**Biological activity of the mammalian RAP genes in yeast**

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We have screened expression libraries for mammalian cDNAs capable of suppressing defects in ras1∗ Schizosaccharomyces pombe. Both the RAP1A and RAP1B genes were identified in this manner. They suppress defects in cell morphology and sporulation, although not conjugation. In contrast, RAP genes do not suppress phenotypes in the yeast Saccharomyces cerevisiae that are deficient in RAS. Indeed, expression of RAP1A appears to antagonize the activated S. cerevisiae RAS2m18 gene. These results indicate that RAP proteins can interact with RAS targets, sometimes productively, sometimes nonproductively.

**Introduction**

The RAS genes are highly conserved in evolution. Homologs of the mammalian oncogenes are found in simple organisms such as the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*. The two RAS genes of the budding yeast, RAS1 and RAS2, have been studied extensively (Wigler et al., 1988). Despite striking similarities in structure and function between the mammalian and *S. cerevisiae* RAS proteins, the RAS effector signaling systems in the respective hosts probably differ (Birchmeier et al., 1985). In *S. cerevisiae*, RAS proteins regulate adenyl cyclase (Broek et al., 1985; Toda et al., 1985), although this may not be their only function (Toda et al., 1987b; Wigler et al., 1988). In mammals, the RAS effector pathways are unknown. A single RAS homolog, ras1, has been discovered in the fission yeast, *S. pombe* (Fukui and Kaziro, 1985). Ras1 does not appear to function on the adenyl cyclase pathway in *S. pombe* (Fukui et al., 1986). The mammalian H-ras gene can complement ras1∗ *S. pombe* (Nadin-Davis et al., 1986).

We have therefore begun to explore the usefulness of *S. pombe* for the study of the mammalian RAS pathway by searching for other mammalian genes capable of complementing phenotypic defects in *S. pombe* strains lacking the ras1 gene.

*S. pombe* cells lacking the ras1 gene are viable, but are defective in sporulation and conjugation, and have a round rather than an elongated morphology (Fukui et al., 1986). The sporulation phenotype can be readily scored by an iodine staining procedure: colonies containing spores stain brown (Nadin-Davis et al., 1986). We used a ras1∗/ras1∗ diploid strain to screen mammalian cDNA libraries cloned into an *S. pombe* expression vector for cDNAs capable of inducing sporulation. We have isolated several cDNAs by this method, including cDNAs encoding the human RAP1A and RAP1B proteins. The mammalian RAP genes are members of the RAS gene superfamily and were first identified by others by low-stringency hybridization (Pizon et al., 1988). They encode proteins which share extensive amino acid sequence homology with the mammalian oncogenic RAS proteins, particularly in the N-terminal domain. The RAP1A cDNA gene was independently isolated as K-rev-1 by its ability to interfere with malignant transformation by the K-ras oncogene (Kitayama et al., 1989). Our results suggest that the H-ras and RAP proteins can both stimulate some of the same effector pathways in *S. pombe*. Expression of the RAP1A protein in *S. cerevisiae* appears to interfere with the RAS2 function in that organism.

**Results**

*Construction of cDNA libraries*

Human cDNAs were made from the poly A+ mRNA of the glioblastoma cell line U118 MG, and, after ligation to Not I linkers, were inserted into the λ zap vector (see Materials and methods). To express the cDNAs in *S. pombe*, an *S. pombe* ADH promoter expression vector was used (see Figure 1). This vector, pAAUN, was adapted from the vector pART1, the generous gift of David Beach. pAAUN contains a Not I

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 restriction endonuclease cloning site just 3' to the S. pombe ADH promoter. The vector also contains the ura4 gene for selection in ura4- yeast, and the bacterial marker for ampicillin resistance for selection in Escherichia coli. cDNA inserts from the λ Zap library were excised by cleavage with Not I and size fractionated on sucrose velocity sedimentation gradients. cDNAs in the size range of 1.5–4 kb were collected, ligated into pAAUN at the Not I site, and transformed into E. coli strain DH5α. A library of complexity of ~10^6 was thus obtained. For optimum expression in S. pombe, cDNA inserts in this library must provide a methionine codon for translation of the proper open reading frame.

**Screening in S. pombe and identification of cDNA candidates**

A ras1^-ras1^- diploid strain, Sp565, was obtained from D. Beach. This strain was made by the self fusion of ras1^- haploid strain Sp525. DNA from the cDNA expression library was prepared and used to transform Sp565 by the lithium acetate method (Ito et al., 1983). About 10^6 transformants were screened and colonies capable of sporulating were identified by iodine staining (Nadin-Davis et al., 1986). The few staining colonies were picked and the plasmids they contained were rescued into E. coli. Most of the plasmids so obtained were capable of efficiently inducing sporulation when transformed back into Sp565. Several of these were grouped by restriction endonuclease mapping and representatives of various groups were sequenced. Two representative clones, pMRS1 and pMRS9, were sequenced in their entirety, and contained cDNAs which were identical in their coding sequences to the published coding sequences of cDNAs for RAP1B and RAP1A, respectively. The cDNA of RAP1A initiated 49 bp upstream of its ATG, and the cDNA for RAP1B initiated 27 bp upstream of its ATG.

**Properties of S. pombe expressing mammalian RAP genes**

We compared RAP1A and RAP1B genes to the human H-ras, S. pombe ras1 and S. pombe byr1 genes for the ability to suppress the phenotypic defects in ras1^- haploid and ras1^-/ras1^- diploid S. pombe strains. The byr1 gene encodes a protein kinase that has been reported to suppress the sporulation defects of ras1^-/ras1^- diploid strains, but not suppress the conjugation defects of ras1^- strains, nor revert the morphological abnormalities of either ras1^- or ras1^-/ras1^- strains (Nadin-Davis and Nasim, 1988). We have repeated all these results with our strains (data not shown). In contrast to byr1, the mammalian H-ras and the S. pombe ras1 gene each suppressed all of the phenotypic defects in ras1^- and ras1^-/ras1^- strains (see Figures 2 and 3). The RAP genes, like H-ras and ras1, suppressed defects in sporulation, as shown in Figure 2, and restored elongated morphology, as seen in Figures 2 and 3. Unlike H-ras and ras1 genes, however, the RAP genes were not able to restore defects in conjugation. This is seen in Figure 3. The ras1^- homothallic haploid strain Sp525 was transformed with various plasmids expressing RAS or RAP genes, and transformed colonies, grown on synthetic medium, were examined microscopically. When Sp525 was transformed with vector alone (panel A), it remained round and showed no evidence of conjugation or sporulation. When Sp525 was transformed with plasmids expressing either H-ras or ras1, normal morphology was restored and multiple sporulating zygotic cells were observed (panels D and E), evidence of conjugal activity. When Sp525 was transformed with plasmids expressing either RAP1A or RAP1B, morphology was restored, but no sporulating zygotes were observed, indicating that conjugation had not occurred.

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**Figure 1. Map of the S. pombe expression vector.** The expression vector pAAUN was derived from pART1 (McLeod et al., 1987) by replacing the leu2 gene with a 1.8-kbp HindIII fragment containing the S. pombe ura4 gene and adding Not I linkers at the Sma I site in the polylinker (PL) derived from pUC118 (Viera and Messing, 1987). The thin line indicates pUC118 sequence. The slashed box indicates an 1.2-kbp ARS sequence of S. pombe. The empty box indicates the 700-bp S. pombe ADH promoter. The direction of transcription from this promoter is counterclockwise. The prototrophic amino acid marker is indicated by the stippled boxes.
Properties of *S. cerevisiae* expressing mammalian RAP genes

We next tested if expression of the mammalian RAP genes could function on the RAS pathway in *S. cerevisiae*. For this purpose we inserted RAP cDNAs into an *S. cerevisiae* ADH1 expression vector, pADNS. A similar vector was used to express the human H-ras gene (Powers *et al.*, 1986). These plasmids were then tested for the ability to alter the phenotype of the *S. cerevisiae* strain SP1 (Kataoka *et al.*, 1984), which is wild type at RAS loci (MATα leu2 his3 ura3 his3 trp1 ade8 can1), the strain STS1 (Powers *et al.*, 1986), which is ras1- ras2ts (MATα his3 leu2 ura3 trp1 ras1- ras2ts), the strain S28.4B which is cdc25ts (MATα his3 leu2 ura3 trp1 ade8 can1R cdc25-1) (Hartwell *et al.*, 1973) and the strain TK161-R2V (Kataoka *et al.*, 1984), which expresses RAS2val19 (MATα leu2 his3 ura3 trp1 ade8 can1 RAS2val19). The STS1 strain first shows a temperature-sensitive growth defect at 36.5°C. CDC25 encodes a protein required for *S. cerevisiae* RAS function (Camonis *et al.*, 1986; Broek *et al.*, 1987). Strains that are RAS2val19, such as TK161-R2V, are typically sensitive to heat shock and nitrogen starvation, due to the activation of RAS targets by the constitutively activated mutant RAS2val19 gene (Kataoka *et al.*, 1984; Sass *et al.*, 1986).

Neither RAP1A nor RAP1B clones were able to restore wild-type growth at 36.5°C to the ras2ts strain STS1, although the H-ras cDNA clone was (data not shown). The temperature sensitivity of another ras2ts strain and a cdc25ts strain were similarly unaffected by RAP clones, although wild-type growth in both was restored by H-ras. Moreover, neither RAP1A nor RAP1B clones made wild-type cells heat-shock sensitive, although the H-ras cDNA clone did (see Figure 4). The RAP1A clone, but not the RAP1B clone, was able to slightly diminish the heat-shock–sensitive phenotype of the RAS2val19 strain (see Figure 5). Expression of RAP1A had no effect on the nitrogen starvation sensitivity of this strain.

These results suggest that RAP1A proteins can antagonize the action of RAS2val19, perhaps by interacting nonproductively with yeast RAS2 target proteins. However, from this data we cannot exclude the possibility that RAP proteins act on a pathway that opposes RAS. To test this hypothesis, we transformed the RAP1A expression vector into the strain RTF1.5prC, which has the genotype bcy1-tpk1-tpk2-tpk3-.

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This strain is heat-shock sensitive since it lacks the BCY1 gene, which encodes the regulatory component of the cAMP dependent protein kinase (Toda et al., 1987a). This strain contains disruptions of two of the three TPK genes, which encode catalytic components of the cAMP-dependent protein kinase (Toda et al., 1987b), and an attenuating mutation in the third TPK gene (Cameron et al., 1988). Hence RTF1.5prC is less heat-shock sensitive than the RAS2_{29619} strain, TK161-R2V, but more sensitive than wild-type strains. The strain TRF1.5prC is insensitive to activated RAS2_{29619}: it is not made more heat-shock sensitive by the expression of RAS2_{29619} (S. Cameron, unpublished data). This is presumably because the disruption of the BCY1 locus “uncouples” the major pathway by which RAS proteins influence the cell. Expression of RAP1A in this strain also has no effect on heat-shock sensitivity. This result suggests that RAP1A protein functions in S. cerevisiae only in strains which are sensitive to RAS proteins and thus does so by competing with RAS2_{29619} protein.

Discussion

There is a large family of low molecular weight proteins that resemble the RAS proteins in their GTP-binding domains. Some members of this superfamily are also highly homologous to RAS proteins in other domains (Chardin and Tavitian, 1986; Lowe et al., 1987; Pizon et al., 1988). The RAP proteins (RAP1A, RAP1B, and RAP2) are among these, since they share >50% identity with RAS overall (Pizon et al., 1988). The RAP1A gene has been independently isolated many times in the recent past: once by low-stringency hybridization with RAS gene probes (Pizon et al., 1988); once as a low molecular weight GTP-binding protein from brain (Kawata et al., 1988); once as a component of a purified neutrophil cytochrome complex (Quinn et al., 1989); once as a cDNA with the ability to partially suppress transformation by the K-ras oncogene (Kitayama et al., 1989); and in the present report. In our functional genetic screen, based on complementation in the yeast S. pombe, we have isolated cDNAs for RAP1A and RAP1B. In a continuation of this project, we have also isolated the RAP2 cDNA (work in progress).

Our results suggest that the mammalian RAP proteins can share effector function with the S. pombe ras1 protein. It is of interest to us that although expression of human H-ras can suppress all the phenotypic defects of ras1{superscript}+ S. pombe strains, expression of human RAP genes
Mammalian RAP in yeast

Figure 4. The effects of RAS and RAP expression plasmids on the heat-shock response of the S. cerevisiae wild-type strain SP1. Yeast cells were patched on synthetic medium plates (SC). Heat-shock experiments were performed by replica plating as described in Materials and methods. Plate I was replica plated onto a cold plate and not subjected to heat shock. Plates II, III, IV, V, and VI were replica plated onto preheated SC plates and subjected to further incubation at 55°C for 0, 15, 30, 45, and 60 min, respectively. In columns A, B, C, D, and E, SP1 was transformed with plasmids: (A) YEp13M4, a S. cerevisiae expression plasmid (Field et al., 1990); (B) pADNSRAP1B, a plasmid expressing the human RAP1B gene; (C) pADNSRAP1A, a plasmid expressing the human RAP1A gene; (D) pADH-H-ras, a plasmid expressing the human H-ras gene (Powers et al., 1986); and (E) pADH-RAS2val19, a plasmid expressing the S. cerevisiae RAS2val19 gene.

fails to correct defects in conjugation. One possible explanation of these observations is that RAS proteins have at least two independent effector functions in S. pombe, as we have found they do in S. cerevisiae (Wigler et al., 1988). RAP proteins might stimulate one effector function (required for morphology and sporulation) but not the other (required for conjugation). Other explanations are also possible.

In contrast to our results in the fission yeast, RAP proteins do not appear to stimulate the RAS pathways of the budding yeast, S. cerevisiae. They fail to complement temperature sensitive alleles of RAS2, and do not induce heat-shock sensitivity, as do both mammalian H-ras and activated S. cerevisiae RAS2val19 proteins. On the contrary, expression of RAP1A actually reduces somewhat the heat-shock sensitivity of RAS2val19 strains. Expression of RAP1A does not reduce the heat-shock sensitivity of yeast with activated cAMP-dependent protein kinases. The simplest interpretation of these results is that the RAP1A protein retains the ability to interact with RAS targets in S. cerevisiae, but that this interaction is ineffective, and thus RAP1A competes with activated RAS2val19 protein. Such nonproductive interactions with effectors might explain why RAP1A can partially block transformation of oncogenic RAS proteins in mammalian systems (Kitayama et al., 1989). We can only speculate on the reason why RAP1A fails to activate RAS pathways in S. cerevisiae. One possibility is that the RAP1A protein interacts with RAS targets but fails to stimulate them. Another possibility is that RAP1A interacts with RAS targets but causes their aberrant localization.

Recently, Frech et al. (1990) have reported that RAP1A protein can interact with mammalian GAP. GAP is a protein that interacts with mammalian RAS proteins to promote GTP hydrolysis (Trahey and McCormick, 1987). It is unknown if GAP is a target or regulator of RAS interactions (Adari et al., 1988; Hall, 1990). Our results indicate that mammalian RAP proteins can indeed interact with RAS targets. In some cases, they can stimulate these targets; in others, they fail to interact productively. In both budding and fission yeasts, the mammalian H-ras protein functions more like the endogenous RAS proteins than do the mammalian RAP pro-

Figure 5. Suppression of heat-shock sensitivity in the S. cerevisiae RAS2val19 strain TK161-R2V by expression of RAP genes. Yeast patches were grown on YPD plates for 2 d and then replica plated to fresh plates at 30°C (I) or to preheated plates and incubated at 55°C for 10 min before returning to 30°C (II). Recovery time at 30°C was 48 h. In columns A, B, C, and D, TK161-R2V was transformed with: (A) YEp13M4, a S. cerevisiae expression plasmid (Field et al., 1984); (B) pADNSRAP1B, a plasmid expressing the human RAP1B gene; (C) pADNSRAP1A, a plasmid expressing the human RAP1A gene; and (D) YEpPDE2, a plasmid expressing the S. cerevisiae cAMP phosphodiesterase gene (Sass et al., 1986).
teins, but it is quite clear that members of the RAS superfamily can be promiscuous in their target interactions.

Materials and methods

**Microbial manipulations and analysis**

*E. coli* strain DH5α (Hanahan, 1983) was used for plasmid preparation, isolation, transformation, and maintenance of the cDNA library (Hanahan, 1983). We used *S. pombe* strains kindly provided by D. Beach, Sp525 (h<sup>ad6</sup> leu<sup>1</sup> ural<sup>4</sup> ras<sup>1</sup> ) (Nadin-Davis et al., 1986) and Sp565, the Sp525/Sp525 diploid formed by the fusion of two haploid spheroplasts during a transformation procedure (Nadin-Davis and Nasim, 1988). The *S. cerevisiae* strains used were TK161-R2V (MATα leu2 his3 ura3 trp1 ade8 can1 RAS2<sup>W19</sup>) (Toda et al., 1985), STS1 (MATα his3 leu2 ura3 trp1 ade8 can1 ras1::URA3 ras2<sup>W19</sup> (Powers et al., 1986), SP1 (MATα leu2 his3 ura3 trp1 ade6 can1) (Kataoka et al., 1984) and RTF1.5 prC (MATα his3 leu2 ura3 trp1 ade8 tpk<sup>W19</sup> tpk2:: HIS3 tpk3::TRP1 bos1::URA3) (S. Cameron, unpublished data). Yeast strains were grown in either rich medium (YEAD, dextrose/yeast extract/adenine, for *S. pombe*; YPD, yeast extract/peptone/dextrose, for *S. cerevisiae*) or synthetic medium with appropriate auxotrophic supplements (PMA for *S. pombe*; SC for *S. cerevisiae*) (Nadin-Davis et al., 1986; Mortimer and Hawthorne, 1969). The iodine vapor staining was performed as previously described (Nadin-Davis et al., 1986). Heat-shock experiments were performed by replica plating onto preheated plates as previously described (Ito et al., 1983). *S. pombe* and *S. cerevisiae* strains were transformed by the lithium acetate procedure (Ito et al., 1983).

**Nucleic acid manipulation and analysis**

Poly A<sup>+</sup> mRNA was purified from the human glioblastoma cell line U-118 MG as previously described (Watson and Jackson, 1984). Double stranded cDNAs were prepared, ligated to Not I linkers, and cloned into the Not I sites of λ Zap. This library will be described in more detail in another publication (Colicelli et al., 1990; in preparation). The cDNAs were excised from the λ zap library by cleavage with Not I and size fractionated by velocity sedimentation in sucrose gradients. The 1.5- to 4.0-kbp fragments were collected and ligated into the Not I site in the *S. pombe* expression vector, pAAU. The vectors containing the 1.8-kbp MR51 and 1.3-kbp MR59 cDNAs were isolated from the extrachromosomal DNA of transformed strains of Sp565 as described (Nasmuth and Reed, 1980). These DNAs were transformed into *E. coli* (Holmes and Quigley 1981), and plasmid DNA was obtained from individual *E. coli* (Katz et al., 1973). Nucleotide sequencing was performed by the dideoxynucleotide chain-termination method by using oligonucleotide primers (Sanger et al., 1977; Biggin et al., 1983).

The cloning vector pAAUN was derived from plasmid pART1 (McLeod et al., 1987) by replacing the leu2 gene with a 1.8-kbp HindIII ura4 fragment from *S. pombe* and adding Not I linkers at the Smal I site (Figure 1). Plasmid pAAUHR was constructed by insertion of a 1.4-kbp BamHI-Bgl II *S. pombe ras1* gene fragment (Nadin-Davis et al., 1986) into the *BamHI* site of pRRT5. pRRT5 is a vector containing a 1.2-kbp *S. pombe* ARS fragment at the EcoRI site and a 1.8-kbp *S. pombe* ura4 fragment at the HindIII site of pUC18. Both pRRT5 and the plasmid containing *S. pombe ras1* gene were kind gifts from D. Beach. pAAUHR was constructed by inserting a 0.7-kbp Spe I-Sal I human H-ras cDNA fragment from plasmid pOFS-2 (Kataoka et al., 1985) into the Smal I site of pART1. The *S. cerevisiae* expression vector pADN5 (Colicelli et al., 1989) was used for constructing expression vectors for the human RAP1α and RAP1B cDNAs in *S. cerevisiae* and was named pADNSRAP1α and pADN2RAP1B, respectively. Yepl3M4 (Field et al., 1990), YeppDE2 (Sass et al., 1986), pADH-H ras (Powers et al., 1986), and pADH-RAS2<sup>W19</sup> were used as controls in transformations of *S. cerevisiae* strains. pADH-RAS2<sup>W19</sup> was prepared by inserting the 1.25-kbp Hpa I fragment of the *S. cerevisiae* RAS2<sup>W19</sup> gene into the blunt ended HindIII site of plasmid pLD95 (Powers et al., 1986).

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