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Identification of a GTPase-Activating Protein Homolog in *Schizosaccharomyces pombe*

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Loss of function of the *Schizosaccharomyces pombe gap1* gene results in the same phenotypes as those caused by an activated *ras1* mutation, i.e., hypersensitivity to the mating factor and inability to perform efficient mating. Sequence analysis of *gap1* indicates that it encodes a homolog of the mammalian Ras GTPase-activating protein (GAP). The predicted *gap1* gene product has 766 amino acids with relatively short N- and C-terminal regions flanking the conserved core sequence of GAP. Genetic analysis suggests that *S. pombe* Gap1 functions primarily as a negative regulator of Ras1, like *S. cerevisiae* GAP homologs encoded by *IRA1* and *IRA2*, but is unlikely to be a downstream effector of the Ras protein, a role proposed for mammalian GAP. Thus, Gap1 and Ste6, a putative GDP-GTP-exchanging protein for Ras1 previously identified, appear to play antagonistic roles in the Ras-GTPase cycle in *S. pombe*. Furthermore, we suggest that this Ras-GTPase cycle involves the *ral2* gene product, another positive regulator of Ras1 whose homologs have not been identified in other organisms, which could function either as a second GDP-GTP-exchanging protein or as a factor that negatively regulates Gap1 activity.

Schizosaccharomyces pombe has only one homolog of mammalian *ras* (14, 31). This gene, *ras1*, is essential for mating but not for vegetative growth (16, 30), whereas *Saccharomyces cerevisiae RAS1* and *RAS2* are essential for cell cycle progression (24, 40). *S. cerevisiae* Ras proteins regulate adenylyl cyclase activity (41), but *S. pombe* Ras1, like mammalian Ras proteins (5, 6, 26), does not (16). Despite these differences, genes involved in activation of Ras proteins, namely, *S. cerevisiae CDC25* (8, 11, 33) and *S. pombe ste6* (22), are homologous in the two distantly related yeasts. Furthermore, the product of *ral2*, whose deficiency causes phenotypes similar to those caused by the *ras1* mutation (18), has been proposed as an additional Ras1 activator in *S. pombe* (17).

The activity of Ras protein is thought to be regulated by both positive and negative factors (see reference 7 for a review). Mammalian cells have a protein that stimulates GTPase activity of Ras (GTPase-activating protein [GAP]) as a negative regulator (42–44) and may also function as an effector of Ras (1, 10, 29). Another protein, encoded by *NF1*, is homologous to GAP and can stimulate Ras GTPase activity (3, 9, 28, 46, 47). *S. cerevisiae IRA1* and *IRA2* encode proteins homologous to both GAP and *NF1* and function as negative regulators of Ras (37–39), but no evidence for their function as an effector of Ras has been obtained. While putative activators of *S. pombe* Ras1, namely, *ste6* and *ral2*, have been identified, no negative regulator of Ras1 has been reported for this eukaryotic microbe. During our attempts to identify elements that may interact with *S. pombe* Ras1, we identified a gene, *gap1*, which apparently regulates Ras1 negatively. The deduced *gap1* gene product has a region homologous to the catalytic domain of mammalian GAP (27, 42–44). We report here the characterization and genetic analysis of *gap1*, which suggest that *S. pombe* Gap1 is likely to be a negative regulator, but not a downstream effector, of Ras1.

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MATERIALS AND METHODS

Strains, media, genetic methods, and transformation of *S. pombe*. The *S. pombe* strains used in this study are listed in Table 1. The media (13, 20, 45) and general genetic methods (20) used for *S. pombe* have already been described. Mutagenesis of *S. pombe* cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was performed essentially as described before (23). Transformation of *S. pombe* cells was done by a high-efficiency protocol recently developed (32).

Cloning of *gap1*. *S. pombe* JZ429 (*h*^{90} *gap1-1 ade6-M210 leu1*) was transformed with a library constructed with *Sau*3AI partial digests of *S. pombe* genomic DNA in vector pDB248′ (4). Leu$^+$ transformants were tested for mating and sporulation by exposure to iodine vapor, which stains spores dark brown (20). Three plasmids with overlapping inserts, one of which is pST200-1 (Fig. 1), were recovered from transformants that formed zygotic asci. A subclone of pST200-1 carrying a 3.4-kb *Eco*RV-*Sau*3AI fragment was able to complement JZ429.

Nucleotide sequence determination. The DNA sequence of the 3.4-kb *Eco*RV-*Sau*3AI fragment was determined by using the dideoxy-chain termination method (35). Subclones for sequencing were generated by progressive deletion with exonuclease III and S1 nuclease (21) from clones in plasmid pUC119. Single-stranded DNA was prepared by using M13-KO7 as a helper bacteriophage. The region shown in Fig. 2 was sequenced in both directions at least once.

Gene disruption. One-step gene disruption (34) of *gap1* was carried out as follows. A 2.0-kb *Kpn*I-*Hin*dIII fragment was removed from the open reading frame (ORF), and an *S. pombe ura4*$^+$ cassette (19) was inserted. An *Xho*I-*Xba*I fragment carrying the disrupted gene was used to transform *S. pombe* JY878 (*h*^{90} *ade6-M216 leu1 ura4-D18*). Most of the stable Ura$^+$ transformants were sterile. Precise replacement of the wild-type allele by the *ura4*$^+$-disrupted gene was confirmed by Southern blot analysis.

Mating assay. Qualitative assay of mating was done by iodine vapor staining (20). Determination of mating effi-

TABLE 1. *S. pombe* strains used

Strain	Genotype
JY450	h^{90} *ade6-M216 leu1*
JY476	h^{90} *ade6-M210 leu1*
JY507	h^{90} *ras1::LEU2 ade6-M216 leu1*
JY878	h^{90} *ade6-M216 leu1 ura4-D18*
SP561[a]	h^{90} *ras1^{Val-17}<<LEU2 ade6-M210 leu1*
JZ265	h^{90} *ral2::LEU2 ade6-M216 leu1*
JZ429	h^{90} *gap1-1 ade6-M210 leu1*
JZ446	h^{90} *gap1::ura4$^+$ ade6-M216 leu1 ura4-D18*
JZ476	h^{90} *gap1::ura4$^+$ ral2::LEU2 ade6-M216 leu1 ura4-D18*
JZ482	h^{90} *gap1::ura4$^+$ ste6::ura4$^+$ ade6-M216 leu1 ura4-D18*
JZ495	h^{90} *gap1::ura4$^+$ ras1::LEU2 ade-M216 leu1 ura4-D18*
JZ510	h^{90} *ste6::ura4$^+$ ade6-M216 leu1 ura4-D18*

[a] The expression *ras1^{Val-17}<<LEU2* indicates that the *S. cerevisiae LEU2* gene is integrated in the vicinity of the *ras1^{Val-17}* allele on the chromosome. This strain was obtained from S. Nadin-Davis (30).

ciency was done in two ways. (i) Cells to be crossed were placed in a patch on plates for mating and incubated at 30°C for 24 to 48 h. Mating efficiency was calculated by counting the numbers of zygotes, asci, spores, and unmated cells in the mixture under a microscope. (ii) A strain to be tested and the mating partner carried the *ade6-M210* and *ade6-M216* markers, respectively, or vice versa. These two *ade6* markers complement intragenically (20). Cells were crossed, and the number of adenine prototrophs, i.e., diploid cells generated by mating, was determined. This method was less quantitative but was essential for detection of the low-frequency mating displayed by *gap1* or *ras1^{Val-17}* cells, which could not be detected by the first method.

RESULTS

Isolation of *gap1* mutants. *S. pombe* cells extend conjugation tubes in response to the mating factor secreted by the

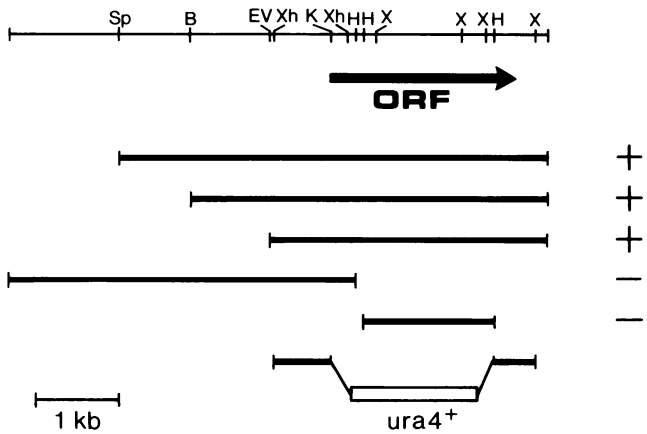

FIG. 1. Cloning and disruption of the *gap1* gene. A restriction map of the insert DNA in plasmid pST200-1 is shown with the extent and direction of the *gap1* ORF indicated by the arrow. Restriction sites: B, *Bam*HI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; Sp, *Sph*I; X, *Xba*I; Xh, *Xho*I. Restriction fragments of pST200-1 were subcloned in pDB248′ (4), and their abilities to complement JZ429 were tested. The ability of a subclone to rescue the mating deficiency of *gap1-1*-carrying cells is indicated as a plus or a minus. The structure of the linear fragment used to disrupt the *gap1* gene in vivo is shown at the foot of the figure. The open box stands for an *S. pombe ura4$^+$* cassette (19).

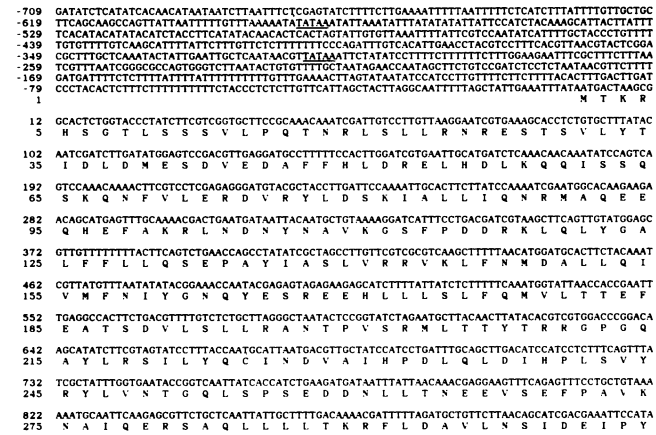

FIG. 2. DNA sequence and corresponding deduced amino acid sequence (single-letter code) of the *gap1* gene. The DNA sequence of a 3,392-base *Eco*RV-*Sau*3AI fragment that could encode a 2,298-base ORF is shown. The deduced amino acid sequence is shown below the DNA sequence. Numbering starts with the first methionine codon in the ORF. Possible TATA elements in the 5′ region of the gene are underlined.

mating partner, although the response of h^+ cells to the M mating factor is more apparent than that of h^- cells to the P mating factor (15, 25). Homothallic *ras1^{Val-17}* mutant cells are hypersensitive to the mating factor and form excessively elongated conjugation tubes, but successful mating is greatly impaired although not entirely inhibited (16, 30). We expected that mutations in elements that are involved in Ras1 activation could exhibit phenotypes similar to those of *ras1^{Val-17}* cells. Thus, we set out to isolate *S. pombe* mutants that exhibit such phenotypes.

Homothallic haploid cells were mutagenized and placed on malt extract-agar plates, on which wild-type cells proliferate to form small colonies and then undergo mating and sporulation because of a limited nitrogen supply. Sporulation-defective mutants were screened by exposure to iodine vapor, which stains spores dark brown (20), and mating-deficient but mating factor-hypersensitive mutants were chosen by inspection under a microscope. Thirteen mutants

TABLE 2. Amino acid identities between GAP homologs in the conserved core region[a]

Protein	% Identity with:			
	Ira1	Ira2	NF1	GAP
S. pombe Gap1	24.6	15.9	19.7	19.7
Ira1		51.7	25.3	22.5
Ira2			26.5	21.3
NF1				27.5

[a] The regions compared are shown as solid boxes in Fig. 3. Original sequence reports: Ira1, reference 37; Ira2, reference 39; NF1, references 9 and 47; GAP, references 43 and 44.

showing phenotypes similar to those of *ras1*$^{Val-17}$ cells were isolated among 80,000 mutagenized cells and genetically classified into three linkage groups (*sxa1*, *sxa2*, and *gap1*). Only one mutant (*gap1-1*) mapped in *gap1* in this analysis. Seven more mutant alleles of *gap1* were isolated as suppressors of the *ral2* mutation (see below).

Cloning of *gap1*. The *gap1* gene was cloned from an *S. pombe* genomic library by complementation of the *gap1-1* mutant with selection for mating proficiency. A restriction map of the original clone, pST200-1, is shown in Fig. 1. The essential region for complementation was delimited by subcloning. A 3.4-kb *Eco*RV-*Sau*3AI fragment was sufficient for complementation (Fig. 1). Nucleotide sequence analysis of this region revealed an ORF of 766 amino acids (Fig. 2). Northern (RNA) blot analysis showed that the *gap1* gene is transcribed into 3.1 kb of mRNA (data not shown).

The deduced amino acid sequence of Gap1 was compared with the EMBL/GenBank/DDBJ data base (Table 2). This gene product had the highest homology (19.7% overall amino acid identity) with *S. cerevisiae* Ira1 (37), which is a yeast homolog of mammalian GAP. It was obvious that *S. pombe* Gap1 has the conserved core sequence seen in mammalian GAP and its homologs (Table 2 and Fig. 3). *S. pombe* Gap1 has relatively short sequences flanking the conserved core, and these sequences are weakly homologous to *S. cerevisiae* Ira1 and Ira2 (37, 39) and human NF1 (9, 47) (Fig. 3). The implication of this structural feature will be considered in the Discussion.

Gene disruption. Disruption of the *gap1* gene by insertion of an *S. pombe ura4*$^{+}$ cassette was carried out in homothallic

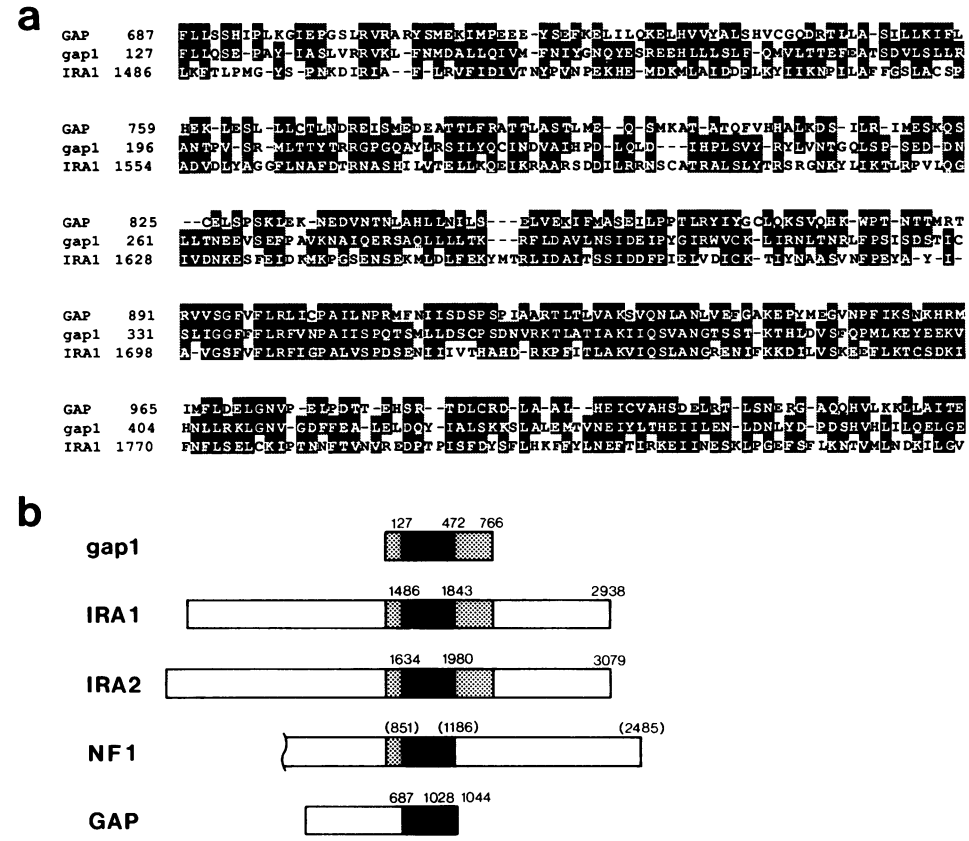

FIG. 3. (a) Comparison of amino acid sequences of the core regions in mammalian GAP, the *S. pombe gap1* gene product, and the *S. cerevisiae IRA1* gene product. Identical amino acids are shown in white against black, and conserved amino acids, in accordance with the mutation data of Dayhoff et al. (12), are shown in white against grey. (b) Schematic illustration of the protein structures of GAP homologs. The solid boxes represent conserved core sequences in which Gap1 and mammalian GAP have homology and regions in other homologs that correspond to them. This region is nearly identical to the catalytic domain assigned in mammalian GAP (27). The homology between each pair of the members in this core region is summarized in Table 2. IRA1 and IRA2 are weakly homologous to gap1 in regions flanking the core (10 to 20% amino acid identity), which are shown as dotted boxes. The leftward flanking region in NF1 also has weak homology to gap1. Outside of these regions, IRA1, IRA2, and NF1 share homology which is not indicated here. Relevant amino acid positions are numbered for each protein. The numbering for NF1 is tentative.

haploid cells by a gene replacement procedure (34), and *gap1*::*ura4*$^+$ derivatives were obtained. The structure of the linear fragment used to disrupt the *gap1* gene is shown at the foot of Fig. 1, and the experimental details are described in Materials and Methods. Precise replacement of the wild-type allele by the *ura4*$^+$-disrupted gene was demonstrated in some of the transformants by Southern blot analysis (data not shown). One such disruptant (JZ446) was fused with JZ429 by protoplast fusion (36), and sporulation was induced in resultant diploid cells. All of more than 500 progeny spores tested were sterile, indicating tight linkage of the disrupted gene and *gap1*.

Genetic interaction of *gap1* with other genes. The *gap1* disruptants showed the same phenotypes as the original *gap1-1* mutant, indicating that loss of *gap1* function confers phenotypes similar to those conferred by activated *ras1*. Cells disrupted in both *gap1* and *ras1* showed the same phenotypes as *ras1* disruptants and were completely sterile (Fig. 4). These observations suggest that the *gap1* null mutation causes its effects through Ras1 and that Gap1 can be an upstream negative regulator of the Ras protein but not an effector regulated by Ras1. The latter is a role proposed for mammalian GAP (1, 10, 29).

Two putative Ras1 activators have been identified in *S. pombe*. One of them, *ste6* (22), is homologous to *S. cerevisiae CDC25* (8, 11, 33), and its product is likely to be a GDP-GTP exchange protein for Ras1. It is unclear how the other, *ral2* (17), is involved in activation of Ras1. Null mutations in either of these genes made cells completely sterile and insensitive to the mating factor (Fig. 4). This insensitivity is suppressed by the activated mutations in *ras1* in both cases, and the resultant cells become weakly mating proficient (17, 22). We examined phenotypes of double mutants defective in *gap1* and *ste6* or *gap1* and *ral2*. Such mutants had essentially the same phenotype as *gap1* mutant cells (Fig. 4) and could mate, although very poorly. Thus, the *gap1* and activated *ras1* mutations were equivalent in these analyses. A *gap1 ste6 ral2* triple mutant also showed the same phenotype as *gap1* mutant cells (data not shown). These observations suggest that the *ste6* and *ral2* gene products are no longer necessary to fix the Ras protein in its GTP-bound form once Gap1 activity is lost.

We isolated eight independent *ral2* suppressor mutants that were weakly fertile and hypersensitive to the mating factor. Consistent with the above-described observations, seven of them had mutations in *gap1*, suggesting intimate interaction between *ral2* and *gap1*, while the eighth had acquired an activating mutation in *ras1* (data not shown).

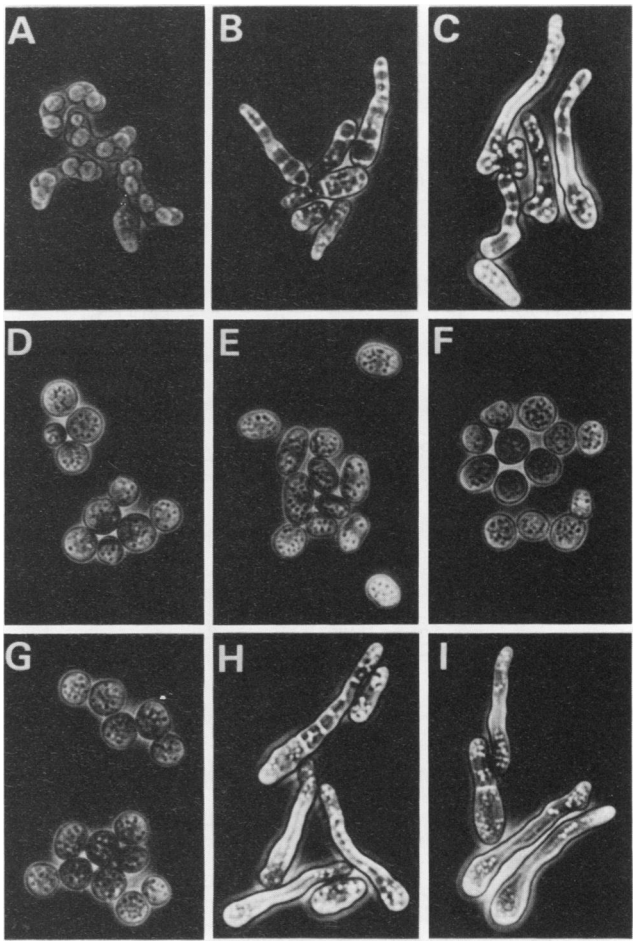

FIG. 4. Cell morphology of various mutants under nitrogen starvation. Panels: A, JY450 (wild type); B, SP561 (*ras1*$^{Val-17}$); C, JZ446 (*gap1* mutant); D, JY507 (*ras1* mutant); E, JZ510 (*ste6* mutant); F, JZ265 (*ral2* mutant); G, JZ495 (*ras1 gap1* mutant); H, JZ482 (*ste6 gap1* mutant); I, JZ476 (*ral2 gap1* mutant). All of the cells shown here are homothallic (h^{90}) haploid, and the mutations indicated stand for disrupted nonfunctional alleles, except for *ras1*$^{Val-17}$. Cells were placed on malt extract-agar plates which contained only a limited nitrogen source and incubated at 30°C for 2 days. Cell morphology was examined by phase-contrast microscopy, and photographs were taken. Mating and subsequent sporulation were successful in control A. The cells shown in panels B, C, H, and I extended conjugation tubes excessively, whereas the cells in panels D, E, F, and G were completely inert. Bar, 10 μm.

DISCUSSION

This work has demonstrated that *S. pombe* has a homolog of mammalian GAP. Genetic analysis strongly suggested that this homolog, Gap1, negatively regulates Ras1 activity. One feature of Gap1 was its small size (766 amino acids). *S. cerevisiae* GAP homologs, namely, Ira1 and Ira2, are much larger, having nearly 3,000 amino acids, and Gap1 corresponds only to their central domain. Mammalian GAP is larger than Gap1 by about 300 amino acids, and its N-terminal region carries two copies of the *Src* homology 2 domain, which has been suggested to be the site for interaction with activated growth factor receptors (2). *S. pombe* Gap1 does not have an *Src* homology 2 sequence. These structural differences suggest that regulation of *S. pombe* Gap1 activity, if any, is different from and probably simpler than that of the other GAP homologs. We found that the putative catalytic domain of Gap1 alone (Met-148 to Arg-492 [Fig. 2]) was sufficient to confer mating proficiency on *gap1* mutant cells (data not shown), although complementation by the truncated gene was slightly weaker than that by the intact gene. On the basis of these observations, we suggest that *S. pombe* Gap1 has a rather simple function not connected with the effector function, i.e., to down regulate the level of Ras GTP.

Combined with our previous finding that Ste6 is an activator of Ras1 homologous to *S. cerevisiae* Cdc25 (22), we can illustrate the Ras GTPase cycle in *S. pombe* as shown in Fig. 5. The cycle is essentially the same as that of *S.*

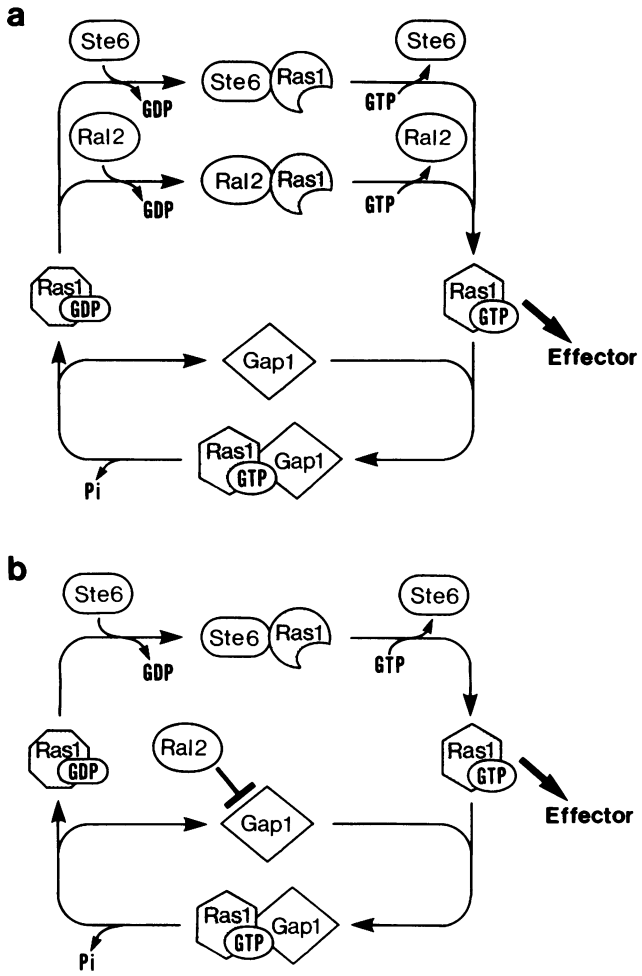

FIG. 5. Two alternative schemes for the Ras-GTPase cycle in *S. pombe*. Now that *ste6* has been identified as an activator (22) and *gap1* has been identified as a negative regulator of Ras1, *S. pombe* has basically the same GTPase cycle as that diagrammed for *S. cerevisiae* (7). Assumption of different roles for another putative Ras1 activator, *ral2*, leads to two possible schemes. (a) Ste6 and Ral2 both function as GDP-GTP exchange proteins for Ras1. Ste6 and Ral2 may respond to different signals and may activate Ras1 to different levels. (b) The *ral2* gene product can be a negative regulator of Gap1. This scheme is also consistent with the genetic data so far obtained.

cerevisiae (see reference 7), with *S. pombe ste6* corresponding to *S. cerevisiae CDC25* (8, 11, 33) and *S. pombe gap1* corresponding to *S. cerevisiae IRA1* and *IRA2* (37, 39), although the physiological role and the overall regulation of Ras are quite different in these two yeasts. However, since Cdc25 and Ste6 have regions with no similarity to each other over 600 amino acids and Ira1 and Ira2 carry long N- and C-terminal sequences which Gap1 does not have, it is conceivable that the two yeasts perform distinct regulation of Ras through these differences. Although little is known about the molecular nature of such regulation, we recently obtained evidence that *ste6* can convey the signal from environmental nitrogen starvation through its transcriptional activation (36a).

It has been reported that in *S. cerevisiae*, *ira1* or *ira2* and *cdc25* function as mutual suppressors (37, 38). However, the *gap1* mutation was epistatic to the *ste6* mutation in *S. pombe*, judging from the hypersensitivity of the cells to the mating factor. Even a combination of the *ste6* and *ral2* mutations did not alleviate the *gap1* mutation-induced phenotype. This suggests that loss of *gap1* function causes stronger effects than that of *IRA1* or *IRA2*. However, a more likely explanation for this is simply that Gap1 is the only Ras GTPase activator in *S. pombe* while Ira1 and Ira2 both function as GTPase activators in *S. cerevisiae*. It is essential to see the phenotype associated with *ira1 ira2 cdc25* to explore this point further, but no report has dealt with it so far.

The role of Ral2, another putative Ras1 activator, in *S. pombe*, is not clear. It appears unlikely that mutations in *ral2* cause effects by inhibiting expression of *ras1*, because point mutations in the *ras1* ORF can suppress *ral2* efficiently, although direct measurement of the amount of Ras1 protein in *ral2* mutant cells has not been done. On the basis of the genetic data obtained in this study, we propose two possible ways in which Ral2 may interact with the Ras GTPase cycle (Fig. 5). (i) Since the roles of *ste6* and *ral2* are genetically indistinguishable, the *ral2* gene product could be another GDP-GTP exchange protein for Ras1. It appears unlikely that Ste6 and Ral2 function cooperatively as a complex, because *ste6* and *ral2* mutant cells have different cell morphologies (Fig. 5). Thus, the Ste6 and the Ral2 pathways may have distinct roles in the activation of Ras1, for instance, responding to different signals and activating Ras1 to different levels. This hypothesis predicts that both pathways should be turned on to induce sensitivity to the mating factor, although we should admit that it has not been substantiated that sensitivity is regulated in a dual manner in *S. pombe*. (ii) The other possible scheme is that the *ral2* gene product causes positive effects on Ras1 because it negatively regulates Gap1 activity, which in turn negatively regulates Ras1 activity. This scheme explains the suppression of a *ral2* mutation by a *gap1* mutation. Because *ste6* and *ral2* encode proteins sharing no homology, this possibility is undoubtedly worthy of examination. However, as discussed above, it is unclear whether *S. pombe* Gap1 is subject to complex regulation. Thus, the role of Ral2 remains a puzzling and intriguing question. Further investigation of the two hypothesized schemes may shed more light on the activation mechanisms of Ras proteins.

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