In Vivo Analysis of the Domains of Yeast Rvs167p Suggests Rvs167p Function Is Mediated Through Multiple Protein Interactions

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ABSTRACT

Morphological changes during cell division in the yeast *Saccharomyces cerevisiae* are controlled by cell-cycle regulators. The Pcl-Pho85p kinase complex has been implicated in the regulation of the actin cytoskeleton at least in part through Rvs167p. Rvs167p consists of three domains called BAR, GPA, and SH3. Using a two-hybrid assay, we demonstrated that each region of Rvs167p participates in protein-protein interactions: the BAR domain bound the BAR domain of another Rvs167p protein and that of Rvs161p, the GPA region bound Pcl2p, and the SH3 domain bound Abp1p. We identified Rvs167p as a Las17p/Bee1p-interacting protein in a two-hybrid screen and showed that Las17p/Bee1p bound the SH3 domain of Rvs167p. We tested the extent to which the Rvs167p protein domains rescued phenotypes associated with deletion of *RVS167*: salt sensitivity, random budding, and endocytosis and sporulation defects. The BAR domain was sufficient for full or partial rescue of all *rvs167* mutant phenotypes tested but not required for the sporulation defect for which the SH3 domain was also sufficient. Overexpression of Rvs167p inhibits cell growth. The BAR domain was essential for this inhibition and the SH3 domain had only a minor effect. Rvs167p may link the cell cycle regulator Pcl-Pho85p kinase and the actin cytoskeleton. We propose that Rvs167p is activated by phosphorylation in its GPA region by the Pcl-Pho85p kinase. Upon activation, Rvs167p enters a multiprotein complex, making critical contacts in its BAR domain and redundant or minor contacts with its SH3 domain.

THE commitment to enter a new cell division cycle occurs during the G_1 phase in most eukaryotic cells (Pardee 1989; Cross 1995). In the budding yeast *Saccharomyces cerevisiae*, the point of commitment to cellular division is termed Start and is analogous to the restriction point in mammalian cells (Planas-Silva and Weinberg 1997). Passage through Start leads to morphological changes in the yeast cell that allow polarized growth toward the budding daughter cell (Lew and Reed 1995). The actin cytoskeleton becomes polarized toward the new bud in late G_1 and is believed to direct growth and secretion toward the bud (Lew and Reed 1995).

Two cyclin-dependent kinase (CDK) complexes, Cln-Cdc28p and Pcl-Pho85p, have been implicated in signaling cell-cycle position to the actin cytoskeleton. Improper expression of Cln-Cdc28p complexes, the master cell-cycle activators in yeast, alters the timing of bud emergence, suggesting that Cln-Cdc28p complexes are involved in regulating this process (Lew *et al.* 1992; Lew and Reed 1993; Nasmyth 1993; Nigg 1995). The Pcl-Pho85p kinase has roles in both cellular metabolism and in cell-cycle regulation (reviewed in Andrews and Measday 1998). Pho85p is activated by association with 10 different Pho85p cyclins (Pcls) that are divided into two subfamilies: *PHO80* and *PCL1,2* (Measday *et al.* 1997). Members of the *PHO80* subfamily (*PHO80, PCL6, PCL7, PCL8,* and *PCL10*) are required to regulate phosphate and glycogen metabolism (Andrews and Measday 1998). Several observations suggest that the *PCL1,2* subfamily (*PCL1, PCL2, CLG1, PCL5,* and *PCL9*) regulates early cell-cycle events. First, the Pcl1,2-Pho85p kinase is essential for cell-cycle progression in the absence of Cln1p and Cln2p, suggesting a role for the Pcl1,2-Pho85p kinases at Start (Espinoza *et al.* 1994; Measday *et al.* 1994). Second, expression of three members of the PCL1,2 subfamily (*PCL1, PCL2, PCL9*) is cell-cycle regulated with peak transcript levels occurring in G_1 (Andrews and Measday 1998). Third, yeast strains lacking the entire *PCL1,2* subfamily display morphological defects that include elongated buds, delocalized actin patches, and abnormal (random) budding in diploids, implying that the *PCL1,2* subfamily may regulate actin polarization during the cell cycle (Measday *et al.* 1997; Lee *et al.* 1998; Tennyson *et al.* 1998).

Recent experiments suggest that Pcl1,2-Pho85p kinases contribute to actin regulation at least in part through the Rvs167 protein. *RVS167* and a similar gene, *RVS161*, were first identified in a screen for mutations that caused reduced viability upon starvation (Crouzet *et al.* 1991; Bauer *et al.* 1993). Deletion of either of

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these genes leads to phenotypes similar to those seen in actin mutants (Ayscough and Drubin 1996; Botstein *et al.* 1997): (1) loss of viability and unusual cell morphology in poor growth or salt-containing media (Crouzet *et al.* 1991; Bauer *et al.* 1993; Lee *et al.* 1998); (2) delocalized actin patches under suboptimal growth conditions (Bauer *et al.* 1993; Sivadon *et al.* 1995; Lee *et al.* 1998); (3) random budding in diploids (Bauer *et al.* 1993; Sivadon *et al.* 1995; Lee *et al.* 1998); (4) endocytic defects (Munn *et al.* 1995; Lee *et al.* 1998); and (5) sporulation defects for *rvs161* (Desfarges *et al.* 1993). Yeast deleted for *RVS161*, but not *RVS167*, also displays cell fusion defects (Dorer *et al.* 1997; Brizzio *et al.* 1998). Deletion of *PHO85* or several members of the *PCL1,2* subfamily shows similar defects to *rvs* and actin mutants, suggesting that Pcl-Pho85p, Rvs167p, and actin lie in the same pathway (Lee *et al.* 1998).

Biochemical evidence also supports the hypothesis that the Pcl-Pho85p kinase regulates Rvs167p. Rvs167p was identified as a Pcl-binding protein in two independent two-hybrid screens using Pcl2p and Pcl9p as bait (Lee *et al.* 1998). Pcl2p and Rvs167p also interact in an affinity chromatography assay *in vitro* and can be coimmunoprecipitated from yeast extracts (Lee *et al.* 1998). *In vitro*, Rvs167p is a substrate of the Pcl2-Pho85p kinase and, *in vivo*, phosphoforms of Rvs167p are reduced in *pho85* and multiple *pcl* mutants, suggesting that Rvs167p is an *in vivo* substrate of the Pcl-Pho85p kinase (Lee *et al.* 1998).

The Rvs proteins likely form part of a multiprotein complex that functions to regulate actin polarization. A mutant allele of *RVS161*, identified in a screen for endocytosis mutants, shows noncomplementation with an actin mutant strain, suggesting that Rvs161p and actin interact (Munn *et al.* 1995). In addition, *rvs161* and *rvs167* mutations are synthetically lethal with a set of actin mutations (Breton and Aigle 1998). Deletion of *RVS167* is also synthetically lethal with mutation of *MYO1*, and growth is severely affected in an *rvs167 myo2* strain (Breton and Aigle 1998). Rvs167p binds actin in the two-hybrid system (Amberg *et al.* 1995); however, the interaction of Rvs167p with actin may be indirect as the two do not appear to interact *in vitro* (Lila and Drubin 1997). *In vitro*, Rvs167p binds actin-binding protein 1 (Abp1p), and it is possible that Abp1p mediates the interaction between Rvs167p and actin (Lila and Drubin 1997). Genetic analysis also suggests that Rvs167p and Abp1p operate in the same pathway. Neither *RVS167* nor *ABP1* is essential, but both mutants display a similar profile of "synthetic-lethal" interactions with genes encoding other cytoskeletal components (*SRV2, SLA1, SLA2/END4, SAC6*; Lila and Drubin 1997). As well, deletion of *RVS167* reduces the poor cell growth and abnormal cell morphology of yeast cells that overexpress Abp1p (Lila and Drubin 1997). In addition to binding actin and Abp1p, Rvs167p interacts with Rvs161p and Sla2/End4p in the two-hybrid system (Navarro *et al.* 1997; Wesp *et al.* 1997). The interaction of Rvs167p and Rvs161p has also been shown by coprecipitation from yeast extracts (Navarro *et al.* 1997). Thus, it appears that Rvs167p is capable of forming several protein contacts and may participate in multiple protein complexes involved in regulating the actin cytoskeleton.

Two mammalian genes, Amphiphysin I and II, are similar to *RVS167* (Wigge and McMahon 1998). Both Amphiphysin I and II are involved in endocytosis of synaptic vesicles at nerve termini (Wigge and McMahon 1998). Amphiphysin I is required for cytoskeleton rearrangement during neurite outgrowth (Mundigl *et al.* 1998). Thus, Rvs167p and the Amphiphysin family have similar functions. Interestingly, Amphiphysin I is an autoantigen in Stiff-Man Syndrome associated with breast cancer, and Amphiphysin I levels are often elevated in breast cancer tissue (David *et al.* 1994; Floyd *et al.* 1998). A splice variant of Amphiphysin II, BinI, negatively regulates Myc and is lacking in several human tumors, suggesting it is a tumor suppressor (Sakamuro *et al.* 1996). Thus, members of the Amphiphysin/*RVS* family appear to be important for proper cell-cycle control.

The structural similarity between the Amphiphysins and the Rvs proteins is primarily in their N termini where they have a common domain, BAR (also known as Rvs and Domain A; Bauer *et al.* 1993; David *et al.* 1994; Sakamuro *et al.* 1996). Rvs161p consists only of the BAR domain. There are two predicted coiled-coil motifs within this domain (Navarro *et al.* 1997). At the C termini of Rvs167p and the Amphiphysins, there is an SH3 domain, a protein module well defined for binding proline-rich sequences (Pawson and Scott 1997). The Rvs167p SH3 domain shows a two-hybrid interaction with actin and binds Abp1p (Amberg *et al.* 1995; Lila and Drubin 1997). The middle portion of Rvs167p and the Amphiphysin proteins is not conserved. In Rvs167p, the central portion of the molecule consists of a GPA (glycine-, proline-, alanine-rich) region that is also found in the actin-binding proteins Abp1p and *Dictyostelium discoideum* myosin I (Bauer *et al.* 1993).

In this study, we analyzed the contribution of the BAR, GPA, and SH3 regions of Rvs167p to its function *in vivo*. First we used the two-hybrid system to map the regions of Rvs167p required for interactions with known partners. We also used a two-hybrid screen to identify Rvs167p as a Las17p/Bee1p binding partner. Second, we tested the ability of the domains of Rvs167p to rescue several loss-of-function phenotypes associated with *rvs167* mutants. Third, we tested the effect of overexpressing various regions of Rvs167p on cell viability. Finally, we suggest models for Rvs167p function based on these and previous results.

MATERIALS AND METHODS

Media and yeast strain manipulations: Yeast strains are listed in Table 1. Standard rich medium (YEP) containing 2% glu-

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
Y153	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3 ade2-101 gal4 gal80 URA3::GAL-lacZ LYS2::GAL-HIS3*	Durfee *et al.* (1993)
Y187	*MAT*α *trp1-901 leu2-3,112 ura3-52 his3 ade2-101 gal4 gal80 URA3::GAL-lacZ*	Harper *et al.* (1993)
BY261[a]	*MAT***a** *TRP1 GAL2 ura3-52 lys2-801*[a] *ade2-107*° *his3*Δ*200 leu2-*Δ*1*	Lee *et al.* (1998)
BY262[b]	*MAT***a**/α *TRP1 GAL2/gal2 ura3-52/ura3-52 lys2-801*[a]*/lys2-801*[a] *ade2-107*°*/ade2-107*° *his3*Δ*200/ his3*Δ*200 leu2-*Δ*1/leu2-*Δ*1*	Lee *et al.* (1998)
BY508	*MAT***a** *rvs167* Δ*TRP1*	Lee *et al.* (1998)
BY561	*MAT***a**/α *rvs167* Δ*TRP1/rvs167* Δ*TRP1 ADE2/ade2-107*°	Lee *et al.* (1998)

[a] This strain and all following haploid strains are isogenic.
[b] This strain and all following diploid strains are isogenic.

cose and supplemented minimal medium containing 2% glucose (SD) or 2% galactose (SG) were used (Kaiser *et al.* 1994). To make SD plus NaCl, NaCl was added to SD to a final concentration of 6% w/v. For the sporulation assay, yeast cells were initially grown in SD containing 10% glucose and then transferred to 1% potassium acetate supplemented with 0.25× amino acid mix minus methionine and histidine (Kaiser *et al.* 1994). Standard methods for yeast transformations were used (Guthrie and Fink 1991). For dot assays, cultures grown to midlog phase were serially diluted and spotted onto the appropriate plates.

Plasmids: Plasmids used in this study were generated by PCR or restriction digest from source vectors and subcloned into the vectors listed below. PCR products were sequenced to confirm their integrity. Details of plasmid construction are available upon request. The following constructs were made as Gal4 activation domain fusions using vector pACT (Durfee *et al.* 1993) or pACT II (Harper *et al.* 1993): (1) pAD-*RVS167* (pBA1117, full length); (2) pAD-P473L (pBA1217, full-length *RVS167* with a mutation in codon 473). This construct was created by *in vitro* overlap-extension PCR (Ling and Robinson 1997). The cytosine at the second position of codon 473 was substituted for thymidine, changing proline 473 to leucine. The primers used for mutagenesis were universal and reverse (from Stratagene, La Jolla, CA) and C2418T 5′ (GGTGTGT TTCTTGGGAACTACG) and C2418T 3′ (CGTAGTTCCCAA GAAACACACC). (Further details of mutagenesis are available upon request.) (3) pAD-BARGPA (pBA1116, *RVS167* codons 1–427 with a stop codon introduced at the end); (4) pAD-GPASH3 (pBA1218, *RVS167* codons 282–482); (5) pAD-BAR (pBA1119, *RVS167* codons 1–281 with a stop codon introduced at the end); and (6) pAD-SH3 (pBA1259, *RVS167* codons 423–482).

The following constructs were made as Gal4 DNA-binding domain fusions using the vector pAS1 (Durfee *et al.* 1993): (1) pAS1-*RVS167*ΔSH3 (pBA1045, *RVS167* codons 12–445); (2) pAS1-BAR (pBA1219, *RVS167* codons 1–281 with a stop codon introduced at the end); (3) pAS1-*RVS161* (pBA1121, full length); (4) pAS1-*PCL2* (pBA668; described in Measday *et al.* 1994); (5) pAS1-ABP1 PRO [pBA1122, *ABP1* codons 444–537; source of *ABP1* PRO is pGABP1-2 (Lila and Drubin 1997)]; and (6) pAS1-*LAS17*/*BEE1* (pBA1101, full length).

The following constructs were made to allow expression of *RVS167* derivatives (see AD fusions for description of constructs) under control of the *MET25* promoter in vector p413 *MET25* (ATCC 87318; Ronicke *et al.* 1997): (1) pMET-*RVS167* (pBA1174); (2) pMET-P473L (pBA1175); (3) pMET-BARGPA (pBA1176); (4) pMET-GPASH3 (pBA1177); (5) pMET-BAR (pBA1213); and (6) pMET-M-SH3 (pBA1215). The pMET-M-SH3 contains a single 5′ Myc epitope tag generated during PCR amplificaton of the SH3 domain using primer Myc 5′-CGACTAGTATGGAGCAAAAGCTCATTTCTGAAGAGGAC TTGAATTCAGATCTAGCCGCGGCCGTAGCG. The following constructs were made to allow expression of *RVS167* derivatives (see AD fusions for description of constructs) under control of the *GAL1* promoter in vector p426 *GAL1* (ATCC 87333; Ronicke *et al.* 1997): (1) pGAL-*RVS167* (pBA1178); (2) pGAL-P473L (pBA1179); and (3) pGAL-GPASH3 (pBA1183).

Two-hybrid assay: Yeast strain Y153 was transformed with plasmids containing the Gal4 DNA-binding domain (pAS1) fusions. Yeast strain Y187 was transformed with plasmids containing Gal4 activation domain (pAD) fusions. Y153 and Y187 containing these plasmids were then mated, and liquid β-galactosidase assays were performed on the resulting diploids as described (Andrews and Moore 1992). The Las17p/Bee1p two-hybrid screen was performed as described using yeast strain Y190 cotransformed with pAS1-*LAS17*/*BEE1* and a λACT yeast cDNA library (Durfee *et al.* 1993; Harper *et al.* 1993). A total of 150,000 independent transformants were selected on medium lacking Trp, Leu, and His but containing 25 mm aminotriazole. Plasmids from positive clones were isolated and sequenced.

Western blotting: To prepare extracts for Western blotting, yeast cells were grown to log phase under inducing conditions (SG minus uracil for GAL vectors and SD minus methionine and histidine for MET vectors). Preparation of cell extracts and Western blot analysis were performed as described (Lee *et al.* 1998).

Fluorescence microscopy: Lucifer yellow accumulation was performed as described except cells were grown in SD medium minus methionine and histidine and incubated with lucifer yellow for 1.5 hr (Lee *et al.* 1998). Calcofluor staining of bud scars was performed as described except the cells were grown in SD medium minus methionine and histidine and examined at a magnification of ×1000 (Tennyson *et al.* 1998).

Sporulation: Cells were grown to midlog phase in supplemented medium (SD) plus 10% glucose minus methionine and histidine. Cells were washed once with distilled water and resuspended in sporulation medium. The cells were shaken at 30° for 4 days. The percentage of cells with two or four spores was determined by examining the cells at ×1000 magnification using a charge-coupled device camera mounted on a Leica DM-LB microscope.

RESULTS

Interaction of Rvs167p with protein targets in the two-hybrid assay: Evidence to date suggests that Rvs167p interacts with several proteins and may be part of a large protein complex that regulates actin (Amberg *et al.* 1995; Lila and Drubin 1997; Navarro *et al.* 1997; Wesp *et al.* 1997; Lee *et al.* 1998). As described earlier, Rvs167p

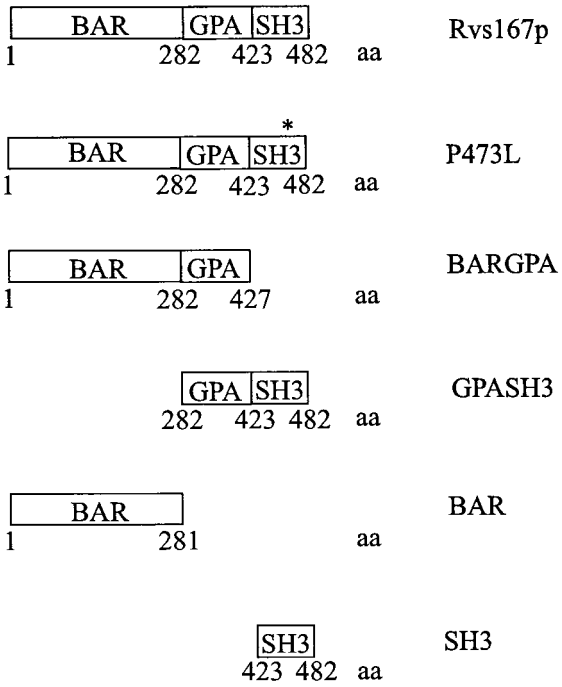

Figure 1.—Schematic diagram of the Rvs167p constructs. At the bottom of each construct is the amino acid position that defines the boundaries between Rvs167p domains. The asterisk on P473L indicates the position of the amino acid substitution of proline 473 to leucine. Although the GPASH3 DNA construct begins at codon 282, the protein is predicted to start at methionine 286 in the *MET25* and *GAL1* vectors. aa, amino acid.

can be divided into three regions: the N-terminal BAR domain, the GPA-rich region, and the C-terminal SH3 domain (Figure 1). We sought to identify binding partners for individual regions of Rvs167p and to correlate the binding partners with the function of that region as determined by phenotypic complementation. To this end, we have used various constructs of Rvs167p in the two-hybrid assay to test the binding of Rvs167p to putative protein targets.

We have previously demonstrated that Rvs167p binds to Pcl2p, a cyclin component of the Pho85p kinase (Measday *et al.* 1994; Lee *et al.* 1998). To better define the interaction between Rvs167p and Pcl2p, we performed liquid β-galactosidase assays on transformants expressing Pcl2p fused to the Gal4 DNA-binding (DB) domain (pAS1-Pcl2p) and various constructs expressing fusions of Rvs167p to the Gal4 activation domain (pAD fusions, Table 2). Pcl2p interacted with full-length Rvs167p as well as the BARGPA and GPASH3 constructs. Pcl2p did not interact with either the BAR or SH3 domains of Rvs167p alone (Tables 2 and 4). We were unable to detect the AD-GPA fusion or the GPA region alone in Western blotting of yeast extracts from transformants expressing the GPA region from various promoters (data not shown). Therefore, we were unable to test whether the GPA region of Rvs167p was sufficient

TABLE 2

Interaction of Pcl2p with Rvs167p in the two-hybrid system

Plasmid	pAS1 Miller units[a]	pAS1-*PCL2* Miller units	Fold increase in activation
pAD	0.07 ± 0.03	0.47 ± 0.07	6.7[b]
pAD-*RVS167*	0.20 ± 0.16	74.5 ± 5.40	372
pAD-BARGPA	0.04 ± 0.03	28.0 ± 5.61	700
pAD-GPASH3	0.06 ± 0.05	3.39 ± 0.09	56.5
pAD-BAR	0.14 ± 0.02	0.64 ± 0.10	4.6

[a] β-Galactosidase activity—see materials and methods and Andrews and Moore (1992); average of three independent cultures.

[b] Fold increase is calculated by dividing the value of the PAS1-*PCL2* pAD-fusion interaction by the pAS1 pAD-infusion interaction value.

for interaction with Pcl2p. Because Pcl2p binds the BAR-GPA and the GPASH3 constructs, but not the BAR or SH3 domains individually, our data suggest that the GPA region of Rvs167p contains a binding site for Pcl2p.

Yeast deleted for either *RVS167* or *RVS161* display similar phenotypes, and Rvs167p and Rvs161p interact in a two-hybrid assay (Bauer *et al.* 1993; Navarro *et al.* 1997). We used our Rvs167p domain fusions to assay Rvs167p-Rvs161p interactions in the two-hybrid system. As well, we tested for Rvs167p-Rvs167p interactions using a Gal4 DB-Rvs167 fusion protein (pAS1-Rvs167ΔSH3) that lacks the SH3 domain of Rvs167p. We were unable to test full-length Rvs167p because it self-activated when fused to the Gal4 DNA-binding domain. In this assay, both Rvs161p and Rvs167ΔSH3 interacted with full-length Rvs167p, the BARGPA region, and the BAR domain by itself (Table 3). A smaller construct expressing a Gal4 DB-BAR domain fusion of Rvs167p also bound full-length Rvs167p, the BARGPA region, and the BAR domain (Table 3). Therefore, it appears that Rvs167p is capable of forming hetero- or homodimers through its BAR domain. As noted above, other two-hybrid results have shown Rvs161p-Rvs167p heterodimers but failed to show evidence of Rvs167p homodimers (Navarro *et al.* 1997).

The SH3 domain of Rvs167p binds directly to a proline-rich region of the actin-binding protein Abp1p in a far-Western blot assay (Lila and Drubin 1997). We used the two-hybrid assay to test the interaction of the SH3 domain of Rvs167p with the proline-rich region of Abp1p *in vivo* (pAS1-Abp1 PRO, Table 4). Abp1 PRO interacted with full-length Rvs167p, the GPASH3 region, and the SH3 domain alone, confirming the *in vitro* data (Lila and Drubin 1997). We also constructed a point mutation, P473L, within the SH3 domain of Rvs167p that is predicted to inactivate the SH3 domain (Clark *et al.* 1992; Musacchio *et al.* 1992; Rozakis-Adcock *et al.* 1993). Abp1 PRO did not interact with the Rvs167 P473L mutant, indicating that this mutant

TABLE 3

Interaction of Rvs167p and Rvs161p with Rvs167p in the two-hybrid system

Plasmid	pAS1 Miller units[a]	pAS1-*RVS161* Miller units	pAS1-*RVS161* Fold increase	pAS1-*RVS167*ΔSH3 Miller units	pAS1-*RVS167*ΔSH3 Fold increase	pAS1-BAR Miller units	pAS1-BAR Fold increase
pAD	0.05 ± 0.02	0	0[b]	0.09 ± 0.04	1.8	0.11 ± 0.04	2.2
pAD-*RVS167*	0	26.0 ± 10.5	521	12.3 ± 4.79[c]	247	2.40 ± 0.59	48.0
pAD-BARGPA	0	5.43 ± 0.69[d]	109	5.08 ± 0.42	102	2.35 ± 0.81	47.0
pAD-GPASH3	0.03 ± 0.04	0	0	0.08 ± 0.11[c]	1.6	0.09 ± 0.03	1.8
pAD-BAR	0.10 ± 0.06	8.57 ± 3.61	171	3.36 ± 0.96	67.2	1.31 ± 0.12	26.2
pAD-*PCL2*	0	0	0	ND[e]		ND	

[a] β-Galactosidase activity—see materials and methods and Andrews and Moore (1992); average of three independent cultures.
[b] Fold increase is calculated by dividing the Miller units of the pAS1-fusion pAD-fusion interaction value by the interaction value of pAS1-pAD (0.05).
[c] Average of best three out of five values.
[d] Average of best three out of six values.
[e] Not done.

disrupts the function of the SH3 domain (Table 4). The P473L mutant remained competent to bind proteins that interact with Rvs167p outside the SH3 domain (see Pcl2p, Table 4).

Las17p/Bee1p, a proline-rich protein involved in actin regulation and endocytosis (Li 1997; Naqvi *et al.* 1998), may interact with SH3 domain-containing proteins involved in actin regulation. We performed a two-hybrid screen with full-length Las17p/Bee1p and identified Rvs167p as a Las17p/Bee1p-interacting protein (data not shown). Seventeen clones encoding Rvs167p were isolated ranging from full length to one lacking the first 212 amino acids. We tested the Gal4 DB-Las17p/Bee1p against our AD-Rvs167p fusions and found that the Las17p/Bee1p-Rvs167p interaction was dependent on the SH3 domain of Rvs167p (Table 4). We also used the two-hybrid assay to demonstrate that the binding site for Rvs167p on Las17p/Bee1p is within the proline-rich region between amino acids 294 and 534 (data not shown). Thus, we propose that the SH3 domain of Rvs167p binds the proline-rich region of Las17p/Bee1p *in vivo*.

Expression analysis of Rvs167p constructs *in vivo*: Our two-hybrid results suggest that Rvs167p can form several protein complexes and that the individual regions of Rvs167p are all likely to contribute to its function. Yeast deleted for *RVS167* displays several phenotypes including reduced viability upon starvation, salt sensitivity, random budding in diploids, and endocytic defects (Bauer *et al.* 1993; Munn *et al.* 1995; Sivadon *et al.* 1995; Lee *et al.* 1998). To test the functional importance of the domains of Rvs167p, we expressed various *RVS167* constructs (Figure 1) *in vivo* under control of either the *MET25* or *GAL1* promoter. We anticipated that both the length of the Rvs167p constructs and their expression levels would affect the ability of the various

TABLE 4

Interaction of Abp1 PRO and Las17p with Rvs167p in the two-hybrid system

Plasmid	pAS1 Miller units[a]	pAS1-*ABP1* Pro Miller units	pAS1-*ABP1* Pro Fold increase	pAS1-*LAS17/BEE1* Miller units	pAS1-*LAS17/BEE1* Fold increase	pAS1-*PCL2* Miller units	pAS1-*PCL2* Fold increase
pAD	0.05 ± 0.02	0.01 ± 0.02	0.2[b]	0	0	0.06 ± 0.09	1.2
pAD-*RVS167*	0.10 ± 0.11	365 ± 240[c]	7306	346 ± 159[c]	6915	ND[d]	
pAD-P473L	0.10 ± 0.07	0	0	0.32 ± 0.49	6.4	91.9 ± 17.0	1838
pAD-BARGPA	0.02 ± 0.03	0	0	0	0	ND	
pAD-GPASH3	0.07 ± 0.13	3.39 ± 0.49	67.8	270 ± 118	5406	ND	
pAD-BAR	0	0.01 ± 0.02	0.2	0	0	ND	
pAD-SH3	0.30 ± 0.07	3.43 ± 1.43	68.6	179 ± 163	3584	0.23 ± 0.09	4.6

[a] β-Galactosidase activity—see materials and methods and Andrews and Moore (1992); average of three independent cultures.
[b] Fold increase is calculated by dividing the Miller units of the pAS1-fusion pAD-fusion interaction value by the interaction value of pAS1 pAD interaction (0.05).
[c] Average of best three out of four values.
[d] Not done.

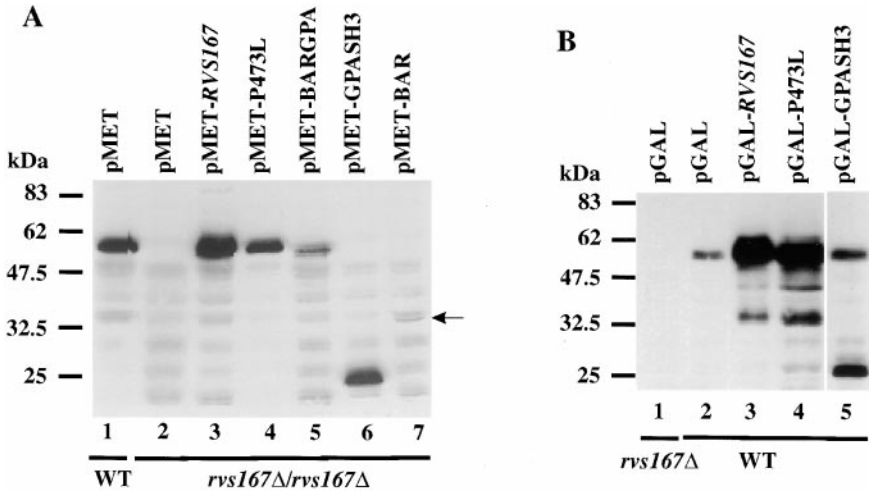

Figure 2.—Expression of *RVS167* constructs *in vivo*. (A) Western blot analysis, using Rvs167p antiserum, of yeast extracts from transformants expressing various *RVS167* constructs under control of the *MET25* inducible promoter. Lane 1, wild-type diploid (BY262) transformed with vector (pMET); lane 2, *rvs167*Δ diploid (BY561) transformed with vector (pMET). Lanes 3–7 show the Rvs167p in extracts from an *rvs167*Δ diploid (BY561) transformed with the *RVS167* constructs indicated at the top of the figure. The arrow denotes the position of migration of the BAR protein derivative in lane 7. Equal amounts of total protein (30 μg) were loaded per lane. The position of migration of molecular weight markers is shown on the left. (B) Western blot analysis, using Rvs167p antiserum, of yeast extracts from transformants expressing various *RVS167* constructs from the *GAL1* promoter. Lane 1, *rvs167*Δ haploid (BY508) transformed with vector (pGAL); lane 2, wild-type haploid (BY261) transformed with vector (pGAL). Lanes 3–5 show the Rvs167p in extracts from a wild-type strain (BY261) transformed with the *RVS167* constructs as noted at the top of the figure. The wild-type strain expresses endogenous Rvs167p so the signal for GAL-Rvs167p and GAL-P473L also contains endogenous Rvs167p. Because GAL-GPASH3 is smaller, the signals for endogenous Rvs167p and the GPASH3 region are separated. Equal amounts of total protein (20 μg) were loaded per lane. The position of migration of molecular weight markers is indicated on the left. WT, wild type.

domains to rescue *rvs167* loss-of-function phenotypes. Because Rvs167p is not defined structurally, we used other information to choose our domain boundaries. Rvs161p contains only a BAR domain and is functional *in vivo* (Crouzet *et al.* 1991). Our BAR domain construct of Rvs167p expresses a BAR region the same size as Rvs161p and may define a functional domain *in vivo*. We aligned the C terminus of Rvs167p with structurally solved SH3 domains to choose the length of our SH3 domain construct (A. Davidson, personal communication). A recombinant version of this Rvs167-SH3 domain folds *in vitro* and displays thermodynamic stability expected for an SH3 domain (A. Rath, K. Colwill, B. Andrews and A. Davidson, unpublished results). Finally, we defined the GPA region as the entire sequence between the BAR and SH3 domains of Rvs167p.

Overexpression of Rvs167p in yeast inhibits growth at both 30° and 37°, indicating that the proper expression levels of Rvs167p are important for normal cellular growth (Lee *et al.* 1998). Given the importance of Rvs167p expression levels for our analysis, we expressed the *RVS167* constructs from the *MET25* promoter, which allowed protein levels comparable to endogenous Rvs167p as demonstrated by Western blot analysis [compare lane 1 (endogenous Rvs167p) to lanes 3–7 (plasmid-derived Rvs167p) in Figure 2A]. Both the BARGPA region and the BAR domain were expressed at lower levels than endogenous Rvs167p (lanes 5 and 7, Figure 2A). The GPA region and a myc-tagged SH3 domain were not detected by Western blot analysis when expressed individually (data not shown). Because the small size of the SH3 domain made detection difficult, we still tested the myc-tagged SH3 domain in all assays described, but we only discuss results that showed rescue by the SH3 domain.

The same domains of Rvs167p were expressed from the *GAL1* promoter in the wild-type strain BY261 (Figure 2B). Both full-length Rvs167p and full-length Rvs167p with proline 473 mutated to leucine (P473L; lanes 3 and 4) are overexpressed compared to endogenous levels of Rvs167p (lane 2). The GPASH3 construct is overexpressed to some extent (compare the lower 25-kD GPASH3 band to the endogenous 57-kD band in lane 5). We have included the GPASH3 construct in our overexpression assays as it represents the C terminus of the protein, whereas the P473L with its nonfunctional SH3 domain (see above) represents the N terminus of the protein. The other constructs were not expressed to sufficient levels to be used in overexpression assays (data not shown).

From the Western analysis, we concluded that constructs under control of the *MET25* promoter were suitable candidates for testing rescue of *rvs167* loss-of-function phenotypes, and a subset of these constructs under control of the *GAL1* promoter were useful for assaying overexpression effects of Rvs167p.

Rescue of salt sensitivity: The first phenotype we tested for rescue by our *RVS167* constructs was the salt sensitivity of *rvs167* mutants. Yeast deleted for *rvs167* is unable to grow on 6% NaCl (Bauer *et al.* 1993; Lee *et al.* 1998). Log phase cultures of wild-type cells transformed with the MET vector alone or an *rvs167* mutant transformed with the MET vector or the MET-*RVS167* constructs were serially diluted and spotted onto SD agar media that contained no NaCl or 6% NaCl (Figure 3). All transformants were able to grow when NaCl was not

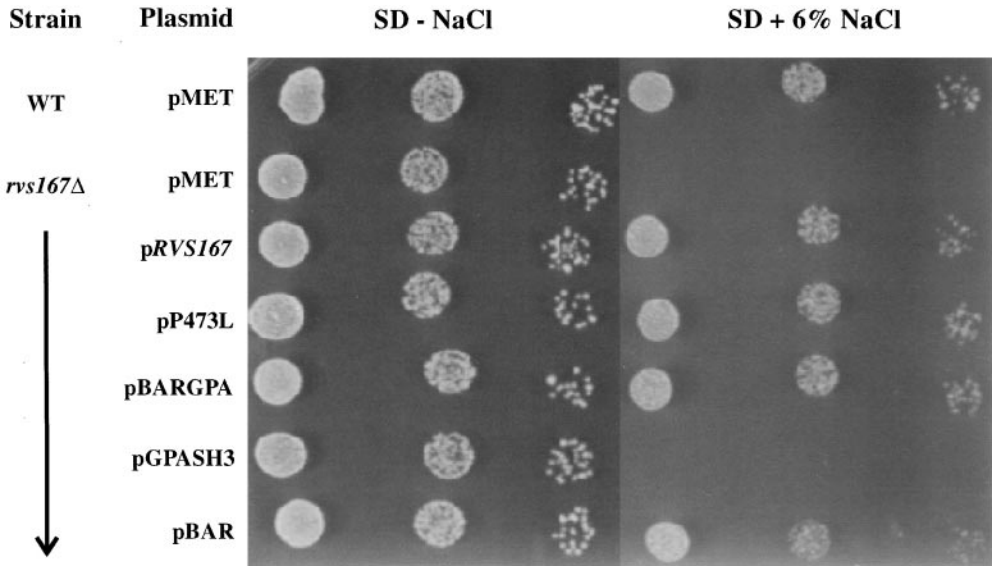

Figure 3.—Effect of *RVS167* constructs on the salt sensitivity of an *rvs167* mutant (BY561, *rvs167*Δ diploid). The viability of yeast transformed with various constructs of *RVS167* under control of the *MET25* promoter in medium containing sodium chloride was tested. Serial dilutions of log phase cultures were spotted on an SD plate lacking NaCl (SD − NaCl), or on an SD plate containing NaCl (SD + 6% NaCl). The strain transformed by the various plasmids is indicated on the far left, followed by the plasmid transformed. WT, wild-type diploid BY262.

included in the medium. When 6% NaCl was added to the medium, the wild-type strain transformed with the MET vector alone was able to grow but the *rvs167* mutant transformed with vector alone did not form colonies (Figure 3). The inability of the *rvs167*Δ yeast strain to grow on 6% NaCl was rescued by transformation with plasmids encoding full-length Rvs167p, the mutant P473L, the BARGPA region, or the BAR domain. The GPASH3 region was unable to rescue the salt sensitivity. The *rvs167* mutant transformed with the BAR domain grew slightly slower than wild type, which may reflect the lower expression levels of the BAR domain (Figure 2A). We conclude that the BAR domain and not the GPASH3 region is required for growth in 6% NaCl. Our results are consistent with a previous report (Sivadon *et al.* 1997).

Random budding assay: Diploid yeast cells bud in a bipolar fashion with new buds forming at either the distal or proximal poles of the yeast cell (Chant and Pringle 1996). In contrast, diploids deleted for *rvs167* tend to bud in a random fashion with the site of new bud selection not confined to the poles (Bauer *et al.* 1993; Lee *et al.* 1998). We tested the ability of the MET-*RVS167* constructs to rescue the random budding phenotype of an *rvs167*/*rvs167* diploid strain (Figure 4). In our strain background, 95% of wild-type cells (BY262 transformed with pMET) showed a bipolar budding pattern. In contrast, only 38% of the *rvs167* mutant cells (BY561 transformed with pMET) budded in a bipolar pattern. The budding defect of the *rvs167* mutant was rescued by transformation with plasmids encoding either full-length Rvs167p or the P473L mutant. The BARGPA region and the BAR domain also rescued the phenotype but not to the same extent. The incomplete rescue by the BARGPA region of the random budding phenotype has been described before and was interpreted as a requirement for the SH3 domain for full rescue

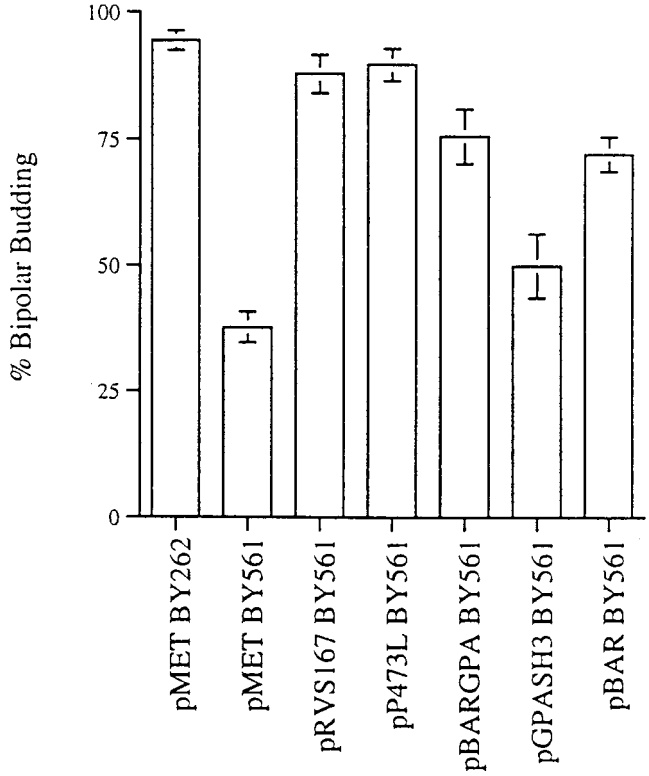

Figure 4.—Effect of *RVS167* constructs on the random budding pattern seen in *rvs167* mutant diploids (BY561, *rvs167*Δ diploid). The percentage of cells showing a bipolar budding pattern is graphed. Bud scars were visualized using Calcofluor, and 150 yeast cells with three or more visible bud scars were counted for each sample. The standard deviations between three independent cultures assayed in this experiment are illustrated by the error bars. The percentage bipolar budding is plotted on the *y*-axis and the strains transformed with various plasmids are indicated on the *x*-axis. BY262, wild-type diploid; BY561, *rvs167*Δ diploid.

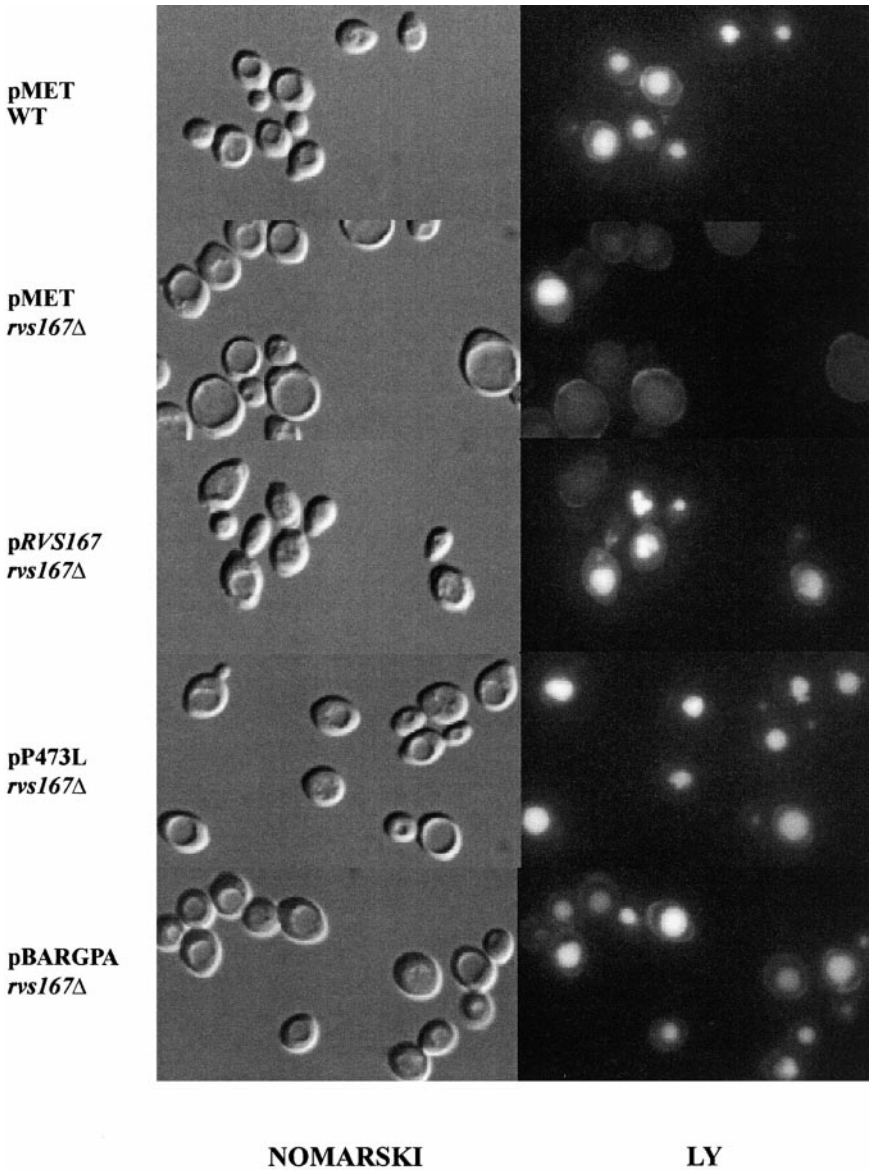

Figure 5.—Effect of *RVS167* constructs on fluid-phase endocytotic uptake of lucifer yellow. Yeast strains (Wild-type diploid, BY262; *rvs167*Δ diploid, BY561) transformed with various constructs of *RVS167* (indicated on the far left) under control of the *MET25* promoter were tested for lucifer yellow uptake. Lucifer yellow accumulation was visualized with fluorescein isothiocyanate (FITC) fluorescence optics and photographed at a magnification of ×630 using an imaging system. Nomarski images are shown on the left and FITC images (LY) of the same field of cells are shown on the right.

(Sivadon *et al.* 1997). In our two-hybrid assay, neither the BARGPA construct nor the P473L mutant bound SH3 targets, suggesting that the two are equivalent in lacking SH3 function (Table 4). Therefore, the lower percentage of rescue by BARGPA as compared to the P473L mutant may be due to inadequate protein expression (Figure 2, lane 7) rather than a requirement for the SH3 domain. However, the GPASH3 region allowed a slight rescue of the random budding phenotype (50 ± 6 for the GPASH3 region *vs.* 38 ± 3 for vector alone), suggesting that the SH3 domain may have a small role in bud placement. We conclude that the BAR domain is required for budding and the SH3 domain may play a minor role.

Endocytosis assay: *rvs167* mutants are defective in both receptor-mediated and fluid-phase endocytosis (Munn *et al.* 1995). We tested the ability of the *rvs167* mutant transformed with the various *RVS167* constructs to take up the fluorescent dye lucifer yellow by fluid-phase endocytosis. The endocytosis defect of the *rvs167* mutant was rescued by expression of full-length *RVS167* or the P473L mutant from the *MET25* promoter (Figure 5). The GPASH3 domain did not rescue the endocytic defect, indicating that the BAR domain is required for rescue (data not shown). Expression of the BARGPA construct also rescued the endocytosis defect but not to the same extent as wild-type Rvs167p or the P473L mutant (Figure 5). Similar to the budding assay, we propose that the lower level of rescue is due to the underexpression of the BARGPA construct.

Sporulation assay: Diploid yeast cells deleted for *RVS161* are sporulation defective (Desfarges *et al.* 1993). Given the similarity in phenotypes between *rvs167* and *rvs161* mutants, we tested *rvs167* mutants for sporulation defects and for rescue by various constructs of *RVS167*. Exponential cultures were transferred to

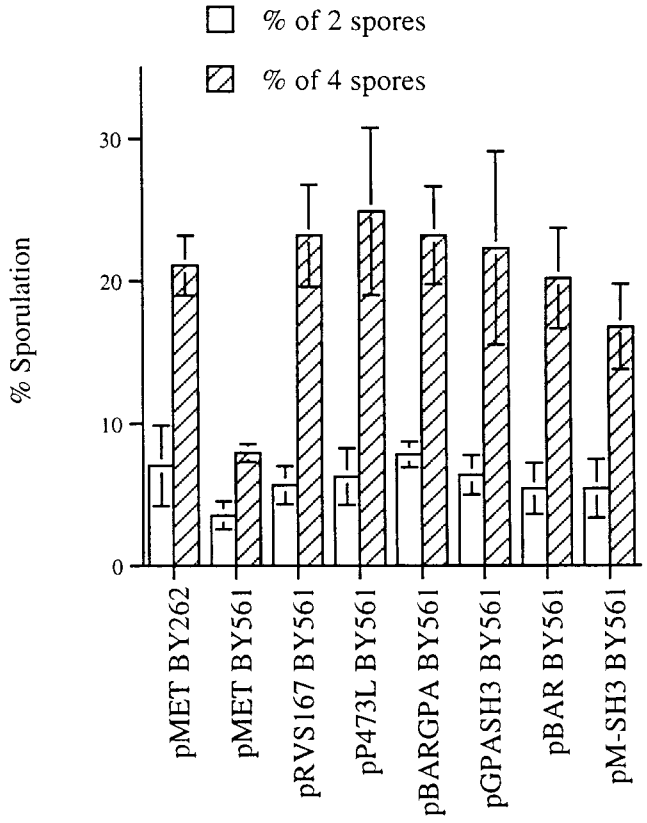

Figure 6.—Effect of various *RVS167* constructs on the sporulation defect of yeast strain BY561 (*rvs167*Δ diploid). The percentage of cells forming spores is graphed. A total of 250 cells from each culture were scored for the percentage of zero, two, or four spores formed. The percentage of two- or four-spore formation is graphed on the *y*-axis, and the strains transformed with various plasmids are displayed on the *x*-axis. The standard deviations between the three independent cultures assayed in this experiment are represented by error bars. BY262, wild-type diploid; BY561, *rvs167*Δ diploid.

sporulation media and incubated for 4 days to allow spore formation. After 4 days, the percentage of cells that were able to form two or four spores was calculated. In this assay, the wild-type strain (BY262 transformed with the *MET25* vector) formed two spores 7% of the time and four spores 21% of the time (Figure 6). The *rvs167* mutant strain (BY561 transformed with the *MET25* vector) formed two spores 4% of the time and four spores 8% of the time, indicating that the strain can sporulate, albeit at a lower efficiency than wild type. All expression constructs of *RVS167* were able to rescue the sporulation defect of the *rvs167* mutant (Figure 6). The lowest efficiency of rescue was conferred by expression of the SH3 domain. This result may be due to low expression levels of the SH3 domain. Therefore, in contrast to the previous assays, either the BAR domain or the SH3 domain was sufficient to rescue the sporulation defect of the *rvs167* mutant.

Overexpression of Rvs167p *in vivo*: Overexpression of Rvs167p *in vivo* leads to growth inhibition and altered cell morphology (Lee *et al.* 1998). We used *RVS167* constructs under control of the *GAL1* promoter to test the effect of overexpressing regions of Rvs167p. As mentioned above, we analyzed only those constructs that allowed significant overexpression of the Rvs167p derivatives compared to endogenous Rvs167p: full-length Rvs167p, the mutant P473L, and the GPASH3 region. We tested the effect of expressing these constructs in a wild-type haploid strain (BY261). Log phase cultures of the various transformants were serially diluted and plated onto SD (noninducing) or SG (inducing) media. The wild-type strain transformed with vector alone was able to grow under all conditions tested (Figure 7). The same strain transformed with any of the three *RVS167* constructs was able to grow on SD agar media at either 30° or 37°. Overexpressing the full-length *RVS167* construct inhibited growth of wild-type cells at 30° or 37°. Previously, we reported that overexpression of Rvs167p inhibited growth at 37° but not at 30° (Lee *et al.* 1998). In this assay, we used a different vector and the expression levels may be high enough that we saw a growth effect at a lower temperature. Overexpression of the GPASH3 construct did not inhibit growth at either temperature, demonstrating that the BAR domain is required for the overexpression phenotype. It is possible, however, that higher expression levels of the GPASH3 region could have affected growth. Expression of the P473L mutant caused poor growth at 30° and prevented colony formation at 37°. Therefore, both the BAR and the SH3 domains are required for the full effect of the Rvs167p overexpression phenotype.

Our rescue and overexpression analyses have demonstrated a function for both termini of Rvs167p. The BAR domain appears essential for all Rvs167p functions except sporulation, whereas the SH3 domain has a more subtle role as assessed in simple complementation assays. Combined with the two-hybrid results, our study suggests that each domain of Rvs167p is involved in protein-protein interactions that are necessary for full Rvs167p function.

DISCUSSION

Rvs167p, a proposed regulator of the actin cytoskeleton, is a modular protein composed of three distinct regions. In this article, we analyzed these regions using two-hybrid assays and phenotypic analyses. Our two-hybrid results indicate that each region of Rvs167p is capable of protein-protein interactions. The BAR domain binds Rvs167p and Rvs161p; the GPA region binds Pcl2p; and the SH3 domain binds Abp1p and Las17p/Bee1p. From our genetic studies, we conclude that the BAR domain is absolutely required for all Rvs167p functions tested except for sporulation. The SH3 domain, on the other hand, can rescue the sporulation defect of *rvs167* mutants but not the other defects associated with *rvs167* loss of function, such as salt sensitivity and

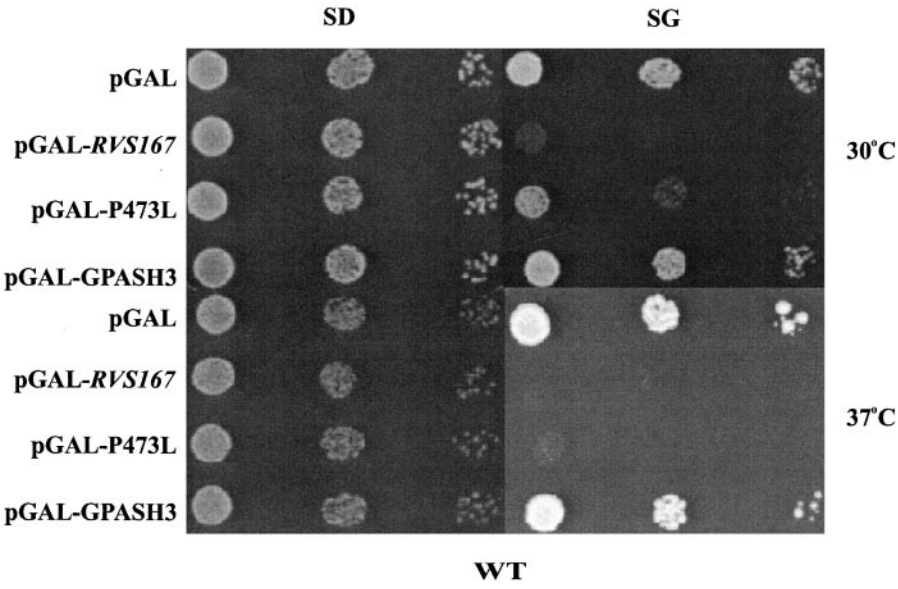

Figure 7.—Effect of overexpression of various *RVS167* constructs on cell growth. Wild-type yeast cells (strain BY261) transformed with various constructs of *RVS167* (shown to the left) under control of the *GAL1* promoter were grown to midlog phase, and serial dilutions were spotted onto SD or SG plates. The ability of these yeast cells to form colonies was tested at both 30 and 37°. The type of medium is indicated at the top and the incubation temperature is shown at the right.

random budding in diploids. We propose that the modular nature of Rvs167p allows it to interact with components of the actin cytoskeleton to signal structural change.

The BAR domain of Rvs167p: The BAR domain at the N terminus of Rvs167p is conserved between Rvs167p, Rvs161p, and the mammalian Amphiphysin proteins (Bauer *et al.* 1993; David *et al.* 1994; Sakamuro *et al.* 1996). The region is not defined structurally although it is predicted to contain two coiled-coil motifs (Navarro *et al.* 1997). BAR domains are not interchangeable; in rescue assays, the BAR domain of Rvs161p and the BAR domain of Rvs167p could not substitute for each other (Sivadon *et al.* 1997). Evidence suggests that the BAR domain may contain more than one protein-protein interaction site. Rvs161p contains only the BAR domain, but the endocytic and cell fusion functions of Rvs161p map to distinct regions of the protein (Brizzio *et al.* 1998). Correlating with these two distinct functions of the BAR domain, only constructs of Rvs161p functional for cell fusion bind Fus2p, a known cell fusion protein (Trueheart *et al.* 1987; Elion *et al.* 1995; Brizzio *et al.* 1998).

In this article, we showed that the BAR domain of Rvs167p binds Rvs161p in the two-hybrid system. Although we have not defined this interaction in detail, it is possible that the BAR domains dimerize through the coiled-coil motifs. A heterodimer between Rvs167p and Rvs161p has already been proposed based on binding assays and the similar phenotypes of yeast deleted for *RVS167* or *RVS161* (Crouzet *et al.* 1991; Bauer *et al.* 1993; Navarro *et al.* 1997). However, yeast deleted for *RVS161* but not *RVS167* displays cell fusion defects, suggesting that the two proteins can perform separate functions for which heterodimerization is not required (Brizzio *et al.* 1998). The mammalian homologues of the Rvs proteins, Amphiphysin I and Amphiphysin II, form heterodimers *in vivo*, and heterodimerization may stimulate the function of Amphiphysin (Wigge *et al.* 1997). *In vitro* assays demonstrate that an Amphiphysin heterodimer stimulates the GTPase activity of Dynamin to a greater extent than either monomeric protein by itself (Wigge *et al.* 1997). We found that the BAR domains of two Rvs167p molecules interact in the two-hybrid system, suggesting that Rvs167p can form homodimers. Our result contrasts with a previous report that showed no interaction between two Rvs167 proteins in the two-hybrid system (Navarro *et al.* 1997). A homodimer of Rvs167p would be more similar to an Amphiphysin heterodimer as both dimer pairs would have SH3 domains. More biochemical studies are required to determine the *in vivo* relevance of homo- or heterodimers of Rvs167p.

The BAR domain is sufficient to rescue all *rvs167* phenotypes tested, suggesting that the BAR domain makes key nonredundant contacts that can compensate for lack of an SH3 domain. To rescue these phenotypes, the BAR domain is likely to be an effector domain as well as a potential dimerization motif. Several experiments suggest that the End4p/Sla2p protein may interact with Rvs167p *in vivo*: End4p/Sla2p is involved in endocytosis and actin regulation (Holtzman *et al.* 1993; Raths *et al.* 1993), *END4/SLA2* deletion is synthetically lethal with *rvs167* (Lila and Drubin 1997), and End4p/Sla2p binds Rvs167p in the two-hybrid system through its coiled-coil domain (Wesp *et al.* 1997). Perhaps End4p/Sla2p binds the coiled-coil motifs within the BAR domain of Rvs167p.

The GPASH3 region of Rvs167: The GPA region of Rvs167p is poorly defined and not conserved among members of the Rvs/Amphiphysin family. A similar region enriched in glycine, proline, and alanine is found in Abp1p and Myosin I upstream of their SH3 domains, suggesting that a GPA-rich region is important for SH3

domain function (Bauer *et al.* 1993). SH3 domains are common protein-protein interaction motifs that bind proline-rich ligands (Pawson and Schlessinger 1993). Although rich in proline, the GPA region in Rvs167p does not contain any PXXP sequences that are targets for SH3 domains (Pawson and Schlessinger 1993). Thus, it is unlikely to bind either to its own SH3 domain or to the SH3 domain of another protein.

From our two-hybrid experiments, we propose that the GPA region is the site for Pcl2p binding. In this case, a probable function of Pcl2p is to deliver Rvs167p to Pho85p for phosphorylation. Substrate targeting by Pcl cyclins has already been demonstrated by the Pho80p and Pcl10p cyclins that target Pho85p to Pho4p and Gsy2p, respectively (Hirst *et al.* 1994; Huang *et al.* 1998). Pho85p does not interact detectably with Rvs167p even though the GPA region contains seven SP/TP sites that are potential Pcl–Pho85p phosphorylation sites and the SH3 domain contains one such site (data not shown; Lee *et al.* 1998). Threonine 454 is the putative Pho85p phosphorylation site within the SH3 domain (Lee *et al.* 1998). Threonine 454 aligns with the n-src loop in other SH3 domains and likely contacts the SH3 ligand (Lee *et al.* 1998). Thus, regulation of the SH3 domain by Pcl-Pho85p may be mediated internally as well as by modification of the upstream GPA region.

The GPASH3 region was able to rescue only the sporulation defect in the *rvs167* mutant. To promote sporulation, Rvs167p may be part of a distinct protein complex in which the BAR or SH3 domain of Rvs167p is sufficient for function. In this model, both the BAR and SH3 domains are redundant with other complex components in the sporulation pathway. We propose that the GPA region is a key linker between the BAR and SH3 domains that is required to position one or both ends of Rvs167p for subsequent target binding. Thus, access of Rvs167p to downstream targets may be controlled by the GPA linker, which in turn is regulated by the Pcl-Pho85p kinase (Lee *et al.* 1998). To test this model, we need to assay Rvs167p mutant derivatives in strains that require both the BAR and SH3 domains for Rvs167p function. Rvs167p is required for viability in strains carrying certain actin mutations or strains deleted for *MYO1*, *SLA1*, *END4/SLA2*, or *SRV2* (Lila and Drubin 1997; Breton and Aigle 1998). In all of these mutant strains, actin is directly or indirectly compromised (Botstein *et al.* 1997). Analysis of the function of the GPA and SH3 domains of Rvs167p may require assessment of *RVS167* mutants in strain backgrounds lacking other actin regulatory proteins such as *SLA2/END4* or *SRV2*.

Potential complexes of Rvs167: We propose that the proper stoichiometry of Rvs167p is critical to cellular growth during stress conditions due to the participation of Rvs167p in protein complexes that become essential during stress. Lack of Rvs167p may cripple the complex,

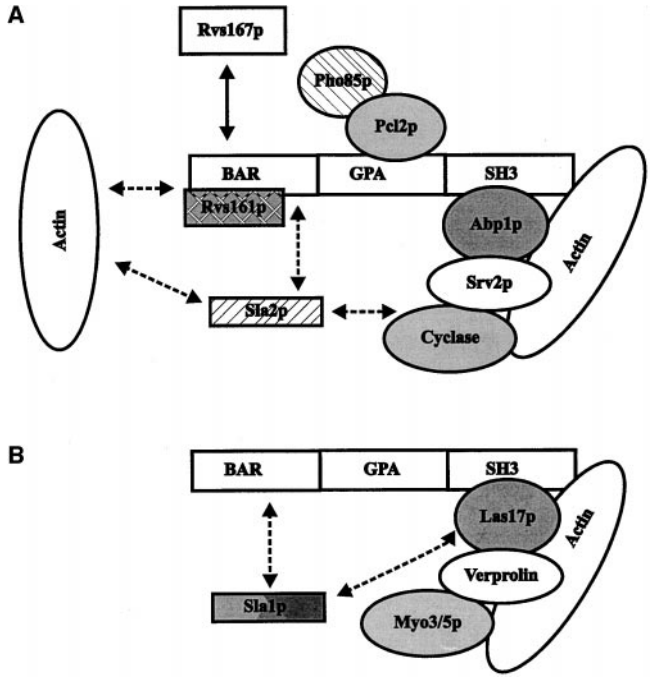

Figure 8.—Potential protein complexes involving Rvs167p. (A) Model of a complex involving Rvs167p based on known binding partners. The solid arrow indicates the potential for an Rvs167p homodimer. The dotted arrows indicate putative binding partners suggested in this article as a means to link the BAR domain to the actin cytoskeleton. (B) An alternate model for a complex involving the SH3 domain of Rvs167p based on the two-hybrid results reported here of an Rvs167p-Las17p/Bee1p interaction. We suggest a link between the BAR domain of Rvs167p and Sla1p that can compensate for the absence of the SH3 domain of Rvs167p.

leading to growth arrest, while excess Rvs167p may alter the timing of complex formation or may titrate out key factors required in the complex. Evidence to date suggests that Rvs167p functions as part of a multiprotein complex (Lila and Drubin 1997; Navarro *et al.* 1997; Wesp *et al.* 1997). A potential complex containing Rvs167p, Abp1p, Srv2p, and cyclase has already been suggested from genetic and biochemical studies (Lila and Drubin 1997; Wendland *et al.* 1998; see Figure 8A). In this complex, the SH3 domain of Rvs167p contacts the proline-rich region of Abp1p. Our results suggest that the BAR domain must make the key nonredundant contacts within this protein complex. The BAR domain interacts with Rvs161p, and it may be through Rvs161p that contacts with actin are made. Alternately, End4p/Sla2p may bind the BAR domain of Rvs167p (see above). In our model, the interaction of the BAR domain with End4p/Sla2p is either sufficient to relay Rvs167p function or the BAR-Sla2p complex can compensate for lack of the Rvs167 SH3 domain by interacting with proteins that the SH3 domain normally contacts (Figure 8A).

We showed that the SH3 domain of Rvs167p binds the yeast protein Las17p/Bee1p in a two-hybrid assay.

LAS17/*BEE1* is the yeast homologue of the mammalian Wiscott-Aldrich Syndrome gene (Li 1997). Several observations suggest a role for Las17p/Bee1p in actin regulation. First, a *las17*/*bee1* mutant displays cytoskeletal defects and Las17p/Bee1p interacts with the actin regulatory protein Sla1p (Li 1997). Second, overexpression of Las17p/Bee1p can suppress the endocytic defects in strains lacking another actin-regulatory protein, End5p/verprolin (Naqvi *et al.* 1998). Third, *las17*/*bee1* mutants, like *end5/verprolin* mutants, are defective in endocytosis and Las17p/Bee1p interacts with End5p/verprolin in a two-hybrid assay (Naqvi *et al.* 1998). End5p/verprolin also interacts with the type I myosin, Myo5p, *in vivo* (Anderson *et al.* 1998). Together, these results suggest another protein complex involving Rvs167p, Las17p/Bee1p, End5p/verprolin, Myo5p, and actin (Figure 8B). As noted above, Las17p/Bee1p interacts with the SH3 domain-containing protein Sla1p (Li 1997). Sla1p may be genetically redundant with Rvs167p in a Rvs167p-Las17p/Bee1p-End5p/verprolin-type complex because both Sla1p and Rvs167p are predicted to bind the proline-rich region of Las17p/Bee1p, and Sla1p is required for viability in an *rvs167* mutant strain.

Another candidate suggested to bind the SH3 domain of Rvs167p is Pan1p (Wendland and Emr 1998). Pan1p is the yeast homologue of eps15 that is involved in endocytosis in mammalian cells (Tang and Cai 1996; Tang *et al.* 1997). Pan1p has been shown to bind yAP180p (homologue of mammalian AP180) and Sjl1p (homologue of mammalian synaptojanin; Wendland and Emr 1998). In mammalian cells, Amphiphysin I has been found in a complex with eps15, AP180, and synaptojanin (Slepnev *et al.* 1998). Thus, an interaction of Rvs167p with Pan1p would demonstrate conservation of binding partners, as well as function, through evolution. Interestingly, Pho85p is most similar to mammalian Cdk5 that has been proposed to regulate cytoskeletal dynamics and neuronal differentiation (Lee *et al.* 1997). Similar to Amphiphysin, Cdk5 co-localizes with actin in developing neurons and is involved in neural outgrowth (Nikolic *et al.* 1996). Thus, following the Pho85-Rvs167p paradigm, Amphiphysins are putative targets for Cdk5 in neuronal cells.

In summary, we propose that Rvs167p is part of a multivalent complex that is regulated by the Pcl-Pho85p kinase in response to environmental stress. Phosphorylation by the Pcl-Pho85p kinase changes the conformation of Rvs167p to allow access of the BAR and/or SH3 domains to their downstream targets.

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