Raf-1 Interferes with Ras and Rap1A Effector Functions in Yeast

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Raf-1 is a serine/threonine kinase that acts downstream of Ras in mitogenic signal transduction pathways, but the mechanism by which Ras transmits signals to Raf-1 is not known. We have examined the interaction between Raf-1 and human H-ras in three different systems that utilize H-ras-induced phenotypes in *Saccharomyces cerevisiae*. In each system, the effects of H-ras depend on guanosine triphosphate and appear to be mediated through the H-ras effector binding region. H-ras effector function was blocked in each case by expression of the N-terminal regulatory domain of Raf-1. These inhibitory effects did not require the Raf-1 kinase domain. Raf-1 also blocked Rap1A effector function in *S. cerevisiae*. Raf-1, therefore, appears to interact with H-Ras and Rap1A in these in vivo systems with properties that suggest it is an immediate downstream effector.

INTRODUCTION

Ras proteins play an essential role in transducing signals from receptors to nuclear factors that regulate cell growth and differentiation. Mitogenic activation of cells generally leads to increased levels of active guanosine 5'-triphosphate (GTP)-bound Ras proteins, and most mitogens depend on Ras activation for signal transmission. A pathway from the epidermal growth factor (EGF) receptor to Ras has recently been elucidated (Buday and Downward, 1993; Egan *et al.*, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). In contrast, the precise molecular interactions occurring downstream of Ras have yet to be identified. Genetic and biochemical evidence has identified a cascade of serine/threonine kinases, from Raf-1 to MEK (mitogen-activated protein [MAP] kinase) and ERKs (MAP kinases) that lies downstream of Ras (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Robbins *et al.*, 1992; Wood *et al.*, 1992). This pathway may be the major route by which Ras proteins send signals to the cell nucleus to provoke cell division. Raf-1 is essential for Ras function: c-raf-1 antisense RNA and kinase-defective Raf-1 mutants revert Ras-transformed cells and inhibit proliferation of normal NIH3T3 cells (Kolch *et al.*, 1991). Analysis of oncogenic v-raf and structural studies of cellular c-raf-1 have indicated that the N-terminus domain provides

a regulatory function for the kinase (Bruder *et al.*, 1992) and phosphorylation of Raf-1 has been associated with its activation (Li *et al.*, 1991).

A direct link between Ras and Raf-1 has been provided by the recent finding that Raf-1 is part of a complex that includes other components of the MAP kinase family and binds specifically to Ras • GTP (Moodie *et al.*, 1993). The possibility of a direct interaction between Ras and Raf-1 has been recently explored using the two-hybrid system that detects physical protein-protein interactions within the nucleus of the yeast *Saccharomyces cerevisiae*. In this system, Ras and Raf-1 form a complex that depends on the effector region of Ras and the N-terminal regulatory region of the Raf-1 protein (Van Aelst *et al.*, 1993). More recently, other groups have demonstrated this interaction in the two hybrid system and shown that direct binding of Ras and Raf-1 occurs in vitro (Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). Furthermore, Zhang and colleagues showed that mutations around the effector domain of Ras that impair Ras-transforming activity prevent Ras binding to Raf-1. These findings strongly suggest that Raf-1 is a direct effector of Ras in higher eukaryotes. However, the consequences of binding in vivo are not known and the exact mechanism by which a signal generated by Ras leads to Raf-1 activation is not yet understood. For example, Raf-1 kinase cannot be stimulated in vitro by simple addition of Ras proteins in their active, GTP-

bound state (Macdonald *et al.*, 1993). On the other hand, Raf-1 activated by co-expression with v-ras or v-src in insect cells appears to be stably activated by a covalent modification and is not dependent on Ras proteins for maintenance of kinase activity (Macdonald *et al.*, 1993; Williams *et al.*, 1993).

To test whether the interaction between Raf-1 and Ras has functional consequences in vivo, we have chosen to study the effect of Raf-1 on effector functions of H-Ras in *S. cerevisiae*, where both yeast Ras and H-ras interact with different effectors than in mammalian cells. We show that in three different systems expression of Raf-1 interferes with and reverts phenotypes induced by mammalian H-Ras. Similarly, the function of Rap1A, which shares a common effector region with Ras proteins, is affected by expression of Raf-1.

MATERIALS AND METHODS

Strains and Culture Conditions

Strain MRY2-16C-D (*MAT α /MAT α ade8/ade8 ura3/ura3 leu2/leu2 his3/his3 RAS2^{val19}/RAS2^{val19}*) was derived by crossing the RAS2^{val19} strain TK161-R2V (Toda *et al.*, 1985) with wild-type strain JRY438 (*MAT α ura3 his4 leu2*), obtained from Georjana Barnes (U.C. Berkeley). A haploid segregant carrying the RAS2^{val19} mutation, the required selective markers and wild type for the ability to grow on galactose, was diploidized by transformation with the HO gene. Strain IR-1 is *MAT α leu2 trp1 his3 ade8 ura3 ira1::HIS3* (Ballester *et al.*, 1989). Strain LRA26 (*MAT α leu2 ura3 his4 cdc25-5*) (Petitjean *et al.*, 1990) was obtained from Scott Powers (Onyx Pharmaceuticals, Richmond, CA). Strain SP1-D is a derivative of wild-type SP1 (*MAT α leu2 ura3 his3 ade8 trp1*) (Toda *et al.*, 1985) that was found to be sensitive to H-ras. Strain Y147 is *MAT α ura3 leu2 his3 cdc24-4* (Bender and Pringle, 1989) and RAY3A-D is *MAT α /MAT α ura3/ura3 leu2/leu2 trp1/trp1 his3/his3* from Kunihiro Matsumoto (Nagoya University, Nagoya, Japan).

Standard genetic methods (Sherman *et al.*, 1987) were used to introduce plasmids into yeast strains. Cells were grown on rich (YPD), or defined complete medium (SC) at various temperatures as noted below. Selective media for isolation of transformants were SC lacking the appropriate requirements.

Plasmids

pNV7-RAF contains the full length c-raf-1 gene, subcloned from pAcC13-Raf (Macdonald *et al.*, 1993) as a 2-kb *Xho* I-*Bam*HI fragment into the *Xho* I-*Bam*HI pNV7 vector (Ninomiya-Tsuji *et al.*, 1991). This plasmid expresses the Raf-1 gene from the GAL1-10 promoter. Plasmids pNV7-RAF-KA and pNV7-RAF Δ N were similarly subcloned from the Sf9 vector pAcC13-Raf. The mutant RAF-KA gene was constructed by site-directed mutagenesis that changed lysine 375 to alanine (Macdonald *et al.*, 1993). Plasmid pNV7-RAF Δ N contains a Raf-1 gene deleted from amino acid 1-315. Plasmid pNV7-RAF Δ C was obtained from pNV7-RAF as a 1.1-kb *Eco*RI-*Sal* I fragment and was subcloned into pNV7-RI-A, a derivative of pNV7 in which one of the two *Eco*RI sites was mutagenized to leave a unique *Eco*RI site after the promoter. Plasmid AAH5-HA-ras (DeFeo-Jones *et al.*, 1985) expresses the wild-type H-ras gene from the ADH promoter. Plasmid YT7 was constructed from YEplac181 (Gietz and Sugino, 1988) in several steps. First, the H-ras^{ser186} gene from plasmid pZIP-H-ras(186-Ser), kindly obtained from Channing Der (University of North Carolina, Chapel Hill NC), was subcloned as a *Sal* I-*Sac* I fragment between the ADH promoter and terminator of plasmid pAD4M (Martin *et al.*, 1990). Then, the *Sph* I-1.85-kb fragment, containing the ADH promoter, the H-ras^{ser186} gene, and the ADH terminator, was introduced into the *Sph* I site of YEplac181. This created plasmid YT3. Finally,

the *ColEI* ori and the Ap-r gene from YT3 were replaced with the Cm and origin of replication from plasmid pACYC184. Plasmid pNV7-GAP and pNV7-NF1 were derived from pADGAP and pADNF1 (Martin *et al.*, 1990), respectively, by introducing *Sac* I-*Not* I linkers at the 3' end of the genes and subcloning the two genes as *Sal* I-*Not* I fragments in the *Sal* I-*Not* I sites of pNV7. Plasmid pADRP1 (McCabe *et al.*, 1992), obtained from Peter McCabe (ONYX Pharmaceuticals) contains the Rap1A sequences under the control of the ADH promoter.

Image Analysis of Cell Growth on Plates

Strains grown as patches on plates were photographed using a Polaroid (Polaroid, Cambridge, MA) camera. The pictures were scanned with a UMAX UC630 scanner (UMAX Technologies, Santa Clara, CA) and the Adobe Photoshop program (Adobe Systems, Mountain View, CA). Analysis of the patch growth was performed by using the National Institutes of Health Image 1.44 program. The average pixel intensity of each patch was determined using a fixed-size region of interest that included the whole patch. For each individual row the background intensity was determined as the average pixel intensity of the areas between patches. This background value was subtracted from the average intensity of the patches. For each strain we determined the average value of 12-16 patches each derived from independent transformants of three to four transformations. Statistical significance of differences between strains was calculated using the Student's *t* test.

Budding Pattern Detection

Yeast budding pattern was determined by visualization of bud scars. Cells were stained with Calcofluor (Fluorescent Brightener 28; Sigma Chemical Co., St. Louis, MO) and observed on a Zeiss Axiovert microscope (Zeiss, Oberkochen, Germany), using a DAPI-filter set and a $\times 100$ Plan-Neofluar 1.3 NA objective. Images were captured with a cooled CCD camera (Photometrics, Tucson, AZ) containing a Kodak-Videc 1340 $\times$ 1037 pixel chip (Eastman Kodak Co., Rochester, NY) used in the bin-2 mode.

RESULTS

Raf-1 Interferes with Suppression of the RAS2^{val19} Phenotype by H-ras^{ser186}

Activating mutations in the yeast *S. cerevisiae* RAS2 gene generate a constitutive signal downstream in the cyclic AMP (cAMP) pathway (Figure 1A), causing an array of phenotypes typical of an active growth state, such as sensitivity to heat shock and nitrogen starvation and inability to accumulate storage carbohydrates (Toda *et al.*, 1985; Sass *et al.*, 1986). Mutations in human H-ras that compromise posttranslational modifications responsible for its membrane localization cause the cytoplasmic protein to interfere with the RAS2^{val19} phenotype. This effect is presumably mediated by diverting essential components of the RAS2^{val19} effector complex to the cytosol (Michaeli *et al.*, 1989) (Figure 1B). For our studies, we constructed the RAS2^{val19} strain MRY2-16C-D, which carries plasmid YT7 expressing human H-ras^{ser186} under the control of the strong alcohol dehydrogenase 1 (ADH1) promoter. This strain behaves normally with respect to several phenotypes affected by RAS2 activity: the cells accumulated glycogen in late log phase, as shown by the typical brown staining of cells upon exposure to iodine vapors (Figure 2A, row

Figure 1. Model of Raf-1 effect on H-ras^{ser186} suppression of the RAS2^{val19} phenotypes. (A) The yeast-activated allele of RAS2, RAS2^{val19}, constitutively signals through the cAMP pathway, leading to heat-shock sensitivity and inability to accumulate glycogen. (B) Cytoplasmic H-ras^{ser186} reverts the RAS2^{val19} phenotypes by titrating some essential component of the RAS2 effector complex. (C) Raf-1 competes with the yeast effector for binding to H-ras^{ser186}, restoring the RAS2^{val19} phenotypes.

3), and became heat-shock resistant (Figure 2B, row 3). Consistent with previous reports (Michaeli *et al.*, 1989), we found that the ability of H-ras^{ser186} to suppress the RAS2^{val19} phenotype is dependent upon H-ras^{ser186} being bound to GTP. Expression of GTPase-activating protein (GAP) or neurofibromin (NF1) (this study), which switch Ras to the GDP-bound inactive state, abolished the H-ras^{ser186} effect and restored the RAS2^{val19} phenotype (Figure 2, A and B, rows 1 and 2). The suppression by GAP was, however, stronger than that shown by NF1.

This was most evident when heat-shock sensitivity was tested (Figure 2B, row 2).

We reasoned that GAP proteins or molecules binding to the H-ras effector binding site would counteract suppression by H-ras^{ser186}. To test if Raf-1 in this strain performed such a role, we introduced a plasmid that expresses Raf-1 from the Gal1-10 promoter, pNV7-RAF, into the RAS2^{val19} strain suppressed by H-ras^{ser186}. We found that this Raf-1 plasmid reduced accumulation of glycogen (Figure 3A, row 1) and recovery from heat shock (Figure 3B, row 1). Although acting less effectively than GAP (Figure 2, row 1), these results are consistent with Raf-1 binding to the effector region of H-ras^{ser186}.

Raf-1 could exert this effect through at least two possible mechanisms: 1) it could bind directly to the effector region of H-ras^{ser186} and 2) its effects could be mediated through Raf-1 kinase activity. To investigate these possibilities, a plasmid carrying a mutant Raf-1 deleted in its regulatory domain and containing only the catalytically active C-terminus (Stanton *et al.*, 1989), pNV7-RAFΔN, was expressed in the RAS2^{val19} strain carrying the H-ras^{ser186} plasmid. pNV7-RAFΔN did not affect H-ras^{ser186} suppression of RAS2^{val19} (Figure 3, A and B, row 4). Western blot analysis revealed that it did direct expression of the truncated Raf protein. These data suggest that the effect of Raf-1 is mediated by its N-terminus regulatory domain rather than by its kinase activity. To further prove this, we tested two other Raf-1 mutants: RAF-KA, in which the Raf-1 gene carries an alanine to lysine substitution at position 375 that is known to affect the ATP-binding site and destroys Raf-

Figure 2. Effect of GAP and neurofibromin on suppression of RAS2^{val19} by H-ras^{ser186}. Strain MRY2-16C-D was transformed with plasmid YT7 that carries the H-ras^{ser186} gene. The resulting strain was transformed with plasmid pNV7-GAP (row 1), pNV7-NF1 (row 2), or control plasmid pNV7 (row 3). When transformants appeared, several independent colonies from each group were picked and tested. Two representative transformants for each strain are shown. Cells were grown on selective plates lacking leucine and uracil to maintain the plasmids that carry the LEU2 and URA3 markers. They were, then, patched on fresh selective plates and incubated for 2 d at 30°C, when they were exposed to iodine vapors for 5 min and photographed (A) or patched on rich plates, heat-shocked for 20 min at 56°C, incubated at 30°C for 3 d and photographed (B).

Figure 3. Raf-1 interference with the H-ras^{ser186} suppression of RAS2^{val19} phenotypes. Strain MRY2-16C-D carrying plasmid YT7 was transformed with several Raf-1 constructs and tested for glycogen accumulation (A) and heat-shock sensitivity (B) as described in the legend to Figure 2. Plasmids were as follows: pNV7-RAF (1), pNV7-RAF-KA (2), pNV7-RAFΔC (3), pNV7-RAFΔN (4), and pNV7 (5).

1 kinase activity (Macdonald *et al.*, 1993); and RAFΔC, which expresses only the amino terminal regulatory domain of Raf-1. In both cases transformants showed a reduced ability to accumulate glycogen (Figure 3A, rows 2 and 3). A less pronounced but detectable effect was also observed on the sensitivity to heat shock (Figure 3B, rows 2 and 3). We conclude that it is the N-terminal domain of Raf-1 that is responsible for counteracting the effects of H-ras^{ser186}.

Raf-1 Partially Suppresses the *ira1*⁻ Mutant

The results described above suggest that Raf-1 interferes with H-ras^{ser186} effector functions. Due to the high degree of conservation between yeast and human Ras proteins, we reasoned that Raf-1 might also affect yeast RAS proteins directly. We therefore tested the effect of Raf-1 expression in the *ira1*⁻ strain IR-1. This strain has lost one of the yeast Ras-GAP genes, IRA1, and shows phenotypes typical of an activated yeast RAS2 allele (Tanaka *et al.*, 1989). Figure 4 shows that expression of full-length Raf-1, but not its kinase domain, is able to partially suppress the inability of *ira1*⁻ cells to accumulate glycogen. In other experiments we confirmed this result in the RAS2^{val19} strain, MRY2-16C-D. Therefore, in the RAS2^{val19} strain carrying H-ras^{ser186}, it seems likely that Raf-1 is affecting the function of both yeast and human Ras proteins. This would explain the weak phenotype caused by Raf-1. Because the expression of RAS2^{val19} leads to decreased glycogen accumulation,

whereas the expression of H-ras^{ser186} leads to increased glycogen levels, by interfering with the activity of each of the Ras proteins, Raf-1 would reduce the observed difference between the phenotypes.

Raf-1 Reduces Suppression of *cdc25* Temperature Sensitivity by H-ras

To test whether Raf-1 affected H-ras function in a different genetic system, we examined a temperature-sensitive (ts) *cdc25* mutant suppressed by overexpression of wild-type H-ras (DeFeo-Jones *et al.*, 1985). Growth inhibition at high temperature is caused by loss of function of the yeast GDP-GTP exchange factor for RAS, Cdc25 (Figure 5A). Because H-ras does not respond to the yeast GAPs, encoded by IRA1 and IRA2, H-ras can compensate for loss of exchange activity and allow growth of the cells at the nonpermissive temperature (Figure 5B). The *cdc25-5* strain LRA26-carrying plasmid AAH5-Ha-ras (DeFeo-Jones *et al.*, 1985), which contains the H-ras gene controlled by the ADH1 promoter, was transformed with either the control plasmid pNV7 or the different Raf-1 constructs. Because the growth phenotype showed some variability (Figure 6A), for each plasmid we tested 12 independent transformants and analyzed growth of the patches by image analysis (see MATERIAL AND METHODS) (Figure 6B). Figure 6 shows that at 37°C, strains containing pNV7-RAF grew poorly, whereas a control plasmid, pNV7, did not affect the H-ras-mediated suppression of the ts defect (Figure 6, A, rows 1 and 5, respectively, and B). The regulatory domain of Raf-1 also interfered with growth (Figure 6A, row 3). The kinase-inactive mutant showed a weaker but detectable effect (Figure 6, A, row 2, and B). These results indicate that Raf-1 affected H-ras function in this genetic system through its regulatory domain. However, the truncated catalytic domain of Raf-1 seemed to have some interference (Figure 6, A,

Figure 4. Raf-1 suppression of *ira1*⁻ mutant. Strain IR-1 was transformed with the following plasmids: pNV7-RAF (1), pNV7-RAFΔN (2), and pNV7 (3). Cells were grown on selective media lacking uracil for plasmid maintenance and tested for glycogen accumulation as described in the legend to Figure 2.

Figure 5. Models for effects of H-ras and Raf-1 on the *cdc25* temperature sensitivity. Solid boxes and arrows indicate active proteins and processes; hatched boxes and arrows indicate defective proteins and processes. Full arrows indicate a transduced growth signal and empty arrows indicate no growth signal. See text for explanation.

row 4, and B). This effect is likely to be attributed to the kinase activity of Raf. These experiments indicate that counteracting the ability of H-ras to restore growth in the *cdc25* mutant could be caused either through the kinase activity of Raf-1 or by direct interference with H-ras effector functions by the regulatory domain of Raf-1 (Figure 5C).

Raf-1 Alleviates the Toxicity Caused by H-ras in a Wild-Type Background

The phenotype shown by the kinase domain of Raf-1 in the *cdc25^{ts}* strain led us to consider the possibility that H-ras, by its contribution to the activation of Raf-1 kinase, would be toxic in combination with Raf-1. Neither Raf-1 nor its kinase domain are toxic to cells when expressed alone (Ruggieri, unpublished observations). We then asked whether H-ras, by contributing

to the stimulation of Raf-1 kinase activity, was toxic in combination with Raf-1. The SP1-D strain carrying either pNV7-RAF, pNV7-RAF Δ N, or the vector control was transformed with plasmid AAH5-Ha-ras or the vector control, AAH5. Surprisingly, we found that H-ras alone

Figure 6. Raf-1 reversion of the H-ras suppression of the *cdc25* temperature sensitivity. (A) Strain LRA26 was transformed with plasmid AAH5-Ha-ras, and then re-transformed with the following plasmids: pNV7-RAF (1), pNV7-RAF-KA (2), pNV7-RAF Δ C (3), pNV7-RAF Δ N (4), and pNV7 (5). Four independent transformants representative of each strain are shown. They were patched on selective plates lacking leucine and uracyl, incubated at 37°C for 2 d and then photographed. (B) Quantitation of the effect of Raf-1 on the H-ras suppression of *cdc25* temperature sensitivity. Twelve independent transformants for each of the strains described above were treated as described in the legend to Figure 6A and then quantitated for growth as explained in MATERIALS AND METHODS. Values were normalized to the Control. Bars, indicate SE. Student's *t* test of the data indicated that the differences between the strain carrying the control plasmid and each of the other strains are statistically significant.

Figure 7. Raf-1 suppression of the toxic effect of H-ras in a wild-type strain. Strain SP1-D was co-transformed with the different Raf-1 constructs and plasmid AAH5-Ha-ras, as indicated, or plasmid AAH5 (Control). Sixteen independent transformants for each strain were patched on selective media and grown at 37°C for 2–3 d and then photographed. Quantitation of the data was carried out as described in MATERIALS AND METHODS. Values were normalized to the Control. Differences between the H-ras-containing strain and each of the strains containing the various Raf-1 constructs were statistically different, as calculated by the Student's *t* test.

was toxic in this strain and caused a slow-growth phenotype at high temperature. The kinase domain of Raf-1 (RAFΔN) in this context did not affect this Ras-induced phenotype. On the other hand, full-length Raf-1 was able to suppress the toxicity caused by H-ras (Figure 7). A reversion of the H-ras-induced toxic effect was also obtained with the kinase-inactive Raf-1 (RAF-KA) and with the regulatory domain of Raf-1 (RAFΔC). This is consistent with the data we obtained in the strains described above that the Raf-1 effects are mediated by its regulatory domain.

Raf-1 Interferes with the Suppression of *cdc24^{ts}* by RAP1A and with Bud Site Selection in Wild-type Cells

Rap proteins share an identical effector binding domain with the Ras proteins (Kawata *et al.*, 1988) and have been shown to interfere with Ras effector functions when overexpressed in cells transformed by oncogenic Ras (Pizon *et al.*, 1988). To investigate whether the interfering effects of Rap1A are mediated by competition with Ras effector(s), we tested the ability of Rap1A to interact with Raf-1. We made use of yeast strains where Rap1A has phenotypes due to its homology with the yeast ras-like protein, Rsr1, which is involved in bud site selection. Like Rsr1 (Bender and Pringle, 1989),

overexpression of Rap1A partially suppresses loss of viability of a *cdc24^{ts}* strain. In addition, overexpression of Rap1A in wild-type cells interferes with proper bud localization, probably by forming nonproductive complexes with the yeast budding proteins (McCabe *et al.*, 1992; Ruggieri *et al.*, 1992). These phenotypes of Rap1A in yeast are abolished by co-expression of Rap-GAP (McCabe *et al.*, 1992), indicating that they depend on the active conformation of this GTPase. We expressed Raf-1 in the *cdc24^{ts}* strain, Y147, which carries Rap1A on plasmid pADRP1 (McCabe *et al.*, 1992), and tested cell growth at the nonpermissive temperature of 35.5°C. Figure 8 shows that at this temperature the *cdc24^{ts}* strain grew poorly (Control), while as expected, Rap1A improved cell growth (RAP1A + Control). Full length Raf-1 or its kinase domain had no significant effect by themselves (Ruggieri, unpublished observations). However, when co-expressed with Rap1A, Raf-1 interfered with the ability of Rap1A to support full growth in these cells (Figure 8). The same level of interference was observed with plasmid pNV7-RAF-KA and plasmid pNV7-RAFΔC, while the kinase domain alone, from plasmid pNV7-RAFΔN, did not seem to affect growth significantly. Therefore, the interference effect seems to be mediated by the N-terminus regulatory domain of Raf-1.

Because the RSR1 gene also shares the identical effector binding site with Ras, we expected a possible effect of Raf-1 on Rsr1 function. Rsr1 loss of function

Figure 8. Raf-1 effect on suppression of the *cdc24^{ts}* growth defect by Rap1A. Strain Y147 was transformed with the indicated plasmids and tested for growth at 35.5°C. Growth was quantitated as described in MATERIALS AND METHODS. Values were normalized to the RAP1A + Control sample. The differences between the effects of control plasmid and plasmids RAF, RAF-KA, and RAFΔC on the RAP1A suppression are statistically significant.

causes a random budding phenotype (Bender and Pringle, 1989). Interaction of Raf-1 with the effector domain of Rsr1 could affect proper function of this protein and lead to a random budding phenotype. We, therefore, introduced the different Raf constructs in the wild-type strain RAY3A-D and visualized the budding pattern by fluorescence staining. Figure 9 shows that the axial budding pattern, typical of diploid cells (panel 1), was disrupted by expression of Raf-1 (panel 2). Interestingly, the kinase domain of Raf-1 was also able to affect bud localization (panel 3). Quantitation of the effect indicated that Raf-1 significantly affected budding site selection, although to a smaller extent than RAP1A (Table 1). The kinase-inactive Raf-1 gave the same result while the amino and carboxy terminal domains affected budding to a lower extent. Therefore, although there seems to be a contribution of the kinase domain to the interference with Rsr1 functions, clearly the regulatory domain of Raf-1 is sufficient for this effect. These results strongly indicate that Raf-1 protein recognizes the effector domain in Ras and the Ras-related proteins Rap1A and Rsr1.

DISCUSSION

We have investigated interaction between Ras and Raf-1 proteins exploiting different *S. cerevisiae* systems that provide readouts for H-ras effector function. The phenotypes induced by Ras are distinct in the three systems used, and at least in two cases they have been demonstrated to depend on Ras effector function. In the

Figure 9. Effect of Raf-1 on wild-type budding pattern. Wild-type strain RAY-3A-D was transformed with plasmid pNV7 (1), pNV7-RAF (2), or pNV7-RAFΔN (3). Cells were grown at 30°C overnight and observed by fluorescence microscopy after staining with Calcofluor.

Table 1. Effect of Raf-1 constructs on budding pattern in wild-type cells

Plasmids	Genes	% Random budded cells ^a
pNV7	<i>Vector</i>	17
pADRP1	<i>Rap1A</i>	79
pNV7-RAF	<i>Raf-1</i>	65
pNV7-RAF-KA	<i>Kinase inactive Raf-1</i>	66
pNV7-RAFΔC	<i>Raf-1 regulatory domain</i>	40
pNV7-RAFΔN	<i>Raf-1 kinase domain</i>	36

^a Ability to affect normal budding was assayed in the diploid strain RAY3A-D as described in MATERIALS AND METHODS. Numbers indicate the percentage of cells that displayed the random pattern.

RAS2^{val19} strain cytoplasmic H-ras^{ser186} attenuates the effects of activated yeast Ras. This phenotype is dependent on the active GTP-bound state of Ras, because GAP or neurofibromin can readily revert the effects. In the *cdc25^{ts}* strain, the ability of H-ras to complement loss of yeast Ras activity has been clearly documented (Sigal *et al.*, 1986), and mutations in the effector-binding region of H-ras in mammalian cells compromise the ability of H-ras to substitute for yeast Ras function (Gibbs and Marshall, 1989). We found that despite these different phenotypes of H-ras in yeast, co-expression of Raf-1 interfered with H-ras function. Although our results do not rule out the participation of yeast components in the interaction between Raf-1 and Ras, they are consistent with the recently reported direct protein-protein interaction between Raf-1 and Ras at the effector binding region (Van Aelst *et al.*, 1993; Vojitek *et al.*, 1993; Zhang *et al.*, 1993). In vitro direct binding has also been reported (Vojitek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). The interaction we have observed of Raf-1 with Rap1A and its yeast homologue, Rsr1, which share identical effector regions with Ras proteins, strongly supports the idea that the interaction between Ras and Raf-1 is mediated by the effector domain of Ras. Moreover, our results indicated that the N-terminal domain of Raf-1 is responsible for this interaction with Ras. This region of Raf-1 has been implicated in regulating the kinase, because deletions that remove it confer constitutive stimulation of the Raf-1 kinase activity (Stanton *et al.*, 1989), and overexpression of this domain blocks activation of the Raf signaling pathway in mammalian cells (Kolch *et al.*, 1991). The effect of the amino terminus of Raf-1, therefore, suggests that Raf-1 binds to H-ras in the systems studied and prevents its interaction with the yeast effector(s).

In addition, we have shown that this interaction is functionally relevant and has physiological consequences. In these in vivo systems, where Raf-1 is not the endogenous effector of Ras, Raf-1 can bind to Ras and compete with the yeast effector(s). Therefore, our data strengthen the hypothesis that Raf-1 is a direct downstream effector of Ras in higher eukaryotes.

The data we have reported do not address the question of whether Ras binding to Raf-1 affects its kinase activity. In some cases, like the *cdc25^{ts}* strain carrying the H-ras gene, the truncated kinase domain of Raf-1 produced phenotypes similar to full-length Raf-1. This observation might suggest that Raf-1 kinase activity is responsible for these effects and that a consequence of Ras binding to Raf-1 might be the stimulation of the kinase. However, we cannot conclude from this that H-ras interaction is sufficient to activate the kinase. The reported correlation of an active Raf-1 kinase with phosphorylation at serine and threonine residues suggests the participation of a kinase in the activation mechanism (Li *et al.*, 1991). In addition, the presence of a cysteine-rich region in the amino terminal domain of Raf-1, homologous to that of the lipid-binding site in protein kinase C, indicates the possibility that a lipid second messenger might be involved in Raf-1 activation (Li *et al.*, 1991). A similar region is also implicated in protein-protein interaction in the case of the CD4/LCK complex and the Tat dimer (Bruder *et al.*, 1992). The functional interaction between Ras and Raf-1 demonstrated by our data suggests that Ras, if not directly activating Raf-1 on its own, is part of a complex that leads to Raf-1 activation. Identification of the factor(s) needed for completing Raf-1 activation in the presence of Ras will be crucial to the understanding of the role that Ras seems to play in integrating different growth signals.

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