using an anti-HA antibody confirmed that similar amounts of Slt2p were present in every lane (Figure 6A, bottom).

The function of Slt2p in ensuring cellular integrity is mediated in part through the transcriptional induction of genes encoding enzymes involved in cell wall biosynthesis (for review, see Heinisch *et al.*, 1999). Slt2p activates the transcription factors Rlm1p and SBF, a heterodimer composed of Swi4p and Swi6p (Andrews and Herskowitz, 1989), leading to expression of specific subsets of genes (Madden *et al.*, 1997; Watanabe *et al.*, 1997; Jung and Levin, 1999; Baetz *et al.*, 2001). Thus, one possible explanation for the requirement for the Slt2p pathway components in *rvs* mutants is that genes whose expression is Slt2p-dependent are essential in the absence of Rvs161p and Rvs167p. Consistent with this hypothesis, we had identified two genes whose transcription is known to be dependent on Slt2p, *CCW12* and *CHS3* (Jung and Levin, 1999; Baetz *et al.*, 2001), in our screen for genes that had synergistic growth defects in combination with deletions of *rvs161Δ* and *rvs167Δ* (Figure 2A).
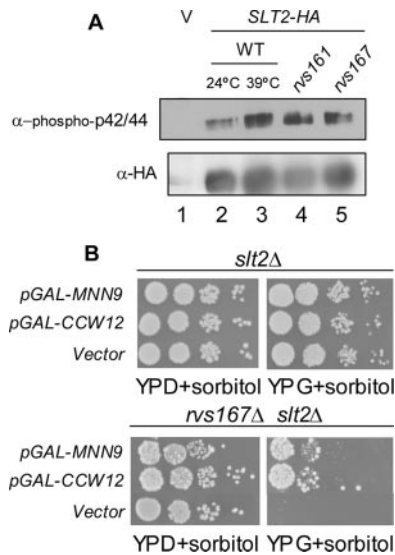
Figure 6. Slt2p is activated in *rvs161Δ* and *rvs167Δ* mutants. (A) Immunoblot assay of Slt2p activation. Slt2p-HA was immunoprecipitated with α-HA antibodies from strains harboring either vector (lane 1) or a plasmid containing *SLT2*-HA (BA1019; lanes 2–5). Lane 2, wild-type strain (BY4741a) grown at 24°C; lane 3, wild-type strain grown at 39°C; lane 4, *rvs161Δ* strain grown at 24°C; and lane 5, *rvs167Δ* strain grown at 24°C. Top, immunoblot assays of immunoprecipitated proteins probed with α-phospho-p42/44 MAPK (Thr202/Tyr204) antibodies. Bottom, immunoblot assays of the same samples probed with 12CA5 α-HA antibodies. (B) Suppression of growth defects of an *slt2Δ rvs167Δ* strain by overexpression of putative downstream targets of the Slt2p pathway. Growth defects of an *slt2Δ rvs167Δ* strain are suppressed by overproduction of *MNN9* and *CCW12* but not by vector. *slt2Δ* or *slt2Δ rvs167Δ* cells transformed with vector, or plasmids expressing *GST-MNN9* or *CCW12* under control of the *GAL* promoter were grown in synthetic dextrose lacking uracil with 1 M sorbitol for plasmid selection. Cells were then assayed by spotting 10-fold serial dilutions on YPD + 1 M sorbitol and on YP + galactose + 1 M sorbitol and incubated for 3 d (*slt2Δ*) or 6 d (*slt2Δ rvs167Δ*) at 30°C.

We hypothesized that overproduction of transcriptional targets of Slt2p might rescue the synthetic lethality of the *slt2Δ rvs167Δ* mutant. The double mutant strain is unable to grow on galactose-containing medium, even in the presence of the osmotic stabilizer sorbitol, but we could keep it alive on glucose-containing medium containing 1 M sorbitol (Figure 6B; Breton *et al.*, 2001). We transformed an *slt2Δ* strain and an *rvs167Δ slt2Δ* strain with overexpression plasmids containing the various genes involved in cell wall maintenance that had come out of our synthetic lethality screen and looked for suppression on galactose. Overexpression of two of the genes, *MNN9* and *CCW12*, but not vector alone, could partially suppress the lethality of an *rvs167Δ slt2Δ* strain (Figure 6B). These data suggest that the essential role of the Slt2p–MAP kinase pathway in the absence of *RVS167* may be to activate transcription of genes involved in cell wall maintenance, presumably through the transcription factor Swi4p.

The SH3 Domain of Rvs167p Does Not Bind Actin But Is Required in the Absence of SLA1

The SH3 domain of Rvs167p interacts with many actin cytoskeleton proteins in two-hybrid studies, including Abp1p, Las17p, and actin (Amberg *et al.*, 1995; Lila and Drubin, 1997; Wendland *et al.*, 1998; Colwill *et al.*, 1999; Bon *et al.*, 2000;

Landgraf *et al.*, 2004). A biological role for the SH3 domain remains unclear because it is not required to complement any *rvs167Δ* defects; the BAR domain of Rvs167p is largely sufficient to rescue all *rvs167Δ* phenotypes (Colwill *et al.*, 1999). One possible role for the SH3 domain is to bind actin. An interaction between the carboxy-terminal region of Rvs167p and actin has been demonstrated in two-hybrid assays, but it is unclear whether this interaction is direct or whether it is mediated through interactions with other actin-binding proteins (Amberg *et al.*, 1995; Lombardi and Riezman, 2001). To test the carboxy-terminal portion of Rvs167p for binding to actin, we asked whether purified GPA-SH3 domain could bind actin in vitro. GPA-SH3 domain was added to preassembled F-actin, and binding was tested in a high-speed cosedimentation assay. Background levels of GPA-SH3 pelleting (~20%) were observed in reactions that contained GPA-SH3 alone (Figure 7A, lanes 3 and 4). No increase in GPA-SH3 pelleting was detected in reactions containing F-actin (Figure 7A, lanes 9 and 10). In contrast, despite similar background levels of pelleting, the majority of Crn1p, which was used in this assay as a positive control for F-actin binding (Goode *et al.*, 1999) was detected in the pellet fractions in reactions containing F-actin (Figure 7A, lanes 7 and 8). These data suggest that previous two-hybrid interactions between actin and the carboxy terminus of Rvs167p are indirect (Lila and Drubin, 1997; Lombardi *et al.*, 2001). It is possible, however, that the SH3 domain of Rvs167p has no role in the absence of the BAR domain and depends on interactions between Rvs161p and Rvs167p.

To gain further insight into the biological role of the Rvs167p SH3 domain, we looked for genetic backgrounds in which the SH3 domain of Rvs167p was essential. We tested whether the SH3 domain was required to complement the growth defects of *rvs167Δ xxxΔ* mutants identified in our SGA analysis. Because both *RVS161* and *RVS167* were required in the double mutants, it seemed likely that the BAR domain was the essential portion of Rvs167p; however, it was possible that the SH3 domain was important in addition to the BAR domain. We tested the *rvs167Δ xxxΔ* mutants for complementation using the plasmid that encodes Rvs167p with a mutant SH3 domain (Rvs167p-P473L). We found that *RVS167* and *RVS167-P473L* complemented the synthetic growth defects of most double mutants (our unpublished data), even when we tested for growth on salt-containing medium. However, *RVS167-P473L* did not complement the synthetic growth defects of the *sla1Δ rvs167Δ* strain as efficiently as wild-type *RVS167* (Figure 7B). In addition, we saw a partial defect in complementation by *RVS167-P473L* of the *end3Δ rvs167Δ* strain (our unpublished data). These results show that the SH3 domain of Rvs167p is required for viability in the absence of *SLA1* and *END3* and provides the first genetic evidence for a role for the Rvs167p SH3 domain.

Our observation that the SH3 domain of Rvs167p is essential in the absence of *SLA1* and *END3* suggests that the protein that binds to the SH3 domain would also be essential in the absence of these genes. To look for the essential SH3-binding protein, we tested the genes encoding eleven putative SH3-interacting proteins for synthetic lethality with deletion of *SLA1* using tetrad analysis: *ABP1*, *ACF2*, *ACF4*, *APP1*, *GYL1*, *BSP1*, *GYP5*, *HUA1*, *HUA2*, *YGL060w* (*YBP2*), and *YBR108w*. We chose these proteins based on their predicted or known interactions with Rvs167p and the presence of SH3 binding motifs (PXXP) in the protein sequences (Pawson and Gish, 1992; Mayer and Eck, 1995; Gavin *et al.*, 2002; Ho *et al.*, 2002; Tong *et al.*, 2002). Some mutants of genes encoding putative Rvs167p SH3 binding proteins, such as Las17p, could not be tested for synthetic lethality
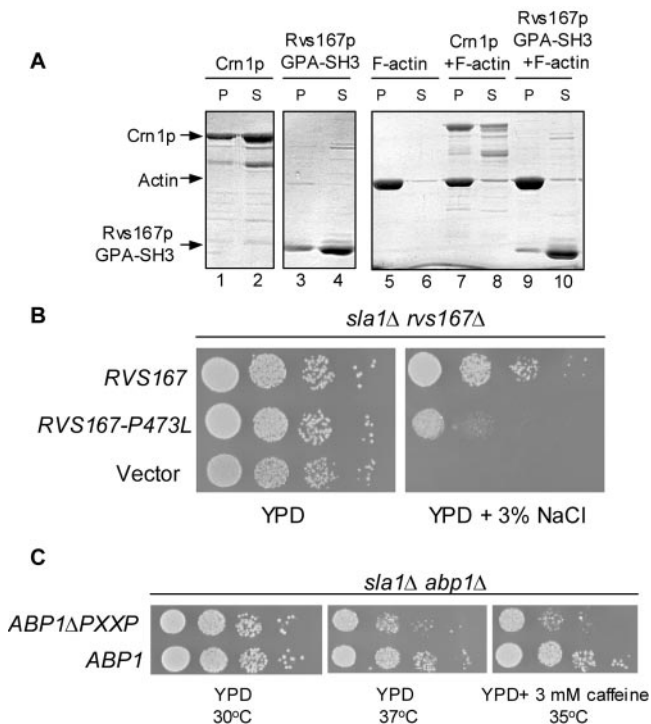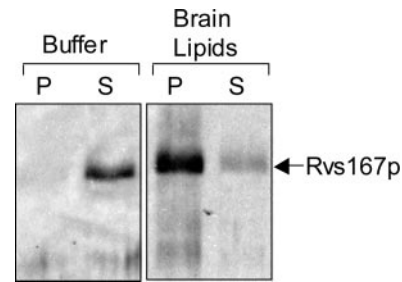
Figure 8. Membrane binding by Rvs167p-161p. Sedimentation assay with liposomes made from total bovine brain lipids or buffer alone. After centrifugation, supernatants were removed immediately and pellets were resuspended in a volume of buffer equal to that of the supernatant and then analyzed by PAGE and Western blotting with α-Rvs167p antibodies.

Figure 7. Role of the SH3 domain of Rvs167p. (A) F-actin binding assay for the GPA-SH3 region of Rvs167. Crn1p (2.5 μM) or His-tagged GPA-SH3 polypeptide (2.5 mM) was incubated with buffer (lanes 1–4) or preassembled actin filaments (5 mM; lanes 7–10). Lanes 5 and 6, F-actin alone. After incubation, the reactions were pelleted at high speed, and the pellet (P) and supernatant (S) fractions were analyzed by SDS-PAGE and Coomassie blue staining. Crn1p (2.5 mM) was included as a positive control for F-actin binding. (B) Biological role of the SH3 domain of Rvs167p. An *sla1Δ rvs167Δ* strain is complemented by wild-type *RVS167* but not by *RVS167-P473L* or by vector. Ten-fold serial dilutions of BY2805 transformants containing vector, wild-type *RVS167* (BA1656) or *RVS167-P473L* (BA1658) were spotted on YPD or YPD containing salt as indicated. (C) The *sla1Δ abp1Δ* strain is complemented by *ABP1* but not by *ABP1ΔPXXP*. The *sla1Δ abp1Δ* cells were kept alive with a wild-type *ABP1-URA3* (BA1695) plasmid, transformed with *LEU2*-based plasmids containing wild-type *ABP1* (BA1697) and *ABP1ΔPXXP* (BA1732) and vector (our unpublished data), and then plated on 5-FOA to select cells that had lost the wild-type *ABP1-URA3* plasmid. No colonies containing *LEU2*-based vector were able to grow on 5-FOA. Survivors were then assayed by spot dilution on YPD or YPD + 3 mM caffeine at the temperatures indicated.

because they were extremely slow growing on their own. Ten of 11 of the genes encoding putative Rvs167p SH3-interacting proteins had no synthetic interaction with *SLA1* (our unpublished data). Only one of the genes, *ABP1*, had a synthetic lethal interaction with *SLA1*, a genetic interaction that has been reported previously (Holtzman *et al.*, 1993). A synthetic interaction between *END3* and *ABP1* was also detected when double mutants were grown at 37°C (our unpublished data). These data suggested that the interaction between Abp1p and the SH3 domain of Rvs167p might be essential in absence of the cytoskeleton proteins Sla1p and End3p.

To test whether the interaction between Abp1p and the SH3 domain of Rvs167p is essential, we created an allele of *ABP1* that encodes Abp1p with a deletion of the putative SH3-binding region (*ABP1ΔPXXP*). We next asked whether PXXP-dependent interactions with Rvs167p are essential by testing whether *ABP1ΔPXXP* could complement the growth defect of the *sla1Δ abp1Δ* cells. We predicted that if the interaction between Rvs167p and Abp1p was essential in the *sla1Δ* background, a version of Abp1p that no longer contains the putative Rvs167p-SH3 binding region should not complement the growth defects of this strain. Because a *sla1Δ abp1Δ* strain is dead, we assayed complementation using a plasmid shuffle method (see *Materials and Methods*). We found that the growth defects of the *sla1Δ abp1Δ* strain grown at 37°C or grown in the presence of 3 mM caffeine at 35°C were complemented less efficiently by *ABP1ΔPXXPI* than by wild-type *ABP1* (Figure 7C). The *end3Δ abp1Δ* strain was also complemented less efficiently by *ABP1ΔPXXPI* than by wild-type *ABP1*, although the degree of complementation of the *ABP1ΔPXXP* plasmid in the *end3Δ abp1Δ* strain was transformant-dependent and variable in penetrance (our unpublished data), possibly because of variability in levels of expression of *ABP1ΔPXXP* from the plasmid. Thus, under certain growth conditions, the putative Rvs167p-SH3 interaction domain of Abp1p is essential in the absence of Sla1p and End3p. These data support a model in which the interaction between Rvs167p and Abp1p is essential under some physiological conditions.

Membrane Binding Activity of Rvs167p-161p

Although Rvs167p GPA-SH3 was not able to bind to F-actin in an actin pelleting assay, we still searched for a biochemical activity associated with Rvs167p. The mammalian homologues of Rvs167p, amphiphysin-1 and amphiphysin-2, and the *Drosophila* homologue dAmph, all of which act as homodimers, have been shown to bind to lipid membranes and to promote membrane curvature in vitro (Takei *et al.*, 1999; Razzaq *et al.*, 2001; Lee *et al.*, 2002; Peter *et al.*, 2004). We tested whether our purified Rvs161p-167p heterodimer could bind to liposomes using a sedimentation assay as described by Peter *et al.* (2004). We preincubated Rvs161p-167p with liposomes from brain lipids or with buffer and then subjected the mixture to high-speed centrifugation to pellet the liposomes. We then assayed the supernatant and the pellet for the presence of Rvs167p by Western blot. In this experiment, Rvs167p (and presumably associated Rvs161p) was pelleted in the presence of vesicles from brain lipids but not with buffer alone (Figure 8). Thus, like its homologues, Rvs167p is able to bind to phospholipid membranes.

DISCUSSION

Rvs161p and Rvs167p are members of the conserved BAR-domain family of proteins and have established roles in the organization of the actin cytoskeleton and in endocytosis. However, since their identification 14 years ago (Crouzet *et al.*, 1991), the biological function of the Rvs proteins has remained poorly understood. In this work, we have shown that, unlike the *Drosophila* homologue amphiphysin, which acts as a homodimer, Rvs161p and Rvs167p form an obligate heterodimer. Consistent with this, our genetic experiments reveal that Rvs161p and Rvs167p have a shared role in both growing and mating cells. Our comprehensive analysis of synthetic lethal interactions reveals an important role for the Rvs proteins in actin polarity, cell wall integrity and maintenance, and vesicle trafficking. Finally we show that, like the amphiphysins, the Rvs161p-167p heterodimer is able to bind to liposomes in vitro.

Analysis of Synthetic Lethal Interactions of RVS161 and RVS167

Our synthetic lethal analyses show that 1) *RVS161* and *RVS167* have synthetic lethal interactions with 49 of 49 of the same genes; 2) the Rvs proteins have roles in cell polarity, cell integrity, cell wall synthesis and maintenance, and vesicle trafficking; and 3) the Slt2–MAP kinase pathway is activated in *RVS* mutants, and the transcriptional induction of Slt2 targets is required in the absence of *RVS* genes. Our SGA analyses have significantly expanded the results of previous conventional genetic approaches and tests that have revealed 10 genetic interactions with *RVS167* mutants (Lila and Drubin, 1997; Singer-Krüger and Ferro-Novick, 1997; Breton and Aigle, 1998; Breton *et al.*, 2001; Germann *et al.*, 2005); *VPS21*, *SLT2*, and *SLA1* were identified by both methods. Several previously identified synthetic interactions with *rvs167Δ* were not uncovered in our screen, in part because some *rvs167Δ xxxΔ* mutants have only subtle growth defects, for example, *kre6Δ rvs167Δ* mutants that exhibit growth defects when grown at 37°C (Breton *et al.*, 2001). As well, some mutant strains, such as *sla2Δ*, *srv2Δ*, and *sac6Δ* single mutants are too slow growing to survive the SGA procedure (our unpublished data) and were therefore not present on the final selection plates. We have uncovered 46 new *RVS167*-requiring genetic backgrounds and have shown that all of these also require *RVS161*.

Cell Polarity

We identified genetic interactions between *RVS161* and *RVS167* and genes that encode proteins involved in the regulation of actin filaments (*CAP1*, *CAP2*, *SLA1*, and *MYO5*) as well as several proteins that have been implicated in the control of the actin assembly through regulation of the Arp2p/3p complex (*SLA1*, *END3*, *BBC1*, and *MYO5*; Goode and Rodal, 2001). Similarly, the defects in the actin cytoskeleton caused by the decreased yield of folded actin in mutants with defects in the prefoldin complex (*pac10*, *yke2*, *gim3*, *gim4*, and *gim5*) may be exacerbated by the loss of Rvs protein expression (Siegers *et al.*, 1999). These data are consistent with a general requirement for Rvs proteins when the actin cytoskeleton is compromised and suggest that loss of more than one function that contributes to actin organization causes inviability. Consistent with this idea, two of the *rvs167* double mutants tested (*rvs167Δ myo5Δ* and *rvs167Δ yke2Δ*) had a severely depolarized actin cytoskeleton compared with either single mutant (Figure 5A).

Cell Wall Synthesis and Regulation

Results from our SGA analyses show that *RVS161* and *RVS167* have synthetic lethal interactions with genes that encode machinery for cell wall construction, including 1) components of the Anp1p-Hoc1p-Mnn9p-Mnn10p-Mnn11p mannosyltransferase complex of the Golgi, encoded by *MNN2*, *MNN9*, *MNN10*, and *HOC1* (Jungmann and Munro, 1998) (*ANP1* had no synthetic interaction with *RVS161* or *RVS167*); 2) *CHS3*, *CHS5*, *CHS6 CHS7*, *SKT5*, and *BNI4*, which encode proteins involved in chitin synthase III activity (DeMarini *et al.*, 1997); and 3) *KRE1* and *KRE11*, which encode proteins involved in cell wall β-glucan assembly (Brown *et al.*, 1993). Subtle defects in cell wall morphology have previously been reported for *rvs161* and *rvs167* mutants (Breton *et al.*, 2001). Thus, *rvs* mutants likely have a compromised cell wall and are sensitive to additional perturbations in their cell wall caused by deletion of cell wall genes. Consistent with this, Calcofluor White staining revealed that several of the *rvs167Δ xxxΔ* mutants (*rvs167Δ myo5Δ*, *rvs167Δ sla1Δ*, *rvs167Δ vps21Δ*, and *rvs167Δ yke2Δ*) had more severe cell wall defects than either of the single mutants (Figure 5B).

The cell wall defects in *rvs167Δ* and *rvs161Δ* strains may be a consequence of defects in endocytosis. Actin patches, which are thought to be sites of endocytosis (Kaksonen *et al.*, 2003), localize near sites of secretion, suggesting that they have a role in compensatory endocytosis to recycle plasma membrane and proteins that have been delivered to the plasma membrane during secretion. In mutants with defects in endocytosis, cell wall synthesis may go unchecked and recycling of plasma membrane will not occur. Many mutants with defects in endocytosis (e.g., *sla2Δ* [Mulholland *et al.*, 1997], *sla1Δ* [Ayscough *et al.*, 1999], *pan1-4* and *end3Δ* [Tang *et al.*, 2000], and *myo3Δ myo5Δ* [Goodson *et al.*, 1996]) have defects in cell wall assembly, often having thickened cell walls.

A related category of genes that have synthetic genetic interactions with *RVS167* and *RVS161* contains genes encoding components of the Sin3p/Rpd3p histone deacetylase complex. *PHO23* (Loewith *et al.*, 2001, Gavin *et al.*, 2002), *SDS3* (Lechner *et al.*, 2000; Gavin *et al.*, 2002), *SAP30* (Zhang *et al.*, 1998; Gavin *et al.*, 2002), *DEP1* (Lamping *et al.*, 1994; Gavin *et al.*, 2002), and *RXT2* (Gavin *et al.*, 2002) encode proteins associated with the Sin3p-Rpd3p histone deacetylase complex (Kasten *et al.*, 1997). Mutants with defects in components of the Sin3p-Rpd3p histone deacetylase complex have been shown to have defective cell walls (Vannier *et al.*, 2001), presumably because of insufficient or inappropriate expression of cell wall genes.

Cell Integrity MAP Kinase Pathway

A role for the Rvs proteins in cell wall integrity is further supported by our identification of synthetic lethal interactions between *rvs* mutants and components of the Slt2p–MAP kinase pathway: *BCK1*, which encodes the MAPK kinase kinase (Lee *et al.*, 1993), and *SLT2*, which encodes the MAPK. Environmental stress or defects in the cell wall activate the Slt2p pathway leading to the activation of downstream transcription factors and the expression of cell wall genes, which may allow cells to compensate for the cell wall defects (Igual *et al.*, 1996; Madden *et al.*, 1997; Jung and Levin, 1999; Lagorce *et al.*, 2003). We found that deletion of *RVS161* or *RVS167* causes constitutive activation of the Slt2p MAP kinase and that the growth defects of an *slt2Δ rvs167Δ* mutant are partially suppressed by overexpression of *MNN9* or *CCW12*, two genes that may be transcriptionally induced

by the Slt2p pathway. Thus, *rvs161Δ* and *rvs167Δ* mutants require Slt2p activation and the transcriptional induction of at least one Slt2p target. These data also predict that Slt2p target genes may be synthetic lethal with *rvs* mutants; further analyses of the genes identified in the *RVS* synthetic lethal screens may allow identification of additional Slt2p transcriptional targets.

Activation of Slt2p results in activation of two transcription factors, Rlm1p (Jung and Levin, 1999) and the heterodimeric transcription factor SBF (composed of Swi4p and Swi6p), leading to expression of downstream targets, including cell wall biosynthesis genes (Madden *et al.*, 1997). We found that *RLM1* and *SWI6* are not synthetic lethal with *rvs167Δ* (our unpublished data), suggesting that *SWI4* is the only essential downstream effector of Slt2p in the response to cell wall damage in *rvs* mutants. These data are in agreement with a previous report that revealed a Swi6-independent role for Swi4p and Slt2p in the regulation of a subset of target genes (Baetz *et al.*, 2001). Thus, deletion of *RVS161* and *RVS167*, like other forms of cell wall perturbation, may lead to activation of the Slt2p pathway acting *via* Swi4p, in response to cell wall damage.

Vesicle Trafficking

Another category of genes showing synthetic interactions with *RVS* genes encodes proteins involved in vesicle trafficking. We have found that the *rvs* delete strains have synthetic growth defects in combination with two genes involved in vesicle trafficking from endoplasmic reticulum (ER) to Golgi, *sec22Δ* and *rud3Δ* (Newman and Ferro-Novick, 1990; Kim, 2003), suggesting that the Rvs proteins may be required for proper ER-to-Golgi trafficking. Additional evidence to support a role for Rvs167p in ER-to-Golgi vesicle trafficking comes from screens for proteins that interact with the SH3 domain of Rvs167p: the Rvs167p SH3 domain has been shown to bind to at least one protein involved in vesicle trafficking from ER to Golgi: Gyp5p (Bon *et al.*, 2000; Ho *et al.*, 2002; Chesneau *et al.*, 2004; Friesen *et al.*, 2005) as well as the Gyp5p-interacting protein Gyl1p (Chesneau *et al.*, 2004; Friesen *et al.*, 2005). In addition, the finding that *RVS167* and *RVS161* have synthetic genetic interactions with *VPS21*, a gene involved in the delivery of vacuolar and endocytosed proteins to the vacuole (Gerrard *et al.*, 2000; interaction with *RVS167* reported previously by Singer-Krüger and Ferro-Novick, 1997) suggests a role for Rvs161p-167p in multiple vesicle-mediated functions.

The classes of genes that have synthetic genetic interactions with *RVS161* and *RVS167*, genes required for cell wall synthesis and maintenance and vesicle trafficking, may be connected through a need for proper vesicle formation or fusion. The recent finding that the Rvs homologue amphiphysin binds to liposomes and promotes membrane curvature in vitro (Takei *et al.*, 1999; Razzaq *et al.*, 2001; Lee *et al.*, 2002; Peter *et al.*, 2004), and our observation that purified Rvs167p-161p binds to liposomes in vitro (Figure 8) is consistent with a role for Rvs161p-167p in vesicle biogenesis or fusion. It is not clear whether a role for the Rvs proteins in vesicle biology would be distinct from their role in the actin cytoskeleton and endocytosis.

Both Rvs161p and Rvs167p Have a Role in Mating

In mating assays with a subset of the genes identified in our mating screen, we found that, like *rvs161Δ*, *rvs167Δ* has synthetic mating defects in combination with deletion of *PRM9* and *FIG2*, but unlike *rvs161Δ*, it has no defects with *FUS1* and *AXL1*. Our results are in agreement with a previous report, which showed no defects in mating of an *rvs167Δ*

fus1Δ mutant (Brizzio *et al.*, 1998). However, we found a requirement for efficient mating for both *RVS161* and *RVS167* in certain genetic backgrounds, revealing a probable role for the Rvs161p-Rvs167p heterodimer during mating. Recently, others have also reported a defect in mating in *rvs167Δ* cells (Talarek *et al.*, 2004). Consistent with a role for Rvs167p in mating, we find that Rvs161p requires Rvs167p for stability during mating (Figure 3, A and B), and Rvs167p becomes hyperphosphorylated in cells exposed to pheromone (Figure 3B; Lee *et al.*, 1998; Friesen *et al.*, 2003). In addition, Rvs167p localizes to the shmoo tip and the cell fusion zone in mated cells and *rvs167Δ* mutants have delayed cell fusion in prezygotes (Balguerie *et al.*, 1999; Breton *et al.*, 2001).

Our data suggest that the Rvs proteins have two roles in mating. The first role is seen for Rvs161p, is essential in the absence of Fus1p, and is Rvs167p independent (Brizzio *et al.*, 1998; Figure 4A). The second role, for which we provide evidence in this manuscript, requires both Rvs161p and Rvs167p and is essential in the absence of Prm9p and Fig2p (Figure 4A). The defect in mating in the *rvs167Δ* strain was not suppressed by overexpressing *RVS161* (Figure 4C), consistent with a requirement for both Rvs proteins. This role in mating does not seem to be a role for endocytosis per se, because two other genes required for endocytosis, *SLA1* and *END3*, are not required for mating in the absence of Prm9p and Fig2p (Figure 4C).

A Role for the SH3 Domain of Rvs167p

Although the SH3 domain of Rvs167p is conserved among amphiphysins, its biological function in *S. cerevisiae* remains unknown. We found that a version of Rvs167p with a defective SH3 domain was able to complement almost all of our *rvs167Δ xxxΔ* mutants, indicating that the SH3 domain was not important for Rvs167p activity under standard growth conditions. In contrast, we found that the SH3 domain was important in the absence of *SLA1* and *END3* under certain unfavorable growth conditions (on YPD + 3% NaCl; Figure 7B) and that this requirement likely requires a functional interaction between the SH3 domain of Rvs167p and Abp1p. We found previously that the phosphorylation sites of Rvs167p are important for growth on salt-containing medium in the absence of *SLA1* or *END3* and that phosphorylation of Rvs167p affects binding to the SH3 domain of some ligands (Friesen *et al.*, 2003). These data are consistent with a model in which the interaction with Rvs167p and Abp1p (and the regulation of this interaction by phosphorylation) is essential in a *sla1Δ* or an *end3Δ* background. Sla1p and End3p form a complex with a third (essential) protein Pan1p (Tang *et al.*, 2000), which activates the Arp2p/3p complex to stimulate actin polymerization (Duncan *et al.*, 2001). Abp1p has also been found to stimulate the Arp2p/3p complex (Goode *et al.*, 2001), so one possibility is that in the absence of the Sla1p-End3p-Pan1p Arp2p/3p-activator complex, it becomes essential for Rvs167p to bind to the alternate Arp2p/3p activator Abp1p.

In summary, we have demonstrated that the yeast amphiphysin homologues Rvs161p and Rvs167p function together in vegetative cells and that the interaction with Rvs161p is the main interaction for Rvs167p. We have shown that Rvs167p plays a role in the mating response that is not a consequence of its stabilizing Rvs161p. We have presented evidence that the interaction between the proline-rich region of Abp1p and the SH3 domain of Rvs167p may be important for actin cytoskeleton organization. The results of our study identify an important link between Rvs161p and Rvs167p and the organization of the actin cytoskeleton,

cell wall, and vesicle trafficking during vegetative growth and reveal a requirement for the Rvs161p-167p heterodimer during the mating response. These finding implicate heterodimerization between other amphiphysins as an important interaction for the function of these molecules in various cellular processes.

ACKNOWLEDGMENTS

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