Functional assessment of the yeast Rvs161 and Rvs167 protein domains

Pierre Sivadon[a], Marc Crouzet[b], Michel Aigle[a,*]

[a] *Laboratoire de Biologie Cellulaire de la Levure, IBGC UPR CNRS 9026, 1 rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France*
[b] *Laboratoire de Biologie Moléculaire et de Séquençage, UPR CNRS 9026, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France*

Received 18 September 1997

Abstract Mutations in *RVS161* and *RVS167* yeast genes induce identical phenotypes associated to actin cytoskeleton disorders. The whole Rvs161 protein is similar to the amino-terminal part of Rvs167p, thus defining a RVS domain. In addition to this domain, Rvs167p contains a central glycine-proline-alanine rich domain and a SH3 domain. To assess the function of these different domains we have expressed recombinant Rvs proteins in *rvs* mutant strains. Phenotype analysis has shown that the RVS and SH3 domains are necessary for phenotypical complementation, whereas the GPA domain is not. Moreover, we have demonstrated that the RVS domains from Rvs161p and Rvs167p have distinct roles, and that the SH3 domain needs the specific RVS domain of Rvs167p to function. These results suggest that Rvs161p and Rvs167p play distinct roles, while acting together in a common function.

Key words: *RVS167*; *RVS161*; SH3 domain; Actin cytoskeleton; Budding pattern; *Saccharomyces cerevisiae*

1. Introduction

The *Saccharomyces cerevisiae rvs161* and *rvs167* mutants were initially selected in order to elucidate the mechanisms involved in the establishment of the stationary phase upon nutrient starvation [1,2]. The mutants show a reduced viability associated with a high budded cell ratio and a morphological heterogeneity in response to carbon or nitrogen exhaustion. Additional studies have put forward a more general sensitivity of the *rvs* cells to several growth conditions such as high salt concentrations or the presence of drugs [1,2].

Cytological studies of the *rvs161* and *rvs167* mutant strains have shed light on several other phenotypes. Microscopic observations have revealed that the actin cytoskeleton is disorganized in the *rvs* cells, with an increased alteration upon glucose starvation or high salt concentration [2,3]. It has also been observed that **a**/α diploid homozygous mutant cells bud with a random budding pattern, whereas the diploid wild-type cells have a bipolar distribution of the bud-emerging sites [2,3]. Finally, the *rvs161* and *rvs167* strains have been shown to be impaired in endocytosis, precisely during the internalization step at the plasma membrane level [4]. In fact, it seems that both the budding pattern and the endocytosis defects could result from actin cytoskeleton disorganization [5,6]. This functional relationship observed between the *RVS* genes and the organization of the actin cytoskeleton has been confirmed by genetic interactions. Indeed, the *end6-1* mutation, which is allelic to *RVS161*, exhibits non-allelic non-complementation when associated with the *act1-1* mutation, therefore strongly suggesting that the two proteins form a complex in vivo [4]. Furthermore, two-hybrid and biochemical studies have shown that Rvs167p is an actin-binding protein [7] and that it binds to an other actin-binding protein, namely Abp1p [8].

The Rvs161 and Rvs167 proteins present similar sequences to each other [2]. The whole Rvs161p sequence is 42% similar to the amino-terminal part of Rvs167p, with 21% identity. This similarity allowed us to define the RVS domain which has also been found in other proteins such as amphiphysins [3,9]. This RVS domain also contains putative coiled-coil structures that are known to allow protein-protein interactions [10]. In addition to this RVS domain, the Rvs167 protein contains a glycine-proline-alanine rich region (GPA domain) associated with an SH3 domain at its carboxy-terminal part [2]. This structure has been found in other actin-binding proteins like yeast Abp1p and Myo3p [11,12]. The SH3 domain is found in numerous proteins and seems to act in signalling pathways by protein-protein interactions. The Rvs-like proteins amphiphysins also contain an SH3 domain at their C-terminal part [9].

To date, little information on the role played by the different Rvsp domains is available. However, the identity of the phenotypes presented by both the *rvs161* and *rvs167* mutant strains [1–3], in addition with the fact that suppressor genes that were selected against one of the mutated *rvs* genes were able to suppress the defects of either *rvs161* or *rvs167* single mutation, or both the *rvs161 rvs167* double mutation [13–15], indicate that the *RVS* genes may be involved in the same cellular process. This was recently supported by Navarro et al. [16] who demonstrated a physical interaction between Rvs167p and Rvs161p in vivo through the RVS domain. In a similar way, it has been shown that the GPA-SH3 region of Rvs167p mediates the Rvs167p-actin interaction as well as the binding between Rvs167p and Abp1p [7,8].

Here we describe the construction of several recombinant and chimeric Rvs proteins that were thereafter expressed in the *rvs161* and *rvs167* mutant strains. The analysis of the phenotypes suggests that Rvs161p and Rvs167p play distinct roles, while acting together in the same function through a protein-protein complex.

2. Materials and methods

2.1. Strains, media, and growth conditions

All of the *S. cerevisiae* strains used in this study were derivatives of X2180 wild-type strain (Table 1). Yeast cells were grown at 30°C.

Yeast strains were cultivated either in YPD complete medium or YNB minimal medium [17]. The YNBGE respiratory medium contained glycerol (2%) and ethanol (2%) as carbon sources. YNBS medium was obtained by addition of NaCl to YNB, and YNBT medium was YNB medium containing 75 mM 3-amino-1,2,4-triazole (3-A-T;

*Corresponding author. Fax: +33 5 56994299.
E-mail: Michel.Aigle@ibgc.u-bordeaux2.fr

PII S0014-5793(97)01248-9

Sigma). G1 was YNB medium limited for carbon source (0.1% glucose). The test medium G1+E was obtained by adding the vital dye Erythrosin B (7.5 μM final concentration) in G1 medium. Mutant strains with low viability give red colonies whereas wild-type colonies stay white [18]. The cell viability was also evaluated by microscopic observation using methylene blue as previously described [15].

2.2. DNA manipulations

Restriction enzymes and other DNA modification enzymes were purchased from Boehringer Mannheim and used according to the specifications of the manufacturer. Plasmid DNA from *E. coli* was prepared by the method of Birnboim and Doly [19]. DNA sequences were determined by the dideoxy chain termination method [20] directly on double-stranded plasmid DNA using the A.L.F. automatic sequencer (Pharmacia). Amplifications of DNA fragments were done by polymerase chain reaction (PCR) on a DNA Thermal Cycler (Perkin Elmer Cetus). Custom oligonucleotides were provided by EuroGentec (Belgium). Yeast cells were transformed by the LiCl method [21]. Transformation of *E. coli* was done by the $CaCl_2$ method [22].

2.3. Construction of the recombinant RVS genes

The pSP167 plasmid was constructed by insertion of the 2.5 kb *Bgl*II-*Sal*I fragment of the pUKC200-134 plasmid containing the *RVS167* gene [2] into the *Bam*HI-*Sal*I-digested centromeric pUKC200 vector [2]. The pCD1S5 plasmid carrying the *RVS161* gene was obtained by inserting the 1.4 kb *Hpa*I fragment of the pUB1-2 plasmid which contains *RVS161* [1], into the *Sma*I site of the pFL44 vector [23].

The different *RVS* recombinant and chimeric genes (see Fig. 1), in which one of the coding sequences for either the RVS, GPA or SH3 domains of Rvs167p is lacking or added, were constructed by directed mutagenesis using PCR-amplification from the *RVS* genes carried by either pSP167 or pCD1S5. These amplifications generated DNA fragments that were thereafter ligated to each other according to the desired recombinant genes. Oligonucleotides were synthesized to generate the appropriate mutations. The resulting recombinant *RVS167* genes are carried by the pUKC200 and the chimeric *RVS161GPASH3* gene is carried by pFL44. The pSP167ΔSH3 plasmid carries a recombinant *RVS167ΔSH3* gene, containing a stop codon (TAA) overlapping the 424th codon between the GPA and SH3 sequences, and which expresses a Rvs167ΔSH3 protein (Fig. 1). The pSP167ΔGPASH3 plasmid carries the *RVS167ΔGPASH3* gene which contains a stop codon (TAA) overlapping the 297th codon between the RVS and GPA sequences. This recombinant *RVS167* gene encodes the Rvs167ΔGPASH3 protein composed of the RVS domain alone (Fig. 1). The pSP167ΔGPA plasmid carries the *RVS167ΔGPA* gene which expresses the Rvs167ΔGPA protein deleted for the GPA domain (from Ala296 to Thr427) (Fig. 1). The pSP167ΔRVS plasmid carries the *RVS167ΔRVS* gene which encodes the Rvs167ΔRVS protein (Fig. 1). The coding sequence starts from an ATG codon and is continued by the GPASH3 sequence starting from Lys290. The pCDGPASH3 plasmid carries a chimeric *RVS161GPASH3* gene, which encodes the Rvs161 protein with the GPA-SH3 domains of Rvs167p at its C-terminal part (Fig. 1). In this gene, the *RVS161* stop codon and terminating sequence are replaced by the GPASH3 (from Tyr291) sequence and terminator of *RVS167*.

All recombinant and chimeric *RVS* genes were verified by DNA sequencing (not shown). Western-blotting experiments using anti-Rvs161p and anti-Rvs167p antisera showed that the expression of all these recombinant proteins is similar to that observed for wild-type Rvs proteins (not shown).

2.4. Visualization of polymerized actin

Cells grown in liquid culture according to the conditions described were fixed by formaldehyde treatment and stained with TRITC-conjugated phalloidin (Sigma) as described previously [3]. The organization of the actin cytoskeleton was then observed by fluorescence microscopy.

2.5. Visualization of chitin rings

Yeast cells were grown as indicated. Chitin rings were visualized by fluorescence microscopy after staining cells in 0.1% Calcofluor (Sigma) and washing them in distilled water [24].

3. Results

3.1. Different involvements of the domains of Rvs167p in the rvs growth phenotypes

To determine the importance of the different domains of Rvs167p, we checked the growth phenotypes of the LG440-1A (*rvs167-1*) mutant strain transformed either by pUKC200, pSP167, pSP167ΔSH3, pSP167ΔGPASH3, pSP167ΔGPA or pSP167ΔRVS (Fig. 2). These transformed strains were cultivated in YNB, YNBGE, YNBT and YNBS media on which the *rvs* mutant strains present growth sensitivities [1,2].

Concerning the growth sensitivities (Fig. 2a), contrary to what is observed with the pUKC200 vector that does not contain any insert, the pSP167 plasmid carrying the *RVS167* gene restored a wild-type phenotype in the *rvs167-1* mutant strain on the different media tested. Recombinant Rvs167 proteins lacking the SH3 domain, or both GPA and SH3 domains, partially complemented the *rvs167-1* mutation (Fig. 2a). Indeed, these strains had light *rvs* phenotypes with growth sensitivities between those observed for the wild-type and mutant strains. Furthermore, the expression of an Rvs167 protein containing only the GPA and SH3 domains did not restore the wild-type phenotype at all (Fig. 2a). On the other hand, an Rvs167ΔGPA protein lacking the GPA domain seemed to have a normal activity since no particular sensitivity was observed in the different growth conditions (Fig. 2a). The same results were obtained when the percentage of cell viability was determined in YNB and G1 media (Fig. 2b).

These different recombinant Rvs167 proteins were also expressed in the wild-type strain LG100-1A (not shown). No dominant effect was observed, these strains showing a wild-type phenotype.

3.2. Both RVS and SH3 domains are necessary for wild-type organization of the actin cytoskeleton

Another phenotypical aspect characterizing the *rvs* strains concerns the organization of the actin cytoskeleton [2,3]. In

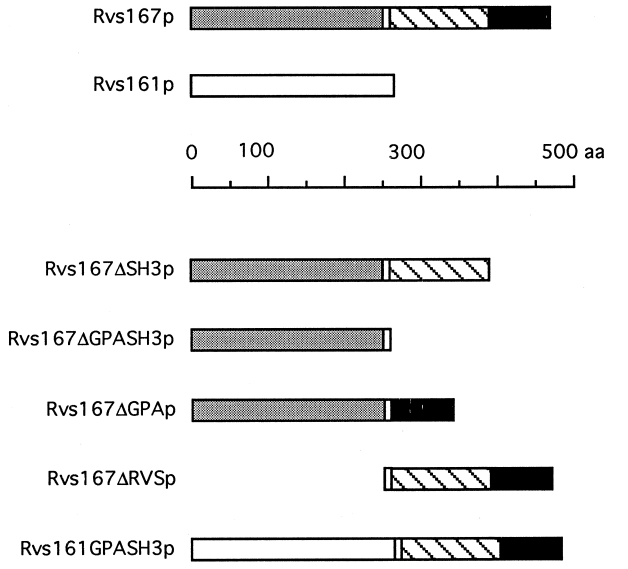

Fig. 1. Schematic representation of the different recombinant Rvs proteins. The wild-type Rvs161 and Rvs167 proteins are also shown. The domains of the proteins are represented as boxes (white box: RVS domain of Rvs161p; grey box: RVS domain of Rvs167p; hatched box: GPA domain; black box: SH3 domain).

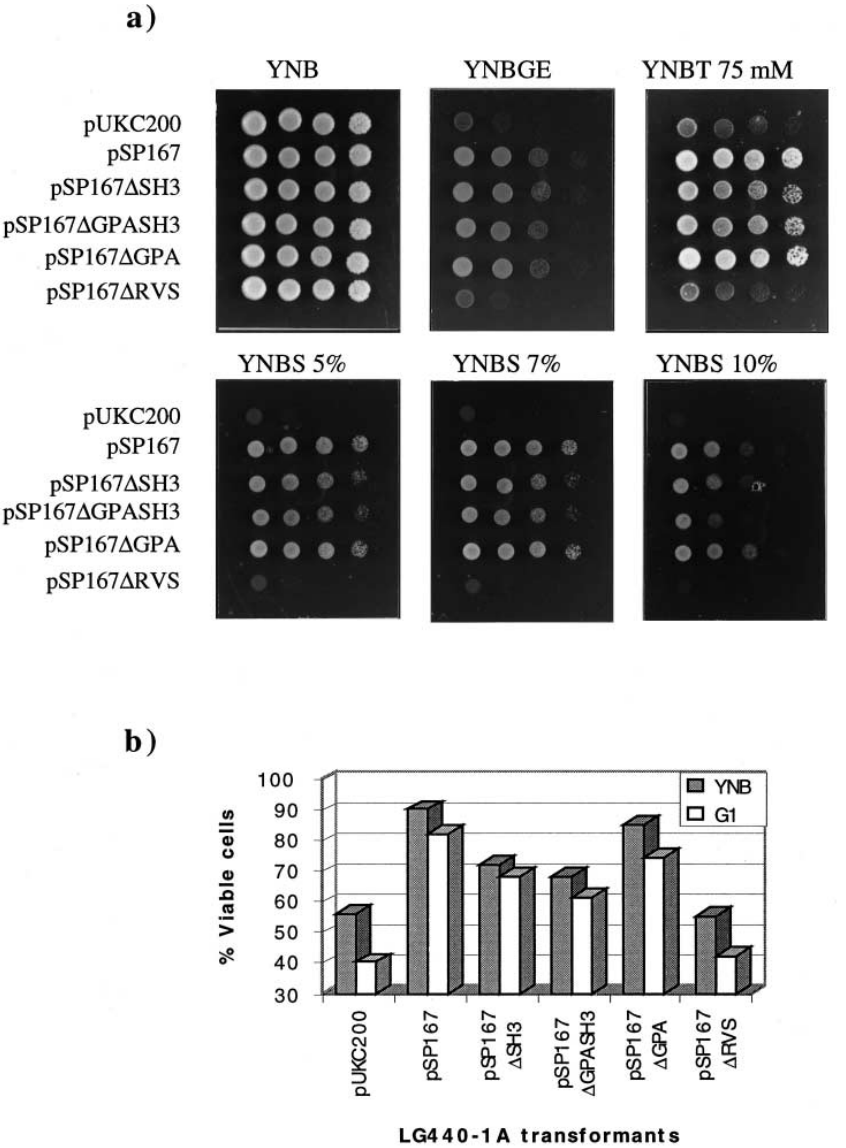

Fig. 2. Phenotypic analysis of the expression of the recombinant Rvs167 proteins in the *rvs167-1* mutant strain. The LG440-1A (*rvs167-1*, *trp1*) strain was transformed either with pUKC200, pSP167, pSP167ΔSH3, pSP167ΔGPASH3, pSP167ΔGPA or pSP167ΔRVS plasmids. a: Growth of these transformed strains was monitored on YNB, YNBGE, YNBT (75 mM 3-A-T) and YNBS (5%, 7% and 10% NaCl) media. b: The cell viability upon nutrient starvation of these transformed strains was evaluated in YNB and G1 media by methylene blue staining. The results are given as percentages of viable cells after 48 h in stationary phase.

the wild-type strain the yeast actin cytoskeleton is found in two forms: a set of cortical actin patches and a network of cytoplasmic bundles of actin filaments [25]. The cellular distribution of these structures is coordinated with cell cycle

Table 1
S. cerevisiae strains

Strains	Relevant genotype	Source
X2180-1A	*MAT***a**, *SUC2*, *mal*, *mel*, *gal2*, *CUP1*	YGSC[a]
X2180-2N	*MAT***a**/MATα, *SUC2*/*SUC2*, *mal*/*mal*, *mel*/*mel*, *gal2*/*gal2*, *CUP1*/*CUP1*	YGSC[a]
LG100-1A	*MAT***a**, *trp1*	Our laboratory
LG400-1A	*MAT***a**, *rvs167-1*	[2]
LG470-2N	*MAT***a**/MATα, *rvs167-1*/*rvs167-1*	[2]
LG440-1A	*MAT***a**, *rvs167-1*, *trp1*	Our laboratory
LG420-1A	*MAT***a**, *rvs167-1*, *ura3*	Our laboratory
LG440-2N	*MAT***a**/MATα, *rvs167-1*/*rvs167-1*, *trp1*/*trp1*	Our laboratory
LG300-1A	*MAT***a**, *rvs161*Δ	[12]
LG503-1A	*MAT***a**, *rvs161*Δ, *ura3*	Our laboratory
LG502-1A	*MAT***a**, *rvs161*Δ, *trp1*	Our laboratory
LG540-1A	*MAT***a**, *rvs167-1*, *rvs161*Δ, *ura3*, *trp1*	Our laboratory

[a]Yeast Genetic Stock Center, California.

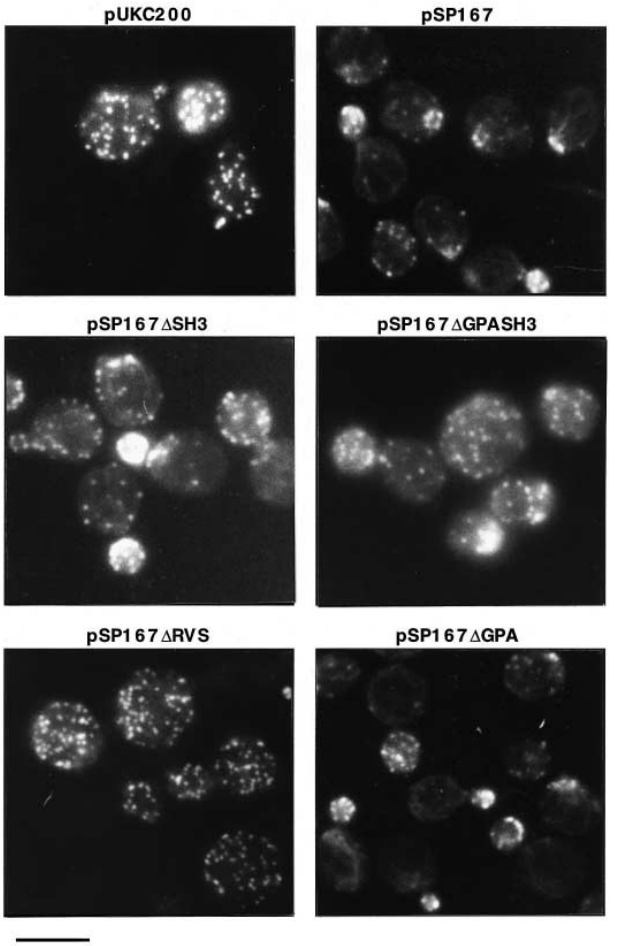

Fig. 3. Actin cytoskeleton distribution in the *rvs167-1* mutant strain (LG440-1A) transformed either with pUKC200, pSP167, pSP167ΔSH3, pSP167ΔGPASH3, pSP167ΔGPA or pSP167ΔRVS. Cells cultivated in YNBS (3% NaCl) were stained with rhodamine-conjugated phalloidin and were observed by epifluorescence microscopy. Cells representative of the bulk of the population are shown (scale bar, 5 μm).

events [26]. Actin patches are located to the bud and actin filaments are oriented towards the bud tip. Microscopic observation of the actin cytoskeleton distribution in *rvs167* and *rvs161* mutant cells showed a delocalization of actin patches and filaments [2,3]. Moreover, the severity of the delocalization depended on the growth conditions; it was more obvious in media containing either a high salt concentration or a limited carbon source.

The organization of the actin cytoskeleton was examined by fluorescence microscopy using rhodamine-conjugated phalloidin. The analysis was carried out on the LG440-1A mutant strain transformed by pUKC200, pSP167, pSP167ΔSH3, pSP167ΔGPASH3, pSP167ΔGPA or pSP167ΔRVS (Fig. 3). The cells were cultivated in YNBS (3% NaCl) medium. Cells carrying the pUKC200 vector presented a delocalization of the actin cytoskeleton similar to that already described for the *rvs* mutant cells (Fig. 3) [2]. The actin patches were distributed homogeneously in both the mother cell and the bud. There was no particular concentration of the cytoskeletal structures in the growth regions. The same phenotype was observed for cells expressing the Rvs167ΔRVS protein (Fig. 3). On the other hand, the expression of Rvs167p from the pSP167 plasmid, as well as Rvs167ΔGPAp from pSP167ΔGPA, restored a wild-type phenotype (Fig. 3). In agreement with observations reported above for growth sensitivities, the recombinant Rvs167 proteins lacking the SH3 domain, namely Rvs167ΔSH3p and Rvs167ΔGPASH3p, partially complemented the defects (Fig. 3). The *rvs167* mutant cells expressing these SH3-deleted proteins presented a delocalization of the actin cytoskeleton halfway between the mutant and wild-type phenotypes.

3.3. *Both RVS and SH3 domains are necessary for the bipolar budding pattern*

Two budding patterns have been described in *S. cerevisiae* [27]. A haploid **a** or α cell buds in contact with the previous site of budding, with an axial pattern, whereas a diploid **a**/α cell buds in a bipolar manner, either near the previous division site or at the opposite pole. It has been shown that the budding pattern is altered in **a**/α diploid *rvs* homozygous mutant cells which exhibit a random distribution of the budding sites [2,3,28].

Budding patterns were determined by fluorescence microscopy, using Calcofluor which binds to chitin. The budding site selection patterns were observed in the **a**/α diploid LG440-2N mutant strain transformed by the same plasmids as aforementioned (Fig. 4). The **a**/α diploid wild-type (X2180-2N) and mutant (LG470-2N) strains were used as controls. The percentage of randomly budding cells was higher than 75% when the pUKC200 vector was introduced into the *rvs167* diploid mutant cells. On the other hand, more than 95% of the mutant cells transformed by the pSP167 plasmid, as well as the wild-type cells, budded with a bipolar distribution of the bud-emerging sites. Similar levels of wild-type budding were obtained when Rvs167ΔGPAp was expressed in the mutant cells. Recombinant Rvs167ΔSH3p and Rvs167ΔGPASH3p could not restore a wild-type bipolar budding pattern in as many cells as in the mutant strain carry-

		bipolar %	axial %	random %
X2180-2N (*RVS167*)		98	0	2
LG470-2N (*rvs167-1*)		25	3	72
LG440-2N (*rvs167-1/rvs167-1, trp1/trp1*)	pUKC200	20	2	78
	pSP167	95	0	5
	pSP167ΔSH3	75	0	25
	pSP167ΔGPA	91	0	9
	pSP167ΔGPASH3	70	0	30
	pSP167ΔRVS	26	1	73

Fig. 4. Budding patterns in wild-type (X2180-2N), *rvs167-1* mutant (LG470-2N) and LG440-2N diploid cells transformed either with pUKC200, pSP167, pSP167ΔSH3, pSP167ΔGPASH3, pSP167ΔGPA or pSP167ΔRVS. The percentages were determined by microscopic observation of log phase diploid cells (about 200) bearing three or more bud scars and grown in YNB medium.

ing the pSP167 plasmid. Indeed, although the percentage of cells budding with a bipolar pattern increased three times with these SH3-deleted proteins in comparison to the mutant strain, more than 25% of the cells still budded randomly.

All these recombinant Rvs167 proteins were also expressed in the haploid LG440-1A (*rvs167*) and LG100-1A (*RVS167*) strains, as well as in the a/α diploid wild-type strain. No particular phenotype concerning bud site distribution was observed (not shown).

3.4. The RVS domains from Rvs161p and Rvs167p have distinct roles

Protein sequence analysis and comparison have revealed that Rvs161p and Rvs167p are quite similar proteins [2,3]. They both contain a RVS domain through which they interact in vivo [2,16]. To test further the possibility that these two RVS domains have similar functions, we constructed a chimeric Rvs161GPASH3 protein (Rvs167p-like) by addition of the GPA-SH3 domains of Rvs167p to the COOH extremity of Rvs161p. Moreover, the structure of the recombinant Rvs167ΔGPASH3 protein we used in the previous studies resembled the Rvs161p composed of the RVS domain only (Fig. 1). Since Rvs167p and Rvs161p have recently been proposed to form a complex in vivo [16], the co-expression of Rvs161GPASH3p (Rvs167p-like) and Rvs167ΔGPASH3p (Rvs161p-like) in the same cells may be considered to allow the formation of a similar complex.

Fig. 5 shows that the Rvs161GPASH3 protein expressed from pCDGPASH3 was able to restore a wild-type phenotype, as well as Rvs161p from pCD1S5, when expressed in the LG503-1A (*rvs161Δ*) mutant strain. This indicates that the addition of the GPASH3 domains to the carboxy-terminal part of Rvs161p did not alter the activity of Rvs161p. On the other hand, the expression of Rvs161GPASH3p in a *rvs167-1* genetic background (LG420-1A) did not improve the growth ability of the strain (Fig. 5). Although this protein has a structure similar to Rvs167p, it cannot functionally replace the wild-type Rvs167 protein. Moreover, a *rvs161* strain (LG502-1A) expressing the Rvs167ΔGPASH3 protein had a *rvs* phenotype (Fig. 5). This protein has no Rvs161p-like activity.

Finally, the co-expression of Rvs167ΔGPASH3p and Rvs161GPASH3p was obtained in a *rvs161 rvs167* double mutant strain (LG540-1A). These proteins were supposed to form a pseudo Rvs161p-Rvs167p complex in vivo. However, this strain showed a *rvs* phenotype (Fig. 5) thus indicating that this Rvs161p-like/Rvs167p-like complex is not functionally similar to the wild-type Rvs161p-Rvs167p complex.

Similar results were obtained when both the organization of the actin cytoskeleton and the budding patterns were observed (not shown).

		YNB	G1+E	YNBS	YNBGE	YNBT
X2180-1A (*RVS167*)		+	w	+	+	+
LG400-1A (*rvs167-1*)		+	r	-	-	-
LG300-1A (*rvs161Δ*)		+	r	-	-	-
LG503-1A (*rvs161Δ, ura3*)	pFL44	+	r	-	-	-
	pCD1S5	+	w	+	+	+
	pCDGPASH3	+	w	+	+	+
LG502-1A (*rvs161Δ, trp1*)	pUKC200	+	r	-	-	-
	pSP167	+	r	-	-	-
	pSP167ΔGPASH3	+	r	-	-	-
LG420-1A (*rvs167-1, ura3*)	pFL44	+	r	-	-	-
	pCD1S5	+	r	-	-	-
	pCDGPASH3	+	r	-	-	-
LG540-1A (*rvs161Δ, rvs167-1, ura3, trp1*)	pUKC200 pFL44	+	r	-	-	-
	pSP167 pFL44	+	r	-	-	-
	pUKC200 pCD1S5	+	r	-	-	-
	pSP167ΔGPASH3 pCDGPASH3	+	r	-	-	-
	pSP167 pCD1S5	+	w	+	+	+

Fig. 5. Phenotypic analysis of the expression of the recombinant Rvs167 and Rvs161 proteins in the (*rvs161*), (*rvs167*) and (*rvs161, rvs167*) mutant genetic backgrounds. The LG503-1A, LG502-1A, LG420-1A and LG540-1A strains were transformed with the pFL44, pUKC200, pSP167, pCD1S5, pSP167ΔSH3, pSP167ΔGPASH3, pSP167ΔGPA, pSP167ΔRVS and/or pCDGPASH3 plasmids. Growth of these strains was observed on YNB, YNBT (75 mM 3-A-T), YNBGE and YNBS (5% NaCl) media (+, growth; −, no growth). Cell viability was evaluated on G1+E medium by erythrosine staining (w, white colonies; r, red colonies).

4. Discussion

The *rvs161* and *rvs167* mutant strains present a wide range of apparently unrelated phenotypes from sensitivities to particular growth conditions to morphological defects. To understand which cellular mechanism could account for such pleiotropy, and therefore which role the Rvs161 and Rvs167 proteins play, we expressed modified Rvsp in *rvs161* and/or *rvs167* genetic backgrounds.

The phenotypes presented by the *rvs161* and *rvs167* mutant strains expressing the different modified Rvs proteins show that no specific link can apparently be established between anyone domain of the Rvsp and anyone *rvs* phenotype. For example, because it has been shown that Rvs167p interacts with the actin cytoskeleton through its GPA-SH3 region [7,8] and since several actin-binding proteins have similar structures, namely a GPASH3 domain at their C-terminal part [2], one could consider that the deletion of the SH3 domain would have affected only the organization of the actin cytoskeleton. At this time, although Rvs167p can be split into three clearly separated domains, our results suggest that it is not possible to consider it as a bi- or tri-functional protein. To take into account the fact that any mutation tested on the Rvs proteins gives all the *rvs* phenotypes, although with different intensities, we hypothesize that the Rvs proteins together play a unique role in a central function.

Considering both the pleiotropy of the *rvs* phenotypes and the actin-binding property of Rvs167p, regulation of the actin cytoskeleton organization could correspond to this central mechanism. This hypothesis is in agreement with the central role that the actin cytoskeleton is supposed to play in the cell, on the one hand, and with the phenotypes of the *act1* mutants that were obtained by charged to alanine systematic mutagenesis of the actin molecule, on the other [29]. Indeed, most of these actin mutants have pleiotropic phenotypes including sensitivity to high salt concentrations, reduced cellular viability, an endocytotic defect, an actin cytoskeleton and budding pattern alterations [5,6,29].

Although all the *rvs* phenotypes are obtained when only one domain is removed, except the GPA domain, the intensities of the phenotypes are clearly different. The SH3-deleted Rvs167 protein shows only a partial defect whereas the RVS-deleted form is fully mutant. This indicates that the RVS domain is essential for protein function whereas the SH3 domain is less important. The RVS domain seems to define a minimum functional part of the Rvs167p activity. The GPA-SH3 part of Rvs167p needs the RVS domain to be functional.

No phenotypical effect is detected when the GPA domain is removed, thus indicating that this domain could rather have a minor structural role, perhaps as a non-specific link between the two active RVS and SH3 domains, but without a noticeable impact on Rvs167p function. This has been somewhat confirmed in our lab by the construction of a tagged Rvs167 protein. Indeed, although a 122-amino-acid-long tag is inserted into the GPA domain, the tagged protein is still functional (unpublished data). On the other hand, no functional proteins were obtained when we tried to insert the tag either into the RVS domain or into the SH3 (unpublished data). Finally, it has been shown that Rvs167p is a member of the Rvs167p-like protein family of amphiphysins [9]. All these proteins contain an RVS domain and an SH3 domain at their N-terminal and C-terminal parts, respectively. The sequences located between these domains are quite different between the proteins. These structural features could indicate that both the RVS and SH3 domains have important conserved functions while the role of the central domain, GPA in the case of Rvs167p, could be specific for each member of the family.

Because the RVS domains of both Rvs161p and Rvs167p present significant similarities to each other, we exchanged these RVS domains between both proteins. The expression of the Rvs161p-like protein Rvs167ΔGPASH3p or the chimeric Rvs167p-like protein Rvs161GPASH3p in the *rvs* single and double mutant strains, showed that the RVS domains of both Rvs161p and Rvs167p cannot be functionally exchanged with each other. Furthermore, the co-expression of the Rvs161p-like and Rvs167p-like proteins, which is supposed to allow the formation of a Rvs167ΔGPASH3p-Rvs161GPASH3p complex quite similar to the wild-type Rvs161p-Rvs167p one, cannot restore a wild-type phenotype. Thus, although structurally comparable, these complexes are not functionally similar. Therefore, Rvs161p and Rvs167p each have distinct roles in a common functional complex.

Moreover, these results also show that the GPA-SH3 domains from Rvs167p do not modify the function of Rvs161p when located at the C-terminal part of this protein. This suggests that not only the GPA-SH3 domains of Rvs167p need an RVS domain to be active, but also that the latter must be that of Rvs167p. Hence, there is a specific functional link between both the RVS and SH3 domains of Rvs167p.

On the basis of the present results, some characteristics concerning Rvs protein function can be proposed. Although having distinct and non-redundant activities, Rvs161p and Rvs167p could in association play a unique role. Moreover, this role could be involved in the regulation of actin cytoskeleton organization through the GPASH3 domain of Rvs167p. This SH3 function seems to be dependent on the RVS domain of Rvs167p. Because some of the *rvs* phenotypes are associated with particular growth conditions, the interaction between the SH3 domain, the RVS domain and the actin cytoskeleton could depend on environmental changes, perhaps through the phosphorylation of Rvs167p. Indeed, some preliminary results obtained in our lab indicate that Rvs167p is phosphorylated in vivo (unpublished results). However, the phosphorylation sites are still unknown. Of the 60 serines and threonines present on the Rvs167p sequence, two putative cAMP-dependent phosphorylation sites and two Pho85-dependent phosphorylation sites located in the GPA-SH3 domains are interesting, since the Ras/cAMP and the cyclin-dependent Pho85 kinase pathways in *S. cerevisiae* regulate many responses to nutrient limitation [2,30,31]. Identification of the phosphorylation sites may not only shed light on the kinase pathway but also on intramolecular mechanisms of interaction between domains of the Rvsp complex.

Acknowledgements: This work was supported by the Centre National de la Recherche Scientifique, the Université Victor Ségalen-Bordeaux2 and the Conseil Régional d'Aquitaine. P.S. was supported by a grant from the French Ministère de la Recherche et de la Technologie.

References

[1] Crouzet, M., Urdaci, M., Dulau, L. and Aigle, M. (1991) Yeast 7, 727–743.
[2] Bauer, F., Urdaci, M., Aigle, M. and Crouzet, M. (1993) Mol. Cell. Biol. 13, 5070–5084.

[3] Sivadon, P., Bauer, F., Aigle, M. and Crouzet, M. (1995) Mol. Gen. Genet. 246, 485–493.
[4] Munn, A.L., Stevenson, B.J., Geli, M.I. and Riezman, H. (1995) Mol. Biol. Cell 6, 1721–1742.
[5] Kübler, E. and Riezman, H. (1993) EMBO J. 12, 2855–2862.
[6] Yang, S., Ayscough, K.R. and Drubin, D.G. (1997) J. Cell Biol. 136, 111–123.
[7] Amberg, D.C., Basart, E. and Botstein, D. (1995) Nature Struct. Biol. 2, 28–35.
[8] Lila, T. and Drubin, D.G. (1997) Mol. Biol. Cell 8, 367–385.
[9] Butler, M.H., David, C., Ochoa, G.C., Freyberg, C., Daniell, L., Grabs, D., Cremona, O. and De Camilli, D. (1997) J. Cell Biol. 137, 1355–1367.
[10] Lupas, A., Van Dyke, M. and Stock, J. (1991) Science 252, 1162–1164.
[11] Drubin, D.G., Mulholland, J., Zhu, Z. and Botstein, D. (1990) Nature 343, 288–290.
[12] Goodson, H.V. and Spudich, J.A. (1995) Cell. Motil. Cytoskeleton 30, 73–84.
[13] Desfarges, L., Durrens, P., Juguelin, H., Cassagne, C., Bonneu, M. and Aigle, M. (1993) Yeast 9, 267–277.
[14] Revardel, E., Bonneu, M., Durrens, P. and Aigle, M. (1995) Biochim. Biophys. Acta 1263, 261–265.
[15] Sivadon, P., Peypouquet, M.F., Doignon, F., Aigle, M. and Crouzet, M. (1997) Yeast 13, 747–761.
[16] Navarro, P., Durrens, P. and Aigle, M. (1997) Biochim. Biophys. Acta, in press.
[17] Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Bonneu, M., Crouzet, M., Urdaci, M. and Aigle, M. (1991) Anal. Biochem. 193, 225–230.
[19] Birnboim, H.C. and Doly, T. (1979) Nucleic Acids Res. 7, 1513–1523.
[20] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[21] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[22] Okayama, H. and Berg, P. (1982) Mol. Cell. Biol. 2, 161–170.
[23] Bonneaud, N., Ozier-Kalogeropoulos, O., Li, G., Labouesse, M., Minvielle-Sebastia, L. and Lacroute, F. (1991) Yeast 7, 609–615.
[24] Pringle, J.R. (1991) Methods Enzymol. 194, 732–735.
[25] Adams, A.E.M. and Pringle, J.R. (1984) J. Cell Biol. 98, 934–945.
[26] Kilmartin, J.V. and Adams, A.E.M. (1984) J. Cell Biol. 98, 922–933.
[27] Freifelder, D. (1960) J. Bacteriol. 80, 567–568.
[28] Durrens, P., Revardel, E., Bonneu, M. and Aigle, M. (1995) Curr. Genet. 27, 213–216.
[29] Wertman, K.F., Drubin, D.G. and Botstein, D. (1992) Genetics 132, 337–350.
[30] Thevelein, J.M. (1994) Yeast 10, 1753–1790.
[31] O'Neill, E.M., Kaffman, A., Jolly, E.R. and O'Shea, E.K. (1996) Science 271, 209–212.