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Mutations in the *SHR5* Gene of *Saccharomyces cerevisiae* Suppress Ras Function and Block Membrane Attachment and Palmitoylation of Ras Proteins

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We have identified a gene, *SHR5*, in a screen for extragenic suppressors of the hyperactive *RAS2*$^{Val-19}$ mutation in the budding yeast *Saccharomyces cerevisiae*. *SHR5* was cloned, sequenced, and found to encode a 23-kDa protein not significantly homologous to other proteins in the current data bases. Genetic evidence arguing that Shr5 operates at the level of Ras is presented. We tested whether *SHR5*, like previously isolated suppressors of hyperactivated *RAS2*, acts by affecting the membrane attachment and/or posttranslational modification of Ras proteins. We found that less Ras protein is attached to the membrane in *shr5* mutants than in wild-type cells and that the Ras proteins are markedly underpalmitoylated, suggesting that Shr5 is involved in palmitoylation of Ras proteins. However, *shr5*^{null} mutants exhibit normal palmitoyltransferase activity measured in vitro. Further, *shr5*^{null} mutations attenuate Ras function in cells containing mutant Ras2 proteins that are not palmitoylated or farnesylated. We conclude that *SHR5* encodes a protein that participates in the membrane localization of Ras but also interacts in vivo with completely unprocessed and cytosolic Ras proteins.

In the budding yeast *Saccharomyces cerevisiae*, Ras (31, 44) is regulated positively by the gene product of *CDC25* (5, 8), which encodes its guanine nucleotide exchange factor, and negatively by the gene products of *IRA1* and *IRA2* (54), which encode homologs of mammalian GTPase-activating proteins (GAPs). Ras is involved in the regulation of nutrient sensing and mitotic growth through the adenylyl cyclase pathway (4, 58). Hyperactivation of the Ras-adenylyl cyclase pathway in *S. cerevisiae* results in several phenotypes, most notably an inability to arrest at G_1, heat shock sensitivity, nutrient starvation sensitivity, and an inability to sporulate (32, 58). Although the adenylyl cyclase pathway accounts for much of the Ras effects in *S. cerevisiae*, there is genetic evidence for an alternate Ras pathway that remains, at present, ill-defined (60).

In both yeasts and mammals, a progression of modifications of the CAAX motif at the carboxyl terminus of RAS is critical for protein-protein interactions and membrane localization (for reviews, see references 13, 14, 34, 37, 39, and 42). The initial modification of the CAAX motif is the isoprenylation (farnesylation) of the cysteine (26), which is followed by proteolytic cleavage of the last three amino acids and subsequent carboxy methylation of the terminal polyisoprenylated cysteine (25). For many Ras proteins (26), acylation (palmitoylation) of the penultimate cysteine (61) results in the fully matured form of the protein. Whereas farnesylation of mammalian Ras has been shown to be critical for its function (11, 33), the role of palmitoylation appears to be limited to the efficient attachment of Ras proteins to membranes (26). Similarly, farnesylation of yeast Ras2 protein significantly increases its ability to activate and associate with adenylyl cyclase, whereas palmitoylation promotes its effective membrane localization (35) and its specific targeting to the plasma membrane (43).

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Elements involved in the effector function, regulation, and modification of Ras proteins have previously been identified in this and other laboratories by selection for mutants that suppress *RAS2*$^{Val-19}$ function in *S. cerevisiae*. Suppressor mutations in *CYR1*, which encodes adenylyl cyclase (30), and *CAP*, which encodes a protein that binds to adenylyl cyclase (16), were isolated. Suppressors of *RAS2*$^{Val-19}$ also led to the initial identification of genes involved in Ras protein modification, *RAM1* and *RAM2*, that encode the subunits of the heterodimeric Ras farnesyltransferase (19, 22, 27, 45, 52). Here, we present genetic and biochemical characterization of another gene that we identified from analysis of phenotypic revertants of a *RAS2*$^{Val-19}$ mutant. We designate this gene *SHR5* (suppressor of hyperactive RAS2^{Val-19}), since it represents the fifth gene identified as an extragenic suppressor of *RAS2*$^{Val-19}$.

MATERIALS AND METHODS

Yeast strains and plasmids. The yeast strains and plasmids used in this study are described in Table 1. Various strains were disrupted with plasmid pΔF-H3 or pΔF-L2 as described below. *BCY1* in strain SPΔFB was disrupted with plasmid p*bcy1*::*URA3* (56). Construction of *SHR5* plasmids is described below.

Isolation of extragenic suppressors of *RAS2*$^{Val-19}$. *S. cerevisiae* strains carrying an activated *RAS2*$^{Val-19}$ allele do not become heat shock resistant upon reaching stationary phase (50). We exploited this phenotypic difference to isolate revertants of the *ras1*^{null} *RAS2*$^{Val-19}$ strain PT1-6. A genetic screen previously used for isolation of the *ram1* and *ram2* mutants (27, 45) was modified as follows. Individual colonies were incubated in liquid medium at saturation at 30°C for 4 days and then heat shocked for 30 min at 50°C in glass tubes. The heat-shocked cultures were then plated, and surviving colonies were isolated and retested for heat shock resistance by a plate assay (50).

Isolation of *SHR5* and genetic manipulations. *RAS2*$^{Val-19}$-containing strains are more heat shock resistant when they reach stationary phase if they contain an *shr5* allele. Therefore, the gene for *SHR5* was isolated by selection for reversal of the heat shock resistance in the *RAS2*$^{Val-19}$ *shr5* strain RS44-5B. Briefly, these cells were transformed with an *S. cerevisiae* genomic DNA library constructed in the centromeric vector YCp50 (libraries AB493, AB494, AB495, and AB496 [kindly provided by Mark Rose]), and approximately 200 transformants per plate were grown on selective medium. One week later, 15,000 colonies were replicated onto plates previously equilibrated to 55°C for heat shocks of 5- and 10-min

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
IR-1	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ira1*::*HIS3*	2
IR1ΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ira1*::*HIS3 shr5*::*LEU2*	This paper
IR-2.1	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ira2*::*URA3*	1
IR2ΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ira2*::*URA3 shr5*::*LEU2*	This paper
KP-2	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ras2*::*URA3*	32, 58
KPΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ras2*::*URA3 shr5*::*HIS3*	This paper
LI-1-3A	*MAT***a** *leu2 ura3 his3 trp1 ade8 ras1*::*TRP1 RAS2*$^{Ser-318}$	S. Powers
LIΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 ras1*::*TRP1 RAS2*$^{Ser-318}$ *shr5*::*HIS3*	This paper
PT1-6	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 RAS2*$^{Val-19}$ *ras1*::*URA3*	45
RS44-5B	*MATα ura3 his3 trp1 ade8 can1 RAS2*$^{Val-19}$ *shr5-1*	S. Powers
RS81-1B	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 RAS2*::*URA3 shr5*::*HIS3*	S. Powers
SN12	*MAT***a** *leu2 ura3 his3 trp1 ade8 RAS2*$^{Val-19}$ *ras1*::*URA3 shr5-1*	S. Powers
SN31	*MAT***a** *leu2 ura3 his3 trp1 ade8 RAS2*$^{Val-19}$ *ras1*::*URA3 shr5-2*	S. Powers
SN38	*MAT***a** *leu2 ura3 his3 trp1 ade8 RAS2*$^{Val-19}$ *ras1*::*URA3 shr5-3*	This paper
SP1	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1*	58
SPΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 shr5*::*HIS3*	This paper
SPΔFα	*MATα leu2 ura3 his3 trp1 ade8 can1 shr5*::*HIS3*	This paper
SPΔFB	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 shr5*::*HIS3 bcy1*::*URA3*	This paper
ST-1	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ras1*::*URA3*	32, 58
ST1ΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ras1*::*URA3 shr5*::*HIS3*	This paper
STS1	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ras2*^{ts} *ras1*::*URA3*	10, 46
STS1ΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ras2*^{ts} *ras1*::*URA3 shr5*::*HIS3*	This paper
STS3	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ras1*::*URA3 ras2*$^{Val-19Phe-319}$	S. Powers
STS3ΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 ras1*::*URA3 ras2*$^{Val-19Phe-319}$ *shr5*::*HIS3*	This paper
T158-5A1	*MAT***a** *leu2 ura3 his3 trp1 ade8 cyr*::*URA3* yEp(*ADE8*)*SCH9*	Takashi Toda
T158ΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 cyr*::*URA3 shr5*::*HIS3* yEp(ADE8)SCH9	This paper
TK161-R2V	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 RAS2*$^{Val-19}$	32
TKΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 RAS2*$^{Val-19}$ *shr5*::*HIS3*	This paper
TMRV-25	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 RAS2*$^{Val-19}$ *cdc25*::*URA3*	5
TMΔF	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 RAS2*$^{Val-19}$ *cdc25*::*URA3 shr5*::*HIS3*	This paper
Plasmids		
pAD4Δ	*LEU2*; ADH promoter	2
pAD54	Epitope-tagged expression vector	15
pAD4Δ-SHR5	*SHR5* cDNA expression	This paper

durations. Transformants that recovered their sensitivity to heat shock were isolated. Plasmid DNA was isolated from these cells and tested for its ability to rescue heat shock sensitivity when it was retransformed into strain RS44-5B. Plasmids that rescued *shr5* were mapped by restriction analysis and subcloned into the yeast plasmid pRS416 (53). The ability of subclones to rescue *shr5* allowed localization of the gene to a 1.8-kb fragment which was sequenced bidirectionally by the Sanger method (Fig. 1) (49). The open reading frame (ORF) encoding *SHR5* was amplified by PCR using the oligonucleotide pair 5′-TACTCAATCTAACCC**GTCGAC**GATGTGC-3′ and 5′-CACGATAAAGC CCTG**GTCGAC**CCATAAA-3′, which contain internal *Sal*I sites (in boldface), to facilitate cloning into identical sites in the *S. cerevisiae* expression plasmids pAD4Δ (2) and pAD54 (15), creating plasmids pAD4Δ-SHR5 and pAD54-SHR5, respectively.

Plasmids designed for a one-step gene disruption (47) of *SHR5* were constructed that contained deletions from the *Xba*I site to the *Bsa*BI site in the ORF of *SHR5*, with insertions of a 1.8-kb *HIS3* fragment (28), creating plasmid pΔF-H3, or of a 2-kb *LEU2* fragment (28), creating plasmid pΔF-L2. This effectively deleted all but the first four amino acids of Shr5. *SHR5* was disrupted by transformation of various yeast strains with a *Sal*I-*Eco*RI fragment derived from pΔF-H3 or a *Sph*I-*Sac*I fragment derived from pΔF-L2. Successful disruptions were confirmed by PCR using an oligonucleotide primer pair that flanked the deletion.

Integrative mapping was performed by mating SPΔFα (*MATα shr5*::*HIS3*) and strain SN12 (*MAT***a** *shr5*) transformed with plasmid YEp13-PDE2.2 (50). High-level expression of a yeast phosphodiesterase gene allows sporulation in diploids that express hyperactive *RAS2*$^{Val-19}$ (58).

Heat shock and temperature sensitivity assays. Heat shock assays (50, 58) were performed by replica plating patches of cells that had been starved for 2 to 3 days onto plates that were preheated for 1 h at 55°C and then by incubation for 2.5, 5, and 10 min at the same temperature and transfer to 30°C for 2 to 3 days. Temperature sensitivity assays were performed by replica plating patches of cells onto plates that were then incubated at the temperatures indicated in the respective figures.

Metabolic labeling of Ras proteins with [^{3}H]palmitic acid. Metabolic labeling

```
1     ATG TGC GAT AGC CAT CAA AAG GAA GAA GAT AAC GCA AAT ACG AGC GAA AGG
1     Met Cys Asp Ser His Gln Lys Glu Glu Asp Asn Ala Asn Thr Ser Glu Arg

52    GCG TTA TTT TTT AAT TAC CAT GAG TTT TCG TAT TCA TTC TAC GAA GAC CTC
18    Ala Leu Phe Phe Asn Tyr His Glu Phe Ser Tyr Ser Phe Tyr Glu Asp Leu

103   GGT TCC GAA GAC GCT AAA CCC ACA GAG CAC GAC GAA GAC CAC AAA TTG TGT
35    Gly Ser Glu Asp Ala Lys Pro Thr Glu His Asp Glu Asp His Lys Leu Cys

154   ATT ACA CAT TTC CCG AAT GTG TAT GCT GCT CGG GGC TCT GCC GAG TTC CAG
52    Ile Thr His Phe Pro Asn Val Tyr Ala Ala Arg Gly Ser Ala Glu Phe Gln

205   GTG ACC CGG GTG GTA CGA GTG CCC CGG CGG TTC GAT GAG TCT CGC AGC AGC
69    Val Thr Arg Val Val Arg Val Pro Arg Arg Phe Asp Glu Ser Arg Ser Ser

256   CTT GAA ACG CCA CAA TTT AGT ACA CAG CTT CCC GGT AGC GAG CCG GCG GCA
86    Leu Glu Thr Pro Gln Phe Ser Thr Gln Leu Pro Gly Ser Glu Pro Ala Ala

307   ATC GTG GGC GAC GAT GGC ACT AGC TTT GTG CGG TGC GGG CGT TAC GAC ATT
103   Ile Val Gly Asp Asp Gly Thr Ser Phe Val Arg Cys Gly Arg Tyr Asp Ile

358   GGG GAT CAC GTG TTT GGC TGC TCC TCC GTC TCG CCT CTG TCA GAA TAT CTT
120   Gly Asp His Val Phe Gly Cys Ser Ser Val Ser Pro Leu Ser Glu Tyr Leu

409   AGT GCG GCA GAG CTC GCG GAG GTT GTG CAC CGG GTA AAC GGA TTC TTG CTG
137   Ser Ala Ala Glu Leu Ala Glu Val Val His Arg Val Asn Gly Phe Leu Leu

460   CGT GAA GAA GGT GAG GTG TTC GGG TGG CGT AAC TTA AGT GGC CTG TTG CTC
154   Arg Glu Glu Gly Glu Val Phe Gly Trp Arg Asn Leu Ser Gly Leu Leu Leu

511   GAT ATG CTT ACG GGC GGT CTG TGG AGC TGG GTT TTG GGG CCC CTT CTT TCT
171   Asp Met Leu Thr Gly Gly Leu Trp Ser Trp Val Leu Gly Pro Leu Leu Ser

562   AGA CCT GTG TTT CAG GAG TCT CTC GCG TTA GAG CAG TAC GTG GCG CAG CTA
188   Arg Pro Val Phe Gln Glu Ser Leu Ala Leu Glu Gln Tyr Val Ala Gln Leu

613   AAC TCG CCG GGA GGT CTG CTT CAC GAG CGC GGT GTG CGC CTA GTA TTG CCC
205   Asn Ser Pro Gly Gly Leu Leu His Glu Arg Gly Val Arg Leu Val Leu Pro

664   CGA CGG TCC GGG TGC CTA TCC CTA GAT TTC GTC GTG CCC CGA CCC AAA TAG
222   Arg Arg Ser Gly Cys Leu Ser Leu Asp Phe Val Val Pro Arg Pro Lys *
```

FIG. 1. Sequence of the *SHR5* ORF. The start and stop codons are in boldface. Multiple in-frame stop codons occur upstream of the initial ATG. The GenBank accession number for *SHR5* is U18313. *, termination codon.

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TABLE 1—*Continued*

Strain or plasmid	Genotype or description	Reference or source
pAD54-SHR5	Epitope-tagged SHR5 expression	This paper
p*bcy1*::URA3	BCY1 disruption vector	56
pRS416	URA3; centromeric vector	53
pSHR5-19.7	SHR5 in vector YCp50	This paper
pΔF-H3	SHR5 disruption by *HIS3*	This paper
pΔF-L2	SHR5 disruption by *LEU2*	This paper
YCplac22	TRP1; CEN	21
YCp22-SHR5	SHR5	This paper
YEp112	TRP1; 2μm	21
YEp112-RAS2^{Val-19}	RAS2^{Val-19}	This paper
YEp13	LEU2; 2μm	3
YEp13-CDC25	Guanine nucleotide exchange factor	8
YEp13-CYR1-11	Adenylyl cyclase	30
YEp13-PDE2.2	Phosphodiesterase	50
YEp13-SCH9	cAMP-related protein kinase	55
YEp13-TPK1	cAMP-dependent protein kinase	57

of Ras proteins with [^{3}H]palmitic acid was performed exactly as described by Powers et al. (45). The labeled Ras proteins were detected with antibody Y13-259 (20) as the primary antibody. Colorimetric detection of Ras proteins was quantitated by densitometry.

Fractionation of yeast extracts and immunoblotting of epitope-tagged Shr5 proteins. One-hundred-milliliter cultures of SP1 cells transformed with plasmids expressing either Shr5, Ste18, or GST, each as hemagglutinin HA1 epitope-tagged (15, 23, 62) proteins, were grown in synthetic medium with galactose to late log phase. The cells were pelleted and washed with ice-cold buffer E (0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM Tris [pH 7.4]), resuspended in 500 μl of buffer E containing 1 mM phenylmethylsulfonyl fluoride and 1 μg of aprotinin per ml, and lysed with glass beads and vortexing. The lysate was cleared by centrifugation at 500 × *g* for 10 min to obtain the supernatant and pellet fractions. Fifteen microliters of each fraction was mixed with 15 μl of 2× Laemmli loading buffer (36), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose paper. Immunoblotting with 12CA5 antibody (41) was performed as described elsewhere (15) and visualized with enhanced chemiluminescence reagents (Amersham, Arlington Heights, Ill.).

In vitro palmitoylation assay. In vitro acylation (palmitoylation) assays were performed essentially as described by Gutierrez and Magee (24) in a buffer containing 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 2 mM $MgCl_2$, 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM NaCl, 130 mM KCl, 1 mM ATP, 200 μM coenzyme A, 1 mM dithiothreitol, 10 μCi of [^{3}H]palmitate {[9,10^{-3}H(N)] palmitic acid; 47 Ci/mmol; DuPont-New England Nuclear, Boston, Mass.}, and 5 μg of yeast extract in a total volume of 100 μl. Incubations were carried out for 1 h at 37°C and stopped by the addition of 500 μl of cold acetone. Acetone precipitates were resuspended in Laemmli loading buffer (36) containing 100 mM dithiothreitol and were subjected to electrophoresis in SDS-12% PAGE. The gels were treated with ENTENSIFY (DuPont-New England Nuclear) according to the directions supplied by the manufacturer and were exposed directly to Kodak XAR-5 film at −85°C.

RESULTS

Identification of *shr5* as an extragenic suppressor of *RAS2*$^{Val-19}$ and cloning of the wild-type *SHR5* gene. *RAS2*$^{Val-19}$ mutants, unlike wild-type yeast cells, are not heat shock resistant upon reaching stationary phase (50). We exploited this phenotypic difference to isolate revertants from the *ras1*^{null} *RAS2*$^{Val-19}$ mutant strain PT1-6 (45). Sixty-four independently derived revertants were obtained in a heat shock screen as described in Materials and Methods. On the basis of complementation analysis, 55 of 64 of these revertants were due to either recessive mutations in the adenylyl cyclase gene or intragenic suppressor mutations in the *RAS2*$^{Val-19}$ gene itself. Three of the remaining nine revertants formed a single complementation group, *supF*, that we now call *shr5*. In plate assays, *RAS2*$^{Val-19}$ *shr5* revertants were not as heat shock resistant as wild-type cells and, unlike wild-type cells, had low levels of glycogen (29). By these criteria and in contrast to *ram1* (45), *ram2* (45), or *cyr1* (30) suppressor mutations, *shr5* is only a partial suppressor of *RAS2*$^{Val-19}$.

The *SHR5* gene was isolated by screening a centromeric genomic DNA library for plasmids that reversed the heat shock resistance of *RAS2*$^{Val-19}$ *shr5* cells. One of 15,000 transformants that reversed heat shock resistance in a plasmid-dependent manner was isolated. Plasmid pSHR5-19.7 was recovered from the revertant and contained a 19-kbp insert. Deletion analysis led to the subcloning of a 1.8-kb *Msc*I-*Cla*I fragment that could rescue *shr5*. The fragment was completely sequenced (Fig. 1), and a 765-bp ORF was identified. In-frame stop codons were found upstream of the first ATG. The ORF can encode a product of about 26.7 kDa (see below). Comparison with available databases failed to identify significant homology to other proteins.

Several genetic criteria indicating that the isolated gene was indeed *SHR5* were satisfied. First, plasmids were constructed for the disruption of *SHR5*. Disruption of *SHR5* in a *RAS2*$^{Val-19}$ strain resulted in heat shock-resistant cells (Fig. 2, rows 3 and 4). Disruption of *SHR5* in a wild-type strain had no effects on its heat shock sensitivity (Fig. 2, rows 1 and 2). Second, we found that expression of the predicted ORF from

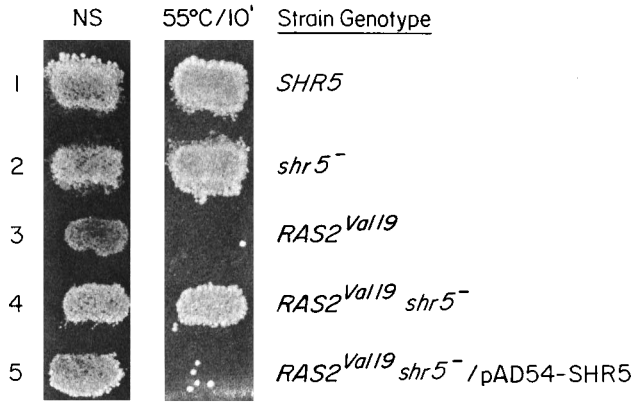

FIG. 2. Reversal of heat shock sensitivity by *SHR5* disruption and reconstitution of heat shock sensitivity by expression of the *SHR5* cDNA. The *SHR5* strain is wild-type SP1 (row 1); the *shr5*$^-$ strain is SPΔF (row 2); the *RAS2*$^{Val-19}$ strain is TK161-R2V (row 3); the *RAS2*$^{Val-19}$ *shr5*^{null} strain is TKΔF (rows 4 and 5). The rescue of the *shr5* disruption in strain TKΔF (row 4) was with plasmid pAD54-SHR5, expressing a cDNA for *SHR5* (row 5). Cells were heat shocked at 55°C for 10 min (10′).

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TABLE 2. Genetic analysis of *SHR5*[a]

Strain	Relevant genotype	*+SHR5*	*−SHR5*
IR1ΔF	*ira1*^{null} *shr5*^{null}	+	−
IR2ΔF	*ira2*^{null} *shr5*^{null}	+	−
TKΔF	*RAS2*$^{Val-19}$ *shr5*^{null}	+	−
TMΔF	*cdc25*^{null} *RAS2*$^{Val-19}$ *shr5*^{null}	+	−
SPΔFB	*bcy1*^{null} *shr5*^{null}	+	+
SPΔF	*shr5*^{null} *TPK1* ↑	+	+
SPΔF	*shr5*^{null} *CYR1* ↑	+	+

[a] Dependence of heat shock sensitivities on *SHR5*. All strains used contained disruptions of the *SHR5* gene. These strains were transformed with plasmid YCp22-SHR5 for expression of *SHR5* (+*SHR5*) or the parental plasmid YC-plac22 as a control (−SHR5). Heat shocks were performed as described in the text. + and −, maintenance or attenuation of heat shock sensitivity, respectively; ↑, overexpression of the gene *TPK1* or *CYR1* from plasmid YEp13-TPK1 or YEp13-CYR1, respectively.

the yeast alcohol dehydrogenase (ADH) promoter can reconstitute the heat shock sensitivity of all *shr5* alleles (strains SN12, SN31, and SN38) (29) as well as of the *shr5*^{null} strains (Fig. 2, rows 4 and 5). Third, tetrad analysis was carried out to determine if the cloned gene was allelic to *shr5*. Strain SPΔFα (*MAT*α *shr5*^{null}), containing a disruption of *SHR5*, was crossed with strain SN12 (*MAT***a** *RAS2*$^{Val-19}$ *shr5*). A total of 76 complete tetrads were analyzed for heat shock sensitivity, and no recombinants exhibited the heat shock sensitivity characteristic of *RAS2*$^{Val-19}$ strains. Therefore, we conclude that the isolated gene is *SHR5*.

***shr5* fails to suppress hyperactive mutations downstream of RAS.** We carried out genetic studies to localize the site of action of *SHR5* in the Ras-adenylyl cyclase pathway (Table 2). We first tested if *SHR5* operates through the upstream regulators of Ras function. Disruption of the GAPs encoded by *IRA1* or *IRA2* (54) can activate Ras. The heat shock sensitivity of an *ira1*^{null}, an *ira2*^{null}, or a *RAS2*$^{Val-19}$ strain is attenuated in an *shr5*^{null} background. Disruption of *SHR5* in a *RAS2*$^{Val-19}$ *cdc25*^{null} strain also attenuated its heat shock sensitivity. We then determined if an *SHR5* disruption can attenuate the heat shock sensitivity created by hyperactivation of downstream elements in the Ras-adenylyl cyclase pathway. *BCY1* (56) encodes the regulatory component of the protein kinase A subunits encoded by the *TPK* genes (57), and its disruption results in hyperactivation of the kinases leading to heat shock sensitivity. A *bcy1*^{null} *shr5*^{null} strain remained heat shock sensitive in the presence or absence of *SHR5*. Disruption of *SHR5* also failed to attenuate the heat shock sensitivity of wild-type strains overexpressing either protein kinase A (*TPK1*) or adenylyl cyclase (*CYR1*). Therefore, disruption of *SHR5* blocks Ras function but fails to block elements downstream of Ras.

***shr5* attenuates hyperactive *RAS2*$^{Val-19}$ in the absence of the effector adenylyl cyclase.** The severe growth defect resulting from disruption of adenylyl cyclase in *S. cerevisiae* T158-5A1 can be rescued by overexpression of *SCH9*, a gene encoding a protein kinase with homology to the *TPK*-encoded protein kinases (55). For convenience, we refer to this strain as *cyr1*^{null} *SCH9* ↑. We tested the consequence of overexpressing activated *RAS2*$^{Val-19}$ in this strain and found that it leads not to heat shock sensitivity but to a severe growth defect (Fig. 3, sector 1) and a starvation sensitivity (29). Since adenylyl cyclase is absent from this strain, it is unlikely that the *RAS2*$^{Val-19}$-dependent effects are mediated through the cyclic AMP (cAMP) pathway. The growth defect resulting from overexpression of *RAS2*$^{Val-19}$ in a *cyr1*^{null} *SCH9* ↑ strain was suppressed by disruption of *SHR5* (Fig. 3, sectors 1 and 2), whereas expression of *SHR5* by itself had no effect on cell growth (Fig. 3, sectors 3 and 4). The growth defect was also suppressed by overexpression of the GAP encoded by *NF1* (1), therefore indicating that the effect is through Ras (29). This result is consistent with previous observations that *NF1* can inhibit the function of activated H-ras^{Val-12} (1). We observed similar results for starvation sensitivity, which can also be attenuated by disruption of *SHR5* or by overexpression of the *NF1* gene (29). These experiments indicate that *shr5* can attenuate Ras function in the absence of adenylyl cyclase.

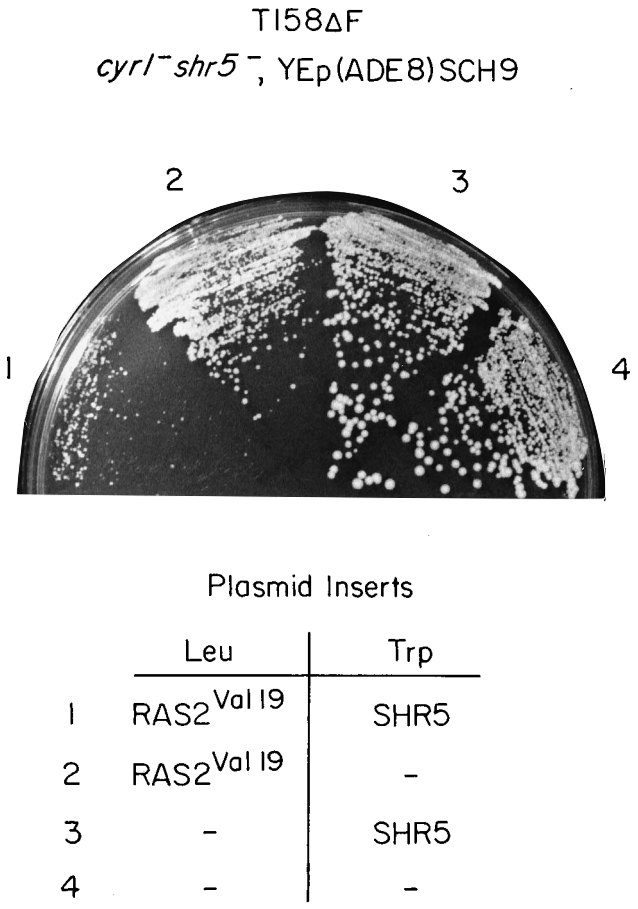

FIG. 3. *SHR5* attenuates *RAS2*$^{Val-19}$ in the absence of the effector adenylyl cyclase. The *cyr1*$^{-}$ strain, whose viability was rescued by overexpression of *SCH9*, was transformed with the indicated plasmids. *RAS2*$^{Val-19}$ was expressed from the *LEU2*-based plasmid YEp112-RAS2^{Val-19}. *SHR5* was expressed from the *TRP1*-based plasmid YCp22-SHR5. Transformants were streaked onto plates, and the plates incubated at room temperature.

***shr5* affects the growth-promoting function of normal Ras.** We had established that disruption of *SHR5* suppresses hyperactive *RAS2*$^{Val-19}$, but could not find any phenotypic effects of disrupting *SHR5* in a wild-type background, suggesting that *SHR5* does not play a role in the normal growth-promoting function of Ras. To address this issue more critically, we needed to test whether SHR5 played a role in promoting growth when Ras was weakened mutationally. Whereas hyperactivation of Ras leads to heat shock sensitivity (50, 58), loss of Ras function manifests as a temperature sensitivity (57, 60). Therefore, we tested whether disruption of *SHR5* had any effects in a *ras1*^{null} or a *ras2*^{null} background, using a temperature sensitivity assay (Fig. 4). Disruption of *SHR5* in a *ras1*^{null} *RAS2* background did not lead to any discernible phenotype. In contrast, disruption of *SHR5* significantly amplified the mild

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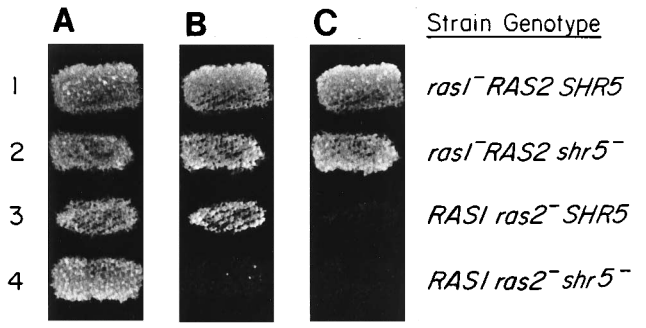

FIG. 4. Increased temperature sensitivity of *shr5*^{null} strains. The *ras1*$^{-}$ *RAS2 SHR5* strain is ST-1 (row 1). The *ras1*$^{-}$ *RAS2 shr5*$^{-}$ strain is STΔF (row 2). The *RAS1 ras2*$^{-}$ *SHR5* strain is KP-2 (row 3). The *RAS1 ras2*$^{-}$ *shr5*$^{-}$ strain is KPΔF (row 4). Temperature sensitivity assays were carried out as described in the text. Triplicate plates were incubated at room temperature (A), at 36°C (B), or at 38°C (C).

temperature-sensitive growth defect of *ras2*^{null} mutants, such that the double *shr5*^{null} *ras2*^{null} mutants did not grow at 36°C (Fig. 4, row 4). This result was confirmed in analysis of a cross between an *shr5*^{null} mutant and a *ras2*^{null} mutant. In 10 complete tetrads, only the eight double *ras2*^{null} *shr5*^{null} mutants were temperature sensitive at 36°C. Thus, Ras1 protein is unable to promote growth at 36°C when *SHR5* is absent.

To test whether *SHR5* affected the growth-promoting function of a mutationally weakened Ras2 protein, we also disrupted *SHR5* in the STS1 strain carrying a *RAS2* allele with a mutation in the effector region (10, 46). This strain, which is also *ras1*^{null}, cannot grow at 37°C but retains the ability to grow at 34°C. Disruption of *SHR5* in this strain blocks the ability to grow at 34°C. This effect of the combined mutations was confirmed by tetrad analysis (29). *SHR5* can, therefore, influence the growth-promoting function of both Ras1 and Ras2 proteins.

We next showed that loss of *SHR5* function results in attenuation of the Ras-adenylyl cyclase pathway. Elements in this pathway were tested to determine if they alter the increased temperature sensitivity of the *RAS1 ras2*^{null} *shr5*^{null} strain. We found that such cells transformed with a control plasmid or a *CDC25* expression plasmid remained temperature sensitive at 37°C, whereas expression of *RAS2*, *SHR5*, or *CYR1* was able to reverse the temperature sensitivity (Fig. 5).

***shr5* mutants are defective in the membrane attachment and palmitoylation of RAS2 proteins.** The epistasis results suggested that *SHR5* might operate at the same level as Ras. Therefore, we sought to determine whether the posttranslational modification or membrane attachment of Ras proteins was altered in *shr5* mutants. We analyzed the membrane attachment of Ras proteins by biochemical fractionation and immunoblotting with anti-Ras antibodies (Fig. 6A). A significant proportion of Ras2 proteins in *Shr5* mutants is found in the soluble fraction. This contrasts with the undetectable Ras2 protein from the soluble fraction in wild-type cells and the much more severe mislocalization to the soluble fraction seen in *ram1* mutants. This partial mislocalization of Ras2 proteins to the soluble fraction in *Shr5* mutants was observed in repeated experiments.

We sought to determine whether the mislocalized protein was processed in *shr5* mutants. The Ras2 protein in *shr5* mutants is likely to be polyisoprenylated, since in other experiments it clearly comigrated with fully processed Ras2 from wild-type cells and did not comigrate with unprocessed Ras2 from *ram1* mutants (29). (This difference is not evident in the experiment depicted in Fig. 6, in which the proteins were separated on a minigel of insufficient resolving capacity.) Then, we decided to examine whether the mislocalization of Ras2 protein in *shr5* mutants might be due to a defect in palmitoylation of Ras2 protein. It has been established that the efficient attachment and specific targeting to plasma membranes for mammalian H-ras is dependent on this final processing step, and similar results have been obtained for yeast Ras2 protein (43). Therefore, we performed metabolic labeling with [^{3}H] palmitic acid and immunoprecipitated Ras2 proteins for analysis by SDS-PAGE and autoradiography. As seen in Fig. 6B, there is a significant defect in the palmitoylation of Ras2 protein in *shr5* mutants. The metabolically labeled extracts were immunoblotted for the detection of Ras2 protein to show that the amount of Ras2 protein did not differ substantially between the wild-type and *shr5* mutants (Fig. 6C). Results from Fig. 6B and C are quantitated in Table 3.

We conclude from this series of experiments that Ras2 protein is mislocalized in *shr5* mutants and has diminished palmitoylation.

Evidence that Shr5 is membrane associated and is not the Ras protein palmitoyltransferase. We examined the subcellular localization of HA1 epitope-tagged Shr5 protein by bio-

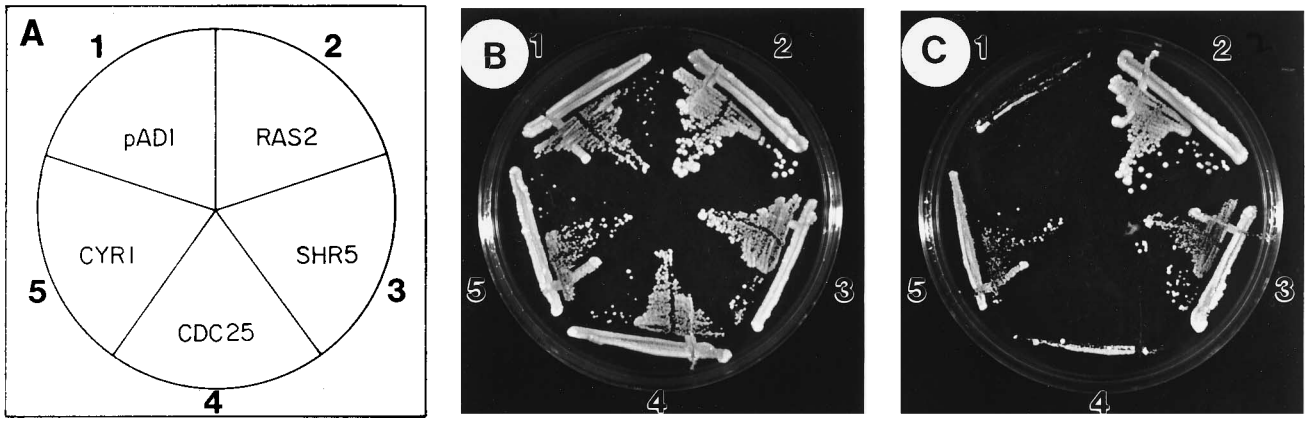

FIG. 5. Reversal of temperature sensitivity of *RAS1 ras2*$^{-}$ *shr5*$^{-}$ strain RS81-1B by elements of the RAS-adenylyl cyclase pathway. The indicated strains were transformed with plasmids expressing various indicated genes (see Table 1) (A). Transformed cells were streaked onto duplicate plates and then incubated at 30°C (B) and 37°C (C).

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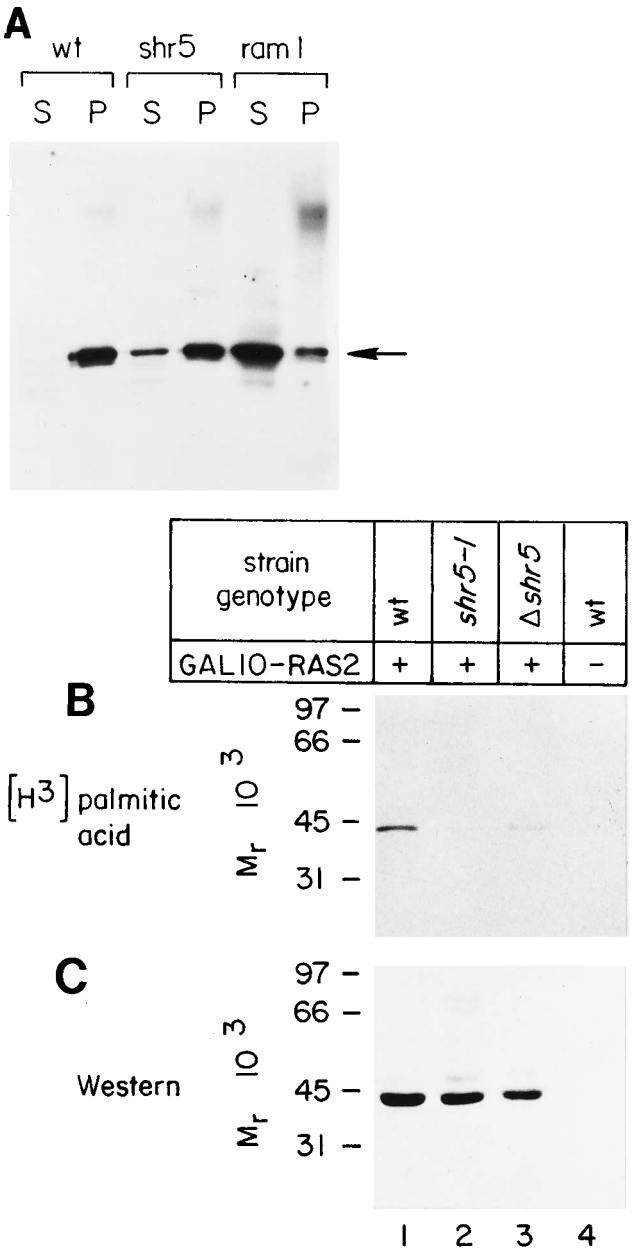

FIG. 6. (A) *shr5* mutants defective in membrane localization of Ras protein. Fractionation of yeast extracts into soluble (S) and membrane (P) fractions and detection of Ras proteins by Western blot analysis were performed as described in the text. The wild-type (wt) strain is SP1. The *shr5* strain is SPΔF. The *ram1* strain is STS11 (45). The arrow indicates the approximately 42-kDa band for Ras protein. (B and C) *shr5* mutants defective for palmitoylation of Ras protein. (B) Autoradiographic detection of immunoprecipitated Ras2 protein labeled with [^{3}H]palmitic acid; (C) Western blot quantitation of Ras2 protein for the corresponding ^{3}H-labeling experiments. The wild-type (wt) cell line is SP1. The *shr5-1* strain is RS44-5B. The Δ*shr5* strain is SPΔF. Ras2 protein was expressed from a galactose-inducible plasmid, pGAL10-RAS2 (31).

chemical fractionation and immunoblotting (Fig. 7A). HA1-tagged GST and Ste18 protein (18) were used as controls for cytosolic and membrane-bound proteins, respectively. The HA1-Shr5 protein was detected as an ≈35-kDa band only in the particulate fraction. Hence, Shr5 protein may be a membrane-bound protein where it could directly participate in the membrane targeting of Ras proteins. Incidentally, HA1-Shr5 migrates significantly more slowly than predicted from its mass (≈28.7 kDa). Recombinant Shr5 expressed in bacteria also migrates slowly (29).

TABLE 3. Palmitoylation of overexpressed Ras2 proteins in *shr5* strains[a]

Strain	Plasmid	Amt of protein		Efficiency
		Total	Palmitoylated	
SP1	YEp51-RAS2	11,567	5,893	0.509
shr5-1	YEp51-RAS2	11,975	44	0.00367
SPΔF	YEp51-RAS2	6,769	336	0.049
SP1	YEp51	Undetectable	Undetectable	

[a] Identical amounts of protein extracts (prepared from [^{3}H]palmitate-labeled yeast strains) were immunoprecipitated, resolved by SDS-PAGE, and subjected to Western blot (immunoblot) and autoradiographic analysis as described in Materials and Methods. The quantitation of Ras2 protein and palmitoylated Ras2 protein was by densitometry, and the results are expressed as relative units. The palmitoylation efficiency was calculated by dividing the units of palmitoylated Ras2 by the units of Ras2 protein.

To determine if *shr5* mutants are defective for Ras protein palmitoyltransferase activity, we performed an in vitro assay for palmitoylation of Ras proteins according to the procedure developed by Magee and coworkers (38). Whole-cell extracts were prepared from strains which overexpressed Ras2 protein and were incubated with [^{3}H]palmitate in the presence of cofactors to promote in vitro palmitoylation of proteins. As shown in Fig. 7B, *shr5* mutants display as many palmitoylated protein bands as do wild-type cells. In fact, for the *shr5* mutant, there is increased labeling of a band that migrates at the molecular mass predicted for Ras2, 42 kDa, and that is only present in experiments performed with strains overexpressing Ras2 protein. This effect of increased labeling of the 42-kDa protein in *shr5* mutants is more pronounced with shorter incubation times and with lower amounts of added extract (29). This result indicates that in *shr5* mutants, there is a larger pool of nonpalmitoylated Ras2 protein available for palmitoylation in the in vitro assay. This is consistent with our findings of a defect in palmitoylation of Ras2 protein by metabolic labeling. There is certainly no evidence for a defect in protein palmitoylation by this in vitro assay.

Evidence that Shr5 interacts in vivo with nonpalmitoylated and with completely unprocessed Ras2 proteins. The results from the previous section suggested that SHR5 does not encode the Ras palmitoyltransferase. To test whether the major phenotypic effect of *SHR5* is mediated by its effects on Ras2 palmitoylation, we asked whether *SHR5* could interact genetically with *RAS2* alleles missing the unique cysteine palmitoylation site at position 318 (6, 27, 44). Indeed, we found by a temperature sensitivity assay that the absence of *SHR5* blocked the growth of *RAS2*$^{Ser\text{-}318}$ strains at 38°C (Fig. 8). This effect is specific to the Ras-adenylyl cyclase pathway since it can be suppressed by overexpression of protein kinase A. We showed similar results for the hyperactivated *RAS2*$^{Val\text{-}19Ser\text{-}318}$ allele which, although attenuated, is still capable of producing a significant heat shock-sensitive phenotype (44). We found that the *shr5*^{null} mutation suppressed the heat shock sensitivity induced by this nonpalmitoylatable version of hyperactive *RAS2* (29). Thus, the ability of the *shr5* deletion to suppress hyperactive RAS2 does not depend on its palmitoylation status.

We decided to take this analysis one step further and ask if the ability of *SHR5* to influence Ras function required that Ras proteins be farnesylated. *RAS2* alleles encoding a serine instead of a cysteine at position 319 within the CAAX box are blocked for farnesylation but can supply essential function if

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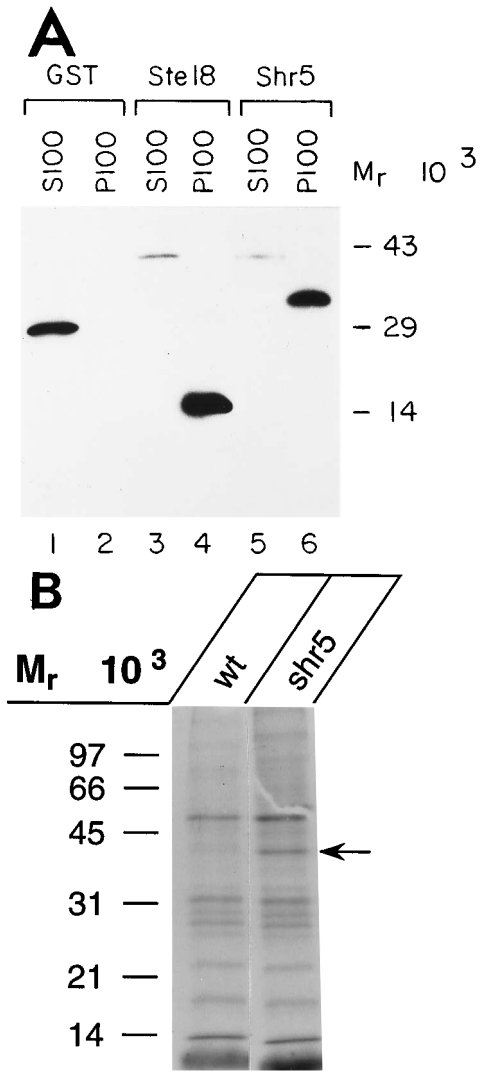

FIG. 7. (A) Membrane or cytoskeletal association of Shr5 protein. Soluble (S) and insoluble (P) fractions of cells expressing HA1 epitope-tagged forms of GST, Ste18, or Shr5 protein were prepared as described in the text. For Western blot analysis, antibody 12CA5 was used for detection of the HA1 epitope. (B) Comparison of in vitro palmitoylation of proteins in extracts prepared from wild-type (wt) and *shr5* mutant cells. Extracts were prepared from wild-type strain SP1 and *shr5* mutant strain SPΔF; both were transformed by the high-copy-number plasmid expressing *Ras2* and assayed according to the method described by Magee et al. (38). The arrow indicates an approximately 42-kDa band corresponding to Ras2 protein. This band is not present in untransformed cells.

they are overexpressed (12). We have found that the nonfarnesylated $RAS2^{Val\text{-}19Phe\text{-}319}$ allele can promote growth at room temperature and weakly at higher temperatures (10). To test if *SHR5* influenced the ability of nonfarnesylated Ras2 protein to promote growth in this background, we deleted *SHR5* in a $ras1^{null}$ $RAS2^{Val\text{-}19Phe\text{-}319}$ strain and examined its growth properties (Fig. 8). Deletion of *SHR5* blocks the ability of the strain to grow at 34°C. This effect is specific to the Ras-adenylyl cyclase pathways, since it can be reversed by overexpression of protein kinase A. The synergistic temperature-dependent growth defect was also confirmed by analysis of a cross between a *shr5* strain and a $ras1^{null}$ $RAS2^{Val\text{-}19Phe\text{-}319}$ strain (29).

The ability of Shr5 to influence the function of Ras does not appear to depend on its modification status.

DISCUSSION

We have isolated a novel gene whose product is involved in Ras function. Although disruption of *SHR5* in wild-type *S. cerevisiae* did not lead to any apparent phenotypic defects, it did suppress $RAS2^{Val\text{-}19}$ cells and did influence the normal growth-promoting function of strains in which Ras was attenuated.

By genetic analyses, we deciphered where *SHR5* functions relative to *RAS*. Disruption of *SHR5* suppressed the hyperactivation of the Ras-adenylyl cyclase pathway in $ira1^{null}$, $ira2^{null}$, and $RAS2^{Val\text{-}19}$ strains. We interpret this to mean that Shr5 functions independently of the GAPs. In addition, disruption of *SHR5* attenuated $RAS2^{Val\text{-}19}$ regardless of the presence or absence of the guanine nucleotide exchange factor encoded by *CDC25*. This indicates that Shr5 does not regulate Ras function by mediating its interaction with Cdc25. These genetic experiments indicate that Shr5 does not function through the upstream Ras regulators, leading us to test elements downstream of the Ras pathway. It was found that disruption of *SHR5* failed to attenuate downstream activation of the adenylyl cyclase pathway caused by overexpression of the *CYR1* or *TPK1* gene or by disruption of the negative regulator of protein kinase A encoded by *BCY1*. We showed that the attenuation of Ras function in *shr5* mutants can be relieved by overexpression of downstream elements in the Ras pathway. Taken together, these studies place the function of Shr5 at the level of Ras and upstream of adenylyl cyclase.

We then showed by a novel assay that *SHR5* disruptants exhibit attenuated Ras pathway function even in the absence of adenylyl cyclase. This assay was based on the observation that overexpression of $RAS2^{Val\text{-}19}$ conferred a phenotype in a $cyr1^{null}$ strain whose viability had been rescued by overexpression of a downstream kinase encoded by *SCH9* (55). $cyr1^{null}$ *SCH9* ↑ $RAS2^{Val\text{-}19}$ ↑ cells are not heat shock sensitive but are growth inhibited and starvation sensitive. The observation of a $RAS2^{Val\text{-}19}$-dependent phenotype in a $cyr1^{null}$ strain supports the alternate-Ras pathway hypothesis that was originally proposed to explain the lethality of a ras^{null} strain versus the viable but sick $cyr1^{null}$ strain (60). Disruption of *SHR5* attenuated the $RAS2^{Val\text{-}19}$ phenotype in this strain, indicating that Shr5 functions at the level of Ras and not on adenylyl cyclase or its downstream targets.

We investigated the possibility that Shr5 is involved in Ras modification, since previously isolated suppressors of $RAS2^{Val\text{-}19}$ had defects in genes encoding the subunits of Ras farnesyltransferase, *ram1* and *ram2*. Indeed, fractionation studies indicate that *shr5* mutants are partially defective in Ras localization; a larger portion of Ras in *shr5* mutants is cytosolic than in wild-type cells, in which Ras is predominantly localized to the membrane. In addition, there is a defect in the palmitoylation of Ras protein in *shr5* mutants. The comigration of Ras protein from *shr5* mutants with the proteolytically processed Ras protein from wild-type cells makes it unlikely that *shr5* mutants are defective in farnesylation, since mutants defective in farnesylation display altered migration of Ras proteins on SDS-PAGE.

The defect in palmitoylation of Ras suggests that *SHR5* encodes a component of a Ras palmitoyltransferase. Therefore, we tested if extracts prepared from *shr5* mutants are defective for palmitoylation of endogenous proteins, including Ras. By a cell-free assay, no palmitoylation defect was observed in *shr5* mutants. In fact, in such cells we observed increased labeling of a band that coincides with Ras2, perhaps because, as we demonstrated, *shr5* mutants have an increased amount of unpalmitoylated Ras that can serve as a substrate

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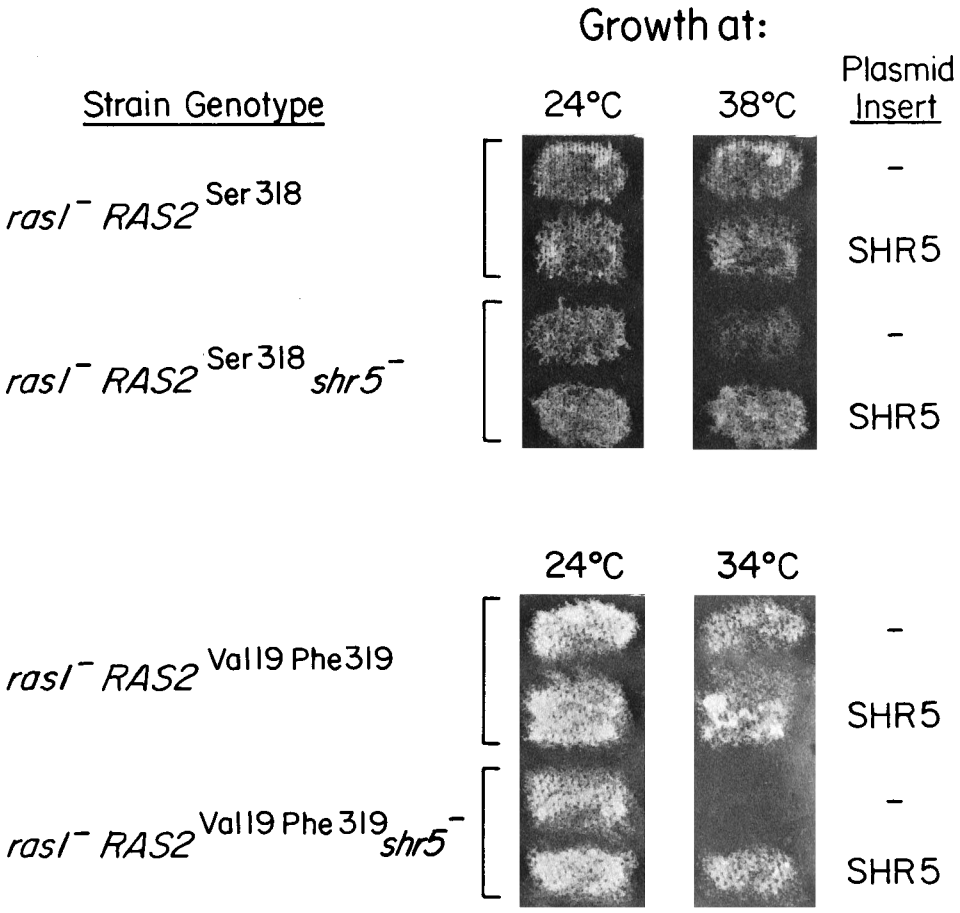

FIG. 8. *SHR5* interacts genetically with nonpalmitoylated $RAS2^{Ser\text{-}318}$ and nonfarnesylated $RAS2^{Val\text{-}19Phe\text{-}319}$ mutants. The $RAS2^{Ser\text{-}318}$ and $RAS2^{Ser\text{-}318}$ $shr5^{null}$ strains are LI-1-3A and LIΔF, respectively. The $RAS2^{Val\text{-}19Phe\text{-}319}$ and $RAS2^{Val\text{-}19Phe\text{-}319}$ $shr5^{null}$ strains are STS3 and STS3ΔF, respectively. *SHR5* was expressed from plasmid YCp22-SHR5. Temperature sensitivity assays were carried out as described in the text.

for the in vitro palmitoylation assay. There was no apparent alteration in the in vitro labeling of other proteins by palmitoylation. *SHR5* is, therefore, unlikely to encode a major cellular palmitoyltransferase. We do not believe that *SHR5* encodes a Ras-specific palmitoyltransferase, because the ability of the *shr5* disruption to suppress hyperactive Ras2 or influence the growth-promoting function of Ras2 does not depend on the palmitoylation status of the Ras protein. In fact, the ability of Shr5 to influence Ras function did not even require that Ras proteins be polyisoprenylated. Therefore, Shr5 can functionally interact with Ras regardless of its modification state.

To date, the data suggest but do not prove that Shr5 is specific for Ras. First, in the comparison of *shr5* mutants and wild-type cells, the in vitro palmitoylation defect appeared to be specific only for Ras but not other protein bands (Fig. 7B), even though similar levels of Ras protein were expressed (Fig. 6C). However, it is possible that Shr5 modifies the palmitoylation of other proteins that are expressed at levels too low to detect in this assay. Second, there are other G proteins in *S. cerevisiae* that might be affected by Shr5 function: Rsr1 (Bud1) (9, 48), which is involved in budding, and Ste18 (18, 59), which is involved in mating. We found that $shr5^{null}$ mutants are neither budding nor mating defective (29), suggesting that the absence of Shr5 did not attenuate the activities of these other G proteins. It remains possible that Shr5 has wider functions, even though there are no apparent differences between $shr5^{null}$ and wild-type cells. The role of *SHR5* becomes manifest only under synthetic conditions, such as in our case, when mutant forms of *RAS* are being used. It is possible that other roles for Shr5 can be discerned under other synthetic conditions. Although the evidence indicates that Shr5 might be specific for Ras, more confirmatory work is necessary.

What might be the function of Shr5? Although more complex models are possible, the most compelling indication of Shr5 function is suggested by its ability to influence localization and palmitoylation of Ras. Our data are consistent with a model in which Shr5 promotes attachment to the membrane and the defect in palmitoylation is a secondary consequence of this defect in membrane attachment. This is consistent with our observation of no defect in palmitoyltransferase activity per se. The ability of Shr5 to influence completely unmodified Ras proteins suggests that even for the function of nonmodified Ras, at least some proper localization to the membrane is important, and this is supported by the recent report of Deschenes and coworkers (40). Membrane localization might be important if the interaction of Ras with either active Cdc25 protein or effector took place exclusively in the membranes. The ability of Shr5 to functionally interact with unprocessed Ras and to influence Ras localization to the membrane suggests that it might be involved in the plasma membrane targeting of Ras proteins (7, 51) and that its function is not solely

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dependent on recognition of the lipid groups. Thus far, we have not detected interaction of Shr5 with Ras by the two-hyrid method (17), thus suggesting that Shr5 functions as an element in a protein complex, that its effects on Ras is indirect, or that its interaction is too transient to observe. The isolation of proteins that function in concert with or that directly interact with Shr5 should further our understanding of how Ras localization is controlled and how this might impact Ras function.

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