Genetic Analysis of Mammalian GAP 
Expressed in Yeast

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Summary
We have designed a vector to express the mammalian GAP protein in the yeast S. cerevisiae. When expressed in yeast, GAP inhibits the function of the human H-ras*12 protein, but not that of the H-ras*12 protein, and complements the loss of IRA1. IRA1 is a yeast gene that encodes a protein with homology to GAP and acts upstream of RAS. Mammalian GAP can therefore function in yeast and interact with yeast RAS. Because expression of GAP complements ira1 mutants, we propose that GAP shares some biochemical functions with IRA1. Other studies indicate that IRA1 controls the level of RAS activity, presumably by regulating GTP hydrolysis. By analogy, we propose that GAP may play a similar role.

Introduction
The yeast Saccharomyces cerevisiae has two closely related RAS genes (DeFeo-Jones et al., 1983; Powers et al., 1984; Kataoka et al., 1984; Dhar et al., 1984). In yeast, RAS proteins control the activity of adenylate cyclase (Toda et al., 1985; Broek et al., 1985). Although activation of adenylate cyclase does not appear to be the role of vertebrate RAS proteins (Beckner et al., 1985; Birchmeier et al., 1985), there are substantial biochemical similarities between mammalian and yeast RAS proteins (for review, see Barbacid, 1987). Thus, mammalian RAS protein expressed in yeast can supply essential RAS function in that organism and stimulate adenylate cyclase (Kataoka et al., 1985; DeFeo-Jones et al., 1985). Preliminary evidence suggests that mammalian RAS can also interact with the CDC25 gene product, a protein believed to catalyze the exchange of nucleotides bound to yeast RAS proteins (Broek et al., 1987; Powers et al., 1989). Finally, a gene, IRA1, has been found in yeast that encodes a protein with a region of homology to mammalian GAP (Trahey et al., 1988; Tanaka et al., 1989). Genetic evidence indicates that IRA1 protein acts upstream of and inhibits wild-type yeast RAS (Tanaka et al., 1989). Cells deficient in IRA1 function show the same phenotypes as cells with the activating RAS2val9 mutation: they are sensitive to heat shock and nitrogen starvation and deficient in sporulation (Toda et al., 1985; Sass et al., 1986; Matsumoto et al., 1985; Tanaka et al., 1989).

Mammalian GAP is a ubiquitous cellular protein that catalytically accelerates GTP hydrolysis of the normal mammalian RAS proteins but not that of mutant, "activated" RAS proteins with oncogenic potential (Trahey and McCormick, 1987; Gibbs et al., 1988). Two hypothetical models for the cellular role of GAP have been proposed. In the first model, GAP would function to regulate RAS by virtue of inducing GTP hydrolysis. The failure of GAP to accelerate GTP hydrolysis of mutationally activated forms of RAS could then be invoked to explain the growth transforming properties of oncogenic RAS. In the second model, GAP would be the effector of RAS or part of an effector complex involved in transmitting the signal of activated RAS (Adari et al., 1988; Cales et al., 1988; Sigal, 1988; McCormick, 1989). This model, framed by analogy to the distantly RAS-related GTP binding protein EF-Tu, finds some support in the observation that GAP does not induce GTP hydrolysis in many mutant RAS proteins incapable of stimulating their effector (Adari et al., 1988; Cales et al., 1988; Vogel et al., 1988). To help distinguish the first and second model and gain insight into the possible role of GAP in cells, we have sought to analyze the function of mammalian GAP when expressed in yeast.

Results
To express mammalian GAP in yeast we constructed a high copy yeast extrachromosomal plasmid in which the coding sequences of the full-length human GAP cDNA were transcribed from the powerful yeast ADH1 promoter (Figure 1). This plasmid, PADGAP, also contains the LEU2 gene. We chose first to introduce GAP into yeast cells that depend upon the expression of mammalian H-ras protein for viability. Two yeast strains were transformed: TTRB-G1, which is cdc25- and expresses the wild-type human H-ras protein; and TTRB-V2, which is cdc25- and expresses the mutant H-rasVa2* protein. In both strains, the H-ras genes are expressed under the control of the ADH1 promoter and are contained in a high copy plasmid that also contains the HIS3 gene (see Experimental Procedures). The CDC25 gene encodes an essential yeast protein, thought to be required for the activation of wild-type yeast RAS proteins, by catalyzing nucleotide exchange (Broek et al., 1987; Robinson et al., 1987; Camonis et al., 1986). Cells deficient in CDC25 can be rendered viable if they express the mammalian H-ras or H-rasVa2* genes (Marshall et al., 1987). Such cells are not temperature sensitive. However, cdc25- cells containing the wild-type H-ras plasmid become temperature sensitive for growth if transformed by the GAP plasmid (Figure 2A). Of a total of 36 independent transformants, 31 containing a control plasmid (vector with auxotrophic marker) are able to grow at 37°C, whereas all 36 transformants containing the GAP plasmid are temperature sensitive (Table 1). In contrast, cdc25- cells containing the mutant H-rasVa2* plasmid are not affected by the presence of the GAP plasmid (Figure 2D). Of 36 independent
Figure 1. Construction of the Plasmid pADGAP

(A) The entire coding sequence of the human GAP gene was isolated from pUC101a as a 4.0 kb Smal-PvuII endonuclease fragment. This fragment contains nucleotides from position -36 to 3844 of clone 101 (Mahey et al., 1988) and 180 nucleotides from pUC18 at the 3' end. pUC18 sequences are indicated by a hatched bar, GAP sequences by an open bar.

(B) pAD4A, a 2um-based LEU2 plasmid that contains the yeast alcohol dehydrogenase (ADH1) promoter and terminator (see Experimental Procedures) was used as the vector to express mammalian GAP in yeast. This plasmid was used previously to express the H-ras (Powers et al., 1989), SCH9 (Toda et al., 1988), CDC25 (T.M., unpublished data), and CYTof alleles (Field et al., 1988) in yeast.

(C) The 4.0 kb fragment isolated from pUC101a was inserted into the Smal site of pAD4A. The direction of the GAP insert was confirmed by digestion with the restriction enzymes SalI (a single site is present in the polylinker region) and NcoI (a single site is present in the GAP-containing fragment). The resulting plasmid pADGAP was used to transform yeast strains.

Figure 2. Effect of GAP on the Temperature Sensitivity of Strains Lacking CDC25 and Expressing the Wild-Type Human H-ras or the Mutant H-ras<sup>va</sup> Proteins

Cells completely lacking the CDC25 gene (see Experimental Procedures for description of strains) but containing pAHRG-H1, a plasmid with the H160 gene and the wild-type H-ras (A, strain TTRB-G1), or the plasmid pAHRC-H2, containing the H197 gene and H-ras<sup>va</sup> (R, strain TTRB-V2), were transformed with a control plasmid pAD4A (LEU2; rows 1 and 2), or a plasmid expressing GAP, pADGAP (LEU2; rows 2 and 4), and selected on SC-Leu-His plates. Independent transformants were patched onto selective plates and incubated at room temperature for 3 days and then replicated onto YPD plates and incubated at 37°C for 2 days. Each set (1-4) shows eight independent transformants.
Table 1. Effect of GAP on the Growth at 37°C of Yeast Strains Lacking CDC25 and Expressing H-ras or H-ras\textsuperscript{v12}

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype\textsuperscript{a}</th>
<th>Transforming Plasmid\textsuperscript{b}</th>
<th>Growth at 37°C (number of transformants growing/total number of transformants tested)\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTRB-G1</td>
<td>HIS3, cdc25\textsuperscript{c}, H-ras</td>
<td>pAD4A</td>
<td>61/66</td>
</tr>
<tr>
<td>TTRB-Q1</td>
<td>HIS3, cdc25\textsuperscript{c}, H-ras</td>
<td>pADGAP</td>
<td>60/66</td>
</tr>
<tr>
<td>TTRB-V2</td>
<td>HIS3, cdc25\textsuperscript{c}, H-ras\textsuperscript{v12}, pAD4A</td>
<td>35/36</td>
<td>35/36</td>
</tr>
<tr>
<td>TTRB-V2</td>
<td>HIS3, cdc25\textsuperscript{c}, H-ras\textsuperscript{v12}, pADGAP</td>
<td>35/36</td>
<td>35/36</td>
</tr>
</tbody>
</table>

\textsuperscript{a} See Experimental Procedures for the complete genotype of the yeast strains.
\textsuperscript{b} See Figure 1 for description of the plasmids.
\textsuperscript{c} The different strains were transformed with a control plasmid (pAD4A) or GAP-containing plasmid (pADGAP). Thirty six independent transformants were tested for temperature sensitivity, as described in Figure 2, and scored for the ability to grow at 37°C in YPD.

RAS2\textsuperscript{v12} strain remains heat shock sensitive (Figure 3, C2). Similar results were observed when we scored resistance to nitrogen starvation (data not shown). These results indicate that expression of mammalian GAP can functionally complement IRA\textsuperscript{1} deficiency and suggest that GAP can inhibit the wild-type yeast RAS2 protein but not the activated RAS2\textsuperscript{v12} mutant protein.

**Discussion**

GAP accelerates the GTP hydrolysis catalyzed by wild-type mammalian RAS proteins (Trahey and McCormick, 1987; Gibbs et al., 1988). From this, one predicts that GAP has the potential to inhibit the activity of RAS proteins. Our studies in yeast lead us to conclude that GAP can indeed inhibit wild-type H-ras protein. Within the limits of the genetic system we have tested, GAP does not appear to inhibit the activity of mutant H-ras\textsuperscript{v12} protein. Thus, GAP neither effectively down regulates H-ras\textsuperscript{v12} protein nor effectively competes for the binding of H-ras\textsuperscript{v12} protein to its target effector in yeast.

GAP expression can restore the heat shock and nitrogen starvation resistance of ira\textsuperscript{1} mutant strains. GAP expression has no discernible effects on these phenotypes in RAS2\textsuperscript{v12} strains. These results suggest that mammalian GAP can effect the function of wild-type yeast RAS protein but not that of the mutationally activated yeast RAS protein. Thus, GAP protein interaction with yeast RAS proteins appears similar to its interaction with mammalian RAS proteins. GAP expression does not complement ira\textsuperscript{1} mutant strains perfectly, as such strains are somewhat more heat shock sensitive than wild-type strains (data not shown).

We cannot infer that GAP and IRA proteins have the same biochemical function. It is reasonable to infer, from the ability of GAP to affect wild-type but not mutationally activated RAS proteins, that GAP effects are due to its induction of GTP hydrolysis. IRA proteins, however, need not act through this mechanism. For example, IRA proteins might block nucleotide exchange on RAS. However, since both GAP and IRA proteins can interact with yeast RAS and share a domain with clear sequence homology, both...
may bind to the same site on RAS proteins. The homologous region may represent a common site for IRA1 protein interactions.

Since GAP can complement ira-1 mutants, it is reasonable to speculate that GAP and the IRA1 protein have a conserved cellular role. In yeast, CAMP production is under feedback control (Nikawa et al., 1987). Wild-type yeast cells show a rapid biphasic change in CAMP levels in response to glucose (Thevelein and Beullens, 1985). However, if cells are ira-1 mutants (Tanaka et al., 1989), or if they contain attenuated CAMP-dependent protein kinase activity, CAMP levels rise and remain elevated in response to glucose (Wigler et al., 1988). Thus, an argument can be made that the IRA1 protein is a component of a feedback pathway that controls RAS protein activity. In yeast, RAS2wt* protein is refractory to feedback inhibition (Nikawa et al., 1987). In mammalian cells there is also evidence from microinjection experiments that wild-type H-ras protein activity is subject to feedback control, while the H-rasmut* protein is not (Bar-Sagi and Feramisco, 1989). These considerations lead us to suggest that, in mammalian cells, GAP is a component of a feedback pathway down regulating RAS proteins by accelerating hydrolysis of RAS-bound GTP. This model is not particularly consonant with GAP, also serving as an effector for RAS proteins.

Experimental Procedures

Yeast and Escherichia coli Strains, Media, Genetic Manipulations, and Nomenclature

The S. cerevisiae strains used for this study are: SP1 (MATa his3 leu2 ura3 trpl ade8 canl; Toda et al., 1985); TTA1 (MATa his3 leu2 ura3 trpl ade8 canl pUC25:URA3 pCDC25(TRPl)-1; Broek et al., 1987); and TK161R2V (MATa leu2 ura3 his3 trpl ade8 can1 RAS2va*; Toda et al., 1987). Yeast cells were grown in YPD and synthetic media and grown for 2 days at 30% for 1 hr at 55°C. This plate was then incubated for 10 min at 55°C and transferred to 30°C for 2 days. Yeast cells were starved for nitrogen by transferring to 30% for 2 days. Yeast cells were starved for nitrogen by transferring to 30% for 2 days.

Plasmids

The cloning vector pAD4A is similar to the plasmid pADNS previously described (Colicelli et al., 1989). It contains the LEU2 gene, an Hpal-HindIII fragment from 2um including the origin of replication, an Sphi-EcoRI fragment containing the ampicillin resistance gene from the plasmid pUC18, a HindIII-BamHI fragment containing the S. cerevisiae alcohol dehydrogenase (ADH1) promoter, and a HindIII-BamHI fragment containing the ADH1 terminator sequences. The promoter and terminator sequences are separated by a polylinker from pUC18 that lacks the SpfI and HindIII sites (Figure 1). The human GAP gene was inserted into the Smal site in the polylinker region pAD4A as described in Figure 1.

The plasmid pAHRG-H1 was constructed by inserting an Sphi fragment that contains the human H-ras CDNA driven by the ADH1 promoter into the vector pHVI. pHVI is a high copy number vector that contains the polylinker from pUC18, the HIS3 gene, and an HpaI-HindIII fragment from 2um, including the origin of replication but lacking Rep3 (Brouch et al., 1985). The plasmid pAHRG-H2 is the same as pAHRG-H1 except that it contains the mutant H-rasmut2 instead of the wild type H-ras. This mutation was obtained by oligonucleotide-directed mutagenesis performed by a modification of a procedure of Zoller and Smith (1984), which utilizes uracil-containing template DNA (Kuniet, 1985). The oligonucleotide 5'-GTGGTGGCGCGCGCGGGTGTA3' was used to change codon 12 to encode valine. Mutagenesis was confirmed by sequencing.

The YepPDE2 plasmid contains the yeast PDE2 gene on the high copy LEU2 plasmid Yep13 (Sass et al., 1986). The PDE2 gene encodes a cAMP-phosphodiesterase. The plasmid pUC101a contains an EcoRI fragment of clone 101 of the GAP gene (Trahey et al., 1988) inserted into pUC18 (Figure 1). This fragment contains the entire coding region of the human GAP.

Construction of Yeast Strains

TTA1 is a strain that lacks a functional chromosomal CDC25 gene but is viable because it has the CDC25 gene in a multicopy plasmid containing the TRP1 marker (pCDC25(TRP1)-1; Broek et al., 1987). Cells deficient in CDC25 are viable if they express the mammalian H-ras or H-rasmut2 genes (Marshall et al., 1987). To construct a strain dependent upon the expression of mammalian H-ras proteins, TTA1 was transformed with pAHRG-H1 or pAHRG-H2, and His+- transformants were selected. These were grown without selection in YPD medium for 48 hr and plated onto SC-His plates. The resulting colonies were replica plated onto SC-His, YPD, and SC-Tp plates. Colonies that had lost the pCDC25(TRP1)-1 plasmid but that were His+- were presumed to contain the plasmid with the wild-type H-ras or H-rasmut gene. This "plasmid exchange" procedure has been used successfully to suppress the loss of CDC25 by the RAS2mut, the TK1, and the CYR1 genes (Broek et al., 1987). The cdc25- strain TTRB-G1 (MATa his3 leu2 ura3 trpl ade8 cdc25:URA3 pAHRG-H1) expresses the mutant H-rasvtir2 instead of the H-rasmut2* gene, whereas the strain TTRB-V2 (MATa his3 leu2 ura3 trpl ade8 cdc25:URA3 pAHRG-H2) expresses the mutant H-rasmut2* protein. To test whether the GAP gene carried on a multicopy plasmid has an effect on mammalian H-ras proteins expressed in yeast, we transformed these strains with the plasmids pAD4A and pADGAP (see Figure 1). His+-, Leu+ transformants were selected and tested for temperature sensitivity.

The yeast strain IR-1 (MATa his3 leu2 ura3 trpl ade8 can1 irr1::HIS3) contains a disruption in the IRA1 gene. This strain was constructed as follows: an Xbal DNA fragment from YIp-cd15 (a plasmid containing the entire IRA1 sequence; Tanaka et al., 1989) was inserted into pUC18 (Viola and Messing, 1987), which had been digested with Xbal. The resulting plasmid was digested with BglII and ligated to a BamHI fragment of the HIS3 gene. This results in a 3.2 kb deletion of IRA1 coding sequences. (A similar deletion/insertion was used previously by Tanaka and co-workers, 1989, to generate a mutant irra-1 allele.) The resulting plasmid was used to carry out gene replacement experiments (Rothstein, 1983). The yeast haploid auxotroph SP1 was transformed with the Xbal fragment of the deleted IRA1 gene, and transformants were selected by histidine prototrophy. Southern hybridization analysis was used to verify that the IRA1 gene was replaced by the disrupted gene.

Heat Shock and Nitrogen Starvation of Yeast Cells

Heat shock sensitivity and nitrogen starvation sensitivity were determined as described previously (Ioda et al., 1986; Sass et al., 1986). Heat shock was performed by replica plating cells to a plate preheated for 1 hr at 55°C. The plate was then incubated for 10 min at 55°C and transferred to 30°C for 2 days. Yeast cells were starved for nitrogen by replica plating cells to medium lacking a source of nitrogen. This replica was incubated for 9 days at 30°C and then replica plated onto YPD or synthetic media and grown for 2 days at 30°C.
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of *S. cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP dependent protein kinase catalytic subunits. Genes Dev. 2, 517–527.


