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Rvs167p, the budding yeast homolog of amphiphysin, colocalizes with actin patches

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SUMMARY

In this report, we have shown that the yeast amphiphysin-like protein Rvs167p was localized mainly in small cortical patches throughout the cell in unbudding cells. During budding, the patches were polarized at bud emergence site. During mating, Rvs167p was concentrated at the tip of the shmoo. Rvs167p colocalized with actin patches during yeast vegetative growth and mating. Complete disruption of the actin cytoskeleton using Latrunculin-A did not affect Rvs167p localization in patches throughout the cell. In *rvs167* mutant cells, actin patches are mislocalized and in *rvs161* or *abp1* mutant cells, Rvs167p localization is not affected. These observations suggest that Rvs167p may localize the actin cortical complex properly. Finally, the amphiphysin-conserved N-terminal domain of Rvs167p, called the BAR domain, was required but not sufficient for the correct localization of the protein.

Key words: Actin cytoskeleton, Amphiphysin, GFP, *RVS167*, *Saccharomyces cerevisiae*

INTRODUCTION

Mammalian amphiphysin I and amphiphysin II, yeast Rvs161p and Rvs167p, fission yeast and nematode isoforms, constitute a family of conserved proteins (David et al., 1994; Sakamuro et al., 1996; Wigge and McMahon, 1998). Indeed, except for Rvs161p, all these proteins share sequence and structural similarities consisting of three domains: (1) an N-terminal part containing putative coiled-coil structures able to mediate protein-protein interactions (Navarro et al., 1997; Sivadon et al., 1997) and called the BAR domain (BIN/Amphiphysin/RVS domain; Sakamuro et al., 1996), (2) an Src Homology 3 (SH3) domain at their C-terminal part and (3) an intermediate domain, which varies among members of protein family. The Rvs161p is restricted in the BAR domain.

Amphiphysin I was first identified as a brain protein associated with synaptic vesicles (Lichte et al., 1992), and has since been implicated in the autoimmune Stiff-Man syndrome disorder associated with breast cancer (Folli et al., 1993; David et al., 1994). Recently, amphiphysin I has been shown to play a more general role in non-neuronal endocytosis (Volchuk et al., 1998; Owen et al., 1998). Amphiphysin II is found, like amphiphysin I, to participate in clathrin-mediated endocytosis at the plasma membrane in the brain, despite having a different tissue distribution (Sakamuro et al., 1996; Sparks et al., 1996; Butler et al., 1997; Leprince et al., 1997; Tsutsui et al., 1997; Ramjaun et al., 1997; Wigge et al., 1997). Indeed, several amphiphysin II variants have been found expressed from the same amphiphysin II gene through a tissue-specific alternative splicing. Recently, an alternatively spliced variant of neuronal amphiphysin I has been described outside the brain (Floyd et al., 1998). The largest isoforms of both amphiphysins are brain-specific and form a stable heterodimer (Wigge et al., 1997). However, surprisingly, the two proteins were found to localize differently in brain (Wigge and McMahon, 1998). More precisely, amphiphysin II is localized to areas of actin fibres in axon initial segments and nodes of Ranvier and amphiphysin I is concentrated in nerve terminals (Butler et al., 1998).

In budding yeast only two genes, *RVS161* and *RVS167*, share homology with the mammalian amphiphysins. These two genes were first identified in a screen for mutations that caused reduced viability and abnormal morphology upon nutrient starvation (Crouzet et al., 1991; Bauer et al., 1993). The two *rvs* mutants exhibit the same phenotypes except for a defect in mating only found in the *rvs161* mutant (Brizzio et al., 1998). *rvs* mutant cells are affected in endocytosis, cell polarization and budding pattern with a random budding site selection in diploid cells (Bauer et al., 1993; Munn et al., 1995; Durrens et al., 1995). Consistent with this, Bauer et al. (1993) and Sivadon et al. (1995) showed that the actin cytoskeleton is affected in these *rvs* mutant cells, with depolarization of the actin network and actin patches. This relationship between the organization of the actin cytoskeleton and the function of the *RVS167* gene was further confirmed by genetic studies that showed synthetic lethalities between *rvs167* and a subset of actin *act1* alleles

(Breton and Aigle, 1998), and by two-hybrid experiments that revealed an interaction between Rvs167p and actin (Amberg et al., 1995) and between Rvs167p through its GPA/SH3 domains and the actin binding protein Abp1p (Lila and Drubin, 1997). Two-hybrid studies have shown that Rvs161p and Rvs167p form a heterodimer in vivo through the BAR domain (Navarro et al., 1997).

Comparison of phenotypes, as well as specific genetic relationships associated with *rvs161*, *rvs167* and *abp1* mutations, strongly suggest that the functions of these proteins are closely related (Bauer et al., 1993; Breton and Aigle, 1998; Crouzet et al., 1991; Durrens et al., 1995; Lila and Drubin, 1997; Sivadon et al., 1997) and are likely to participate in multiprotein complexes involving actin.

All these relationships suggest that Rvs167p may play a role together with actin, Rvs161p and Abp1p in cytoskeleton-associated mechanisms. Rvs161p has already been detected mainly in the cytoplasm, with some patches distributed at the neck of the small bud (Brizzio et al., 1998). As vertebrate amphiphysin I and amphiphysin II localize differently in brain, we decided to observe the cellular localization of Rvs167p. We constructed a reporter protein by inserting a synthetic jellyfish *Aequorea victoria* Green Fluorescent Protein (GFP), optimized for expression in yeast cells (Cormack et al., 1997), into the GPA domain of the Rvs167 protein.

In this report we describe the cellular localization of the Rvs167 protein in cortical patches that colocalize with actin cortical patches. Observation of the cellular localization of Rvs167p in cells with a Latrunculin-A (LAT-A)-disrupted actin network allowed us to specify the relationships existing between Rvs167p and the cortical protein complex. In addition, we have analyzed the cellular localization of Rvs167p in *rvs161* or *abp1* mutant backgrounds. Finally, we have assessed the role of the different Rvs167p domains in its cellular localization.

MATERIALS AND METHODS

Strains, media and growth conditions

All the *Saccharomyces cerevisiae* strains used in this study are derivatives of X2180 wild-type strain and are described in Table 1. Yeast cells were grown at 30°C according to the methods described by Sherman et al. (1991).

DNA manipulations

Restriction enzymes and other DNA modification enzymes were purchased from Promega and used according to the specifications of the manufacturer. Amplifications of DNA fragments were done by PCR on a DNA Thermal Cycler (Perkin Elmer Cetus). Custom oligonucleotides were provided by Genset. Transformation of *E. coli* was carried out by the $CaCl_2$ method (Okayama and Berg, 1982). Yeast cells were transformed either by the LiCl method (Ito et al., 1983) or by the One-Step method (Chen et al., 1992).

Plasmid construction

To construct the *RVS167-YGFP* (Yeast-enhanced Green Fluorescent Protein) fusion gene, a 736 bp PCR product containing the YGFP open reading frame from the pYGFP3 plasmid (a gift from B. Cormack) was obtained using the following primers: N-Bgl, 5′-GA-AGATCTTG**ATG**TCTAAAGGTGAAGAAT-3′; C-Sac, 5′-CCGG-GAGCTCAATTTGTACAATTCATCCATACC-3′. N-Bgl introduced a *Bgl*II site (underlined) before the start codon (bold). C-Sac introduced a *Sac*I site in place of the stop codon of *YGFP*. The pMA167-YGFP plasmid carrying the *RVS167* gene fused to the *YGFP* was obtained by replacing the two Z domains of protein A from *Staphylococcus aureus* (Moks et al., 1987) in the *RVS167-ZZ* fusion gene carried by the pMA3A-167ZZ plasmid (Navarro et al., 1997). This plasmid was digested by *Bgl*II and *Sac*I and then ligated with the 736-bp PCR fragment. The different proteins are represented schematically in Fig. 1A. The pMA3A167-YGFP plasmid carries the *RVS167-YGFP* gene in a 3.3 kb *Eco*RI-*Sph*I fragment. A multicopy plasmid and an integrative plasmid containing the fusion gene *RVS167-YGFP* were obtained by insertion of the 3.3 kb *Eco*RI-*Sph*I fragment of the pMA167-YGFP plasmid into the *Eco*RI-*Sph*I-digested multicopy pFL45 vector or into the *Eco*RI-*Sph*I-digested integrative pFL34 vector (Bonneaud et al., 1991), and were named respectively pFL45-167 and pFL34-167.

The resultant Rvs167p-GFP fusion protein was able to complement a null mutation of *RVS167* gene (data not shown). In particular, this fusion protein restored the *rvs167* mutant phenotypes described by Bauer et al. (1993). We also analyzed the effect of the *RVS167-GFP* gene copy number on the cellular localization of Rvs167p-GFP using the two plasmids pFL45-167 and pFL34-167. The protein was localized in patches when expressed from either the multicopy plasmid or from an integrated gene. However, the intensity of fluorescence was much higher with the multicopy vector than with the integrated gene. Indeed, to obtain the same fluorescent signal, the exposure time had to be 40 times longer with the integrated gene (20 seconds) than with the multicopy vector (0.5 seconds).

The pFL45-ΔBAR plasmid (carrying the *RVS167-YGFP* fusion gene deleted for the BAR domain) was constructed by inserting a 529 pb PCR fragment containing the *RVS167* promoter into the *Eco*RI-*Bam*HI-digested pFL45 plasmid. This 529 bp PCR fragment was obtained by PCR using the following primers: p167E, 5′-GGAATTCCGAATAAAGCCCACCAAT-3′; p167B, 5′-CG-GGATCCATTGGCTCCTTGATTCTTG-3′. p167E introduced an *Eco*RI site (underlined) at the 5′ end of the promoter and p167B introduced a *Bam*HI site (underlined) at the 3′ end of the promoter. The 1.4 kb *Bgl*II-*Sph*I fragment of the pMA167-YGFP plasmid corresponding to the YGFP-GPA-SH3 domains of the Rvs167p-YGFP fusion protein was then inserted into the pFL45 plasmid containing the *RVS167* promoter. To construct the pFL45-ΔSH3 plasmid (carrying the *RVS167-YGFP* fusion gene deleted for the GPA/SH3 domains), the pFL45-167 plasmid was digested by *Sac*I and *Sac*II, and the 3′ protruding ends were degraded with the Klenow fragment of the DNA polymerase I. The ligation of these blunt-ended extremities creates an in-frame stop codon 8 amino acids downstream from the ligation site. The different proteins are represented schematically in Fig. 1B.

Visualization of polymerized actin

Rhodamine-phalloidin staining of actin was carried out using a modification of the method of Kaiser et al. (1994). Cells (4×10^7) grown in liquid culture according to the described conditions, were fixed by adding formaldehyde to a final concentration of 3.7%. After 30 minutes, cells were collected by centrifugation, washed twice in PBS (8 g/l NaCl, 0.2 g/l KCl, 1.14 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, adjusted to pH 7.3) and resuspended in 50 μl of PBS containing 3.3 μM TRITC-conjugated phalloidin (Sigma). Cells were washed immediately three times with PBS and resuspended in 20 μl of a mounting medium containing 1 mg/ml p-phenylenediamine (Sigma). The organization of the actin cytoskeleton was then observed by fluorescence microscopy.

Latrunculin-A treatment of cells

To analyse Rvs167p localization in cells with a disrupted actin cytoskeleton, cells expressing the fusion protein Rvs167p-GFP were treated with latrunculin-A (LAT-A). Haploid cells (4×10^7) were treated for 15 minutes with either 200 μM LAT-A (Molecular Probes

Inc., Eugene, OR) or with DMSO alone as control. Cells were then fixed and prepared for rhodamine-phalloidin staining as described above.

Microscopic imaging and analysis

GFP was visualized either in living cells or in cells that had been fixed with formaldehyde as described above. Epifluorescence microscopy was carried out with a Leica DMRXA microscope fitted with a 100× immersion and standard FITC or TRITC filter sets.

Mating was performed by mixing about 10^7 cells of each mating type for 4 hours on YPD at 30°C. The mating mixture was then treated for phalloidin staining as described above.

RESULTS

Rvs167p-GFP localizes in cortical patches during vegetative growth and mating

The intracellular localization of Rvs167p was studied using the Rvs167p-GFP fusion protein (see Materials and methods). As this fusion protein was able to rescue the *RVS167* disruption (data not shown), we assumed that Rvs167p-GFP localized like wild-type protein. Moreover, we found that the quantity of fusion protein (i.e. expression with an integrative or a multicopy vectors) had no effect on the cellular localization of Rvs167p-GFP (data not shown). As shown in Fig. 2, Rvs167p was localized mainly in a set of small cortical patches throughout the cell during the vegetative growth. Cells in G_1 phase (i.e. unbudded cells) had patches distributed throughout the cell, and were unpolarized (Fig. 2A). In cells where no bud could be detected but in which the emergence of a bud was impending, the patches were concentrated at one pole (i.e. at the future bud emergence site) (Fig. 2B). In cells with small buds, patches were concentrated in the developing bud (Fig. 2C). Towards the end of budding, when bud expansion had essentially ceased, patches were uniformly distributed throughout the mother and the bud (Fig. 2D), or patches could be seen essentially at the neck between the mother and the bud (Fig. 2E). Moreover, during mating, the Rvs167p patches were concentrated at the tip of the shmoo (Fig. 2F).

These cellular localizations were unaffected by the presence or absence of the wild-type protein Rvs167p (data not shown). As the *rvs167* mutant strain exhibits particular budding phenotypes according to the cell type (Durrens et al., 1995), we specified the localization of Rvs167p in haploid and diploid cells and observed no difference (data not shown).

Table 1. Yeast strains used in this study

Strain	Genotype
X2180-1A	*MAT***a***
X2180-1B	*MAT*α*
X2180-2n	*MAT***a**/*MAT*α*
LG100-1B	*MAT*α, trp1*‡
LG100	*MAT***a**/*MAT*α, trp1/trp1*‡
LG67-2C	*MAT***a**, rvs167-1, ura3, trp1*‡
LG67	*MAT***a**/*MAT*α,rvs167-1/rvs167-1, ura3/ura3, trp1/trp1*‡
LG502-1C	*MAT*α, rvs161Δ, trp1*‡
LG502	*MAT***a**/*MAT*α, rvs161Δ /rvs161Δ, trp1/trp1*‡
LG776-4D	*MAT***a**, abp1::LEU2¶, leu2,3-112, trp1-1*‡
LG806	*MAT***a**/*MAT*α, abp1::LEU2¶/abp1::LEU2¶,leu2/leu2,trp1/trp1*‡
LG846-2D	*MAT*α, act1-129::HIS3¶, his3Δ200, trp1Δ63*‡

*Obtained from the Yeast Genetics Stock Center
‡Obtained from our laboratory.
¶Actin and Abp1 alleles originate from D. Botstein's laboratory.

Rvs167p patches colocalize with actin patches

Numerous evidence, including *rvs167* mutant phenotype, synthetic lethalities between *rvs167* and a subset of actin *act1* alleles, interaction between Rvs167p and actin by two-hybrid experiments, indicated a close relationship between the actin cytoskeleton and Rvs167p. The cellular localization of Rvs167p suggests that it colocalizes with actin patches. Therefore we examined whether Rvs167p would colocalize with some actin structures. We stained F-actin with TRITC-phalloidin in cells expressing the *RVS167-GFP* fusion gene. This actin staining was carried out during vegetative growth and during mating. During vegetative growth, Rvs167p patches colocalized strictly with actin patches (Fig. 3A). This colocalization was also observed in cells during mating (Fig. 3B). As expected, considering the motility of actin patches, time-lapse analyses stated that Rvs167p patches moved (data not shown). These movements were similar to those of actin

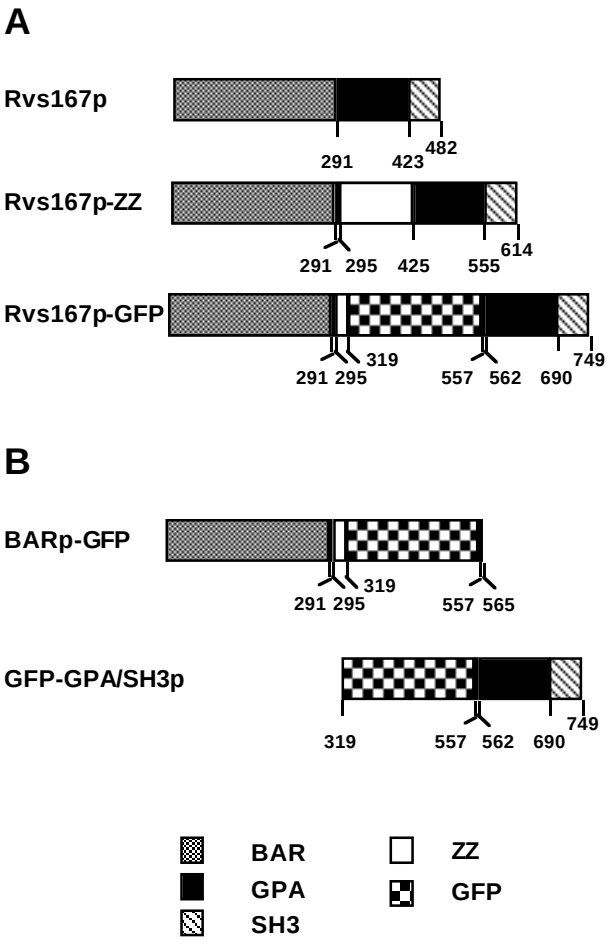

Fig. 1. (A) Schematic structures of Rvs167p, Rvs167p-ZZ and Rvs167p-GFP proteins. (B) Schematic structures of the BARp-GFP and GFP-GPA/SH3p fusion proteins deleted for the GPA/SH3 domains or the BAR domain, respectively. In each case, the N terminus is at the left. Numbers indicate amino acid positions. BAR, Bin/Amphiphysin/RVS domain; GPA, glycine-proline-alanine rich region; SH3, Src Homology 3 domain; ZZ, two Z domains of protein A from *Staphylococcus aureus*; GFP, Green Fluorescent Protein.

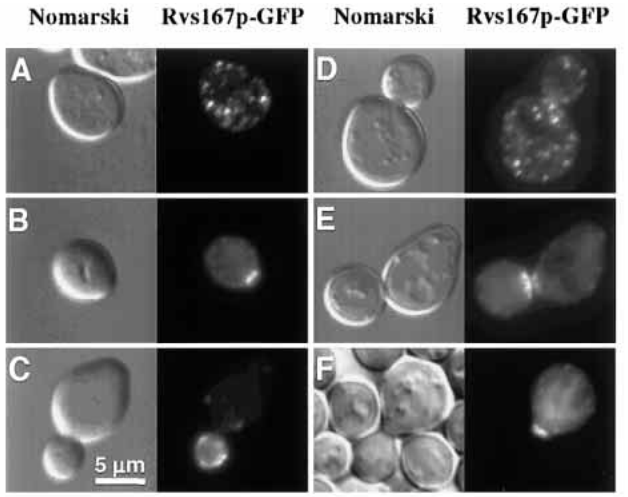

Fig. 2. Cellular localization of Rvs167p-GFP fusion protein. Representative wild-type vegetative cells expressing the *RVS167-GFP* fusion gene were visualized by Normarski optics or with an FITC filter set. Acquisition time with FITC filter set was 0.5 seconds. (A) Unbudding cell with patches distributed throughout the cell; (B) polarization of Rvs167p-GFP patches at the bud emergence site; (C) polarization of Rvs167p patches in the small bud; (D) patches distributed uniformly in the mother and the bud cells; (E) patches concentrated at the neck between the mother and the bud cells; (F) the polarization of Rvs167p at the tip of the shmoo (only one sexually compatible cell type containing *RVS167-GFP* fusion gene was fluorescent).

patches previously described by Waddle et al. (1996) and Doyle and Botstein (1996).

Surprisingly, when we analyzed an overlay in detail (Fig. 3C), we noticed that the relative intensities of GFP and TRITC-phalloidin were not constant. As indicated in the overlay presented in Fig. 3C, the GFP/TRITC fluorescence ratios varied from 0.87 to 5.31. Because the quantum yield of the camera used in this study is different under green or red light, a GFP/TRITC fluorescence ratio of 1 does not mean that the similar quantities of GFP and TRITC-phalloidin colocalize. In any case, this GFP/phalloidin ratio variation indicates that the relative quantities of Rvs167p-GFP and phalloidin are variable. One possible explanation for this result is that Rvs167p binds actin near or at the phalloidin-binding site, leading to exclusion of phalloidin near Rvs167p already positioned on the actin patches. The phalloidin-binding site has been previously identified by Drubin et al. (1993). Notably, two actin residues (R177 and D179) are involved in the binding of phalloidin on actin. Indeed, the *act1-129* mutant protein, which contains a mutation in amino acids R177A and D179A, is unable to bind phalloidin, even if actin cables and cortical actin patches are still detected with an anti-actin antibody (Drubin et al., 1993). This *act1-129* mutant forms filaments of normal appearance, but is unable to be stained with conjugated phalloidin. This effect is specific because defects in phalloidin binding were not observed for any other *act1* alleles (Drubin et al., 1993). Thus, we recorded the localization of Rvs167p in *act1-129* mutant at the permissive temperature. We observed that in *act1-129* mutant cells, Rvs167p was still localized in patches, while as expected, the phalloidin could not bind to actin patches (data not shown). Thus, this result shows that the two amino acid residues involved in the binding of phalloidin are not essential for the interaction of Rvs167p with actin. However, it does not rule out the possibility that Rvs167p might obstruct the phalloidin-binding site on actin.

Rvs167p patches are present and localize in the absence of F-actin structures

As Rvs167p is localized with actin patches and because these proteins interact physically, we wondered whether actin or Rvs167p were responsible for this specific cellular localization. Thus, we used the drug latrunculin-A (LAT-A) that causes the rapid and reversible disassembly of cellular filamentous actin structures in vivo (Ayscough, 1998). Wild-type cells expressing Rvs167p-GFP were treated with LAT-A and stained with TRITC-phalloidin to check the disassembly of the structure of actin patches. Rvs167p remained localized in patches when actin patch structures were disrupted (Fig. 4). The control experiment without LAT-A (Fig. 4, column 1) allowed us to confirm the colocalization and the variation in the relative intensities between GFP and phalloidin as described above. In contrast, cells treated with 200 μM LAT-A showed a complete disruption of actin patches (Fig. 4, middle line, columns 2-6) whereas Rvs167p was still localized in patches throughout the cell in G_1 phase (Fig. 4, bottom line, column 2) or at the bud emergence site in budding cells (Fig. 4, bottom line, column 4), indicating that the localization of Rvs167p was not dependent on the presence of actin patches. However, when Rvs167p was still localized in patches to the correct sites, disruption of actin cytoskeleton led to Rvs167p patch immobilization (data not shown). When LAT-A was washed out and then reassembly of the actin cytoskeleton was observed, Rvs167p patches recovered mobility (data not shown).

The cellular localization of Rvs167p-GFP is not dependent on Rvs161p and Abp1p

Previous studies indicated that *RVS161* and *RVS167* gene products interact functionally and structurally in vivo (Navarro et al., 1997). Moreover, the cellular localization of Rvs161p has already been described by Brizzio et al. (1998). Although the Rvsp proteins exhibit two different cellular localizations, that of Rvs167p could be directed by this protein-protein interaction. We therefore observed the cellular distribution of Rvs167p in null *rvs161* mutant cells. In this genetic context, we noted no clear delocalization of the Rvs167p-GFP protein (data not shown).

Two-hybrid experiments had previously revealed that Rvs167p interacts with numerous proteins (P. Recordon-Navarro, personal communication). Among these interacting proteins is the actin-binding protein, Abp1p (Lila and Drubin, 1997). To assess the impact of these physical interactions on the cellular position of Rvs167p-GFP, we investigated it in the *abp1* mutant. The absence of Abp1p in the cells did not particularly affect the localization of Rvs167p-GFP as compared to the wild-type background (data not shown).

The BAR domain is required but not sufficient for correct localization of Rvs167p

We have shown in this report that Rvs167p is always localized in patches. In contrast, unbudded and large budded cells expressing Rvs161p-GFP present mainly cytoplasmic fluorescence with some punctate fluorescence, while in small

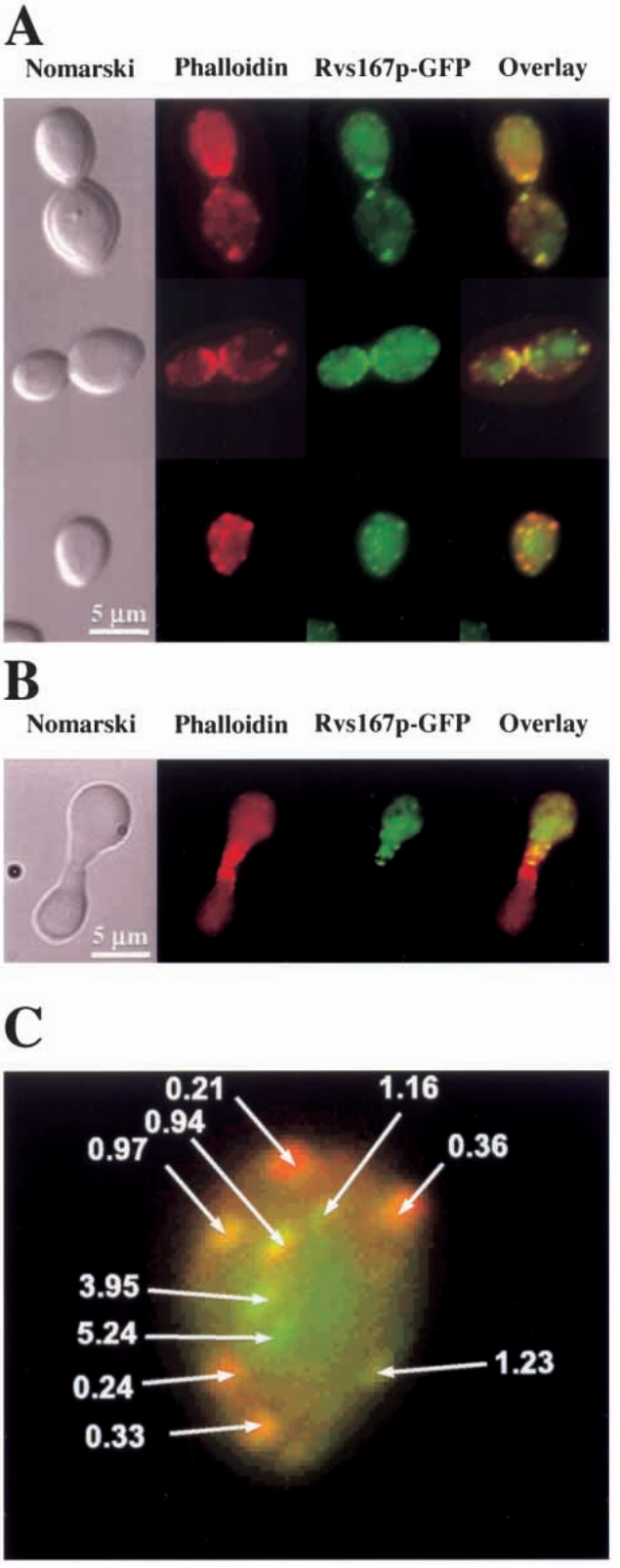

Fig. 3. Colocalization of actin and Rvs167p. (A,B) Cells were fixed and prepared for phalloidin staining as described in Materials and methods. From left to right, columns show Nomarski images of the cells, actin stained with TRITC-phalloidin, the green fluorescence of Rvs167p-GFP and an overlay of the red and green images. (A) Vegetative wild-type growing cells. (B) Two shmoos during mating (only one sexually compatible cell type containing *RVS167-GFP* fusion gene was fluorescent). (C) An overview of the last cell presented in A. Numbers correspond to the GFP/TRITC-phalloidin ratios for the major patches. The GFP/TRITC-phalloidin ratio corresponds to the ratio of fluorescence intensities determined with FITC and TRITC filter sets in the same areas. These fluorescence intensities were separately measured using Metamorph software and ratios were calculated.

budded cells Rvs161p-GFP is localized in patches mainly at the mother-bud neck (Brizzio et al., 1998). Considering that the two proteins have been shown to work together in a complex (Navarro et al., 1997), it is possible that this complex appears only during bud emergence. As the two proteins have a BAR domain with a similar amino acid sequence (Bauer et al., 1993), we hypothesized that the GPA and SH3 domains may determine the specific localization of Rvs167p, and that the isolated BAR domain of Rvs167p may localize like Rvs161p (i.e. in patches only in small budded cells). To test this hypothesis, we constructed two fusion proteins (Fig. 1B): the first only contained the BAR domain fused to the GFP at the C-terminal end (BARp-GFP) and the second contained the GPA and SH3 domains fused to the GFP at the N-terminal end (GFP-GPA/SH3p). The cellular distribution of these two new fusion proteins is presented in Fig. 5. BARp-GFP protein localization was observed in four typical distributions (Fig. 5A-D). Table 2 gives the number of cells in each class. In most cases the fusion protein was localized like Rvs167p (Fig. 5A) or at the periphery of the cell with one or two patch(es) inside, as previously described for Rvs167p patches (Fig. 5B). A few cells had some remaining patches but most of the BARp-GFP was delocalized and clearly excluded from the vacuole (Fig. 5C), while others had the BARp-GFP totally delocalized and also excluded from the vacuole (Fig. 5D). These results are independent of the budding state of the cells. GFP-GPA/SH3p, however, was either delocalized with some remaining patches throughout the cell (Fig. 5E) or totally delocalized (Fig. 5F), and always excluded from the vacuole. About 80 vegetative cells were analyzed (Table 2) but none showed any typical wild-type Rvs167p cellular localization. These results show that the BAR domain is required but not sufficient for the correct localization of Rvs167p and that the GPA and SH3 domains are not responsible for the specific Rvs167p localization. Moreover this BAR domain, which is an Rvs161-like protein, is not localized like Rvs161p.

DISCUSSION

This report describes the cellular localization of the yeast amphiphysin-like protein Rvs167p using an Rvs167p-GFP fusion protein. The use of this fusion protein has enabled us to show that Rvs167p localizes in cortical patches in vivo. Observation of several cells expressing the *RVS167-GFP* gene pointed to an heterogeneity in the cellular distribution of the tagged protein. We were able to identify roughly two classes of cells: cells with small patches distributed homogeneously, and budding cells or cells in which the emergence of the bud is impending, with polarization of the patches. These observations are in perfect agreement with previous descriptions of the distribution of actin patches during the cell

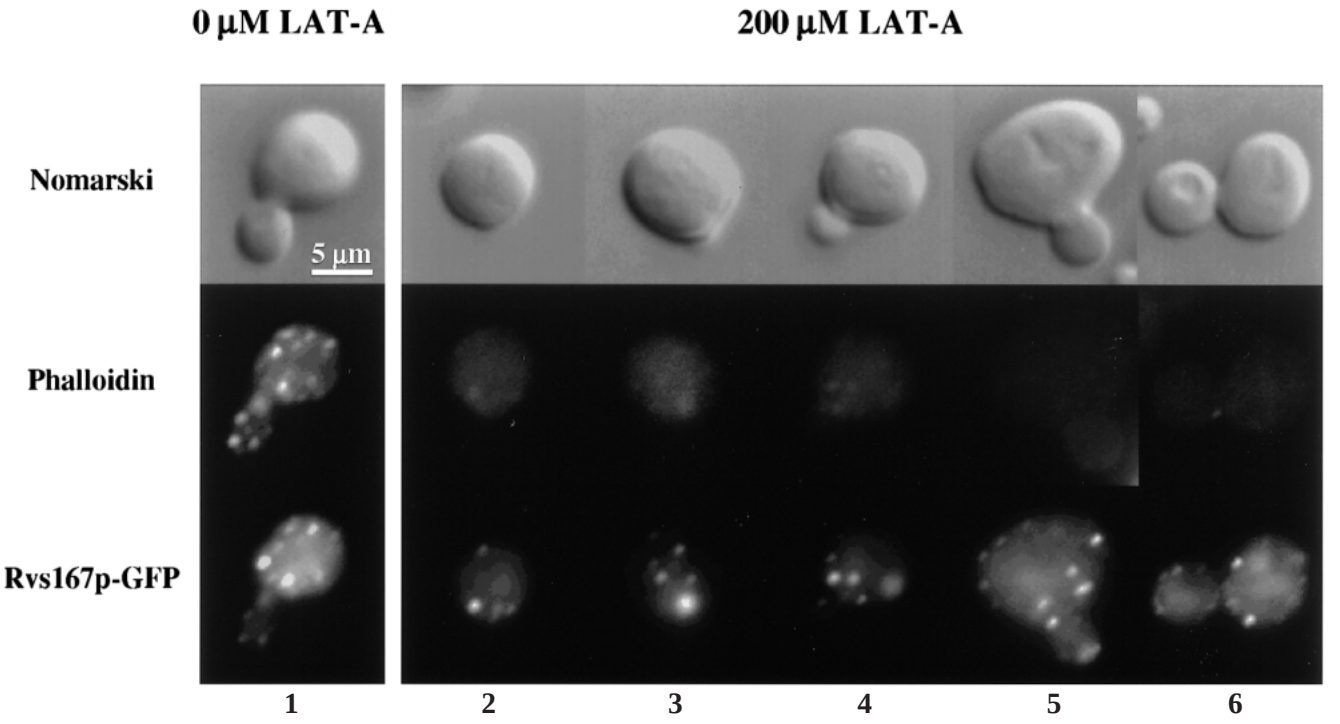

Fig. 4. Cellular localization of Rvs167p-GFP and F-actin-phalloidin staining in wild-type haploid cells in the absence (0 μM LAT-A, column 1) or presence (200 μM LAT-A, columns 2-6) of LAT-A, which was used to disrupt the actin cytoskeleton. Cells were fixed and prepared for phalloidin staining as described in Materials and methods. Acquisition time was 2 seconds for Rvs167p-GFP images and for phalloidin images of cells not treated with LAT-A, and was 6 seconds for phalloidin images of cells treated with LAT-A.

cycle (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Botstein et al., 1997; Winsor and Schiebel, 1997).

As *rvs161* and *rvs167* mutants have the same pleiotropic phenotype (Bauer et al., 1993) and two-hybrid experiments have shown that these two proteins interact in vivo (Navarro et al., 1997), we expected a similar cellular localization for Rvs161p and Rvs167p. Surprisingly, the two Rvs proteins seem to have two different cellular distributions: according to Brizzio et al. (1998), Rvs161p is localized in patches only during bud emergence, whereas Rvs167p localizes in patches throughout the vegetative cell cycle (this work). These results imply that during vegetative growth these two proteins may interact only during bud emergence. However, they may also interact during mating since Brizzio et al. (1998) and we (this work) found Rvs161p and Rvs167p, respectively, localized at the tip of the shmoo. A different cellular localization has also been described for the two mammalian amphiphysins. Indeed, in brain cells, amphiphysin II was reported to be concentrated to areas of actin fibres in axon segments and nodes of Ranvier, whereas amphiphysin I is located in the cortical cytoplasm of nerve terminals (Butler et al., 1997). This result is at odds with another study which established that the brain form of amphiphisin II forms heterodimers with amphiphysin I (Wigge et al., 1997).

The cellular localization of Rvs167p is in perfect agreement with our previous hypothesis based on numerous arguments

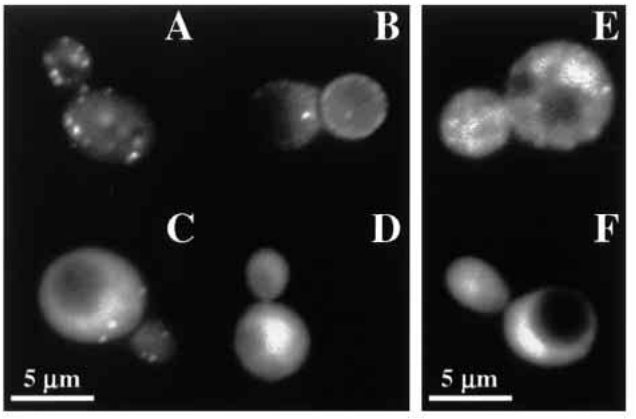

Fig. 5. Localization of the BARp-GFP and GFP-GPA/SH3p fusion proteins. (A-D) The four typical distributions detected in cells expressing the *BAR-GFP* fusion gene. (E,F) Representative wild-type cells expressing the *GFP-GPA/SH3* fusion gene. Acquisition time was 0.5 seconds.

Table 2. Cellular localization of Rvs167p domains

Fusion protein	Cell state			
BARp-GFP	32	28	8	10
GFP-GPA/SH3p	0	0	22	60

Wild-type cells were analyzed during vegetative growth. The BARp-GFP is a fusion protein containing only the BAR domain of Rvs167p while the GFP-GPA/SH3p is a fusion protein containing the GPA and SH3 domains.

About 80 vegetative cells were analyzed in each experiment.

Schematic representations of cell state correspond to the different states described in Fig. 5. From left to right the cells correspond to Fig. 5A, 5B, 5C,E and 5D,F. Values are the number of cells counted in each state.

that would indicate a closely relationship between actin and Rvs167p. Using TRITC-phalloidin to stain F-actin in cells expressing an *RVS167-GFP* fusion gene, we have demonstrated that Rvs167p colocalizes with actin patches during vegetative growth and during mating. Analyses of overlay frames demonstrated variations in the GFP/TRITC fluorescence ratio, which means that the relative quantities of Rvs167p-GFP and phalloidin are variable from patch to patch. On the one hand, if phalloidin is present in homogeneous quantities in F-actin, then a variation of the GFP/phalloidin ratio from patch to patch could indicate that Rvs167p-GFP is distributed heterogeneously in F-actin. On the other hand, the presence of Rvs167p-GFP in the cell could tend to disturb phalloidin staining, suggesting that the binding of Rvs167p on actin occurs at the phalloidin-binding site or includes it. To test this hypothesis, we analyzed the cellular localization of Rvs167p in the *act1-129* mutant, whose protein carries two mutations in residues R177A and D179A in the phalloidin-binding site (Drubin et al., 1993). Our results indicate that Rvs167p is still localized in patches in these mutant cells, while the phalloidin cannot bind to actin patches, thus demonstrating that the two amino acid residues involved in the binding of phalloidin are not essential for the binding of Rvs167p to actin. However, it does not rule out that the prior interaction of Rvs167p-GFP with actin obstructs the phalloidin-binding site in our conditions. The relationship between Rvs167p and actin mutated in residues R177 and D179 has been confirmed by genetic studies demonstrating a synergic effect between the *act1-129* mutant and the *rvs167* mutant (Breton and Aigle, 1998) and by two-hybrid experiments showing interaction between actin and Rvs167p but not with the actin mutated in the two residues R177 and D179 (Amberg et al., 1995).

Although we were able to detect actin cables by TRITC-phalloidin staining, we have not been able to colocalize Rvs167p-GFP with these actin cables, even after overexpression. This might indicate that Rvs167p does not interact with filamentous polymerized actin in cytoplasmic actin cables, but rather interacts with the actin involved in cortical actin patches. Another hypothesis is that Rvs167p is bound to actin in low quantities along the cable, so there is not enough fluorescence signal and it is therefore not detected. This problem has already been noted by Doyle and Botstein (1996), who were unable to see any actin cable with an actin-GFP fusion protein.

Having established that Rvs167p patches colocalized with actin patches, we next determined how total disruption of actin filaments would affect the Rvs167p specific localization. Numerous proteins (such as Abp1p, cofilin, Sla1p, Sla2p), known as components of the actin cytoskeleton and involved in cell polarity development, have been shown to require actin to achieve polarized localization (Ayscough et al., 1997). These proteins are unable to polarize properly when the actin cytoskeleton is completely disrupted by LAT-A. Other proteins (such as Cdc42p, Bem1p, Bud6p, Myo2p, calmodulin) also involved in the actin cytoskeleton achieve polarized localization even if filamentous actin is totally disrupted (Ayscough et al., 1997). Our results showed that in the presence of the drug LAT-A, when the actin cytoskeleton was completely disrupted, Rvs167p remains localized in immobilized patches. These patches remain polarized at the small bud. Thus, the localization of Rvs167p is not dependent on the localization of the actin in patches. In contrast, in an *rvs167* mutant there is a small concentration of actin patches at the bud emergence; when the bud enlarges, actin patches are distributed throughout the cell (Bauer et al., 1993). When Rvs167p is absent, the actin cytoskeleton is partially independent of the cell cycle. Thus, we can assume that Rvs167p, with other proteins, may be responsible for the polarization of actin patches during the cell cycle. Moreover, it is known that (1) Abp1p interacts with actin and colocalizes to the cortical actin patches (Drubin et al., 1988); (2) Abp1p binds to the SH3 domain of Rvs167p; and (3) Abp1p binds to Srv2p by its SH3 domain (Lila and Drubin, 1997). Amberg et al. (1995) using two-hybrid experiments demonstrated an interaction between Rvs167p and actin. Our results show that the BAR domain alone can localize properly to a partial extent, while the GPA/SH3 domains are totally unable to do it. And recently, Ayscough et al. (1997) showed that in the presence of LAT-A, when the actin cytoskeleton is disrupted, Abp1p is unable to achieve a polarized localization. In summary, it may be that Rvs167p localizes first to polarized patches via its BAR domain. This Rvs167p localization might be then responsible for the Abp1p correct position in collaboration with actin, and consequently for the polarization of the proteins involved in the actin patch complex.

Two-hybrid experiments have shown that Rvs167p and Rvs161p interact in vivo (Navarro et al., 1997). It seemed interesting to observe the localization of Rvs167p in null *rvs161* mutant cells because Rvs167p cellular localization could be mediated by this protein-protein interaction. Our results demonstrate that the tagged protein is still localized in the same sites in a *rvs161* mutant genetic background. This suggests that Rvs167p cellular localization is not dependent on the presence of Rvs161p. Moreover, Lila and Drubin (1997) have demonstrated that the SH3 domain of Rvs167p interacts with Abp1p, so we checked the cellular localization of Rvs167p in *abp1* mutant cells. We found that the localization of Rvs167p was not modified in absence of Abp1p in the cell. As Drubin et al. (1988) have shown that Abp1p interacts with actin, and considering the model proposed by Lila and Drubin (1997), who clearly demonstrated that Abp1p interacts with Srv2p by its SH3 domain and also with the SH3 domain of Rvs167p, we can hypothesize that the localization of Rvs167p in cortical patches is due to itself or to other proteins involved in this cortical complex like Sla1p, Sla2p, Sac6p and Srv2p, but not to Abp1 (Lila and Drubin, 1997). Moreover, although Abp1p and Srv2p are still localized in the cortical actin patches in the *rvs167* mutant (Lila and Drubin, 1997), actin patches are delocalized in *rvs167* mutant cells (Bauer et al., 1993), suggesting that Rvs167p may localize the actin cortical complex properly.

As the sequence comparison of the Rvs161p and Rvs167p revealed that the whole sequence of Rvs161p shows significant similarities to the BAR domain of Rvs167p (Bauer et al., 1993), we hypothesized that the specific localization of Rvs167p might be due to its GPA/SH3 domains. The cellular localization of two truncated fusion proteins, one with the BAR domain only and one with the GPA/SH3 domains only, clearly demonstrated that the GPA/SH3 domains were not responsible for the specific localization of the Rvs167p. In fact, our results indicate that the BAR domain is required but not sufficient for the cellular localization of Rvs167p. Most of the cells

expressing the *BAR-GFP* fusion gene show a correct localization of the corresponding truncated protein. This result is in agreement with the fact that the BAR domain alone is able to partially complement a *rvs167* null mutant (Sivadon et al., 1997). The difference in Rvs167p localization between cells from the same population could be explained by cellular physiological differences. We speculate that the different Rvs167p domains are involved in different specific protein-protein interactions in association with specific physiological states. It has been shown that Rvs167p can form different complexes with proteins through its SH3 domain (Amberg et al., 1995) or through the predicted coiled-coil structure of the BAR domain (Navarro et al., 1997). Two-hybrid experiments have shown that Rvs167p interacts at least with about 8 or 18 partners, through its BAR or GPA/SH3 domains, respectively (Lila and Drubin, 1997; Navarro et al., 1998). The relationship between these protein interactions and the physiological state of the cell could be mediated through the phosphorylation state of Rvs167p by the cyclin-dependent kinase Pho85p and other kinases (Lee et al., 1998).

The insertion of the GFP into Rvs167p between the BAR domain and the GPA domain resulted in a functional protein, indicating that these domains can be separated by 267 additional amino acids without affecting the Rvs167 protein function. Two hypotheses are possible: either the three-dimensional barrel structure of GFP, with both ends protruding at the same side of the cylinder (Ormö et al., 1996; Yang et al., 1996) does not modify the functional structure of Rvs167p (i.e. the distance between the BAR and the SH3 domains); or the size and the structure between the BAR and SH3 domains are not crucial for Rvs167p function. The latter seems more likely since the insertion of two Z IgG binding units (130 amino acids) from *Staphyloccocus aureus* protein A in the GPA domain of Rvs167p does not affect its cellular function (Sivadon et al., 1997). This would also be in agreement with previously published findings that have demonstrated that a GPA-deleted Rvs167 protein, which contains the BAR and the SH3 domains only, is fully functional (Sivadon et al., 1997). Moreover, Rvs167p is a member of the amphiphysin/Rvs family, which contains proteins with a BAR domain and an SH3 domain highly conserved at their N-terminal and C-terminal ends, respectively (Sakamuro et al., 1996; Butler et al., 1997). Interestingly, the internal domain (i.e. between BAR and SH3 domains) is poorly conserved between all these proteins. These structural features could indicate that both BAR and SH3 domains are important for the functionality of these proteins, whereas the internal domain could be specific for each member of the family. In the specific case of Rvs167p, it might only represent a link between the functional BAR and SH3 domains.

The high degree of sequence similarity between the terminal regions of the amphiphysin/Rvs family proteins and their overall conserved organization, is some evidence that they share similar molecular and perhaps cellular functions. Recently, new results have shown that amphiphysins are also associated with actin. More precisely, amphiphysin II is localized in areas of actin fibers in brain and in skeletal muscle (Butler et al., 1997). This observation is confirmed by two-hybrid data in which amphiphysin I has been shown to interact with yeast actin (P. Recordon-Navarro, personal communication). So, amphiphysin/Rvs family members may have a central function in the polarization of the actin cytoskeleton.

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