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Cooperative Interaction of S. pombe Proteins Required for Mating and Morphogenesis

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Summary

We isolated two S. pombe genes, *scd1* and *scd2*, that are required for normal morphology and mating. scd1 and scd2 are homologous to CDC24 and BEM1, respectively, of S. cerevisiae. Epistasis analyses indicate that scd2 and ras1 converge upon scd1, which, in turn, interacts with cdc42sp, a RHO-like GTPase. Studies with the yeast two-hybrid system indicate that scd2 forms complexes with both scd1 and cdc42sp. Furthermore, biochemical studies indicate that the interaction between scd1 and scd2 is direct. The yeast two-hybrid data further suggest that scd1, scd2, cdc42sp, and ras1, in its GTP-bound state, act cooperatively to form a protein complex.

Introduction

In Schizosaccharomyces pombe, *ras1* is the single known homolog of the mammalian *RAS* genes (Fukui and Kaziro, 1985). Cells that lack *ras1* fail to agglutinate, conjugate, sporulate, or express the pheromone receptor P (mam2) and are round rather than elongated (Fukui et al., 1986; Nadin-Davis et al., 1986; Xu et al., 1994). One of the effectors of ras1 is byr2 (Van Aelst et al., 1993; Wang et al., 1991), a protein kinase homologous to the Saccharomyces cerevisiae STE11 (Neiman et al., 1993) and the mammalian MEKK (mitogen-activated protein kinase kinase kinase). Cells that lack byr2 fail to agglutinate, conjugate, sporulate, or express *mam2*, but have a normal cellular morphology (Wang et al., 1991; Xu et al., 1994). Therefore, ras1 must have at least one additional effector required for morphogenesis. To explore the functions of ras1, we have sought mutants with defects similar to those found in $ras1^{null}$ cells. Such an approach was first taken by Fukui and Yamamoto (1988) who isolated the *ral1*, *ral2*, *ral3*, and *ral4* mutants that are round and defective in sporulation and conjugation. Here, we report two genes (*scd1* and *scd2*) that, like *ras1*, are required for normal cell shape and conjugation, but unlike *ras1*, are not required for sporulation, agglutination, or *mam2* expression. Genetic experiments indicate that scd2 and ras1 converge upon scd1, which interacts with cdc42sp, a member of the RHO family of small GTPases (Fawell et al., 1992). We present data indicating that these S. pombe proteins form complexes that contain and are influenced by ras1.

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Results

Isolation of *scd1* and *scd2*

We mutagenized approximately 10^6 homothallic S. pombe cells of strain SP870 and selected the rare colonies of cells that did not mate, as determined by both iodine staining and microscopic examination. We selected for further study those cells that were also round. Cells that were judged by genetics to be defective in *ras1* were discarded. Six mutant strains (SP870M1–M6) fell into two classes: SP870M3, SP870M5, and SP870M6 had a more rounded morphology than SP870M2, SP870M1, and SP870M4. One mutant strain from each class (SP870M2 and SP870M3) was used to screen a plasmid genomic library for genes that could fully complement the defects of the mutants. Two different plasmids, pSCD1 and pSCD2, were isolated, containing genes subsequently named *scd1* and *scd2*, respectively (for shape and conjugation deficiency). pSCD1 could completely suppress the defects of only SP870M3, SP870M5, and SP870M6, while pSCD2 could completely suppress the defects of only SP870M1, SP870M2, and SP870M4. Deletion analysis of these plasmids was conducted to define their essential genetic regions (see Experimental Procedures).

Sequence Analysis of *scd1*

The sequence of *scd1* revealed an open reading frame (ORF) capable of encoding a peptide of 834 amino acids (Figure 1A). The entire ORF was cloned into the vector pALU, designed to express the ORF fused to the HA1 epitope (Field et al., 1988). The resulting plasmid, pALUSCD1, could restore the conjugation defect of SP870M3. Western blot analysis using monoclonal antibody to the HA1 epitope confirmed that these cells expressed a protein of about 100 kDa, the expected size of scd1. Hence, we conclude that this ORF represents the major coding block of scd1.

Protein sequences homologous to the scd1 ORF were identified by the FASTA program (Pearson and Lipman, 1988). We focused the analysis on proteins that had INITN (initial) scores at least 3-fold over the mean (= 36) and a minimum of 200 residues in the homologous regions. Nine sequences met these criteria, but one stood out, the CDC24/CLS4 protein from S. cerevisiae (Miyamoto et al., 1987; Miyamoto et al., 1991). It gave the highest score at 652. The identity between the scd1 and CDC24 is 32%. The MACAW program (Schuler et al., 1991; see Experimental Procedures) identified a total of 11 homologous blocks scattered over the entire region. These results indicate that scd1 and CDC24 are globally homologous. Another set of proteins with homology included the human BCR (Heisterkamp et al., 1985), the human DBL (Eva and Aaronson, 1985), the mouse and human VAV (Adams et al., 1992; Katzav et al., 1989), the mouse and rat RAS-GRF (Cen et al., 1992; Martegani et al., 1992), and the fly and mouse SOS (Shou et al., 1992; Simon et al., 1991),

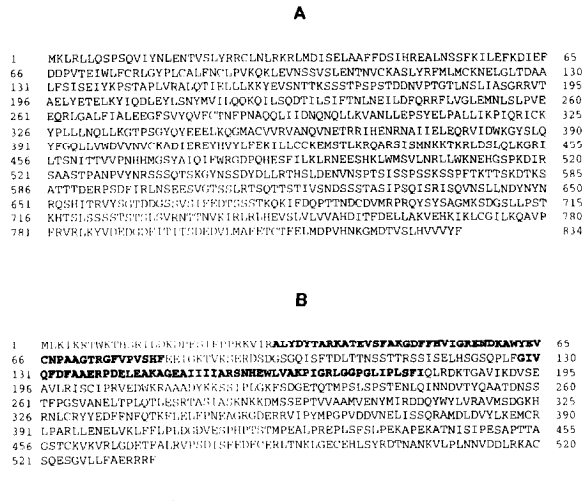

Figure 1. Peptide Sequences

The peptide sequences of scd1 and scd2 are shown in (A) and (B), respectively. The putative SH3 domains in scd2 are shown in bold.

with scores ranging between 131 and 254. These sequences are all homologous to residues 194–426 of scd1, and the identity within this region is about 20%. These sequences have also been found homologous to CDC24 as reported previously (Cen et al., 1992). All these proteins have been shown biochemically to interact with members of the RAS superfamily of GTP-binding proteins (reviewed by Downward, 1992). In particular, CDC24 has been shown to promote nucleotide exchange for the RHO-like G protein CDC42 (Zheng et al., 1994).

Sequence Analysis of *scd2*

scd2 contains a single intron, confirmed by cDNA analysis (see Experimental Procedures), and encodes a peptide of 536 amino acids (Figure 1B). The entire coding sequence was cloned into an expression vector, pARTCM, as a fusion to a c-MYC epitope (Evan et al., 1985). We confirmed that the candidate sequence suppressed the conjugation defect of SP870M2 and, by Western blotting, that a protein of about 60 kDa was produced from this vector. The FASTA program found an outstanding homologous coding region in the *BEM1* gene of S. cerevisiae (Chenevert et al., 1992), with a score of 643. The identity between scd2 and BEM1 coding regions is 36%. The MACAW program detected eight blocks of homology spread over the entire length of the two proteins. Both scd2 and BEM1 proteins contain two putative SH3 domains (in bold, Figure 1B; Rodaway et al., 1990).

Analysis of Mutant Alleles

Deletion of the chromosomal copies of *scd1* or *scd2* in wild-type strain SP870 produced cells with phenotypes indistinguishable from SP870M3 or SP870M2, respectively. During exponential growth, while the wild-type cells maintained an elongated shape (Figure 2A), both *scd1*^{null} and *scd2*^{null} cells (strains SPSCD1U and SPSCD2L, Figures 2B and 2C) were round. *scd1*^{null} cells were more nearly spherical, while *scd2*^{null} cells more closely resembled in shape the *ras1*^{null} cells (SPRN, Figure 2D). Starvation induced mating in wild-type cells, but the deletion mutants, in contrast, did not mate. As with the SP870M1–M6 mutants, *scd1*^{null} and *scd2*^{null} cells displayed no defect in growth rate, or in agglutination and sporulation, two responses to sex pheromones that are dependent upon ras1 and byr2. Additionally, deletion of both *scd1* and *scd2* caused a phenotype that was indistinguishable from that of the *scd1*^{null}. In further contrast with *ras1*^{null} and *byr2*^{null} strains, *scd1*^{null} cells had normal levels of expression of *mam2*, the gene encoding the receptor for mating pheromone P (Figure 3). In addition, overexpression of byr2 did not rescue the phenotype of the *scd1* or *scd2* mutant. Therefore, scd1 and scd2 appear to be exclusively involved in mating and maintaining normal morphology, through pathways distinct from those which are dependent upon byr2.

Protoplast fusions demonstrated that the mutant alleles of SP870M2, SP870M3, and the *scd1* and *scd2* deletions were all recessive. *scd1*^{null} and SP870M3 formed one complementation group while *scd2*^{null} and SP870M2 formed another (See Linkage Analysis in the Experimental Procedures). Tetrad analysis demonstrated that the mutations in SP870M3 and *scd1* were linked, as were the mutations in SP870M2 and *scd2*. Therefore, we named the mutant alleles in SP870M3 and SP870M2 as *scd1-1* and *scd2-1*, respectively. Strains SP870M3 and SP870M2 were chosen for detailed analyses.

Relation of *scd* Mutants to *ral* Mutants

Fukui and Yamamoto (1988) isolated four *ral* mutants that were abnormally round and sterile and reported the DNA sequence of *ral2* (Fukui et al., 1989). We found that the restriction maps for *scd2* and *ral3* were indistinguishable. Expression of scd2 from the genomic pSP2SCD2 restored the abnormal shape of a h^+ ral3^{null} JY806 (A gift from M. Yamamoto [Fukui and Yamamoto, 1988]), and the *ral3*^{null} and *scd2-1* mutations belonged to the same complementation group. These data indicate that *scd2* is the same gene as *ral3*. Furthermore, we determined that *scd1* and *ral1* encode the same protein (C. Kitayama and M. Yamamoto, personal communication).

Cross Species Complementation

We tested whether CDC24 and BEM1 (the S. cerevisiae homologs of scd1 and scd2, respectively, that are involved in bud formation) could also function in S. pombe to restore normal shape and conjugation. The vector expressing *CDC24*, pALUCDC24, restored conjugation and weakly improved the cell shape of the *scd1-1* mutant (also see Table 1 and discussion below). CDC24 also rescued those phenotypes in the *scd1*^{null} strain. The vector expressing *BEM1* (pARTCMBEM1), however, failed to restore conjugation or shape to *scd2-1* mutant. However, *scd2-1* mutant cells expressing *BEM1* do elaborate an extended structure suggestive of a conjugation tube.

Genetic Interactions among *ras1*, *scd1*, and *scd2*

We examined the effects of overexpressing *scd1* and *ras1* on the phenotypes of the *scd2-1* mutant cells (Table 1). Expressing *scd1* from pSCD1 restored detectable conju-

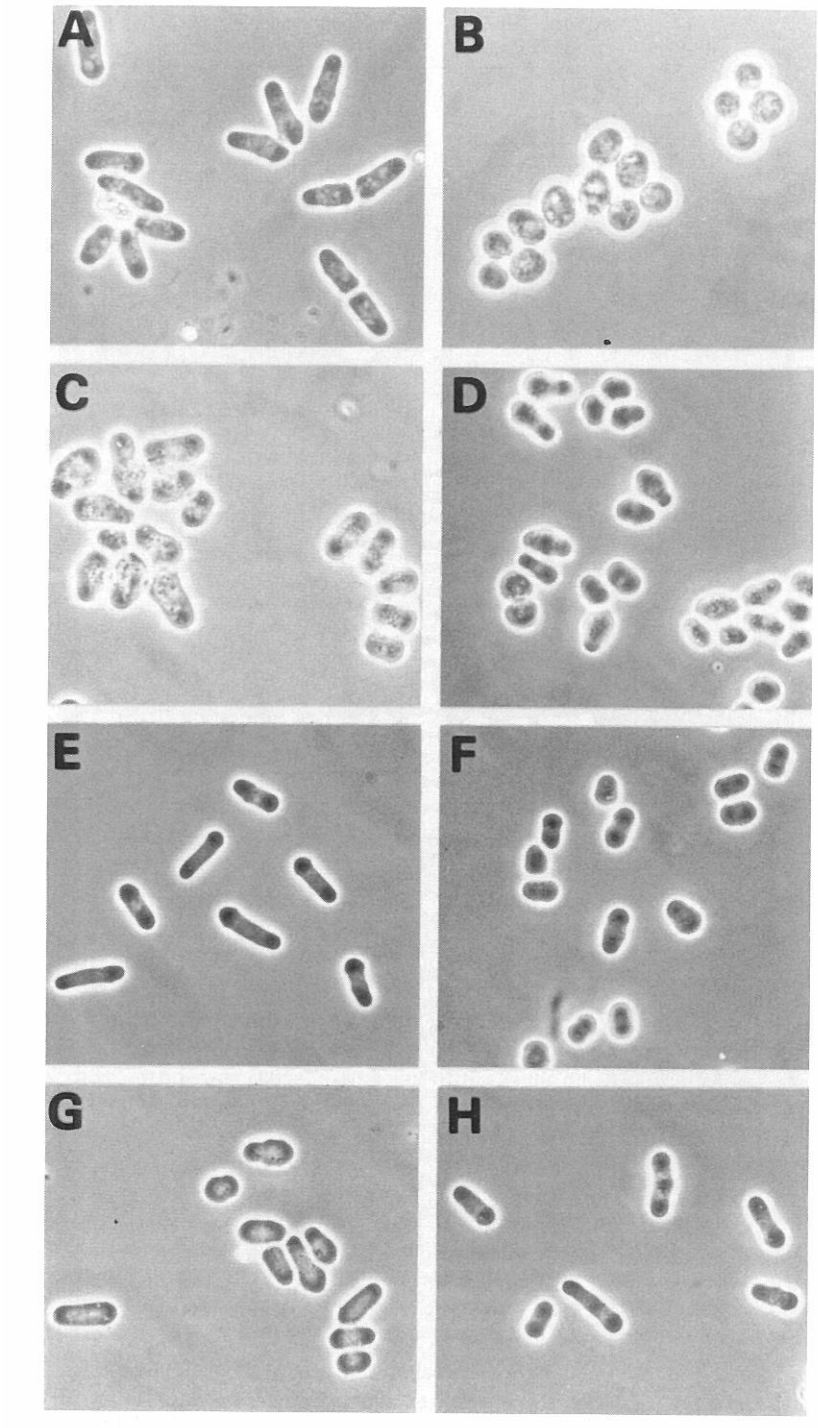

Figure 2. Phenotypes of Wild-Type, Mutant, and Suppressed Mutant Cells

Cells shown in (A)–(C) were grown in liquid rich medium (YEA) to mid-log phase ($\sim 2 \times 10^7$ cells/ml). Strains SP870 (the wild type), SPSCD1U (*scd1*^{null}), and SPSCD2L (*scd2*^{null}) are shown in (A), (B), and (C). In (D)–(H), SPRN (*ras1*^{null}) was transformed with various plasmids, and the transformed cells were examined as soon as the colony size reached 1 mm diameter. The plasmids used in this study were the following: pALU and pART1 (the control vectors) in (D), pART1 and pAUR (expressing ras1) in (E), pART1 and pALUCDC42SP (expressing cdc42sp) in (F), pALU and pSCD1 (expressing scd1) in (G), and pALUCDC42SP and pSCD1 in (H).

gation in the *scd2-1* mutant to 0.5% of the wild-type level and weakly improved its abnormal shape. Expression of *ras1* from the genomic clone pAUR (Xu et al., 1990) weakly complemented the defective mating and morphology of the *scd2-1* mutant. *ras1* and *scd1* functioned synergistically in suppressing the defects in the *scd2-1* mutant: the combination of both induced conjugation three times as effectively as the sum of each one expressed separately (2.4% versus 0.9%). In a separate study, we found that coexpression of *scd1* and *ras1* from pALASCD1 and pAUR, respectively, also suppressed the mating defect of the *scd2*^{null} cells to 3.1% of the wild-type level. Next, we tested whether expression of *scd1* and *scd2* could rescue the phenotypes of the *ras1*^{null} cells. Expressing *scd1* from pSCD1 and *scd2* from the genomic clone pSCD2, either singly or in combination, weakly improved the abnormal shape but not the defect in mating, sporulation, or agglutination seen in *ras1*^{null} cells. The effect of expressing *scd1*

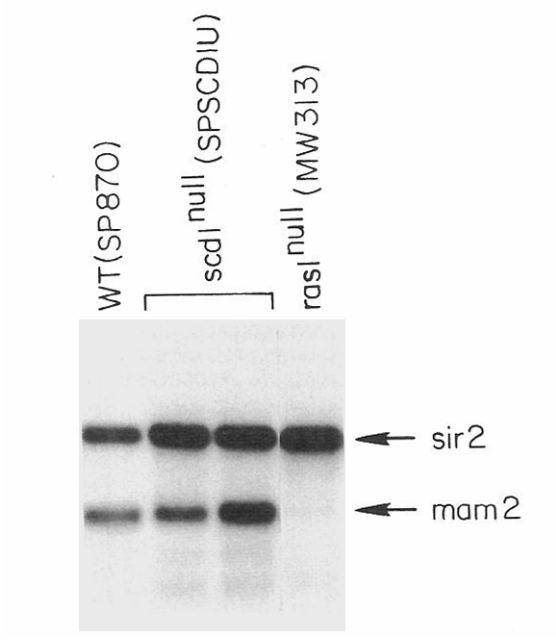

Figure 3. Detection of *mam2* Expression by Northern Blot Analysis

The strains analyzed, along with their relevant genotypes, are indicated on top of each lane. The detailed description of these strains, except SPSCD1U, has been reported previously (Xu et al., 1994). The expression of *sir2* is not altered under our experimental conditions and therefore is routinely used as a positive control. The bands corresponding to the *sir2* and *mam2* mRNA are marked by arrows. WT, wild type.

Table 1. The Effects of Overexpressing Genes on Mating in *scd1* and *scd2* Mutants[a]

	% Wild Type Mating Efficiency in the Indicated Strain[c]	
Genes Tested[b]	*scd1* Mutant	*scd2* Mutant
scd1	100.0	0.5
scd2	<0.5[d]	100.0
ras1	<0.5	0.4
scd1 and *ras1*	ND[e]	2.4
CDC24	8.7	ND
cdc42sp	<0.5	<0.1[d]
CDC24 and *cdc42sp*	27.2	ND
scd1 and *cdc42sp*	ND	4.9
None	<0.5	<0.1

[a] The mutant strains used were SP870M3 (*scd1-1*) and SP879M2(*scd2-1*).

[b] The vectors used were the following: pSCD1 (*scd1*), pSCD2 (*scd2*), pAUR (*ras1*), pALUCD24 (*CDC24*), and ALUCDC42SP (*cdc42sp*). pALU and pAL were the vector controls (None).

[c] Mating efficiency was scored microscopically for the presence of zygotic asci in a given population of cells. Three colonies of each transformed cells were patched on PM plates, and between 500–6000 cells from each colony were examined. The average mating efficiency in the *scd1* mutant rescued by pSCD1 was 19.5% (i.e., 19.5% of the cells had mated), which is defined as 100% wild-type mating efficiency. pSCD2 produced a mating efficiency of 14.2% in the *scd2* mutant. The wild-type strain SP870 mates at an efficiency around 18%.

[d] Zygotic asci were never observed in the *scd1* and *scd2* mutants. The indicated numbers, therefore were the detection limits of this experiment and were derived from examining around 1000 *scd1* mutant cells and around 6000 *scd2* mutant cells, respectively.

[e] ND, not determined.

on the morphology of the *ras1*^{null} cells is shown in Figure 2G. The phenotype of the *scd1-1* mutant was not improved by expressing *ras1* or *scd2* either singly or in combination.

Genetic Interactions between *cdc42sp* and *scd1*, *scd2*, and *ras1*

Previous studies indicate that defects in S. cerevisiae *CDC24* can be suppressed by *CDC42*, encoding a member of the RHO family of G proteins (Bender and Pringle, 1989; Johnson and Pringle, 1990). We investigated whether cdc42sp, a CDC42 homolog (Miller and Johnson, 1994), might suppress the defects in a *scd1-1* mutant. A vector expressing the cDNA for *cdc42sp* (Fawell et al., 1992), pALUCDC42SP, was constructed to express cdc42sp fused to the HA1 epitope under the control of the *adh* promoter. We noted that expressing cdc42sp alone had no effect on the shape or mating of the *scd1-1* mutant. As previously noted, CDC24 could partially suppress the mating defect of the *scd1-1* cells (8.7% versus <0.5%; Table 1), and we found that cdc42sp enhanced the effect of CDC24 in restoring mating (27.2% versus 8.7%). This combination also improved morphology more effectively.

Similarly, we found that expressing cdc42sp had no effect on the sterility and abnormal cell shape of the *scd2-1* mutant, but enhanced the effects of expressing scd1. As indicated in Table 1, the combination of cdc42sp and scd1 rescued the mating defect of the *scd2-1* mutant about 10-fold more efficiently than expressing scd1 alone (4.9% versus 0.5%), and the effect of the combination on morphology was also more profound. In a separate experiment, expression of both scd1 and cdc42sp together, using pALASCD1 and pALUCDC42SP, also suppressed the mating defect of the *scd2*^{null} strain (9.9%). Moreover, while the expression of cdc42sp alone did not rescue the phenotype of *ras1*^{null} cells (Figure 2F), cdc42sp in combination with either scd1 (Figure 2H) or scd2 improved the morphology of the *ras1*^{null} cells to almost that of wild-type cells. Suppression of the conjugation, sporulation, or agglutination defects of the *ras1*^{null} cells was not observed by overexpressing any combination of genes with *cdc42sp*.

To test the role of cdc42sp in the maintenance of cell shape and mating, we sought to block its function. Since cdc42sp has been shown to be essential for viability (Miller and Johnson, 1994), we decided to express an interfering form of cdc42sp, cdc42sp(T17N), containing an asparagine rather than a threonine at position 17. This substitution at analogous positions of other RAS-like GTPases has been shown to produce a mutant protein that inhibits the activity of exchange factors (Feig et al., 1986; Munder and Furst, 1992). We have found that overexpression of cdc42sp(T17N) using pALUT17N in wild-type strain SP870 caused a round morphology. Furthermore, overexpressing either cdc42sp or cdc42sp(T17N) markedly reduced conjugation. Cells containing cdc42sp(T17N) also grew more slowly.

Pairwise Interactions Detectable by the Two-Hybrid System

We investigated whether we could detect the formation of

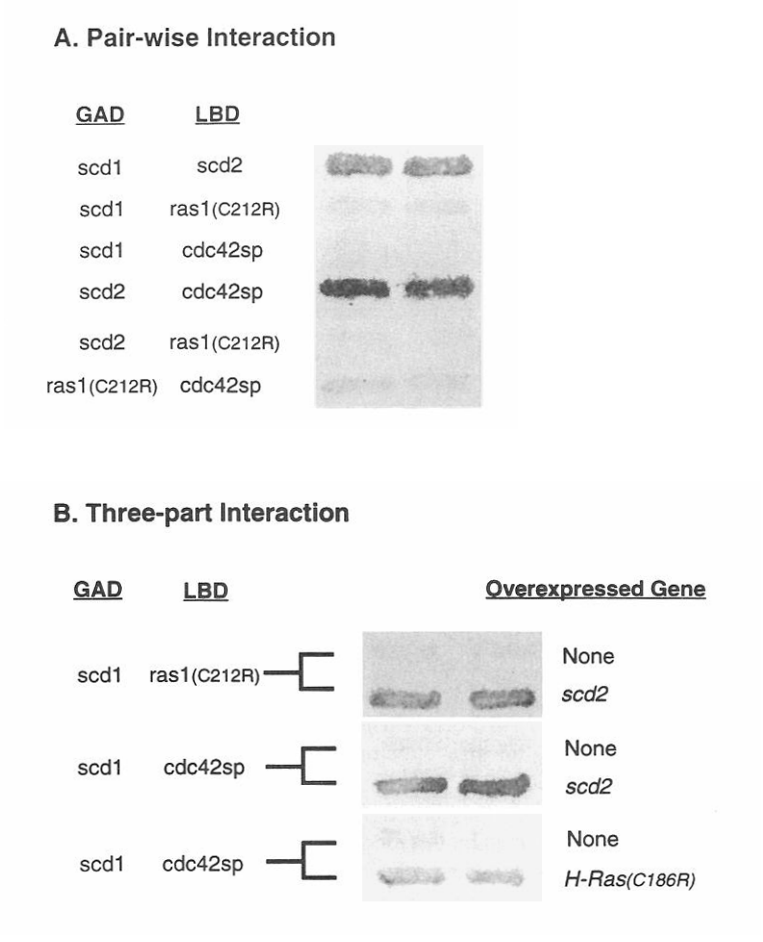

Figure 4. Protein Complex Formation Detected by the Two-Hybrid System

Reporter strain L40 containing the tested hybrids, shown to the left, were patched on Whatman 50 paper on which a β-Gal assay using X-Gal was performed. Those cells containing hybrids that interacted turned blue and therefore photographed darkly. The plasmids expressing GAD–scd1, GAD–scd2, LBD–scd2, LBD–cdc42sp, GAD–ras1(C216R), and LBD–ras1(C216R) were pGADSCD1, pGADSCD2, pLBDSCD2, pLBDCDC42SP, pGADR1, and pLBDR1, respectively. L40 cells in (B) were modified prior to the two-hybrid study by transformation with plasmids expressing the indicated genes, shown to the right. The plasmids used for overexpressing scd2 and H-RAS were pAASCD2 and pAHHR. The plasmid controls (lane marked None in [B]) were pAA and pAH. Cells shown in (C) contained GAD–scd1 and various forms of H-RAS as LBD fusion proteins. LBD–lamin was included as the negative control. The β-Gal activities in cell lysates were measured using ONPG and are shown next to the cell patches. The indicated activities were the average calculated from samples prepared from three independent transformants. SD, standard deviation. The vectors used were the following: pGADSCD1, pLBDHR, pLBDHRV, pLexA-RAS^{A15} (Vojtek et al., 1993), pLBDHRA35, pLexA-Lamin (Vojtek et al., 1993) for expressing scd1, H-RAS(C186R), H-RAS(G12V, C186R), H-RAS(T15A, C186R), H-RAS(T35A, C186R), and lamin, respectively.

Note that the C212R in (A) and (B) should be C216R.

complexes among scd1, scd2, cdc42sp, and ras1 proteins using the two-hybrid system of Fields and Song (1989). This study was carried out using the reporter strain L40 of S. cerevisiae (Vojtek et al., 1993). We performed our study by pairing hybrids that were fused to the DNA-binding domain of the LexA protein (LBD) and the transcriptional activation domain of the GAL4 protein (GAD). As shown in Figure 4A, transformants containing either the LBD–scd2/GAD–scd1 hybrids or the LBD–scd2/GAD–cdc42sp hybrids turned blue in a filter color assay. We did not detect any interaction between scd1 and cdc42sp. We found that none of the tested proteins interacted with the negative control, lamin (Vojtek et al., 1993). These results indicated that scd2 can complex with scd1 and with cdc42sp.

To test whether any of these three proteins could interact with ras1, we constructed the ras1 hybrid containing the mutant ras1(C216R), with a cystine to arginine mutation in the CAAX motif for membrane localization (Willumsen et al., 1984). As shown in our previous study, this alteration

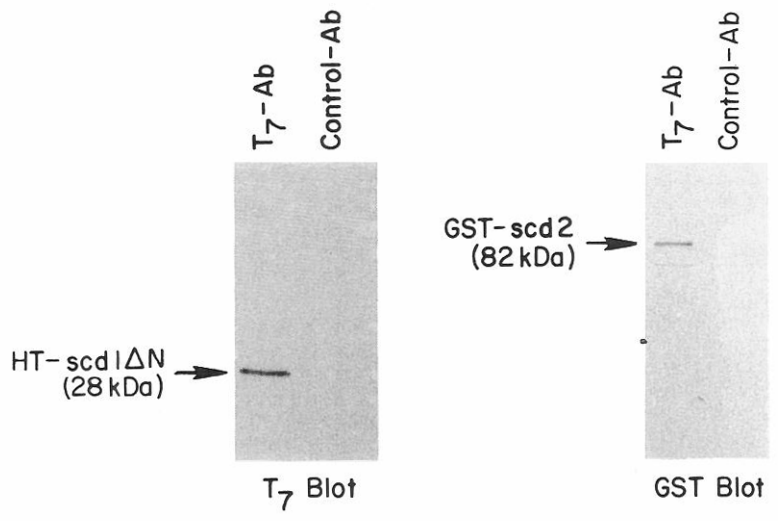

Figure 5. Protein Complex Formation Detected by Immunoprecipitation

Lysates containing GST–scd2 or HT–scd1ΔN were mixed and immunoprecipitated by indicated antibodies as shown on top of each lane. T7-Ab is the antibody directed against the T7 tag on HT–scd1ΔN, while Control-Ab is purified rabbit IgG used as the antibody control. The blot on the left was probed with the anti-T7 antibody, and the one on the right was probed with an antibody directed to GST. The bands corresponding to GST–scd2 and HT–scd1ΔN are marked by arrows, and their apparent molecular masses are also indicated.

can enhance the detection of interactions between RAS proteins and their effectors (Van Aelst et al., 1993). As a positive control for the functionality of the ras1(C216R) hybrid, we tested it against a byr2 hybrid. We found that this pair transactivated the *lex* reporter genes, and therefore formed a complex, as we previously reported (Van Aelst et al., 1993). scd1, scd2, or cdc42sp hybrid proteins, however, did not interact with the ras1(C216R) hybrid protein (Figure 4A).

Pairwise Interactions Induced by Third Proteins

Efficient interactions among these components might require three-part interactions. The effect of expressing scd2 on the formation of complexes was tested first (Figure 4B). When scd2 was expressed from pAASCD2, interaction between scd1 and ras1(C216R) and between scd1 and cdc42sp could be readily observed. Expression of scd2 did not induce nonspecific interaction between other hybrid proteins. The effects of scd2 on scd1 and cdc42sp can be readily explained by scd2 acting to bridge interactions between scd1 and cdc42sp proteins through distinct binding sites on scd2. We previously observed such "bridging" interactions through the use of the two-hybrid system. In particular, we demonstrated that overexpression of RAF, which has separate binding sites for H-RAS and MEK, could induce interaction between H-RAS and MEK (Van Aelst et al., 1993). Although the two-hybrid system detects protein complex formation in the nucleus, we speculate that either the overexpressed bridging protein is present at sufficient concentration in the nucleus, or else that a three-part complex is formed in the cytosol prior to its entry into the nucleus.

The effect of scd2 on interaction between ras1(C216R) and scd1 is not so readily explained, as we could not initially detect pairwise interactions between ras1(C216R) and scd1 or ras1(C216R) and scd2. Thus, scd1, scd2, or both proteins might contain cryptic binding sites for ras1(C216R). This hypothesis was strengthened when a truncated form of scd1, scd1ΔN, lacking the first 671 residues from the N-terminus, that could interact with ras1(C216R) was found by screening a million S. pombe cDNA clones in the two-hybrid system. The only other positive from the screen was a cDNA encoding byr2.

RAS proteins in the GTP-bound state have a higher affinity for their effectors, and hence we tested whether the full-length scd1 would interact better with RAS proteins when they are GTP bound. Human H-RAS protein was chosen for this study because its interaction with guanine nucleotides has been well studied in S. cerevisiae, and H-RAS can fully complement the loss of ras1 in S. pombe (Nadin-Davis et al., 1986). C186R mutation was introduced to the CAAX motif of H-RAS to enhance the detection of interactions. We studied the effects of H-RAS(C186R), H-RAS(G12V, C186R), H-RAS(T15A, C186R), and H-RAS(T35A, C186R). The first two are inferred to be predominantly GTP bound when expressed in S. cerevisiae (Ballester et al., 1989), and, as shown in Figure 4C, both interacted well with scd1. H-RAS(T15A, C186R), with a mutation that blocks its activation (Powers et al., 1991), and H-RAS(T35A, C186R), with a mutation that disrupts RAS interactions with its known effectors (Sigal et al., 1986), both failed to interact with scd1. We found that all H-RAS hybrids interacted well with the CDC25 hybrid (L. Van Alest, unpublished data; Vojtek et al., 1993). These results suggest that scd1 interacts with RAS preferentially in the GTP-bound state; moreover, this interaction requires a functional effector loop on RAS. In keeping with our results with the H-RAS proteins, we found that the activated ras1(G17V, C216R) showed a weak but detectable interaction with scd1.

We next examined whether H-RAS could affect interactions between full-length scd1 and cdc42sp. Mammalian H-RAS expressed from pAHHR, which did not induce nonspecific two-hybrid interactions, clearly induced complex formation between full-length scd1 and cdc42sp (Figure 4B). In a separate experiment, we found that the isolated scd1ΔN, in contrast with full-length scd1, could also interact unassisted with cdc42sp. These data support the hypothesis that scd1 contains a cryptic binding site for cdc42sp that is enhanced by binding RAS.

Determination of Complex Formation by Immunoprecipation

We sought to express both scd1 and scd2 in Escherichia coli to test whether their interaction was direct. The scd2 was expressed as a GST fusion protein (GST–scd2), and it was soluble. The full-length scd1 fusion protein, however, was not. We learned, through the use of the two-hybrid system, that scd1ΔN retained the ability to interact with scd2. The fusion protein HT–scd1ΔN, comprised of polyhistidine, the leader peptide of the T7 major capsid protein, and scd1ΔN, was soluble. We then mixed lysates from cells expressing GST–scd2 and HT–scd1ΔN, and the mixed lysate was incubated with a monoclonal antibody against the T7 peptide (T7-Ab, Figure 5) that was covalently coupled to protein A beads. The immunoprecipitates were split in two, and both were analyzed by Western blotting. These blots were probed with antibody either directed to the T7 tag (T7 Blot, Figure 5), which detected the presence of HT–scd1ΔN, or probed for GST (GST Blot, Figure 5), which detected the coprecipitated GST–scd2. Purified rabbit IgG was used as the antibody control (Control-Ab, Figure 5), and it did not precipitate any of the tested fusion proteins. No coprecipitation was observed between T7 tagged scd1 and other GST fusion proteins, such as GST–STE11ΔC, or between GST–scd2 and other T7-tagged fusion proteins, such as His–STE11ΔC (data not shown; see Marcus et al., 1994 for the preparation of GST–STE11ΔC and His–STE11ΔC). We conclude that scd2 and scd1 interact directly.

Discussion

We have isolated *scd* mutants with morphogenic and mating defects similar to those observed in *ras1*^{null} cells, but without the ras1-associated defects in gene expression, sporulation, and agglutination; and we have isolated and characterized the corresponding *scd* genes. Fukui and Yamamoto (1988) previously identified mutants in the *ral* loci, which were characterized as having the same phenotype as *ras1* mutant. We have shown that *scd2* and *ral3* denote the same gene, as do *scd1* and *ral1*, yet the *ral* mutant cells are defective in sporulation while our *scd* mutant cells are not. This may be due to differences in the genetic backgrounds of our strains. Our genetic studies also demonstrate interaction among scd1, scd2, and ras1. For example, overexpression of ras1 weakly suppressed the mating and morphological defects of the *scd2-1* mutant; overexpression of scd2 weakly rescued the abnormal shape of *ras1*^{null} mutants; and overexpression of scd1 weakly suppressed the conjugation defect of *scd2-1* mutant and the abnormal morphology of both *scd2-1* and *ras1*^{null} mutants. Additional studies suggest the involvement of cdc42sp, a RHO-like G protein that is the homolog of the S. cerevisiae CDC42 required in that organism for bud formation. We found that overexpression of cdc42sp, although ineffective by itself, markedly enhanced the effect of overexpression of scd proteins. Overexpression of the interfering *cdc42sp(T17N)*, like disruption of the *scd* genes, induced a round cell shape and diminished mating.

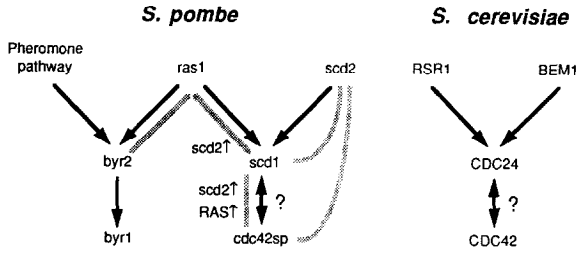

Figure 6. The Proposed Genetic Pathways Involving ras1, scds, and Their Homologs

The ordering of components inferred from the epistasis analysis is indicated by solid lines with arrows. Protein–protein interactions determined by the two-hybrid system are illustrated by stippled bars. In some cases, detection of interaction requires or is enhanced by the overexpression of a third component, which is indicated by a vertical arrow

We can make inferences about the dependence of action of these components upon each other using epistasis analysis of null strains. Because overexpression of either scd1 or scd2 can weakly suppress the morphologic defects of a *ras1*^{null} stain and because coexpression of cdc42sp improves this suppression further, we infer that these components can interact in the absence of ras1. Similarly, expression of scd1 together with either ras1 or cdc42sp can partially suppress the *scd2*^{null} strain, and hence we infer that these components can interact in the absence of scd2. On the other hand, overexpression of ras1 or scd2 (either alone or in combination with cdc42sp) is incapable of suppressing mutants in *scd1*. These data suggest that ras1 and scd2 act through scd1. Consistent with this, we note that the phenotype of a *scd1*^{null} strain is more severe than the phenotype of a *scd2*^{null} strain, and the phenotype of a *scd1*^{null}*scd2*^{null} double null is no more severe than the phenotype of a *scd1*^{null} strain. We infer from all of these relationships that ras1 and scd2 independently converge upon scd1. Because *cdc42*^{null} cells are not viable, we cannot determine whether scd1 acts upon cdc42sp, or whether cdc42sp acts upon scd1. These relationships are summarized in Figure 6.

Our two-hybrid studies confirm our conclusions from genetic analysis that ras1, scd2, scd1, and cdc42sp proteins interact. We observe clear evidence of complex formation between scd1 and scd2 and between scd2 and cdc42sp when they are expressed in S. cerevisiae. Consonant with this, we observe direct physical interaction between scd2 and a C-terminal fragment of scd1 in mixed E. coli extracts. scd1 and activated RAS proteins also form complexes detectable with the two-hybrid system. Complexes between cdc42sp and scd1 are observed when scd2 is coexpressed, suggesting that scd2 may bridge and facilitate scd1 and cdc42sp interactions. Interactions between scd1 and cdc42sp are also observed in the presence of the active RAS proteins, indicating that scd1 contains a cryptic site for interaction with cdc42sp and suggesting that complex formation may be cooperative. Enhanced interactions between ras1 and scd1 are also seen in the presence of scd2, yet further evidence for cooperativity. These results, summarized in Figure 6, suggest a model in which interac-

tion between scd1 and cdc42sp is under the concerted control of ras1 and scd2. We speculate that complex formation leads to the further localization of other components responsible for remodeling the cell. Candidates for these components include ral2 and ral4.

scd1 contains a domain in common with several proteins, including BCR, CDC24, DBL, RAS–GRF, SOS, and VAV. All, except BCR, have been reported to accelerate the guanine nucleotide exchange of certain members of the RAS superfamily. In particular, CDC24, the S. cerevisiae homolog of scd1, acts as an exchange protein for S. cerevisiae CDC42. We would therefore predict that scd1 will promote guanine nucleotide exchange on cdc42sp. While our data are consistent with this as the cellular role for scd1, our data are equally, if not more, consistent with the hypothesis that cdc42sp regulates scd1. In particular, we do not see evidence from the two-hybrid system for enhanced interaction between the interfering cdc42sp-(T17N) and either scd1 or scd2, as one would expect if the latter were exchange factor (unpublished data). Moreover, neither overexpression of the wild-type nor the activated cdc42sp(G12V) rescues the phenotype of *scd1-1* mutant cells. Preliminary data from our two-hybrid studies with truncated proteins indicate that the domain of scd1 that interacts with cdc42sp, while conserved with CDC24, is not conserved with GRF, SOS, VAV, or DBL. Thus, the interactions among cdc42sp and scd1 and scd2 may be more complex than nucleotide exchange, and scd1, through its domain that is homologous with SOS, BCR, GRF, VAV, and DBL, may have additional functions that are dependent upon its interaction with cdc42sp, ras1, and scd2.

scd2, like its homolog, BEM1, contains two SH3 domains in the N-terminus. Our preliminary data indicate that deletion of SH3 domains in scd2 markedly reduces its ability to rescue the mating defect in the *scd2-1* mutant (unpublished data) and hence that the SH3 domains are essential for the function of scd2. However, neither scd1 nor cdc42sp contains a prominent proline-rich region, one that resembles the potential binding site for other SH3 domains (Ren et al., 1993). It seems unlikely therefore that scd2 interacts with cdc42sp and scd1 through its SH3 domains, and this is supported by work in progress using the two-hybrid system to analyze the interaction of truncated proteins. It seems likely to us that the scd "complex" will include other components interacting through the SH3 domains.

Counterparts to each of the components required for maintenance of cell shape and conjugation in S. pombe are found in S. cerevisiae. S. cerevisiae RSR1/BUD1, a RAS-like protein, is required for the selection of bud sites (Bender and Pringle, 1989; Chant and Herskowitz, 1991). CDC24 and CDC42, homologs to scd1 and cdc42sp, respectively, are cell cycle mutants that fail to form buds (Adams et al., 1990; Hartwell et al., 1973). Mutations in BEM1, the homolog of scd2, create a phenotype similar to that seen in cells with mutations in CDC24 (Bender and Pringle, 1991; Chenevert et al., 1992). Cells containing certain alleles of *CDC24* and *BEM1* do not undergo proper morphologic transformation in response to mating pheromones and are also defective in mating (Chenevert et al., 1992; Sloat et al., 1981). Like their S. pombe homologs, these proteins appear to interact: CDC42 and RSR1 are multicopy suppressors of the budding defects of cells containing certain *cdc24*^{ts} alleles (Bender and Pringle, 1989). The ordering of the S. cerevisiae components by genetic means has been hampered because the null alleles of *CDC24* and *CDC42* are lethal (Johnson and Pringle, 1990). We propose that the ordering of these components in S. cerevisiae reflects the ordering of their homologs in S. pombe (Figure 6). One notable difference between S. pombe and S. cerevisiae is that the latter contains multiple RAS-like proteins. Whereas S. cerevisiae RSR1 is specialized and devoted to morphogenesis, RAS1 and RAS2 function in nutrient signaling (Wigler et al., 1988). In S. pombe, on the other hand, a single RAS participates in both morphogenesis and regulation of a protein kinase cascade that mediates pheromone signaling.

The morphogenic functions of bud formation, cell shape, and conjugation all involve remodeling of the whole-cell structure. In both budding and fission yeasts, these processes involve RAS-like and RHO-like proteins. Since RAS proteins can participate in morphogenic events requiring RHO-like proteins in mammalian cells (Ridley and Hall, 1993; Ridley et al., 1993), it is possible that mammalian cells will use similar pathways. The primary structures of the homologous genes of the two highly diverged yeasts provide guides for the identification of such homologs in mammals, and the two-hybrid system provides a genetic tool for the search for functional homologs.

Experimental Procedures

Nomenclature

All genes described are italicized. Genes from S. pombe are in lower case, but genes from other organisms are in uppercase. The nomenclature of the gene products follows the same rule except they are not italicized. DNA sequences are written 5′ to 3′. Experimentally designed restriction endonuclease sites in oligonucleotides are underlined, and the first ATG in an open reading frame is in bold.

Microbial Manipulation and Analysis

All S. pombe strains used in this study were derived from strain SP870 (*h*^{90}, *ade6.210*, *leu1.32*, *ura4-D18*, a gift from D. Beach) unless indicated otherwise. S. pombe cells were grown in either rich medium (YEA) or nutrient-limited synthetic medium (PM) with the appropriate auxotrophic supplements (Nadin-Davis et al., 1986). Agglutination of cells was tested by a sedimentation assay (Wang et al., 1991).

Mutagenesis and the Selection of Mutants

Mutagenesis was performed with 1.5% ethylmethanesulfonate (v/v in 100 mM sodium phosphate [pH 7.4]). The reaction was quenched by diluting treated cells with 5% sodium thiosulfate. These cells were then spread on PM plates and were grown for 5 days before screening for sterile and round mutants.

Plasmids and DNA Sequencing

pSCD1 and pSCD2 were isolated from a described S. pombe genomic library (Molz et al., 1989). *scd1* was localized to a 4.5 kb fragment produced by a PstI and a partial BamHI digestion. This fragment was cloned into both pUC118 and pUC119 to generate single-stranded DNA for sequencing. pSCD1U was made by replacing the 2.4 kb XhoI fragment of *scd1* in pUC118 with a blunt-ended 1.8 kb HindIII fragment containing *ura4*. The *scd2* was localized to an unique 5.2 kb HindIII fragment. A HindIII–SacI digestion produced two fragments of 2.7 kb and 2.5 kb, which we named HS1 and HS2, respectively, and both were

cloned into pUC vectors for sequencing. Subsequently, we determined that HS1 was 5′ to HS2. pSCD2L was constructed by first replacing a 1.8 kb PstI–SacI fragment from HS1 with a *LEU2* of S. cerevisiae. This fragment together with HS2 were cloned into pGEM7Zf(−) (Promega) to create pSCD2L. A PstI–SmaI fragment containing the genomic DNA of *scd1* was cloned into pAL (Wang et al., 1991) to generate pALSCD1. A PstI fragment of the S. cerevisiae *ADE2*, which complements the *ade6* mutation in S. pombe (S. Marcus, unpublished data), was cloned into pALSCD1 to create pALASCD1. pSP2SCD2 was constructed by cloning the 5.2 kb HindIII fragment of *scd2* into the HindIII site of pSP2 (Cottarel et al., 1993). pALU was derived from pALY1, which contains the HA1-tagged *cyr1* gene expressed under the control of an *adh* promoter (Kawamukai et al., 1992). The coding region of *cyr1* was removed by SalI and SmaI, and a single SalI site in-frame with the HA1 epitope was created by a linker. Furthermore, the *ura4* gene of pALU was replaced by *LEU2* to create pALL. PCR was carried out to generate appropriate cloning sites in *scd1*, *CDC24*, and *cdc42sp* for cloning into pALU. The resulting vectors were named pALUSCD1, pALUCDC24, and pALUCDC42SP, respectively. *scd1*, modified by PCR, was also cloned into pALL to generate pALLSCD1. The sequences of all PCR products described in this study were validated by sequencing. The interfering *cdc42sp(T17N)* was constructed by PCR mutagenesis using pALUCDC42SP as template (Ho et al., 1989). The forward primers were GTGGTGGACAGGTGCCTTCG and CTGTAGGAAAGAaCTGTCTG. The C to A substitution on the forward primer is shown in lower case. The reverse primers were CAGACAGTTCTTTCCTACAG and TGCACAGAAGGGTCATCACG. The final PCR generated a fragment around 400 bp that replaced the corresponding fragment in pALUCDC42SP to create pALUT17N. pARTCM was constructed to express an inserted gene as a c-MYC epitope-tagged fusion protein under the control of an *adh* promoter. The c-MYC epitope was created by a linker:

```
        PstI                                        NheI   SalI    BamHI
CCAAGCTTCTGCAGATGGAGCAAAAGCTCATTTCTGAAGAGGACGACTTGCTAGC GTCGAC GGATCCGC
          M  E  Q  K  L  I  S  E  E  D  D  L
```

The linker was digested with PstI and BamHI and cloned into pART1 (McLeod et al., 1987) to create pARTCM. PCR was employed to create cloning sites suitable for cloning into pARTCM in *scd2* and *BEM1* to generate pARTCMSCD2 and pARTCMBEM1. Fusions to the transcription activation domain of GAL4 were constructed using pGADGH (Hannon et al., 1993); fusions to the DNA-binding domain of LexA were made in pVJL11, which was derived from pBTM116 (Vojtek et al., 1993). The described BamHI fragment of *scd2* was cloned into both pGADGH and pVJL11 to create pGADSCD2 and pLBDSCD2. The DNA fragment containing *cdc42sp* suitable for cloning into pGADGH was generated by PCR. A BamHI–XhoI fragment of *cdc42sp* was then obtained from pGADCDC42SP and cloned into pVJL11 to create pLBDCDC42SP. To clone *scd1* into pGADGH, a BamHI fragment of *scd1* was generated by PCR that lacked the last 58 bp of the coding sequence. We named this vector pGADSCD1ΔB. In a separate experiment, we isolated a clone of *scd1* from a cDNA library, named pSIP7, containing the complete C-terminus of the coding region (scd1ΔN, encoding residues 672–834) cloned into pGADGH. pGADSCD1 was generated by swapping the XhoI–ApaI fragments between pSIP7 and pGADSCD1DB. The ras1 hybrids, pGADR1 and pLBDR1, were constructed by cloning a BamHI–SalI fragment of *ras1* created by PCR to introduce a A to G substitution at position 648. pLBDHR, pLBDHRV, and pLBDHRA35 contain previously described DNA fragments (Van Aelst et al., 1993) encoding H-RAS(C186R), H-RAS(G12V, C186R), and H-RAS(T35A, C186R) cloned into pBTM116 (L. Van Aelst, unpublished data). pAA was made by inserting a SacI fragment containing *ADE2* into pUAD6, previously constructed by R. Ballester. The BamHI fragment of *scd2* was cloned into pAA to create pAASCD2. The coding regions for RAF in pAH-RAF (Van Aelst et al., 1993) was replaced by the DNA fragment encoding H-RAS to generate pAH-HR (L. Van Aelst, unpublished data). The expression of gene in pAH, like pAA, is under the control of the *ADH1* promoter. The selectable marker in pAH is a *HIS3*. The described BamHI fragment of *scd2* was cloned into pRP259, a derivative of pGEX-1 (Pharmacia) obtained from M. Gebbink, to generate pGSTSCD2. An EcoRI linker was inserted into the ApaI site 3′ to the coding region of *scd1ΔN* in pSIP7. The EcoRI fragment containing the coding region of *scd1ΔN* was cloned into pTrcHisC (Invitrogen) to create pHTSCD1ΔN.

cDNA Analysis

To verify the position of intron in *scd2*, the cDNA of *scd2* was amplified by PCR using oligos AATTCCGGATCCT**ATG**TTAAAG and TCTTCAAATGGGATCCTGGAACAAAGC and sequenced. The template for this reaction was the cDNA prepared from nutrient-deprived strain SP870 (H.-P. X. and J. Camonis, unpublished data).

MACAW Protein Sequence Analysis

This analysis was carried out using the "segment pair overlap" method, and the "search space N" was calculated using the "effective sequence length." The alignments are not given for the sake of brevity, but they are available upon request.

Construction of Yeast Strains

The *scd1^{null}* strain SPSCD1U was constructed by transforming wild-type strain SP870 with a SmaI–PstI fragment of pSCD1U. The *scd2^{null}* strain SPSCD2L was made by transforming SP870 with the HindIII fragment of pSCD2L. Proper gene deletions were confirmed by either PCR or Southern blotting. The auxotrophic marker in both SP870M3 and SP870M2 was changed from Ade$^-$ to Ade$^+$ to facilitate the selection of diploids for the linkage analysis. SP870M3 was transformed by an *ade6* fragment (a gift from J. Kohli, [Szankasi et al., 1988]), and one Ade$^+$ colony named SPM3A was used for the linkage analysis. Similarly, SPM2A was derived from SP870M2. SPRN was derived from SPRU that was constructed by replacing part of the coding region of *ras1* in SP870 with *ura4* (Wang et al., 1991). SPRN was constructed by inserting a fragment of pUC18 into the coding region of *ura4*.

Linkage Analysis

To determine whether the deletion of *scd1* is allelic to the mutation in SP870M3, strain SPSCD1U and SPM3A were fused, and the Ade$^+$ and Ura$^+$ colonies were selected. One of these diploid cells was sporulated on PM plates. The asci were dissected, and the presence of spores was scored after iodine staining. Similarly, SPM2A was fused with SPSCD2L.

Northern Blot Analysis on *mam2* Expression

Cells were pregrown in PM medium to a density about 2 × 10^7 cells/ml and then transferred to fresh PM medium lacking NH_4Cl to allow for nitrogen starvation. After overnight culture, poly(A)$^+$ mRNA was isolated and analyzed as previously described (Xu et al., 1994).

Immunoprecipitation

E. coli strain BL21(DE3) was transformed by either pGSTSCD2 or pHTSCD1ΔN and was induced by 1 mM IPTG for 90 min. The lysis buffer contained 20 mM Tris–HCl (pH 7.5), 0.5% Triton X-100, 1 mM EGTA, 3 mM $MgCl_2$, 1 mM PMSF, 10 μg/ml leupeptin, and 1 μg/ml pepstatin. The protein concentration in the cleared supernatant was about 7 mg/ml. The antibody against the T7 tag (αT7, Novagen) was covalently coupled to protein A beads using dimethylpimelimidate (Harlow and Lane, 1988). Similarly, purified rabbit IgG was coupled to the beads as an antibody control. 400 μg of each cleared lysates containing GST–SCD2 and HT–scd1ΔN were mixed, and then 30 μl of antibody–protein A conjugate was added. The sample was incubated at 4°C for 1 hr with rocking. The washed beads were resuspended in 50 μl of SDS sample buffer, and 5 μl of this was loaded for Western blot analysis.

Western Blot Analysis

All blots were preincubated in 3% nonfat dried milk prepared in TBS (20 mM Tris–HCl [pH 7.6] and 150 mM NaCl). Monoclonal antibody 12CA5 (Field et al., 1988) and 9E10 (Evan et al., 1985) were used for the detection of the HA-1-tagged scd1 and c-MYC-tagged scd2, respectively. The blots containing HT–scd1ΔN and GST–scd2 were probed with antibody αT7 and a goat anti-GST antibody (Pharmacia). All antibodies were diluted in the antibody solution, comprised of 3% BSA and 0.5% Tween 20 prepared in TBS. The substrates for the detection of the conjugated alkaline phosphatases were NBT/BCIP.

Detection of Protein Complex Formation Using the Two-Hybrid System

The color filter assayed was performed using X-Gal as described (Van Aelst et al., 1993). Furthermore, cell lysates were prepared in buffer Z, and the β-Gal activities in these lysates were determined colorimetrically using ONPG (Hoffman and Winston, 1990).

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GenBank Accession Numbers

The accession numbers for the *scd1* and *scd2* sequences reported in this paper are U12538 and U12539, respectively.