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Genetics

Genetic evidence for the roles of the bud-site-selection genes *BUD5* and *BUD2* in control of the Rsr1p (Bud1p) GTPase in yeast

(cell polarity/GTPase-activating protein/GDP-dissociation stimulator/ras/*Saccharomyces cerevisiae*)

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ABSTRACT **Yeast cells normally display either an axial (for *MAT*a or *MATα* cells) or bipolar (for *MAT*a/*α* cells) pattern of bud-site selection. The *RSR1* gene, which was previously identified as a multicopy suppressor of Ts⁻ mutations in the bud-emergence gene *CDC24*, encodes a GTPase of the Ras family that is required for both budding patterns. Mutations in Rsr1p that presumably block its ability to bind or hydrolyze GTP cause a randomized budding phenotype, suggesting that regulators of Rsr1p will prove to be required for proper bud positioning. The *BUD5* gene product is required for proper bud-site selection and contains similarity to GDP-dissociation stimulators (GDS) for Ras-type proteins, suggesting that Bud5p may be a GDS for Rsr1p. Here I report that *BUD5* is required for wild-type *RSR1*, but not for mutationally activated *rsr1*^{val12}, to serve as a multicopy suppressor of *cdc24*, indicating that Bud5p functions as a GDS for Rsr1p *in vivo*. To identify the GAP (GTPase-activating protein) for Rsr1p, a genetic selection was designed based on the observation that mutationally activated *rsr1*^{val12}, but not wild-type *RSR1*, can serve as a multicopy suppressor of yeast *RAS2*(Ts) mutants. Mutants were selected that allowed wild-type *RSR1* to act as a multicopy suppressor of *RAS2*(Ts). Two such mutations proved to be in the *BUD2* gene, suggesting that Bud2p functions as a GAP for Rsr1p *in vivo*.**

The orientation of cell division in the yeast *Saccharomyces cerevisiae* is determined by the position of the bud. Yeast cells normally display either of two patterns of bud-site selection. When only *MAT*a or *MATα* mating-type information is expressed, daughter and mother cells bud adjacent to the site of the previous cytokinesis (axial pattern). When both *MAT*a and *MATα* mating-type information are expressed (as in a normal diploid), daughters bud at a position opposite from the site of the previous cytokinesis, and mothers bud either adjacent to the site of the previous cytokinesis or at the opposite pole of the cell (bipolar budding) (1, 2).

The *RSR1* (or *BUD1*) gene encodes a GTPase of the Ras family that is required for both budding patterns (3, 4). Cells that express either a mutant version of Rsr1p predicted to be defective in GTPase activity (Rsr1p^{Val12}) or one predicted to interfere with the exchange of GDP for GTP on wild-type Rsr1p (Rsr1p^{Asn16}) display a random pattern of budding, suggesting that the cycling of Rsr1p between its GDP- and GTP-bound states is required for proper positioning of the bud site (5). An understanding of how bud-site selection is effected therefore will require elucidation of the mechanisms by which the GTPase cycle of Rsr1p is controlled. Based on analogies with other GTPases, the existence of at least two proteins that regulate Rsr1p can be predicted: a GDP-dissociation stimulator (GDS) to stimulate the exchange of GTP for bound GDP and a GTPase-activating protein (GAP) to stimulate the hydrolysis of GTP to GDP.

For the following reasons, the product of the *BUD5* gene is a strong candidate to be a GDS for Rsr1p: (*i*) *BUD5* was identified during a screen for multicopy suppressors of a dominant-negative *RAS2*(Ts) mutation (6). The only other gene identified in this way was *CDC25*, which encodes a GDS for Ras2p (6, 7). (*ii*) The sequence of Bud5p displays 20% identity with Cdc25p over the portion of Cdc25p that is required for its GDS activity (6, 8). (*iii*) Mutations in *BUD5*, like mutations in *RSR1*, give a randomized-budding phenotype (8). These observations are, however, also consistent with other possible models. For example, Bud5p might interact with, but not activate, Rsr1p, or Bud5p might function as a GDS for a GTPase other than Rsr1p. Similarly, sequence analysis, biochemical studies, and the randomized budding phenotype of *bud2* mutants suggest that the *BUD2* gene product may function as a GAP for Rsr1p (4, 9).

In this paper, I report the results of genetic experiments suggesting that Bud5p and Bud2p function as a GDS and a GAP, respectively, for Rsr1p *in vivo*.

MATERIALS AND METHODS

Yeast Strains. Y597 (*MAT*a *cdc24-4 bud5 ura3 leu2 trp1 his4*) is a segregant from a cross between Y145 (10) and Da2 (8). Strain RS60-15B is *MATα RAS2*(Ts) (*RAS2*^{val19,ala22}) *ura3 leu2 trp1 his3 ade2 ade8* (5). Strain 172 is *MAT*a *bud2 ura3 trp1 his4 HMRα HMLα* (J. Chant and I. Herskowitz, personal communication). Strain Y630 [*MAT*a *RAS2*(Ts) *ura3 leu2 ade2 ade3 his3*] is a segregant from a cross between RS60-15B and Y389. Y389 itself is a segregant from a cross between Y145 and Y367 (10).

Plasmids. YCp(BUD5) is pK1 (8) and contains *BUD5* in a *URA3-CEN4-ARS1* vector; YEp(RSR1) contains *RSR1* in a high-copy-number *LEU2*-2μm vector (5); YEp(rsr1^{val12}) is *rsr1*^{val12} in the same *LEU2*-2μm vector (5); and pPB117 contains *RSR1* in a high-copy-number *URA3*-2μm plasmid (5).

Media and Transformations. Standard rich [yeast extract/peptone/dextrose (YPD)], defined minimal (SD), and defined complete (SC) media were used (11). SC+5FOA is SC plus 5-fluoroorotic acid (1 mg/ml) (12). Yeast transformations were performed by the lithium thiocyanate procedure (13).

Assay for the Ability of *RSR1* to Serve as a Multicopy Suppressor of *cdc24*. Two independently derived transformants for each plasmid or pair of plasmids were grown at 23°C to saturation in SD supplemented with histidine, tryptophan, and/or uracil, and/or leucine (depending on which plasmid or plasmids were being selected). These cultures were then diluted 1:16 into SD medium. Five microliters of each diluted culture was spotted onto duplicate SC plates containing 1 M sorbitol. [The inclusion of 1 M sorbitol

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Abbreviations: GDS, GDP-dissociation stimulator; GAP, GTPase-activating protein; 5FOA, 5-fluoroorotic acid.

previously was found to be needed for the multicopy suppression of *cdc24* by *RSR1* at 36°C (ref. 3).] One plate was incubated in a water bath at 36°C for 40 hr; the other was incubated at 23°C for 40 hr.

Assay for the Ability of *RSR1* to Serve as a Multicopy Suppressor of *RAS2*(Ts). To assay suppression of the *RAS2*(Ts) mutation, cultures were grown to saturation at 23°C in SD medium supplemented with adenine, histidine, tryptophan, uracil, and/or leucine (depending on which plasmid was being selected) and then diluted 1:16 in YPD. Ten microliters of each diluted culture was then plated onto duplicate YPD plates. One plate was incubated in a water bath at 36°C for 40 hr; the other was incubated at 23°C for 40 hr.

Visualization of Bud Scars. Cultures were grown to near saturation in liquid YPD medium at 23°C and then stained with Calcofluor (200 μg/ml) and observed by fluorescence microscopy as described (14).

RESULTS

Testing Whether Bud5p Behaves as Expected for a GDS for Rsr1p. Although the nature of the interaction between Rsr1p and Cdc24p is not known, previous studies have indicated that the binding of GTP by Rsr1p is required for *RSR1* to serve as a multicopy suppressor of *cdc24* (5). Thus, if Bud5p functions *in vivo* to activate Rsr1p, then Bud5p function should be required for *RSR1* to act as a multicopy suppressor of *cdc24*. To test this idea, a *cdc24 bud5* mutant strain was tested for its ability to grow at 36°C after being transformed with a high-copy-number plasmid containing *RSR1*, and/or with a low-copy-number plasmid containing *BUD5* (see *Materials and Methods*). As shown in Fig. 1 (lanes 1–3), both plasmids are required for growth at 36°C, indicating that wild-type *BUD5* function indeed is required for *RSR1* to serve as a multicopy suppressor of *cdc24*. If the role that Bud5p plays in this suppression is to facilitate the exchange of GTP for GDP on Rsr1p, then mutationally activated Rsr1p^{val12}, by having a decreased rate of GTP hydrolysis, should be able to serve as a multicopy suppressor of *cdc24* even in the absence of Bud5p function. As shown in Fig. 1 (lane 4), *BUD5* indeed is not required for *rsr1*^{val12} to serve as a multicopy suppressor of *cdc24*.

Genetic Selection for Mutations Affecting the Rsr1p-GAP. To search for the gene that encodes Rsr1p-GAP, the following rationale was used. When expressed from a high-copy-number plasmid, Rsr1p^{val12}, by activating adenylyl cyclase, can suppress the Ts$^-$ phenotype caused by a dominant-interfering *RAS2*(Ts) allele (Fig. 2, lane 1; ref. 5). In contrast, wild-type *RSR1* normally cannot serve as a multicopy suppressor of *RAS2*(Ts) (Fig. 2, lane 2; ref. 5). However, because acquisition of a mutation that would destroy Rsr1p-GAP function would be predicted to be functionally equivalent to having an activating mutation in *RSR1* itself, wild-type *RSR1* was expected to be capable of serving as a multicopy suppressor of *RAS2*(Ts) in cells that had acquired a mutation in the Rsr1p-GAP gene. To search for such mutations, multiple cultures of *RAS2*(Ts) strain RS60-15B containing plasmid pPB117 (*RSR1* on a *URA3*-containing, high-copy-number plasmid) were incubated on YPD plates at 36°C for 3 days. Forty-two mutants that survived were isolated for further analysis. To test whether any of these mutants required pPB117 for suppression [as opposed to having acquired direct suppressors of *RAS2*(Ts)], the ability of each mutant to grow at 36°C on SC+5FOA medium was analyzed. [Because cells that contain wild-type *URA3* cannot survive in the presence of 5FOA (12), cells that require plasmid pPB117 for survival were expected to be unable to grow in 5FOA at 36°C.] Only two independently derived mutants, RG4.2 and RG6.3, were inviable at 36°C on SC+5FOA medium (data not shown). When cured of the plasmid by growth on SC+5FOA medium at 23°C, RG4.2 and RG6.3 were unable to grow at 36°C (Fig. 2, lanes 3 and 5), confirming that pPB117 was indeed required for the suppression. To determine whether the suppression in these strains was due to a genomic or a plasmid-borne mutation, RG4.2 and RG6.3 were cured of pPB117, transformed with plasmid YEp(RSR1), and tested for growth at 36°C. As shown in Fig. 2 (lanes 4 and 6), wild-type *RSR1* was able to serve as a multicopy suppressor of *RAS2*(Ts) in both strains, indicating that the mutation responsible for the suppression in each strain was genomic. This conclusion was also supported by the observation that the mutation in each strain is recessive (data not shown).

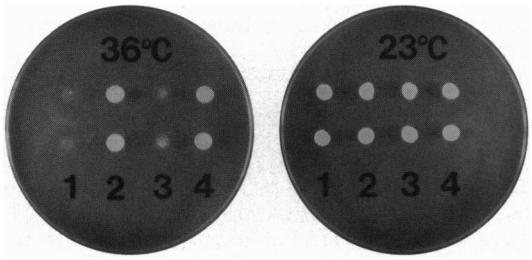

FIG. 1. Requirement for *BUD5* function in the multicopy suppression of *cdc24* by *RSR1* but not by *rsr1*^{val12}. The *cdc24 bud5* strain Y597 was transformed with high-copy-number vectors containing *RSR1* or *rsr1*^{val12} [plasmids YEp(RSR1) and YEp(rsr1^{val12}), respectively] and/or with a low-copy-number vector containing *BUD5* [plasmid YCp(BUD5)]. The resulting transformants were then tested for their ability to grow at 36°C and 23°C (see *Materials and Methods*). Lanes: 1, plasmid YCp(BUD5); 2, plasmids YCp(BUD5) + YEp(RSR1); 3, plasmid YEp(RSR1); 4, plasmid YEp(rsr1^{val12}).

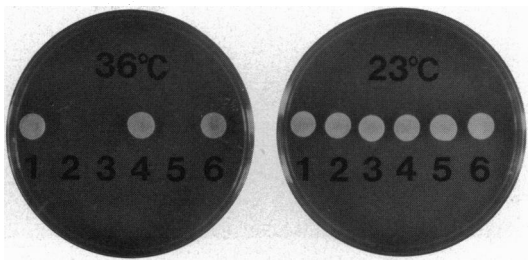

FIG. 2. Ability of RSR1 to serve as a multicopy suppressor of *RAS2*(Ts) in mutant strains RG4.2 and RG6.3. Growth of the following strains was assayed at 36°C and 23°C (see *Materials and Methods*). Lanes: Original *RAS2*(Ts) strain RS60-15B carrying plasmid YEp(rsr1^{val12}); 2, RS60-15B carrying plasmid YEp(RSR1); 3, mutant strain RG4.2 bearing no plasmid; 4, RG4.2 retransformed with YEp(RSR1); 5, mutant strain RG6.3 bearing no plasmid; 6, RG6.3 retransformed with YEp(RSR1).

Testing Whether Mutations Predicted to Affect Rsr1p-GAP Function Are in *BUD2*. Because cells that express mutationally activated Rsr1p^{val12} display a random pattern of budding, mutants lacking Rsr1p-GAP function were also expected to be defective for proper bud-site selection. Indeed, mutants RG4.2 and RG6.3 display a random budding pattern, in contrast to the normal axial pattern displayed by the parent strain (Fig. 3).

The *BUD2* gene was identified during a screen for mutations that alter bud-site selection (4). Recently, the sequence of *BUD2* has been determined and found to predict a product containing a Ras-GAP homology domain (9). In addition, biochemical experiments suggest that Bud2p has GAP activity on Rsr1p (9). To determine whether the mutations in strains RG4.2 and RG6.3 were allelic with *bud2*, genetic complementation and linkage analyses were performed. Diploids formed by crossing RG4.2 and RG6.3 with the *bud2* strain 172 were found to display random patterns of bud-site

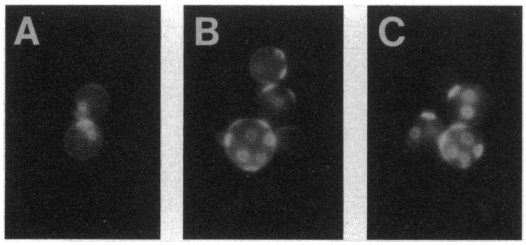

FIG. 3. Patterns of bud-site selection in wild-type and Rsr1p-GAP mutants. Cultures of the following strains were stained with Calcofluor to detect bud scars: Original *RAS2*(Ts) strain RS60-15B (*A*), mutant RG4.2 (*B*), and mutant RG6.3 (*C*).

selection, as did every segregant from six tetrads from each of these diploids (data not shown). These new *bud2* alleles are hereby designated *bud2-43* and *bud2-63*.

To confirm that the loss of Bud2p function itself (as opposed to a mutation in some other gene) was responsible for allowing wild-type *RSR1* to act as a multicopy suppressor of *RAS2*(Ts), strain RG6.3 containing plasmid YEp(RSR1) was crossed to *RAS2*(Ts) strain Y630, and tetrad analysis was performed on the resulting *MAT***a**/*MATα bud2-63*/*BUD2 RAS2*(Ts)/*RAS2*(Ts) strain. Of the segregants that inherited the plasmid (Leu^+), all 26 that displayed a random budding pattern were Ts^+, and all but one of the 26 that had a wild-type budding pattern were Ts^-. Thus, a mutation in *BUD2* is sufficient to allow wild-type *RSR1* to serve as a multicopy suppressor of *RAS2*(Ts), supporting the conclusion that Bud2p functions as a GAP for Rsr1p *in vivo*.

DISCUSSION

The functions of proteins can often be predicted from their sequences and from their biochemical properties as displayed *in vitro*. However, genetic studies provide the best test of whether the proteins play the expected roles *in vivo*. In the present study, the finding that *BUD5* function is necessary for wild-type *RSR1*, but not for mutationally activated $rsr1^{val12}$, to serve as a multicopy suppressor of *cdc24* suggests that Bud5p really does play a role in the activation of Rsr1p *in vivo*. Given that the inferred Bud5p protein sequence contains a Ras-GDS homology domain (6, 8), the simplest view is that Bud5p acts directly as a GDS for Rsr1p. Similarly, the identification of *BUD2* during a screen for mutations that enable wild-type *RSR1* to serve as a multicopy suppressor of *RAS2*(Ts) suggests that Bud2p acts as a negative regulator of Rsr1p *in vivo*. Given the sequence of Bud2p and the finding that Bud2p purified from yeast has GAP activity toward Rsr1p/Bud1p (9), the simplest interpretation is that Bud2p acts as a GAP for Rsr1p *in vivo*.

The *BUD1–BUD4* genes were identified previously during a screen for bud-site-selection mutants (4). The genes *CDC24*, which is required for both proper bud-site selection and bud emergence (15, 16), and *BUD5* were not identified during that screen, indicating that the screen had not been exhaustive and that there were probably other genes involved in bud-site selection that had not yet been identified. The finding that mutations isolated in a screen to identify the GAP for Rsr1p fell in one of the known *BUD* genes was therefore somewhat unexpected but leads to a simplifying view of the role of at least one set of the *BUD* genes. These genes had previously been organized into two groups: those that when mutated randomize bud positioning regardless of cell type, and those that when mutated have the more limited effect of causing cells that would normally display an axial budding pattern to display a bipolar budding pattern (4). The existence of these two specific classes of mutants has led to a model in which the default normal mode of budding gives the bipolar pattern, but there exists a set of genes required for effecting both the bipolar and axial budding patterns (4). It is precisely that set of *BUD* genes (*BUD1*, *BUD2*, and *BUD5*) that now are all implicated as components of the Rsr1p GTPase cycle. This result raises the possibility that with the exception of those proteins that are also required for other aspects of budding, all of the proteins that are required specifically for effecting nonrandom (axial and bipolar) patterns of bud-site selection may prove to be directly involved in either the processing of Rsr1p or the control of the Rsr1p GTPase cycle.

One general model for the role that Rsr1p plays in bud-site selection is that it facilitates the attachment of one protein (bud-initiator protein) that is required for the assembly of a bud site to a second protein (landmark protein) that marks the site at which the bud is to emerge. A variety of more specific models can be imagined in which either Bud5p or Bud2p colocalizes with the putative landmark protein. Because *RSR1* can act as a multicopy suppressor of *cdc24*, and *CDC24* is required both for proper bud-site selection and bud emergence, Cdc24p is a good candidate for the putative bud-initiator protein of this model. Given that Cdc24p contains a Dbl homology domain (17), and the corresponding domain of Dbl can serve as a GDS for human Cdc42p (18), it is likely that Cdc24p is itself a GDS for Cdc42p, a member of the Rho (Ras homologous) family of GTPases that is required for the initiation of bud formation (19, 20). Recent intimations that the regulation of Ras- and Rho-type GTPases in other systems may be tightly coordinated (21, 22) raise the possibility that the processes of bud-site selection and bud emergence may prove to be coupled through interactions between the regulators of the Rsr1p GTPase cycle and regulators of the Cdc42p GTPase cycle.

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