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Ras Regulates the Polarity of the Yeast Actin Cytoskeleton through the Stress Response Pathway

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Polarized growth in yeast requires cooperation between the polarized actin cytoskeleton and delivery of post-Golgi secretory vesicles. We have previously reported that loss of the major tropomyosin isoform, Tpm1p, results in cells sensitive to perturbations in cell polarity. To identify components that bridge these processes, we sought mutations with both a conditional defect in secretion and a partial defect in polarity. Thus, we set up a genetic screen for mutations that conferred a conditional growth defect, showed synthetic lethality with *tpm1*Δ, and simultaneously became denser at the restrictive temperature, a hallmark of secretion-defective cells. Of the 10 complementation groups recovered, the group with the largest number of independent isolates was functionally null alleles of *RAS2*. Consistent with this, *ras2*Δ and *tpm1*Δ are synthetically lethal at 35°C. We show that *ras2*Δ confers temperature-sensitive growth and temperature-dependent depolarization of the actin cytoskeleton. Furthermore, we show that at elevated temperatures *ras2*Δ cells are partially defective in endocytosis and show a delocalization of two key polarity markers, Myo2p and Cdc42p. However, the conditional enhanced density phenotype of *ras2*Δ cells is not a defect in secretion. All the phenotypes of *ras2*Δ cells can be fully suppressed by expression of yeast *RAS1* or *RAS2* genes, human Ha-ras, or the double disruption of the stress response genes *msn2*Δ*msn4*Δ. Although the best characterized pathway of Ras function in yeast involves activation of the cAMP-dependent protein kinase A pathway, activation of the protein kinase A pathway does not fully suppress the actin polarity defects, suggesting that there is an additional pathway from Ras2p to Msn2/4p. Thus, Ras2p regulates cytoskeletal polarity in yeast under conditions of mild temperature stress through the stress response pathway.

INTRODUCTION

Growth during the cell cycle in budding yeast is polarized to ensure correct assembly of a new bud and correct septum formation before cell division. These processes require coordination between the synthesis of cell growth material by the secretory pathway and its delivery by the polarized cytoskeleton. The actin cytoskeleton, consisting of cortical patches and cables, is polarized toward regions of cell growth and is responsible for delivery of the post-Golgi vesicles (Adams and Pringle, 1984; Novick and Botstein, 1985; Ayscough *et al.*, 1997; reviewed by Finger and Novick, 1998; Pruyne and Bretscher, 2000b). The organization of the actin cytoskeleton is under the control of the cyclin-dependent kinase Cdc28p, which directs, in an unknown manner, a cascade of proteins centered on Cdc42p that ultimately sets up and maintains polarity (reviewed by Lew and Reed, 1995; Pruyne and Bretscher, 2000a). For example, when G1 cyclins, Cln1/2p, activate Cdc28p early in the cell cycle, Cdc42p along with several polarisome components, including Bni1p, Spa2, and Bud6p/Aip3p, localizes to the site of bud emergence to direct the assembly of a polarized actin cytoskeleton (Snyder, 1989; Snyder *et al.*, 1991; Ziman *et al.*, 1993; Amberg *et al.*, 1997; Evangelista *et al.*, 1997; Sheu *et al.*, 1998; Jin and Amberg, 2000). Recently, distinct functions have been assigned to cortical patches and cables: many of the components of actin cortical patches are necessary for endocytosis (reviewed by Geli and Riezman, 1998; Wendland *et al.*, 1998; Pruyne and Bretscher, 2000b); whereas actin cables are necessary for Myo2p-dependent polarized delivery of secretory vesicles, vacuolar elements, and factors necessary for initial spindle orientation (Johnston *et al.*, 1991; Govindan *et al.*, 1995; Hill *et al.*, 1996; Catlett and Weisman, 1998; Schott *et al.*, 1999; Yin *et al.*, 2000), as well as for delivery of Ash1p mRNA by Myo4p (Bobola *et al.*, 1996; Takizawa *et al.*, 1997).

So far four components of actin cables have been identified: actin (Adams and Pringle, 1984), fimbrin (Drubin *et al.*, 1988), Abp140p (Asakura *et al.*, 1998), and tropomyosin (Liu

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Abbreviations used: CM, complete minimal; DIC, differential interference contrast; 5-FOA, 5-fluoroorotic acid; LY, lucifer yellow-carbohydrazide; PCR, polymerase chain reaction; YPD, yeast extract-peptone-dextrose medium.

and Bretscher, 1989), of which only tropomyosin is restricted to the cables. Tropomyosins are encoded by two genes, *TPM1* and *TPM2*, which overlap in providing an essential polarizing function (Drees *et al.*, 1995). Disruption of *TPM1*, which encodes the major tropomyosin isoform, is not lethal but results in yeast cells with a greatly reduced number of cables and with a slightly reduced cortical patch polarity (Liu and Bretscher, 1989, 1992). Elimination of all tropomyosin function leads to the complete loss of cables, depolarization of secretion, and depolarization of cortical patches (Pruyne *et al.*, 1998).

In earlier studies aimed at identifying components that participate with tropomyosins in the structure or the regulation of the actin cytoskeleton, we set up a genetic screen for mutations that were lethal in the absence, but not in the presence, of Tpm1p. We analyzed seven mutations that fell into six complementation groups, all of which disrupted the normal polarity of the actin cytoskeleton (Wang and Bretscher, 1995, 1997). Thus, the *tpm1*Δ synthetic lethality screen appears to cast a wide net for mutations affecting polarity of the actin cytoskeleton. We therefore reasoned that an additional constraint to this screen might recover mutations affecting more defined aspects of polarization.

Because secretion and polarization of the actin cytoskeleton are closely coordinated events, there might be molecules that integrate these two processes. To search for such components, we sought to identify mutations that showed both synthetic lethality with *tpm1*Δ and were conditionally defective in secretion. In their classic screen for mutations that defined essential genes necessary for secretion, Novick *et al.* (1980) made use of the fact that cells conditionally defective for secretion become denser at their restrictive temperature. We therefore set out to look for mutations that resulted in synthetic lethality with *tpm1*Δ, conferred a conditional growth phenotype, and caused the cells to become denser at their restrictive temperature.

In addition to the expected isolation of mutations in *SEC* genes to be described elsewhere, this strategy resulted in the unexpected isolation of mutations in the *RAS2* gene. Yeast has two Ras genes, *RAS1* and *RAS2*, which are functional homologues of the mammalian proto-oncogene Ha-ras (DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985). In mammalian cells, Ras signals to multiple pathways that regulate nuclear gene expression as well as the actin cytoskeleton (Vojtek and Der, 1998; Shields *et al.*, 2000). Ras regulation of the actin cytoskeleton is of particular interest in the study of cancers because rearrangements of the actin cytoskeleton are thought to be necessary for metastasis (Barbacid, 1987).

In contrast to the multiple Ras pathways present in mammalian cells, there exists only one well characterized pathway in yeast. This is a regulatory pathway from Ras1/2p through the cyclase-associated protein Srv2p to adenyl cyclase, Cyr1p (Shima *et al.*, 2000). Production of cAMP activates the three protein kinase (PK) A catalytic subunits (Tpk1p, Tpk2p and Tpk3p) by binding to the PKA regulatory unit (Bcy1p) (Broach, 1991). In turn, PKA signals to the nucleus to carry out many functions of which an essential one is G1 progression (Thevelein and de Winde, 1999). The Ras2/PKA pathway has also been found to be a negative regulator of the stress response pathway (Marchler *et al.*, 1993; Gorner *et al.*, 1998). Many pathways, including the PKA pathway, converge on the related transcription factors Msn2p and Msn4p, which bind to the stress response element STRE to induce stress response genes (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996; Gorner *et al.*, 1998).

Here we show that yeast Ras proteins are important for maintaining the polarity of the yeast actin cytoskeleton under conditions of mild heat stress. Moreover, loss of polarity

Table 1. Yeast strains used in this study

Strain	Genotype	Source
NY10	*MAT*α *ura3-52*	P. Novick, Yale University
NY13	*MAT*a *ura3-52*	P. Novick
CUY570	*MAT*a *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1*	T. Huffaker, Cornell University
RCY250	*MAT*α *ura3-52 leu2-3,112 sec5-24*	R. Collins, Cornell University
ABY153	*MAT*α *met4*	B. Tye, Cornell University
ABY1075	*MAT*a *ura3-52 leu2*Δ*::kanMX*	This study
ABY1078	*MAT*α *ura3-52 tpm1*Δ*::kanMX* + pTPM1-URA3	This study
ABY1082B	*MAT*α *ura3-52 leu2*Δ*::kanMX*	This study
ABY1084B	*MAT*α *ura3-52 leu2*Δ*::kanMX tpm1*Δ*::kanMX* + pTPM1-URA3	This study
ABY1087B	*MAT*a *ura3-52 leu2*Δ*::kanMX tpm1*Δ*::kanMX* + pTPM1-URA3	This study
ABY1089	*MAT*α *ura3-52 leu2*Δ*::kanMX RAS1::LEU2*	This study
ABY1204	*MAT*a *ura3-52 leu2*Δ*::kanMX ras2*Δ*::URA3*	This study
ABY1205	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1*	This study
ABY1206	*MAT*a *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1*	This study
ABY1207	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1 ras1*Δ*::LEU2*	This study
ABY1209	*MAT*a *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1 ras2*Δ*::LEU2*	This study
ABY1210	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1 ras2*Δ*::LEU2*	This study
ABY1226	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1 tpm1*Δ*::kanMX*	This study
ABY1228	*MAT*a *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1 ras2*Δ*::LEU2 tpm1*Δ*::kanMX*	This study
ABY1240	*MAT*α *ura3-52 leu2-3,112 his3-*Δ*200 trp1-*Δ*1 ras1*Δ*::LEU2 ras2*Δ*::LEU2* + pTPK1	This study
ABY1354	*MAT*a *ura3-52 leu2*Δ*::kanMX tpm1*Δ*::kanMX tsl7-1* + pTPM1-URA3	This study
SP1	*MAT*α *his3 leu2ura3 trp1 ade8 Can*	Stanhill *et al.*, 1999
SP1*ras2*Δ	Isogenic to SP1 but *ras2::LEU2*	Stanhill *et al.*, 1999
SP1*ras2*Δ*msn2*Δ*msn4*Δ	Isogenic to SP1 but *ras2::LEU2 msn2::HIS3 msn4::URA3*	Stanhill *et al.*, 1999

Table 2. Plasmids used in this study

Name	Characteristics and Source
pTPM1-URA3	*TPM1* in pRS316 (*CEN, URA3*) (Bretscher Lab Stock)
pTPM1-LEU2	= pTW119, *TPM1* in pRS315 (*CEN, LEU2*) (Wang and Bretscher, 1997)
pRAS1	= pJH71, *RAS1* in pRS315 (*CEN, LEU2*). Isolated as a suppressor of *ras2* mutant from a genomic library in pRS315 (Wang and Bretscher, 1997).
pRAS2	= pJH92, *RAS2* in pRS416 (*CEN, URA3*). Made by subcloning a 1.9-kb *Hin*dIII-*Xba*I *RAS2* containing genomic fragment from a genomic library in pRS315 (Wang and Bretscher, 1997) into pRS416.
P_{ADH1}-Ha-ras	= AAH5 H-ras, Ha-ras under the control of the *ADH1* promoter in AAH5 (2μ, *LEU2*) (Marshall et al., 1987)
P_{RAS2}-Ha-ras	= pJH139, Ha-ras under the control of the *RAS2* promoter in pRS414 (*CEN, TRP1*). PCR was used to generate a *RAS2* promoter/Ha-ras fusion. The fusion fragment was amplified by using the primer pair 5′-AACGTTTTCGAATTGAAAGGAGATATACAGAAAAAAAA**ATGACAGAATACAAGCTTGT-3′** spanning the *RAS2* promoter/Ha-ras junction and 5′-AGACTCGAGACTGCCCAGATGTCTTGCTG-3′ downstream of Ha-ras. The *Nsp*V and *Xho*I restriction sites are underlined and the Ha-ras open reading frame is in bold. This PCR product was cloned into the pCR-BluntII-Topo vector (Invitrogen, Carlsbad, CA) and then checked for errors by sequencing. The DNA was then cut with *Nsp*V and *Xho*I and inserted into a plasmid containing a genomic fragment of *RAS2* in pRS414 (*CEN, TRP1*) which replaced the ORF of *RAS2* with Ha-ras but left the promoter intact.
pCDC42	= pPB102, *CDC42* (2μ, *URA3*) (Bender and Pringle, 1989)
$pCDC42^{Val12}$	= pRS315(42/Val12)BH-2, $CDC42^{VAL12}$ in pRS315 (*CEN, LEU2*) (Ziman et al., 1991)
pRHO1	= pWT-URA_E, *RHO1* (2μ, *URA3*) (Madaule et al., 1987)
pRHO2	= pC-186, *RHO2* (2μ, *URA3*) (Madaule et al., 1987)
pRHO3	= pPB1070, *RHO3* (2μ, *URA3*) (Bender et al., 1996)
pRHO4	= pOPR4, *RHO4* (2μ, *TRP1*) (Matsui and Toh, 1992)
pTEM1-HA3	= pSJ56, C-terminally 3× HA tagged *TEM1* in pRS426 (2μ, *URA3*) (Jaspersen et al., 1998)
pHA3-DBF2	= pSJ57, N-terminally 3× HA tagged *DBF2* in pRS426 (2μ, *URA3*) (Jaspersen et al., 1998)
pCDC15-HA3	= pSJ103, C-terminally 3× HA tagged *CDC15* in pRS426 (2μ, *URA3*) (Jaspersen et al., 1998)
pHA-CDC5	= p29, N-terminally HA tagged *CDC5* in pRS426 (2μ, *URA3*) (Jaspersen et al., 1998)
pSPO12	= pSJ19, *SPO12* in pRS426 (2μ, *URA3*) (Jaspersen et al., 1998)
pRSR1	= pJH140, *RSR1* in pRS424 (2μ, *TRP1*). Made by subcloning a *Pst*I-*Spe*I PCR-generated region of genomic *RSR1* into pRS424 (2μ, *TRP1*). The PCR construct was checked for errors by sequencing.
pSRV2	= pJH129, *SRV2* in pRS424 (2μ, *TRP1*). Made by subcloning a *Xho*I-*Sst*I PCR-generated region of genomic *SRV2* into pRS424 (2μ, *TRP1*). PCR construct was checked for errors by sequencing.
pVPS34	= pPHY52, *VPS34* in pRS324 (2μ, *TRP1*) (Schu et al., 1993)
pTPK1	= pJH126, *TPK1* in pRS423 (2μ, *HIS3*). Made by subcloning a *Hin*dIII-*Sph*I *TPK1* containing fragment from B1373 (Toda et al., 1987) in PUC19. Then from this plasmid, a *Hin*dIII-*Sst*I *TPK1* containing fragment was subcloned into pRS416. Then from this plasmid, a *Xho*I-*Sst*I *TPK1* containing fragment was subcloned into pRS423 (2μ, *HIS3*).
pTPK2	= pXP3, *TPK2* in Yeplac195 (2μ, *URA3*) (Pan and Heitman, 1999)
pTPK3	= pXP4, *TPK3* in Yeplac195 (2μ, *URA3*) (Pan and Heitman, 1999)
pPFY1	= p593, *PFY1* in YEp24 (2μ, *URA3*) (Evangelista et al., 1997)

is accompanied by depolarization of some key polarity markers. Because this effect of *ras2*Δ is abrogated in cells lacking Msn2/4p, Ras2p must normally signal through the stress response pathway to regulate cytoskeletal organization and polarization in yeast.

MATERIALS AND METHODS

Strains, Plasmids, and Media

Yeast strains used in this study are listed in Table 1. Plasmids used in this study and their method of construction are described in Table 2. Transformation of yeast cells was performed with the use of Frozen-EZ Yeast Transformation Kit (ZYMO Research, Orange, CA). Standard genetic techniques were used for strain construction and linkage analysis (Guthrie and Fink, 1991). Yeast were grown with the use of standard rich yeast extract-peptone-dextrose (YPD) and complete minimal (CM) dropout media (Ausubel *et al.*, 2000; Difco Laboratories, Detroit, MI)

Strain Construction

ABY1075 was constructed by replacing *LEU2* with *kanMX* in NY13. *LEU2* was replaced by transforming a polymerase chain reaction (PCR)-generated DNA fragment containing the *kanMX* module (Wach *et al.*, 1994) flanked by 50 bases homologous to a region immediately upstream of the *LEU2* start codon and directly downstream of the *LEU2* stop codon. ABY1078 was constructed by replacing *TPM1* with *kanMX* in NY10 and then transforming in pTPM1-URA3. ABY1082B, ABY1084B, and ABY1087B were obtained by mating ABY1075 with ABY1078. Because *kanMX* was used in the disruption of both *LEU2* and *TPM1*, $leu2^-$ and $LEU2^+$ spores were differentiated on the basis of their ability to grow on CM medium lacking leucine, and $tpm1^-$ and $TPM1^+$ spores were differentiated by immunoblot analysis after having grown on 5-fluoroorotic acid (5-FOA) to select for loss of pTPM1-URA3. To tag the *RAS1* locus with the *LEU2* marker in ABY1089, a PCR-generated fragment containing the *LEU2* marker replaced a region of the chromosomal DNA from position −617 to −864, relative to the start of the *RAS1* open reading frame. Proper insertion of the *LEU2* marker was verified by PCR. ABY1204 was constructed by replacing *RAS2* with *URA3* in ABY1075. To disrupt *RAS2*, a segment of the *RAS2* open reading frame (from +42 to +543) was replaced with a PCR-generated DNA fragment containing *URA3* from the pRS series of vectors (Sikorski and Hieter, 1989) flanked by 625 bases homologous to a region upstream and 676 bases homologous to a region downstream of the replaced segment. Proper disruption of *RAS2* was verified by PCR. ABY1205 and ABY1206 were obtained

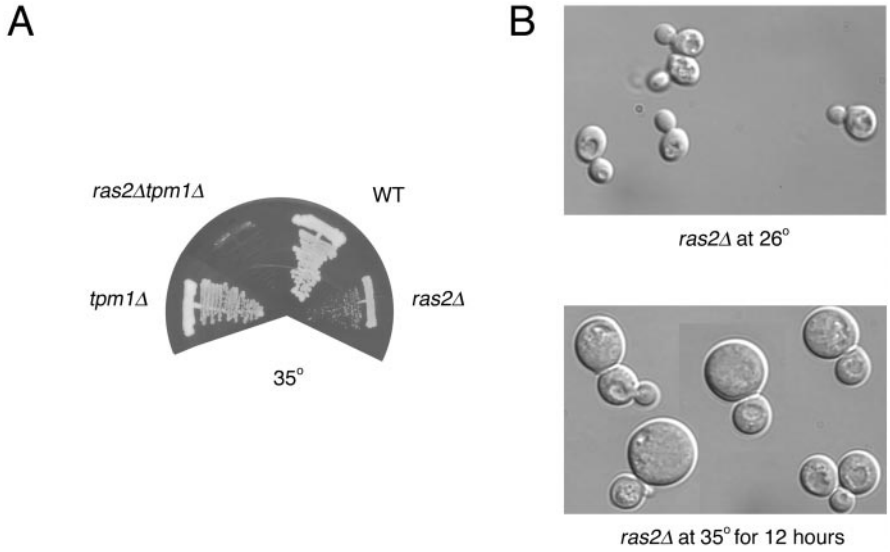

Figure 1. Growth properties of mutants. (A) Wild-type (WT; ABY1205), *ras2*Δ (ABY1209), *tpm1*Δ (ABY1226), and *ras2*Δ*tpm1*Δ (ABY 1228) were first grown on selective media and then streaked onto YPD plates at 35°C. Growth after 2 d is shown. (B) DIC images of *ras2*Δ (ABY1209) grown at 26°C and after a 12-h 35°C temperature shift.

by mating ABY1082B with CUY570 followed by sporulation of the generated diploid. ABY1207 was generated by first replacing *RAS1* with *LEU2* in ABY1082B and then mating the resultant strain to CUY570 followed by sporulation of the generated diploid. To disrupt *RAS1*, the first 480 bases and much of the upstream promoter sequence was replaced with a PCR-generated DNA fragment containing *LEU2* flanked by 575 bases homologous to a region upstream and 428 bases homologous to a region downstream of the replaced segment. Proper disruption of *RAS1* was verified by PCR. ABY1209 and ABY1210 were constructed by replacing *RAS2* with *LEU2* in ABY1082B (method of replacement is the same as the method used to construct ABY1204) and then mating the resultant strain to CUY570 followed by sporulation of the generated diploid. ABY1226 and ABY1228 were obtained in a two-step process: first, ABY1206 was mated to ABY1078 to obtain a strain that was *MAT*a *his3 trp1 leu2 ura3 tpm1*Δ*::kanMX* + pTPM1-URA3; second, the resulting strain was mated to ABY1210 and then sporulated, and the dissected spores were grown on 5-FOA. To construct ABY1240, pRAS2 was transformed into ABY1209; this strain was then mated to ABY1207 and the generated diploid was sporulated. Because a double knockout of Ras is lethal, a *ras1*Δ*ras2*Δ strain was selected on the basis of 5-FOA sensitivity; the selected strain was then transformed with pTPK1 and grown on 5-FOA to select against cells retaining the pRAS2 plasmid.

Isolation of tpm1Δ *Sythetic Lethal Mutants*

Six stationary-phase cultures of ABY1084B and three stationary phase cultures of ABY1087B were harvested, washed twice with water, and resuspended in 5 ml of 0.5 M sodium acetate (pH 4.8) and 20 mg of sodium nitrite to induce mutations. After 10 min at 30°C, 5 ml of 2.7% Na_2HPO_4 containing 1% yeast extract was added, and cells were harvested and washed with water. This treatment resulted in approximately a 50% loss in viability. The mutagenized cells were resuspended in 50 ml of YPD medium to give an OD_{600} of ~0.015 and then allowed to recover at 25°C for 16 h to an OD_{600} of 0.1–0.25. The cultures were then incubated at 37°C for 3 h, placed in an ice bath for 15 min, spun down, and resuspended in 0.8 ml of water. To enrich for dense cells, a sample was mixed with 9.2 ml of Percoll (colloidal silica; Sigma Chemical, St Louis, MO) in a 10-ml Sorvall Polypropylene Oak Ridge bottle (Sorvall, Newton, CT) and centrifuged in an HB-4 swinging bucket rotor (20,000 × *g*, 20 min, 4°C). One-milliliter fractions were taken from the top of the gradient and the two densest fractions, containing ~2% of the cells, were diluted into 50 ml of YPD medium. The cells were then subjected to another round of growth at 25°C, a temperature shift to 37°C, and density enrichment. After the second round of density enrichment, the two densest fractions were diluted into 10 ml of YPD medium containing 25% glycerol and frozen (−80°C).

Cells were thawed, diluted 1:20, and plated onto YPD to give ~100 colonies per plate, with a total of 360 plates used for the nine independent cultures. The plates were incubated at room temperature (20–25°C) for 2 d, and colonies were then replicated onto a CM 5-FOA plate and a CM uracil dropout plate. After 2 d the replica plates were compared, and those colonies that were able to grow on CM lacking uracil and not on CM 5-FOA were saved for further analysis.

Microscopy and Imaging

Fluorescence microscopy of actin was performed as described by Pringle *et al.* (1989). Affinity-purified actin antibodies (Wang and Bretscher, 1995) were used at a 1:25 dilution. Myo2p staining was performed with the use of affinity-purified Myo2p antibody (Schott *et al.*, 1999) at a 1:50 dilution. Cdc42p staining was performed as described by Kozminski *et al.* (2000) with the use of an affinity-purified α-Cdc42p antibody, generously provided by David Drubin. Tpm1p staining was performed with the use of affinity-purified Tpm1p antibody (Pruyne *et al.*, 1998) at a 1:50 dilution. In all cases, a methanol/acetone postfixation step and a goat anti-rabbit IgG fluorescein isothiocyanate (ICN BioChemicals, Aurora, OH) secondary antibody at a 1:150 dilution were used. Staining with lucifer yellow-carbohydrazide (LY; Molecular Probes, Eugene, OR) staining was performed as described by Riezman (1985). *N*-(3-Triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl) pyridium dibromide (FM4–64; Molecular Probes) staining was performed as described by Vida and Emr (1995).

For brightfield microscopy, cells were first fixed in formaldehyde for 2 h and visualized with the use of differential interference contrast (DIC).

DIC and fluorescence images were acquired with a RTC/CCD digital camera (Princeton Instruments, Trenton, NJ) with the use of a Zeiss Axiovert 100 TV microscope (Carl Zeiss, Oberkochen, Germany) and the Metamorph Imaging System (Universal Imaging, West Chester, PA). All images were processed through Abode Photoshop 5.0 (Adobe Systems, Mountain View, CA).

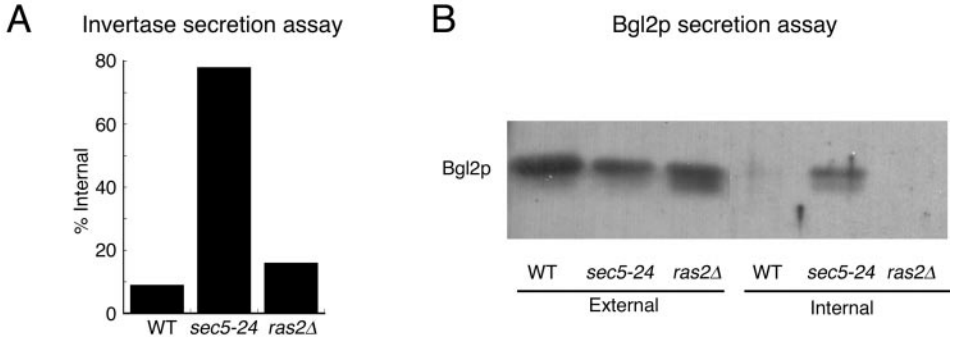

Figure 2. *ras2*Δ mutants accumulate neither invertase nor Bgl2p. (A) *ras2*Δ (ABY1205) mutants shifted to 37°C for 3 h accumulate invertase to the same degree as wild-type (WT) strains (ABY1209) and to a lesser degree than *sec5-24* mutants (RCY250). Percentage of internal invertase is calculated as internal/(internal plus external). (B) Immunoblot analysis of Bgl2p shows that *ras2*Δ (ABY1205) mutants shifted to 37°C for 3 h does not accumulate Bgl2p internally, similar to wild-type strains (ABY1205) but in contrast to *sec5-24* mutants (RCY250).

Determination of Internal Levels of Invertase and Bgl2p

Cells were grown in YPD to midlog phase and preshifted to 37°C for 30 min. The yeast were then washed once with water, pelleted, and resuspended in low-glucose (0.1%) YPD and kept at 37°C for 3 h. Afterward, cells were washed twice in chilled 10 mM NaN_3 solution, once in a basic solution (0.1 M Tris-SO_4, pH 9.4) to loosen the cell wall, once in spheroplast buffer (0.1 M Tris-PO_4, pH 7.5, with 1.2 M sorbitol and 14 mM β-mercaptoethanol), and then spheroplasted by the addition of 0.05 mg/ml 100T zymolyase for 30 min at 37°C. Samples were centrifuged to separate internal from external fractions. The supernatant (external fraction) was separated and the pellet (internal fraction) was resuspended in an equal volume of spheroplast buffer. Triton X-100 was added to one-half of both the external and internal fractions to a 0.5% final concentration and used for invertase assays. The other half was boiled in 6× SDS sample buffer, subjected to SDS-PAGE, and immunoblotted for Bgl2p with an antibody generously provided by Dr. Franz Klebl. Invertase was assayed at 30°C as described by Novick and Schekman (1979).

Quantitation of LY uptake

Quantitation of LY uptake was done through analysis of digitally acquired images with the use of the Metamorph Imaging System. A fixed area smaller than the size of a single yeast vacuole (a circle with a radius of 0.3 μm) was chosen as the standard area of measurement. Within each cell the amount of fluorescence contained in the standard area was measured when the standard area was placed within the vacuole and when it was placed in the cytoplasm. The difference between these two measurements was considered the amount of LY taken up by the cell. This process was repeated for n = 30 *RAS2*$^+$ cells and n = 30 *ras2*Δ cells.

RESULTS

Identification of Conditional Mutants That Show Synthetic Lethality with **tpm1Δ** *and Become Denser at 37°C*

To isolate mutants that are synthetically lethal with *tpm1*Δ, show temperature-sensitive growth, and get denser at the restrictive temperature, we generated *tpm1*Δ strains that carried the pTPM1-URA3 plasmid in both mating types (ABY1084B and ABY1087B). Nine independent cultures were mutagenized with nitrous acid to ~50% viability. After recovering from mutagenesis, exponentially growing cultures were shifted to 37°C for 3 h and fractionated on a Percoll density gradient. The denser cells, representing ~2% of the population, were recovered and then subjected to a second temperature-dependent density enrichment step. After the second enrichment, cells were spread onto YPD plates. After growth at 25°C, ~34,000 colonies were replica plated onto control and 5-FOA-containing plates. A total of

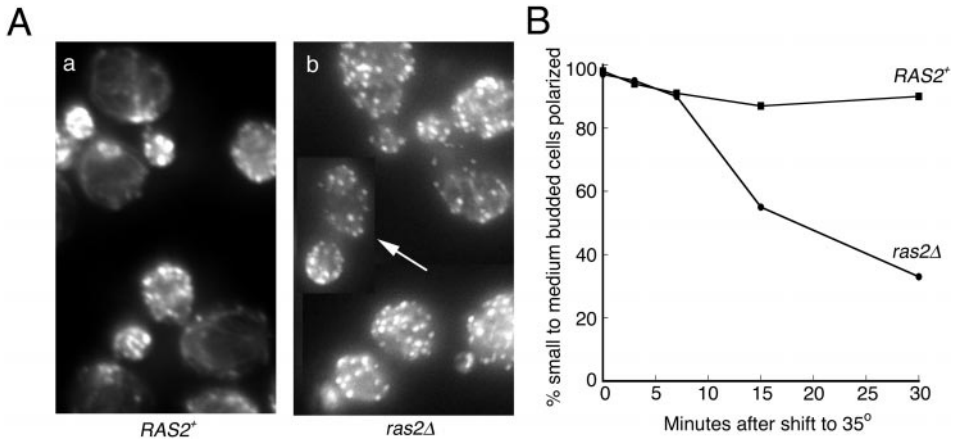

Figure 3. The actin cytoskeleton becomes depolarized in *ras2*Δ cells at 35°C. (A) *RAS2*$^+$ (ABY1205) and *ras2*Δ (ABY1209) cells growing in YPD were shifted to 35°C for 30 min, fixed, and stained for actin. *ras2*Δ mutants became depolarized after a 30-min shift, whereas wild-type cells remained polarized. The arrow indicates a cell that was scored as polarized, although qualitatively the cell is not completely polarized. (B) *RAS2*$^+$ (■, ABY1205) and *ras2*Δ (●, ABY 1209) cells were shifted to 35°C, fixed at 0, 3, 7, 15, and 30 min, and stained for actin. One hundred small and medium budded cells were scored for actin polarization. A cell was scored polarized if >50% of total actin patch fluorescence was concentrated in the bud.

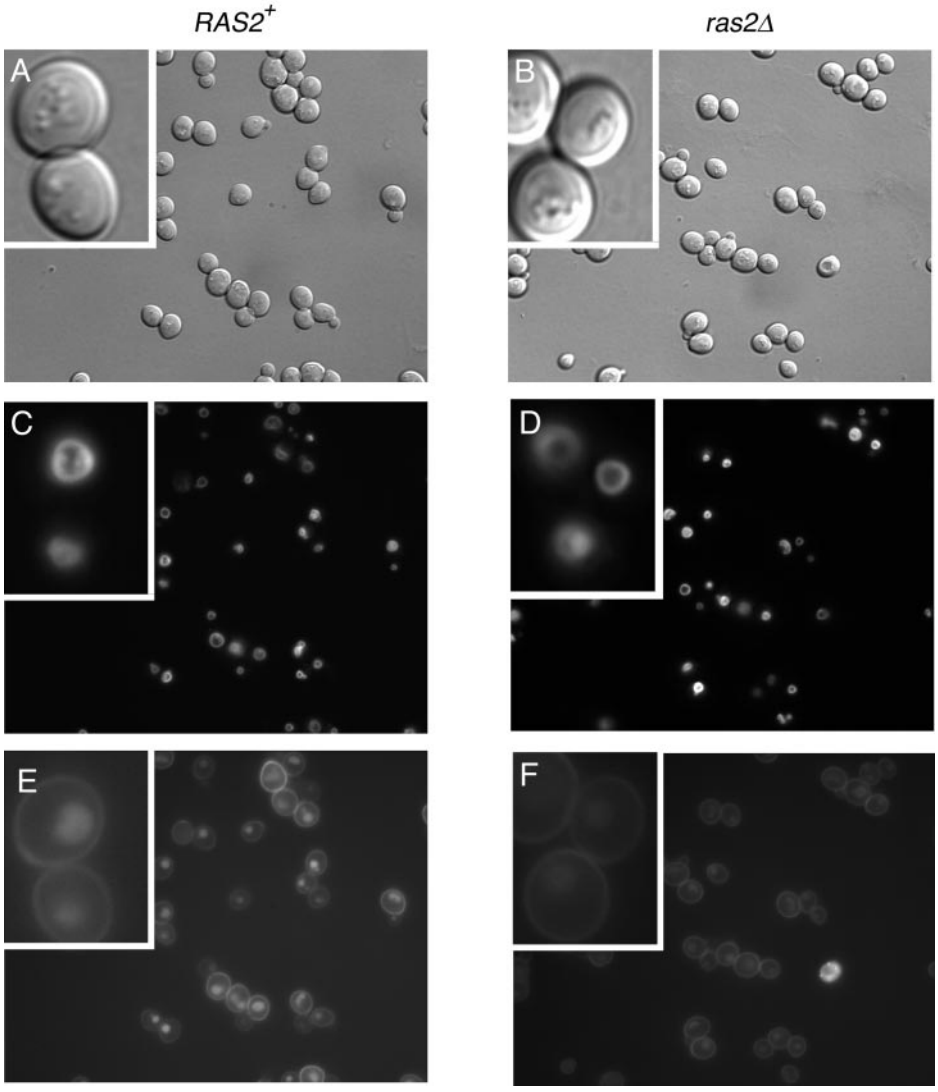

Figure 4. *ras*2Δ cells are partially defective for endocytosis of LY. Corresponding DIC (A and B), FM4-64 (C and D), and LY (E and F) images of wild-type (ABY1205) and *ras*2Δ (ABY1209) cells. Cells were grown and stained with the vacuolar marker FM4-64 at 26°C and then shifted to 37°C for 1.5 h before incubating with LY for 1 h at 37°C.

1064 colonies failed to grow on the 5-FOA plates, indicating that they were probably unable to grow without the pTPM1-URA3 plasmid. Of these colonies, 112 were temperature sensitive for growth at 37°C. From these, 72 were able to grow on 5-FOA if an alternate plasmid containing *TPM1* (pTPM1-LEU2) was introduced, demonstrating a dependence on *TPM1*. On retesting, all 72 potential Tsl (*TPM1* synthetic lethal) mutants showed temperature sensitivity and became denser after growth at 37°C.

Complementation tests were used to assess dominance of the mutations and to place recessive mutants into complementation groups. After this, selected Tsl mutants were backcrossed to the appropriate parental strains and sporulated. Tetrads were analyzed for segregation of the three phenotypes: synthetic lethality with *tpm1*Δ, conditional growth at 37°C, and increased density at 37°C. Thirty-three of the mutants were discarded either because the phenotypes did not cosegregate or were too leaky. The remaining 39 mutants contained recessive mutations that fell into 10 complementation groups, designated *TSL7* through *TSL16*. The two largest groups, *TSL7* and *TSL8*, showed a clear cosegregation of the temperature sensitivity and enhanced density phenotypes, although through successive backcrosses the *tpm1*Δ synthetic lethal phenotype became leaky. Because *TSL7* contained the largest number of independent isolates, we explored it further. In this report we characterize the *TSL7* gene; the other *TSL* complementation groups will be described elsewhere.

TSL7 Is RAS2

The *TSL7* gene was cloned from a *CEN*-based genomic library (Wang and Bretscher, 1995) by complementation of *tsl7-1* temperature sensitivity. Seventeen plasmids were recovered, eight of which contained genomic DNA covering the *RAS1* locus and nine of which contained genomic DNA covering the *RAS2* locus. Further subcloning demonstrated that *RAS2* complemented the *tsl7* mutation but not the adjacent open reading frames. *RAS1* on a *CEN* plasmid was also able to complement the temperature sensitivity of *tsl7*.

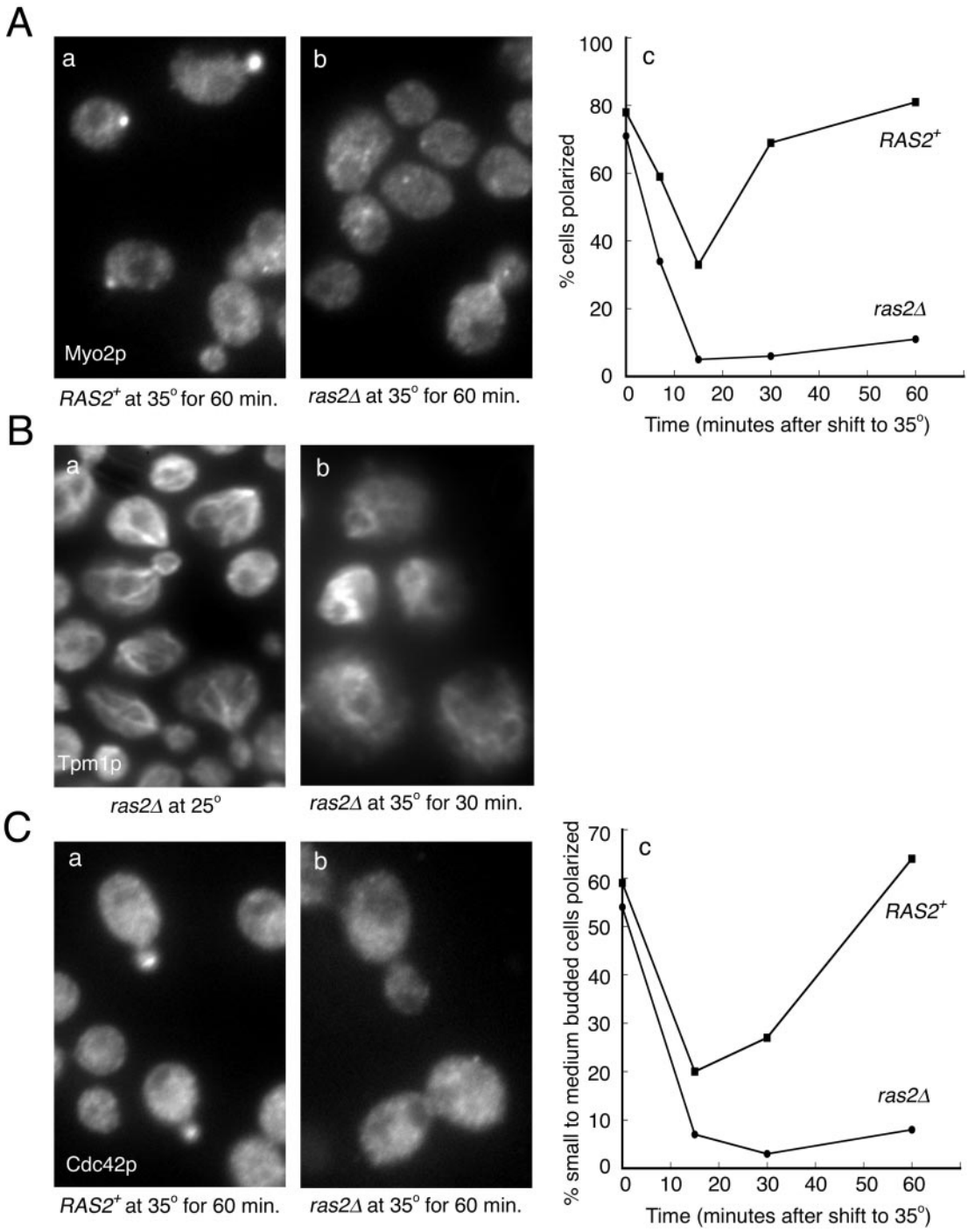

Figure 5. The polarity markers Myo2p and Cdc42p show sustained depolarization in *ras2*Δ cells. The percentage of *RAS2*$^+$ (■, ABY1205) and *ras2*Δ (●, ABY1209) cells showing a polarized distribution of Myo2p and Cdc42p was determined after shifting cells growing in YPD to 35°C for the indicated times and then staining with specific antibodies. (A) For Myo2p staining, cells at all stages of the cell cycle were scored. (C) For Cdc42p staining, cells at the small and medium budded stages were scored. Examples of staining after 60-min shifts (a and b). (B) Tpm1p localization in *ras2*Δ cells (ABY1209), before (a) and after (b) a 30-min shift to 35°C.

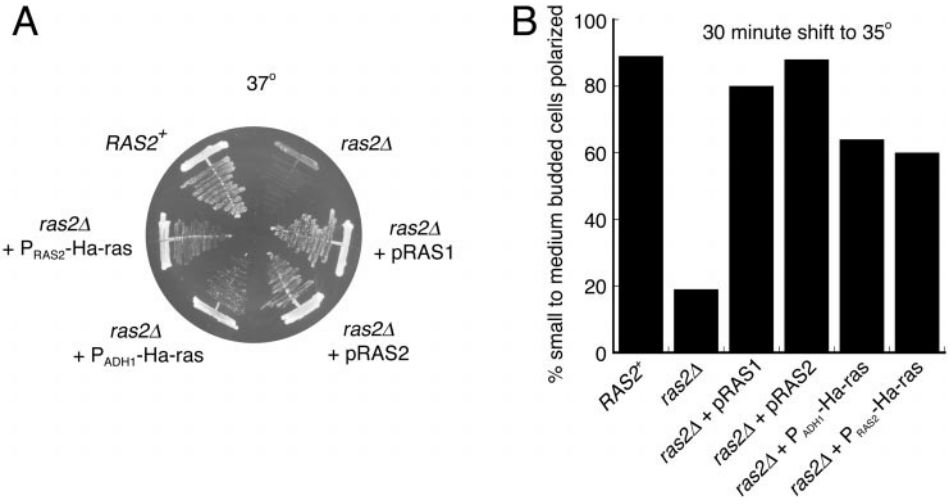

Figure 6. Suppression of both temperature sensitivity and actin depolarization *ras2*Δ phenotypes by yeast and human Ras proteins. (A) Growth of wild-type (ABY1205), *ras2*Δ (ABY1209 or ABY1204) alone or carrying pRAS1, pRAS2, P_{ADH1}-Ha-ras, or P_{RAS2}-Ha-ras on CM selective media at 37°C after 3 d. (B) Cells were also grown in CM selective media, shifted to 35°C for 30 min, fixed, stained for actin, and scored for actin polarization.

To distinguish whether *TSL7* was *RAS1* or *RAS2*, the linkage of *tsl7-1* (ABY1354) to *RAS1::LEU2* (ABY1089) and the linkage of *tsl7-1* to *met4* (ABY153), which is closely linked to *RAS2*, was determined. Analysis of 14 tetrads revealed no linkage between *tsl7-1* and *RAS1::LEU2*, whereas analysis of 17 tetrads revealed a genetic distance of 5.5 cM between *tsl7-1* and *met4*, close to the reported distance of 4.4 cM between *RAS2* and *MET4*. Next, we sequenced the *RAS2* locus in four of the five independent isolates from the *TSL7* complementation group. All four isolates contained mutations (Gln29→Stop, Gly67→Arg, Gln136→stop, Gln156→stop) in *RAS2* that are predicted to destroy Ras2p function.

Consistent with the linkage analysis, we generated a *ras2*Δ strain that conferred both a temperature-sensitive phenotype and a temperature-dependent increase in density; whereas our *ras1*Δ conferred neither a temperature-sensitive phenotype nor a temperature-dependent increase in density. *ras2*Δ cells were crossed to *tpm1*Δ cells and, surprisingly, the double mutant combination was viable at room temperature. However, whereas the *tpm1*Δ and *ras2*Δ strains grew at 35°C, the *tpm1*Δ *ras2*Δ double mutant did not (Figure 1A). From these data, we conclude that *TSL7* is *RAS2* and that *ras2*Δ shows synthetic lethality with *tpm1*Δ at 35°C.

When *ras2*Δ cells are shifted to 35°C for 12 h, cells enlarge and become heterogeneous in size (Figure 1B). This phenotype is in contrast to phenotypes in which there are conditional defects in secretion, where cells stop growing completely, but remarkably similar *tpm1*Δ and other mutants defective in the cytoskeleton, such as *act1*, *myo2,* and *cdc42*, which continue to grow isotropically when shifted to their restrictive temperatures (Novick and Botstein, 1985; Adams *et al.*, 1990; Johnston *et al.*, 1991).

Table 3. Ability of various genes expressed from plasmids to suppress the conditional growth and actin depolarization phenotypes of a *ras2*Δ mutant

Plasmid	Growth at 37°C	Actin polarity at 35°C	Plasmid	Growth at 37°C	Actin polarity at 35°C
Vector	−	−	pTPK2	−	−
pRAS1	+	+	pTPK3	−	−
pRAS2	+	+	pTEM1-HA3	−	−
P_{RAS2}-Ha-ras	+	+	pHA3-DBF2	−	−
P_{ADH1}-Ha-ras	+	+	pCDC15-HA3	−	−
pRHO1	−	−	pHA-CDC5	−	−
pRHO2	−	−	pSPO12	−	−
pRHO3	−	−	pRSR1	−	−
pRHO4	−	−	pSRV2	−	−
pCDC42	−	−	pPFY1	−	−
$pCDC42^{V12}$	−	−	pVPS34	−	−
pTPK1	−	−			

*ras2*Δ (ABY1209) cells were transformed with the various plasmids. After growth on selective media, strains were streaked to YPD media for growth at 37°C and after 3 d were scored to test for suppression of the conditional growth defect. Alternatively, cells were grown in liquid YPD at 26°C before being shifted to 35°C for 30 min and then fixed, stained for actin, and scored for actin polarization. + indicates growth or polarized actin; − indicates no growth or depolarized actin.

The ras2Δ Mutant Is Not Defective in the Secretion of Periplasmic Enzymes Invertase and Bgl2p

To determine whether the density phenotype of *ras2*Δ cells at 37°C was due to a defect in secretion, we examined the ability of *ras2*Δ strains to secrete the periplasmic enzymes invertase and Bgl2p. Wild-type, *ras2*Δ, and *sec5–24* strains were shifted to low glucose to induce invertase expression at 37°C, the restrictive temperature for *ras2*Δ strains. The amount of internal and external invertase was determined in three separate experiments, and the percentage of internal invertase is shown in Figure 2A. As expected, the classical secretion mutant *sec5-24* accumulates internal invertase and the wild-type strain secretes invertase normally. Similar to the wild-type strain, the *ras2*Δ mutant secretes invertase normally.

There are two distinct post-Golgi vesicle populations in yeast, with invertase being specific to the lighter population and Bgl2p, a cell wall endoglucanase, being specific to the denser population (Harsay and Bretscher, 1995). We reasoned that *ras2*Δ mutants might accumulate the denser population of vesicles rather than the lighter population and therefore examined whether Bgl2p accumulated in *ras2*Δ mutants shifted to 37°C for 3 h. Because Bgl2p is constitutively expressed, immunoblots revealed that it was present in all external fractions and, as expected, wild-type strains did not accumulate significant Bgl2p internally, whereas *sec5-24* did (Figure 2B). As with wild-type cells, the *ras2*Δ mutant did not accumulate Bgl2p internally, showing that *ras2*Δ mutants do not have a defect in Bgl2p secretion.

The **ras2Δ** *Mutant Has a Temperature-dependent Actin Cytoskeletal Polarization Defect*

Because the synthetic lethality between *tpm1*Δ and *ras2*Δ suggested that Ras2p might be involved in the organization of the actin cytoskeleton, we examined actin localization in *ras2*Δ cells. At room temperature, the actin organization of *ras2*Δ cells was similar to wild-type. However, upon a 30-min shift to 35°C, the actin cytoskeleton of the *ras2*Δ mutant became disorganized, whereas in wild-type cells the actin organization remained normal (Figure 3A). To quantitate this phenotype, we counted the number of small to medium budded cells that were polarized at various times after shifting to 35°C (Figure 3B). A cell was counted as polarized if >50% of the actin patches detected by fluorescence microscopy were within the bud. Before the temperature shift (0 time point), >95% of both *RAS2*$^+$ and *ras2*Δ cells were polarized. After a 30-min shift, only ~30% of *ras2*Δ cells remained polarized, whereas ~90% of *RAS2*$^+$ remained polarized; of that 30% in *ras2*Δ cells, many cells were qualitatively depolarized but were not counted as depolarized because the majority of actin patches remained in the bud (arrow in Figure 3A shows an example of a cell counted as polarized). This result argues that Ras2p is essential for maintaining actin cytoskeletal organization under mild heat stress conditions but is not essential for actin organization at room temperature. It also suggests that the reason *ras2*Δ and *tpm1*Δ show synthetic lethality only at 35°C is because the *ras2*Δ depolarization phenotype is manifested only at higher temperatures.

The ras2Δ Mutant Has a Partial Defect in the Endocytosis of LY

Mutations in cortical actin patch components frequently cause a loss of actin polarity as well as a defect in endocytosis (reviewed by Geli and Riezman, 1998; Wendland *et al.*, 1998; Pruyne and Bretscher, 2000b). Because *ras2*Δ cells display a loss of actin patch polarity and Ras2p binds directly to the patch component Srv2p, we examined whether *ras2*Δ cells would display a temperature-dependent endocytic defect. Although no defect could be detected in the endocytosis of the lipophilic dye FM4–64 (our unpublished results), *ras2*Δ mutant cells displayed a partial defect in the uptake of LY at 37°C (Figure 4, E and F). Quantitation of fluorescence intensity indicated a 50% reduction of LY staining in *ras2*Δ compared with wild-type cells (an average of 16,620 arbitrary units of fluorescence in *RAS2*$^+$ vs. 9098 U in *ras2*Δ mutants.)

Because it was difficult to see the vacuole clearly in DIC images of mutant cells after the temperature shift (Figure 4, A and B), we were concerned that the reduced level of LY staining might reflect a disruption of the vacuole rather than a defect in endocytosis. To examine this, the vacuole was labeled at room temperature with FM4-64 and then the cells were shifted to 37°C to examine endocytosis of LY. The results showed no significant alteration in vacuole morphology (Figure 4, C and D) and therefore indicated that *ras2*Δ confers a mild defect on endocytosis of LY.

Two Markers of Polarity, Myo2p and Cdc42p, Are Depolarized in **ras2Δ** *Mutants in a Temperature-dependent Manner*

Polarized secretion in yeast has been shown to occur by the delivery of post-Golgi vesicles, carried by the myosin V encoded by *MYO2*, along polarized actin cables (Govindan *et al.*, 1995; Pruyne *et al.*, 1998). In wild-type cells, Myo2p shows a highly polarized distribution (Lillie and Brown, 1994). To examine whether loss of Ras2p affected this distribution, *ras2*Δ cells were shifted to 35°C for various times and Myo2p localized by immunofluorescence microscopy. In wild-type cells, Myo2p underwent a transient depolarization within 15 min and then repolarized back to its former distribution (Figure 5A; Lillie and Brown, 1994). In contrast, *ras2*Δ cells, although initially having a highly polarized Myo2p distribution, depolarized to a greater extent than wild-type cells and remained depolarized for up to 60 min. But consistent with the ability of *ras2*Δ cells to grow in temperatures up to 35°C, after 3 h at 35°C, 60% of *ras2*Δ cells regained a polarized distribution of Myo2p (Ho and Bretscher, unpublished results). This is in contrast to a *ras2*Δ*tpm1*Δ mutant that was unable to live at 35°C and in which Myo2p distribution remained depolarized after 3 h at 35°C (Ho and Bretscher, unpublished results). Because Myo2p translocates down actin cables (Lillie and Brown, 1994; Pruyne *et al.*, 1998), we examined cable organization. Immunolocalization of the cable-specific component Tpm1p in *ras2*Δ cells revealed that they are well organized at 25°C but completely disorganized after shifting to 35°C for 30 min (Figure 5B).

Cdc42p is one of the key molecules directing the polarity of the actin cytoskeleton in yeast. During the cell cycle, Cdc42p is localized to sites of active growth (Ziman *et al.*,

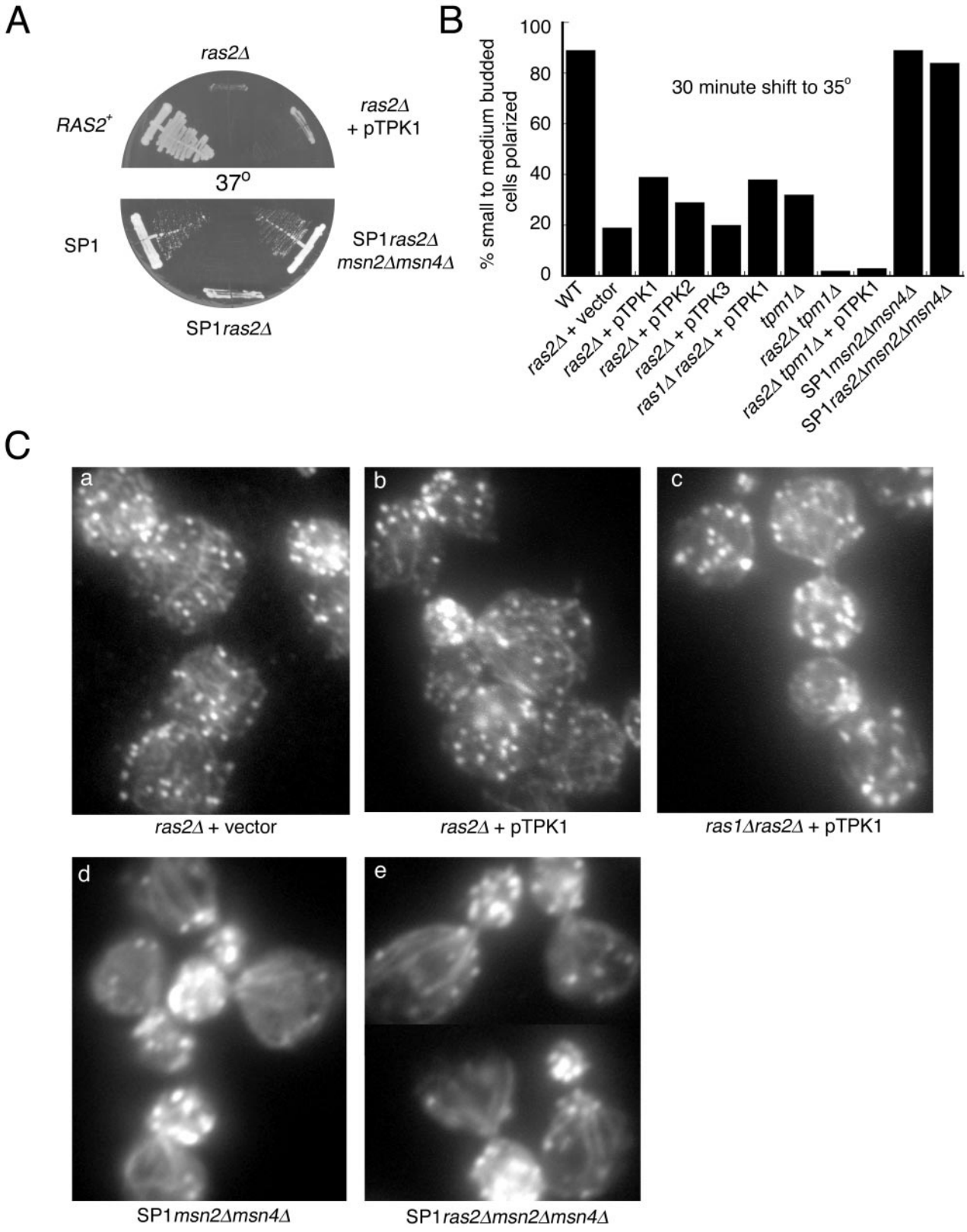
A
ras2Δ
RAS2+
ras2Δ
+ pTPK1
37°
SP1
SP1ras2Δ
msn2Δmsn4Δ
SP1ras2Δ
B
% small to medium budded cells polarized
100
80
60
40
20
0
30 minute shift to 35°
WT
ras2Δ + vector
ras2Δ + pTPK1
ras2Δ + pTPK2
ras2Δ + pTPK3
ras1Δ ras2Δ + pTPK1
tpm1Δ
ras2Δ tpm1Δ
ras2Δ tpm1Δ + pTPK1
SP1msn2Δmsn4Δ
SP1ras2Δmsn2Δmsn4Δ
C
a
b
c
ras2Δ + vector
ras2Δ + pTPK1
ras1Δras2Δ + pTPK1
d
e
SP1msn2Δmsn4Δ
SP1ras2Δmsn2Δmsn4Δ

Figure 7.

1993). To see whether the distribution of this molecule was also affected, Cdc42p was localized by immunofluorescence microscopy in wild-type and *ras2*Δ cells after a temperature shift to 35°C. In wild-type cells, the percentage of cells with a polarized distribution of Cdc42p dropped to 20% in 15 min but then recovered (Figure 5C). In *ras2*Δ cells, Cdc42p was localized at 25°C, but the percentage of cells polarized dropped rapidly after shifting to 35°C and it remained low for up to 60 min (Figure 5C). Thus, two proteins that play key roles in polarized growth, Myo2p and Cdc42p, depend on *ras2*Δ for their polarized distribution at 35°C.

Human Ha-ras Is Able to Complement the ras2Δ *Actin Depolarization Phenotype*

Classic studies have shown that at 25°C human Ha-ras can provide the essential function in yeast normally provided by Ras1p and Ras2p (DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985). We therefore wished to examine whether human Ha-ras could also suppress the growth defect of *ras2*Δ cells at 37°C and/or provide the function necessary for polarization of the actin cytoskeleton at 35°C. The human Ha-ras on a 2 μ plasmid expressed from the strong *ADH1* promoter (P_{ADH1}-Ha-ras) or on a *CEN* plasmid and behind the yeast *RAS2* promoter (P_{RAS2}-Ha-ras), as well as yeast *RAS1* and *RAS2* behind their endogenous promoters on *CEN* plasmids (pRAS1 and pRAS2), were all able to complement the temperature sensitivity at 37°C caused by *ras2*Δ (Figure 6A; Table 3). To test for suppression of the actin polarization defect, cells were shifted to 35°C for 30 min, and the percentage of small to medium budded cells with a polarized actin distribution was determined. Whereas *ras2*Δ mutant cells are 20% polarized under these conditions, Ha-ras behind either promoter or yeast *RAS1* or *RAS2* on *CEN* plasmids were able to rescue the actin depolarization phenotype (Figure 6B). These results indicate that not only can an enhanced level of Ras1p substitute for Ras2p function involving regulation of the polarity of the actin cytoskeleton but also that human Ras can provide this function as well. The reason *ras2*Δ, but not *ras1*Δ, cells display a mutant phenotype may be because Ras2p is more abundant than Ras1p in wild-type cells (cited as unpublished data in Mosch *et al.*, 1999).

Figure 7 (facing page). An *msn2msn4* double disruption suppresses the temperature sensitivity and actin depolarization phenotypes of *ras2*Δ cells, whereas activation of the PKA pathway does not. (A) Growth of *RAS2*$^+$ (ABY1205), *ras2*Δ (ABY1209) alone or carrying pTPK1, SP1, SP1*ras2*Δ, or SP1*ras2*Δ*msn2*Δ*msn4*Δ on YPD medium after 2 d at 37°C. (B and C) Actin phenotype of wild-type (ABY1205); *ras2*Δ (ABY1209) carrying an empty vector, pTPK1, pTPK2, or pTPK3; *ras1*Δ*ras2*Δ carrying pTPK1 (ABY1240); *tpm1*Δ (ABY1226); *ras2*Δ*tpm1*Δ (ABY1228) alone or carrying pTPK1; SP1*msn2*Δ*msn4*Δ; and SP1*ras2*Δ*msn2*Δ*msn4*Δ. (B) Cells were grown in CM selective media or YPD media, shifted to 35°C for 30 min, fixed, stained for actin, and scored for actin polarization. (C) Images of actin localization. *ras2*Δ cells have similar actin phenotypes whether they carried pTPK1 or an empty vector, and *ras2*Δ*msn2*Δ*msn4*Δ cells have well polarized actin similar to *msn2*Δ*msn4*Δ cells. (a) *ras2*Δ (ABY1209) with empty vector, (b) *ras2*Δ (ABY1209) with pTPK1, (c) *ras1*Δ*ras2*Δ with pTPK1 (ABY1240), (d) SP1*msn2*Δ*msn4*Δ, and (e) SP1*ras2*Δ*msn2*Δ*msn4*Δ.

The Ras2p-Mediated Polarization of the Actin Cytoskeleton Is Not Mediated Solely by the PKA Pathway

The best characterized downstream pathway of Ras1/2p in yeast involves the activation of adenylate cyclase, which produces cAMP to activate the cAMP-dependent protein kinases (Tpk1/2/3p; Broach, 1991). Overexpression of Tpk1p rescues the double *ras1*Δ *ras2*Δ mutant at 25°C (Toda *et al.*, 1987). However, *ras1*Δ *ras2*Δ rescued by Tpk1p is still temperature sensitive, indicating a function of Ras that is not provided by Tpk1p (Wigler *et al.*, 1988). In agreement with these results, overexpression of Tpk1p in our *ras2*Δ strain does not rescue the temperature sensitivity at 37°C (Figure 7A; Table 3) but does rescue the temperature-dependent increase in density (Ho and Bretscher, unpublished results).

We next examined the polarization of the actin cytoskeleton after shifting cells to 35°C for 30 min. *ras2*Δ overexpressing Tpk1p and *ras1*Δ*ras2*Δ overexpressing Tpk1p have normally polarized actin cytoskeletons at room temperature, and both show very modest suppression of the temperature-dependent actin depolarization phenotype (Figure 7, B and C). Overexpression of Tpk2p showed similar levels of suppression and overexpression of Tpk3p showed almost no suppression of the actin phenotype (Figure 7B). The suppression by *TPK1* of the *ras2*Δ*tpm1*Δ actin polarization defect was slight (Figure 7B).

The ras2Δ *Actin Depolarization Phenotype Is Not Suppressed by Several Candidate Downstream Effectors but Is Suppressed by Loss of the Stress Response Pathway*

Because the PKA pathway did not seem to be the primary conduit for the regulation of cytoskeletal polarity contributed by Ras2p, we examined other potential pathways. Previous studies have indicated a potential physical link between Ras and the cytoskeleton through Srv2p, the yeast homologue of cyclase-associated protein (Shima *et al.*, 2000). A component of cortical actin patches (Lila and Drubin, 1997; Yu *et al.*, 1999), Srv2p, has been identified as a suppressor of an activated Ras allele (Fedor-Chaiken *et al.*, 1990). In yeast, Srv2p has two independent functions, one linking activated Ras proteins to stimulation of adenylate cyclase and the other having a role in actin filament organization (Gerst *et al.*, 1991). In addition, profilin (*PFY1*) overexpression can suppress the loss of Srv2p's actin filament organization function (Vojtek *et al.*, 1991). Furthermore, overexpression of any one of a group of mitotic regulatory genes, *TEM1*, *DBF2*, *CDC15*, *CDC5,* and *SPO12*, or of the Ras-like gene, *RSR1*, have been reported to suppress the temperature sensitivity of the triple *ras1*Δ*ras2*Δ*cyr1*Δ mutant maintained alive at 25°C by overexpression of Tpk1p (Morishita *et al.*, 1995). In our background, overexpression of these genes, of *SRV2*, or of *PFY1,* was unable to suppress the temperature sensitivity associated with *ras2*Δ or the actin depolarization phenotype (Table 3).

In mammals, there are multiple effectors of Ras, including PI-3 kinase and members of the Rho family of G-proteins that regulate the actin cytoskeleton (Scita *et al.*, 2000; Shields *et al.*, 2000). We therefore tested the ability of overexpression of these candidate genes to suppress the temperature sensitivity of *ras2*Δ or to rescue the temperature-dependent de-

polarization phenotype. Overexpression of genes encoding proteins of the Rho family, *CDC42*, *RHO1*, *RHO2*, *RHO3*, and *RHO4*, of an activated allele of *CDC42*, *CDC42*^{Val12}, or of a PI-3 kinase, encoded by *VPS34*, was unable to suppress the *ras2*Δ temperature sensitivity or the actin depolarization phenotypes (Table 3).

Although PKA is one of the regulators of the stress response pathway (Gorner *et al.*, 1998), we explored whether the temperature dependence of the actin depolarization phenotype in our *ras2*Δ strains might nevertheless be mediated through the transcriptional activators Msn2/4p of the stress response pathway. Recently, Stanhill *et al.* (1999) showed that introduction of *ras2*Δ into the SP1 genetic background eliminated its ability to undergo invasive growth, and this could be restored by deletion of the *MSN2* and *MSN4* genes. We therefore obtained these strains and confirmed that *ras2*Δ in the SP1 background confers both temperature-sensitive growth and temperature-dependent depolarization of the actin cytoskeleton. Remarkably, disruption of *msn2*Δ*msn4*Δ rescued both the temperature sensitivity conferred by *ras2*Δ (Figure 7A) and completely suppressed the actin depolarization phenotype (Figure 7, B and C). This argues that an important function of Ras2p is to suppresses the stress response through the Msn2/4p pathway.

DISCUSSION

There is enormous interest in Ras-signaling pathways in vertebrate cells because mutations in human Ras genes are found in a very large percentage of cancers (Barbacid, 1987). Throughout the years, vertebrate Ras has been implicated in multiple signaling pathways that regulate the actin cytoskeleton as well as nuclear gene expression, thus ultimately contributing to the transformed phenotype (Vojtek and Der, 1998).

In this paper, we show that in yeast Ras also contributes to the maintenance of cytoskeletal polarity in response to mild temperature stresses, and because human Ras can supply this polarity function in yeast perhaps mammalian Ras contributes to the maintenance of mammalian cytoskeletal organization via a similar mechanism. As far as we are aware, this is the first demonstration of the involvement of Ras in regulating the actin cytoskeleton in vegetatively growing yeast.

We identified *RAS2* as a regulator of actin polarity in a screen for genes important to both cytoskeletal polarity and secretion. Mutations in such genes were expected to confer synthetic lethality with deletion of the gene encoding the actin-binding protein tropomyosin (*TPM1*; indicative of a polarity defect) and to confer increased cell density (indicative of a secretion defect). Although the increased density in the *ras2* mutants is not due a secretory defect, the *ras2* mutants show profound defects in cytoskeletal polarity under mild temperature stresses.

The localization of Myo2p is a sensitive indicator of cytoskeletal polarity. Myo2p, a molecular motor that normally localizes to regions of cell growth during bud formation through rapid translocation along polarized actin cables (Lillie and Brown, 1994; Pruyne *et al.*, 1998), shows a transient and reversible depolarization (within 30 min) in wild-type cells upon shifting to 35°C. In contrast, in *ras2*Δ cells shifted to 35°C the normal polarized distribution of Myo2p is lost rapidly, depolarized to a greater extent, and takes up to 3 h to recover. This loss of polarization correlates with the loss of organization of the actin cables, which are necessary for Myo2p polarization.

Another indicator of cell polarity is the small GTPase Cdc42p. Cdc42p is essential for the establishment of actin polarity for bud emergence and regulates polarity through the cell cycle (reviewed by Johnson, 1999; Pruyne and Bretscher, 2000a). Again, wild-type cells show a partial transient delocalization of Cdc42p from growth sites, and the *ras2*Δ mutants show a complete delocalization, indicating Ras2p plays a role in restoring the polarity of Cdc42p during mild temperature shifts.

Studies in yeast have suggested that during normal vegetative growth Ras signals primarily through stimulation of adenylate cyclase with subsequent activation of the PKA pathway—a pathway not used by Ras in mammalian cells. Under normal vegetative growth conditions, loss of both *RAS1* and *RAS2* is lethal. However, the *ras1*Δ *ras2*Δ lethality at room temperature is rescued simply by overexpression of the cAMP-dependent protein kinase Tpk1p, and indeed Tpk1p even suppresses loss of both *RAS* genes and adenylate cyclase. However, overexpression of Tpk1p is unable to rescue the temperature-sensitive growth phenotype at 37°C of *ras2*Δ both in this and in other studies (Wigler *et al.*, 1988; Morishita *et al.*, 1995). Because overexpression of Tpk1p, Tpk2p, or Tpk3p does not restore cytoskeletal polarity to *ras2*Δ cells at 35°C, it appears that the PKA pathway does not play a major role in the maintenance of cytoskeletal polarity by Ras2p in vegetatively growing yeast.

We have shown that inactivation of the stress response pathway by disruption of the *MSN2* and *MSN4* genes is able to rescue both the temperature sensitivity and actin phenotypes of a *ras2*Δ strain. Msn2p and Msn4p are $Zn2^{+}$-finger transcription factors involved in the activation of stress response genes that contain the stress response element or STRE (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). The Msn2/4p pathway is negatively regulated by the PKA pathway (Marchler *et al.*, 1993; Gorner *et al.*, 1998): therefore, it was surprising that activation of the PKA pathway by overexpression of Tpk1p, Tpk2p, or Tpk3p was not able to rescue the *ras2*Δ temperature-sensitive and actin depolarization phenotypes. This suggests that the fully activated PKA pathway is unable to negatively regulate Msn2/4p sufficiently to suppress the phenotype associated with loss of Ras2p. It suggests that another parallel pathway exists to Msn2/4p that allows Ras2p to inhibit the stress response in a cAMP-independent manner.

What is this PKA-independent pathway? One interesting possibility is via the Cdc42p pathway shown to be necessary for yeast pseudohyphal growth. Previous studies have indicated a link among Ras, Cdc42p, and polarity during the pseudohyphal differentiation in yeast (Mosch *et al.*, 1996; Cook *et al.*, 1997). In response to particular nutritional signals, yeast assume a pseudohyphal form, showing exaggerated polarized growth and alterations in gene expression, cell cycle progression, and budding pattern. Pseudohyphal development requires a functional Ras2p, which can act through either of two pathways: 1) via adenylate cyclase and the PKA pathway and 2) via Cdc42p. Although the Cdc42p pathway indicates a link between Ras2p and polarity during pseudohyphal development, it might also be a route

whereby Ras2p polarizes the cytoskeleton during mild temperature stress. Roberts *et al.* (1997) have shown that Ras2p signals to a Bmh1/2p-Ste20p complex during pseudohyphal development. Bmh2p has also been shown to bind and negatively regulate Msn2/4p nuclear localization (Beck and Hall, 1999). An attractive hypothesis is that Ras2p signals through both the PKA pathway and through a PKA-independent pathway such as via Cdc42p and Bmh1/2p. It will be of interest to determine whether such a pathway exists.

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