finity-purified with nitrocellulose strips containing immobilized GST-Ste5p or GST-Bem1p as described (*10*). Antibodies to Ste20p were as described (*10*). Detection of actin was performed with C4 monoclonal antibody to actin (Boehringer Mannheim).
14. Extracts of yeast W303-1A cells were prepared as described (*13*). The supernatant was further fractionated by centrifugations at 10,000*g* for 20 min and 100,000*g* for 90 min. Pellets were combined to yield the particulate fraction. Equal amounts of proteins of the soluble and particulate fractions were subjected to immunoblot analysis (*13*). After densitometric evaluation of immunoblots, relative percentages of Ste20p, Ste5p, Bem1p, and actin were corrected for the total protein content of the two fractions.
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16. Preimmune sera or antibodies to the calnexin homolog Cne1p [F. Parlati, M. Dominguez, J. J. M. Bergeron, D. Y. Thomas, *J. Biol. Chem.* **270**, 244 (1995)] failed to precipitate actin, and antibodies to actin failed to precipitate Cnep1. These results demonstrate the specificity of the immune reactions.
17. The centromere plasmid pBEM324 containing *BEM1* was constructed by converting the Pst I site in *BEM1* in plasmid pCENBEM (*12*) into an Eco RI site. Plasmid pBEM324 was then digested with Hind III and Eco RI within the coding region of *BEM1*, and transformed into *bem1-s1* strain JC-G11 and *bem1-s2* strain JC-F5 (*6*). The repaired plasmids were isolated from transformants that were defective in mating with a *far1* tester strain and sequenced by the dideoxy chain termination method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)].
18. The *bem1-s1* allele contains a G → A mutation at nucleotide position 896, and the *bem1-s2* allele contains a C → T mutation at nucleotide position 1555 of the coding sequence of *BEM1*.
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26. The particulate fraction of yeast W303-1A cells was washed by pipetting for 3 min at 4°C with homogenization buffer (*13*) containing the indicated concentrations of NaCl or detergent. After centrifugation at 100,000*g* for 60 min, proteins in the resulting supernatant were precipitated with 7% (w/v) trichloroacetic acid and both fractions were resuspended in equal volumes of lysis buffer [10% glycerol, 5% β-mercaptoethanol, 2% SDS, and 60 mM tris (pH 6.8)]. Equal volumes were subjected to immunoblot analysis, and the relative amounts of Ste20p, Bem1p, Ste5p, and actin were determined densitometrically.
27. The particulate fraction of W303-1A cells was obtained as described (*14*), except that the homogenization buffer did not contain $Na_3VO_4$. The resulting pellet was resuspended in 2 ml of separation buffer [54% (w/w) sucrose in 20 mM Hepes (pH 7.4)] containing PIC (*13*) and homogenized by eight strokes in a 2-ml Wheaton tissue grinder (Wheaton Scientific). The homogenate was placed at the bottom of a tube (Beckman Instruments) and overlaid with 1.15 ml each of 50, 47.5, 45, 42.5, 40, 37.5, 35, and 32% (w/w) sucrose, all in 20 mM Hepes (pH 7.4). Gradients were centrifuged at 170,000*g* for 16 hours. Eighteen fractions were collected from the bottom. Portions (200 μl) of each fraction were stored for determination of protein amounts (*13*) and sucrose concentrations [with a refractometer (Fisher)]. Assays for plasma membrane ATPase and Golgi GDPase activities were performed as described [B. J. Bowman and C. W. Slayman, *J. Biol. Chem.* **254**, 2928 (1979); C. Abeijon, P. Orlean, P. W. Robbins, C. B. Hirschberg, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6935 (1989)]. Fractions were precipitated with trichloroacetic acid and resuspended in lysis buffer (*26*), and equal volumes were subjected to immunoblot analysis (*13*).
28. Equal volumes of cell cultures were harvested at an optical density at 598 nm of ~1.3, and cells were washed in 1.2 M sorbitol, resuspended in precipitation buffer [50 mM tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM $Na_3VO_4$, and 0.07% Triton X-100] containing PIC (*13*) and 1% (w/v) bovine serum albumin, and disrupted in a Mini Beadbeater. After two sequential centrifugations at 5000 and 10,000*g* (each for 4 min), the supernatant was incubated with antibodies for 1 hour. Antibody-antigen complexes were incubated with protein A or protein A–protein G Sepharose beads (1:1) (Pharmacia) for polyclonal and monoclonal antibodies, respectively. Beads were sedimented at 1000*g*, washed three times with precipitation buffer, and resuspended in equal volumes of lysis buffer (*26*). Equal volumes of the various precipitates were subjected to immunoblot analysis (*13*).
29. The yeast strains used were the *bem1-s1* and *bem1-s2* mutant derivatives of strain JC2-1B (*6*).
30. The two-hybrid protein interaction assays were performed as described (*20*) in a *far1-1* derivative strain of L40. Fusions of Bem1p with the transcriptional activation domain (AD) of Gal4p were constructed by cloning polymerase chain reaction (PCR) fragments of *BEM1* into plasmid pGAD424. Ste20p fusions with the LexA DNA-binding domain (DBD) were constructed by cloning PCR fragments of *STE20* into plasmid pBTM116. The fusion of the AD of Gal4p with the *BEM1* fragment encoding amino acids 157 to 551 was isolated from a genomic DNA library fused to the AD of Gal4p (*20*) in a two-hybrid screen in which the $NH_2$-terminus of Ste20p (amino acids 1 to 497) fused to the LexA DBD was used as a bait. From 189,000 transformants, eight were specifically positive and identified as *BEM1*. β-Galactosidase activity assays were performed as described (*20*).
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# Requirement of *Saccharomyces cerevisiae* Ras for Completion of Mitosis

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In the yeast *Saccharomyces cerevisiae*, Ras regulates adenylate cyclase, which is essential for progression through the $G_1$ phase of the cell cycle. However, even when the adenosine 3′,5′-monophosphate (cAMP) pathway was bypassed, the double disruption of *RAS1* and *RAS2* resulted in defects in growth at both low and high temperatures. Furthermore, the simultaneous disruption of *RAS1*, *RAS2*, and the *RAS*-related gene *RSR1* was lethal at any temperature. The triple-disrupted cells were arrested late in the mitotic (M) phase, which was accompanied by an accumulation of cells with divided chromosomes and sustained histone H1 kinase activity. The lethality of the triple disruption was suppressed by the multicopies of *CDC5*, *CDC15*, *DBF2*, *SPO12*, and *TEM1*, all of which function in the completion of the M phase. Mammalian *ras* also suppressed the lethality, which suggests that a similar signaling pathway exists in higher eukaryotes. These results demonstrate that *S. cerevisiae* Ras functions in the completion of the M phase in a manner independent of the Ras-cAMP pathway.

Ras functions in the regulation of cell proliferation and differentiation in various eukaryotes (*1*, *2*). The yeast *S. cerevisiae* has two *RAS* genes, *RAS1* and *RAS2* (*3*), whose products stimulate adenylate cyclase encoded by *CYR1* (*4*). Ras thereby regulates the cAMP pathway, which is essential for progression through the $G_1$ phase of the cell cycle (*3*, *5*). However, Ras does not appear to regulate adenylate cyclase in other eukaryotes (*6*, *7*). Given the diversity of Ras effectors (*8*), *S. cerevisiae* Ras may have a function other than the regulation of adenylate cyclase. Wigler and colleagues described genetic evidence that predicts the alternative function of Ras (*5*). Here, we show that *S. cerevisiae* Ras and Ras-related protein Rsr1 are functionally redundant and that they jointly regulate the completion of the M phase.

Cells with a single disruption of *CYR1* (*cyr1*Δ) or a triple disruption of *RAS1*,

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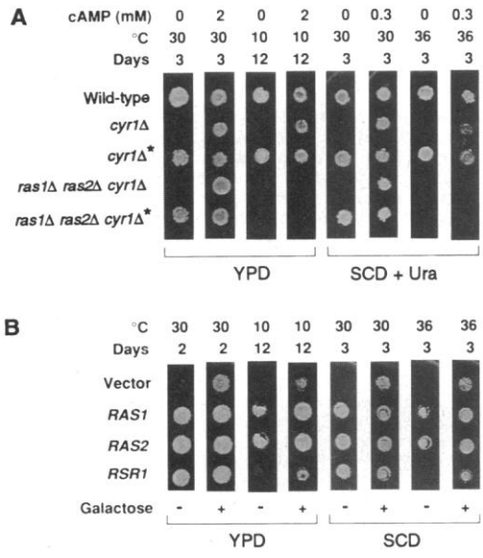


**Fig. 1.** Growth defects associated with *RAS1 RAS2* disruption. (**A**) The isogenic *cyr1*Δ, *cyr1*Δ (YEpT-TPK1) (asterisk), *ras1*Δ *ras2*Δ *cyr1*Δ, and *ras1*Δ *ras2*Δ *cyr1*Δ (YEpT-TPK1) (asterisk) strains (*21*) were grown up on YPD (2% glucose, 2% polypeptone, and 1% yeast extract) plates or on SCD + URA {SCD [2% glucose, 0.67% yeast nitrogen base without amino acids (Difco), 0.003% leucine, 0.002% histidine, 0.002% tryptophan] supplemented with 0.002% uracil} plates containing the indicated concentrations of cAMP at the indicated temperatures for the specified periods. (**B**) TMY12 cells [*ras1*Δ *ras2*Δ *rsr1*Δ *cyr1*Δ (YEpT-TPK1, YCpLeGAL1-RAS2-1)] were transformed with a low-copy vector pRS316 (*22*) carrying *RAS1*, *RAS2*, or *RSR1*, or with the vector alone. The transformants were grown on SCD plates with casamino acids (0.3%) at 30°C for 1 day and then incubated on YPD or SCD plates with (+) or without (−) addition of 5% galactose as in (A).

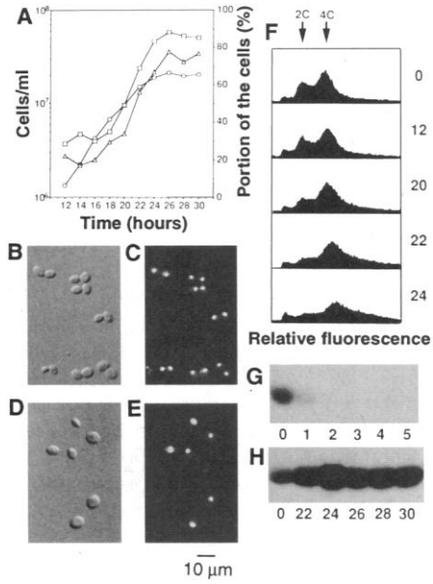


**Fig. 2.** Two terminal phenotypes associated with disruption of two *RAS* genes. (**A**, **B**, **C**, **F**, and **H**) TMD12 cells [*ras*Δ/*ras1*Δ *ras2*Δ/*ras2*Δ *rsr1*Δ/*rsr1*Δ *cyr1*Δ/*cyr1*Δ (YEpT-TPK1, YCpLeGAL1-RAS2-1)] grown in YPGD (YPD with 5% galactose and 1% glucose instead of 2% glucose) medium were shifted to YPD medium and cultured at 30°C. Cells were harvested at the indicated times after the shift. In (A), cell numbers (○) and the ratios of large-budded cells (□) and cells with divided chromosomes (△) were determined. The same sample at 26 hours was stained with 4′6-diamino-2-phenylindole and observed by (B) Nomarski optics or (C) fluorescence microscopy. In (F), DNA content of the same cells was analyzed by flow cytometry for 24 hours. In (H), histone H1 kinase activity associated with Suc1 beads in the same cells was measured as described (*23*) for 30 hours. (**D**, **E**, and **G**) Similarly, TMD9 cells (*ras1*Δ/*ras1*Δ *ras2*Δ/*ras2*Δ) grown in YPD medium containing 1 mM cAMP were shifted to YPD medium without cAMP at 30°C. In (D) and (E), the sample of TMD9 cells at the 3-hour time point was observed microscopically. In (G), the TMD9 cells were harvested each hour for 5 hours after the shift, and the histone H1 kinase activity of each sample was measured.

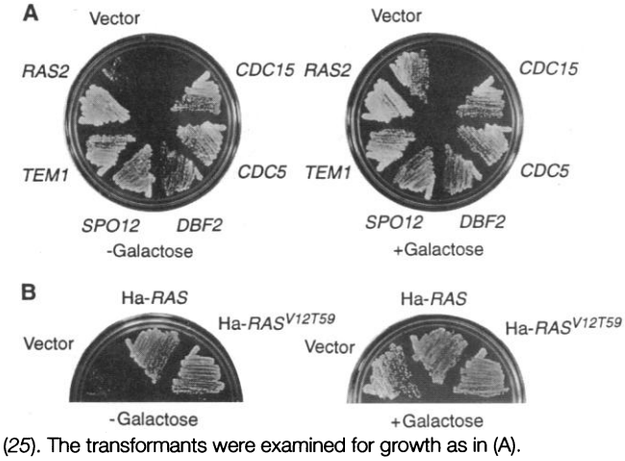


**Fig. 3.** Multicopy suppressors of the *RAS1 RAS2 RSR1* triple disruption. (**A**) TMY12 cells [*ras1*Δ *ras2*Δ *rsr1*Δ *cyr1*Δ (YEpT-TPK1, YCpLeGAL1-RAS2-1)] were transformed with each of the high-copy plasmids carrying *RAS2*, *TEM1*, *SPO12*, *DBF2*, *CDC5*, or *CDC15*, or with the control vector YEp352 (*24*). Transformants were examined for growth on YPD (left) and YPGD (right) plates at 30°C for 2 days. (**B**) TMY12 cells were transformed with vector AAH5 carrying c-Ha-*RAS* or its activated form (Val$^{12}$, Thr$^{59}$) (*25*). The transformants were examined for growth as in (A).

*RAS2*, and *CYR1* (*ras1*Δ *ras2*Δ *cyr1*Δ) are inviable because they cannot synthesize cAMP (*4*, *5*). Exogenous cAMP, or a multicopy plasmid carrying the *TPK1* gene (YEpT-TPK1) that encodes a catalytic subunit of cAMP-dependent protein kinase (*9*), rescued these cells at 30°C (Fig. 1A). However, the *ras1*Δ *ras2*Δ *cyr1*Δ (YEpT-TPK1) cells still could not grow either at 10°C or at 36°C. Because isogenic *cyr1*Δ (YEpT-TPK1) cells did not show such growth defects under the same conditions (Fig. 1A), it appears that temperature sensitivities of the *ras1*Δ *ras2*Δ *cyr1*Δ cells are not the result of the inability of Ras to stimulate adenylate cyclase. Both low- and high-temperature sensitivities were complemented by *RAS1* or *RAS2*, but not by biologically inactive alleles *RAS2*$^{Asp22}$ or *RAS2*$^{Ala42}$ (*10*). These results indicate that Ras may have another essential function besides the stimulation of adenylate cyclase. Wigler and colleagues described similar high-temperature sensitivity of *ras1*Δ *ras2*Δ *cyr1*Δ cells carrying multicopy *TPK1* and its complementation by *RAS* genes (*5*).

To elucidate this other function of Ras, we screened multicopy suppressor genes for the low- and high-temperature sensitivities of the *ras1*Δ *ras2*Δ *cyr1*Δ (YEpT-TPK1) cells. We obtained six genes: *RSR1* (*11*), *TEM1* (*12*), *BUD5* (*13*), *LTE1* (*14*), *DBF2* (*15*), and *SPO12* (*16*). *RSR1* and *TEM1* encode guanosine 5′-triphosphate (GTP) binding proteins of small molecular size. Bud5 is an activator for Rsr1, and Lte1 is a putative activator whose target GTP binding protein has not been identified. *DBF2* and *SPO12* encode a protein kinase and its putative regulator, respectively.

Rsr1 has the same effector domain as Ras and stimulates adenylate cyclase when overexpressed in its activated form (*17*), which suggests that Rsr1 shares some of the alternative Ras functions. We disrupted the *RSR1* gene of the *ras1*Δ *ras2*Δ *cyr1*Δ cells, cells in which the *RAS2* gene is conditionally expressed under the control of a *GAL1* promoter. The resulting *ras1*Δ *ras2*Δ *rsr1*Δ *cyr1*Δ (YEpT-TPK1, YCpLeGAL1-RAS2-1) cells grew in a medium containing galactose, but did not grow when *RAS2* expression was shut off in a galactose-free medium (Fig. 1B), even at 30°C. The lethality of the triple disruption was fully complemented by either *RAS1* or *RAS2* on a low-copy vector, but the cells carrying *RSR1* on the same vector still showed the temperature-sensitive growth defects (Fig. 1B), indicating that the contribution of *RAS1* and *RAS2* to the cell cycle progression is greater than that of *RSR1*. Introduction of the *CYR1* gene into the triple disruption did not suppress the lethality (*10*). Although the disruption of *RSR1* results in cells with random

bud sites (*11*), no growth defect has been reported. The results reveal a functional redundancy of Ras and Rsr1 in spite of their known individual roles.

When the *ras1*Δ *ras2*Δ *rsr1*Δ *cyr1*Δ (YEpT-TPK1, YCpLeGAL1-RAS2-1) cells were shifted to a galactose-free medium at 30°C, they ceased to proliferate as large budded cells with chromosomal DNA evenly distributed in mother cells and buds (Fig. 2, A to C), indicating that the cells were arrested at or near the end of the M phase. This arrest was accompanied by an accumulation of cells with DNA content characteristic of the $G_2$ and M phases (Fig. 2F) and by sustained Cdc28 histone H1 kinase activity (Fig. 2H), consistent with an M phase arrest. Cells carrying an intact *RAS* gene did not accumulate such cells (*10*). This terminal phenotype was different from the $G_1$ arrest of *ras1*Δ *ras2*Δ cells when the cells were starved of exogenous cAMP. In this case, the cells were arrested as unbudded cells (Fig. 2D) with a single nucleus (Fig. 2E), Cdc28 kinase activity decreased (Fig. 2G), and cells with $G_1$ DNA content accumulated (*10*). These results indicate that the cells with the *RAS1 RAS2 RSR1* triple disruption are defective in the completion of the M phase.

We examined genetic interactions between *RAS* and other genes that participate in M phase completion, including *DBF2*, *CDC5* (*18*), *CDC15* (*19*), *SPO12*, and *TEM1*. Both *CDC5* and *CDC15* encode protein kinases. We introduced multicopy plasmids carrying each of these genes into the *ras1*Δ *ras2*Δ *rsr1*Δ *cyr1*Δ (YEpT-TPK1, YCpLeGAL1-RAS2-1) cells, and the resulting transformants were examined for growth on a galactose-free plate. Each of the multicopy plasmids suppressed the growth defects of the cells (Fig. 3A). None of these genes suppressed the cAMP requirement of the *ras1*Δ *ras2*Δ cells (*10*). Mammalian c-Ha-*RAS*, or its activated form ($Val^{12}$, $Thr^{59}$), also suppressed the lethality, indicating that mammalian Ras can substitute for yeast Ras in this other function (Fig. 3B).

We have shown here that *S. cerevisiae* Ras functions in the completion of the M phase. The genetic interactions between *RAS* and other genes involved in M phase completion suggest a network of signal transduction pathways in which low molecular weight GTP binding proteins and various protein kinases are involved. Activation of Ras and downstream protein kinase cascade by growth factors in mammalian cells is necessary for cell cycle progression through the $G_1$ phase to the S phase (*2*). Several reports contain arguments for another function of Ras in the $G_2$-M boundary in vertebrate cells (*7*, *20*). Yeast and mammalian Ras proteins could share the same effector molecule in this signal transduction pathway.

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# Ligand-Induced Autoregulation of IFN-γ Receptor β Chain Expression in T Helper Cell Subsets


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Interferon γ (IFN-γ) responsiveness in certain cells depends on the state of cellular differentiation or activation. Here an in vitro developmental system was used to show that IFN-γ produced during generation of the $CD4^+$ T helper cell type 1 ($T_H1$) subset extinguishes expression of the IFN-γ receptor β subunit, resulting in $T_H1$ cells that are unresponsive to IFN-γ. This β chain loss also occurred in IFN-γ–treated $T_H2$ cells and thus represents a specific response of $CD4^+$ T cells to IFN-γ rather than a $T_H1$-specific differentiation event. These results define a mechanism of cellular desensitization where a cytokine down-regulates expression of a receptor subunit required primarily for signaling and not ligand binding.


Recently it was reported that $T_H1$ and $T_H2$ cells develop opposing patterns of responsiveness to the cytokines interleukin-12 (IL-12) and IFN-γ, with $T_H1$ retaining only IL-12 responsiveness and $T_H2$ retaining only IFN-γ responsiveness (*1*). Because this differential responsiveness significantly affects $T_H1$ and $T_H2$ subset stability in vivo, the developmental mechanism controlling the loss of cytokine receptor signaling becomes central to the understanding of pathogen susceptibility or resistance (*1*, *2*). Using fully differentiated long-term T cell clones, Pernis *et al.* observed that expression of the IFN-γ receptor β chain, the receptor subunit required primarily for signaling and not ligand binding, is limited to $T_H2$ cells, and it was implied that this was important to phenotype differentiation (*3*, *4*). To characterize the mechanism controlling developmental expression of the two IFN-γ receptor subunits and to critically test their


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