



# Critical Function of the Ras-associating Domain as a Primary Ras-binding Site for Regulation of *Saccharomyces cerevisiae* Adenylyl Cyclase

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**Title:** Critical Function of the Ras-associating Domain as a Primary Ras-binding Site for Regulation of *Saccharomyces cerevisiae* Adenylyl Cyclase\*

(Ras- associating ドメインの出芽酵母アデニル酸シクラーゼの活性調節のための主要 Ras 結合部位としての重要な機能)

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**Running title:** Role of RA Domain in Regulation of Adenylyl Cyclase by Ras

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RA Domain, Adenylyl Cyclase, Ras

## SUMMARY

Mammalian candidate effectors of the small GTPase Ras, such as RalGDS, afadin/AF-6, Rin1 and phospholipase C $\epsilon$ , have been shown to share structurally conserved modules termed Ras-associating (RA) domains at their Ras-binding sites. The Ras-binding domains of Raf-1 and phosphoinositide 3-kinase, other Ras effectors, also share a similar tertiary structure with the RA domains. On the other hand, the primary Ras-binding site of *Saccharomyces cerevisiae* adenylyl cyclase, the best characterized Ras effector, has been mapped by mutational studies to the leucine-rich repeats (LRR) domain (amino acid 674-1300), whose structure apparently bears no resemblance to the RA domains. By a computer algorithm-based search we have unexpectedly found an RA domain in the N-terminal 81-amino acid residues (676-756) of the LRR domain. The purified RA-domain polypeptide exhibits an ability to bind directly to Ras in a GTP-dependent manner and to competitively inhibit Ras-dependent activation of adenylyl cyclase *in vitro*, with an affinity comparable to that of the whole LRR domain. The specificity of binding of the RA domain to various Ras effector region mutants is indistinguishable from that of the full-length adenylyl cyclase. The activated *RAS2* (*RAS2*<sup>Val-19</sup>)-dependent heat shock sensitivity of yeast cells is suppressed by overexpression of the RA-domain polypeptide. Further, mutations of the RA domain abolish its Ras-binding activity, and adenylyl cyclase molecules carrying these mutations are rendered unactivatable by Ras *in vitro*. This RA domain bears highest similarity to the Ras-binding domain of Raf-1 based on comparison of its primary and predicted secondary structures with those of other Ras effectors. These results indicate that the RA domain is a primary Ras-binding site for activation of adenylyl cyclase, implicating RA domains as universal modules for interaction of effectors with Ras, conserved from yeast to mammals.

## INTRODUCTION

Ras proteins are small guanine nucleotide-binding proteins cycling between the active GTP-bound and the inactive GDP-bound states. They are conserved from yeasts to mammals and essential signaling components regulating a number of biological responses. The budding yeast *Saccharomyces cerevisiae* has two *RAS* genes, *RAS1* and *RAS2*, that encode proteins highly homologous to mammalian Ras proto-oncogene products (1, 2). The yeast *RAS* proteins are essential regulatory elements of adenylyl cyclase, which catalyzes the production of cAMP, a second messenger vital for yeast cell growth. The Ras-cAMP pathway is implicated in transduction of glucose-triggered signals to intracellular environments where cAMP initiates protein phosphorylation cascades. Yeast cells bearing the activated *RAS2* gene, *RAS2<sup>Val-19</sup>*, exhibit an elevated level of intracellular cAMP and display abnormal phenotypes, including sensitivity to heat shock, sensitivity to nutritional starvation and failure to sporulate (3, 4). Mammalian Ras proteins can substitute for yeast *RAS* to induce activation of *S. cerevisiae* adenylyl cyclase (3, 5, 6).

*S. cerevisiae* adenylyl cyclase consists of 2,026 amino acid residues and includes at least four domains: the N-terminal, middle repetitive, catalytic, and C-terminal domains (7, 8). The middle repetitive domain is composed of a repetition of 23-amino acid amphipathic leucine-rich motifs and hence called the LRR<sup>1</sup> domain. Introduction of insertion or point mutations into virtually any LRR unit abrogated *RAS2*-dependent activation of adenylyl cyclase, implicating the LRR domain as a primary Ras-interaction site (9-11). Adenylyl cyclase forms a complex with 60-kDa CAP through its C-terminal region (12-14). CAP is a multifunctional protein. Its N-terminal region is required for the proper function of the Ras-cAMP pathway, while its C-terminal region is involved in regulation of the actin cytoskeleton (13, 15, 16).

We previously demonstrated that at least two kinds of interaction with Ras are required for efficient activation of adenylyl cyclase (17, 18). One is a GTP-dependent high affinity interaction between Ras and the LRR domain. The other is a GTP-independent weak interaction between Ras and a complex of adenylyl cyclase and CAP, which is dependent upon post-translational modification, in particular farnesylation, of Ras. The latter interaction successfully explains the reason why farnesylation of Ras is essential for the efficient activation of adenylyl cyclase by

Ras (19).

Recently a computer algorithm-based study by Ponting and Benjamin revealed the existence of a motif of roughly 100-amino acid residues, called RAD, which was conserved among Ras-binding regions of mammalian RalGDS, Rin1 and afadin/AF-6 (20). A Ras-homologue Rap1, which possesses the identical effector region with Ras, was shown to bind to almost all of these RADs as well. Subsequent studies identified RADs in a variety of other Ras/Rap1 effector candidates including PLC $\epsilon$  (21–24) and RA-GEF-1 (25, 26). Interestingly, X-ray crystallographic studies revealed that the overall tertiary structure of RalGDS RAD is similar to those of Raf-1 and PI3-K $\gamma$  RBDs, although no extensive sequence similarity is found among them (27–33). These studies as well as various mutational studies on Ras revealed that the effector regions of Ras and Rap1 (amino acid 32–40 of mammalian Ras/Rap1) are mainly responsible for the associations with the RAD/RBDs. Although the studies described above implied a universal nature of RADs as primary Ras-binding sites of the Ras effectors, yeast adenylyl cyclase comprised a notable exception because its LRR domain did not appear to bear any resemblance to RADs.

In this study, a computer algorithm-based search has unexpectedly predicted an RAD in the N-terminal part of the LRR domain of yeast adenylyl cyclase. Evidence is presented for the essential function of the RAD in association with Ras.

## EXPERIMENTAL PROCEDURES

*Cell Strains and Growth Media*----The *S. cerevisiae* strains TK36-1 [*MAT $\alpha$* , *his3*, *leu2*, *trp1*, *ura3*, *ade8*, *cyr1-2*, *ras2::LEU2*, (YEP24-ADC1-CYR1)], TM5-1 [*MAT $\alpha$* , *his3*, *leu2*, *trp1*, *ura3*, *ade8*, *can1*, [pAD4-GST-CYR1(606-1380)]]], and TK161-R2V (*MAT $\alpha$* , *his3*, *leu2*, *trp1*, *ura3*, *ade8*, *ras2::LEU2*, *RAS2<sup>Val-19</sup>*) were described previously (34, 35). Yeast cells were grown in YPD (2% Bacto-peptone, 1% Bacto-yeast extract, 2% glucose) or yeast synthetic medium (0.67% yeast nitrogen base, 2% glucose) with appropriate auxotrophic supplements. Genetic manipulations of yeast cells were performed as described previously (36). Transformation into yeast cells was carried out with lithium acetate (37).

*Construction of Expression Plasmids*----YEP24-ADC1-CYR1 was used for expression of the full-length adenylyl cyclase under the control of the *ADC1* promoter (10). Adenylyl cyclase mutants, which carry 2 (-V-D-) or 4 (-S-T-S-T)-

amino acid insertions in their LRR domains, were prepared by using a 6-bp oligonucleotide pCGTCGA as described previously (10). The resulting mutations were transferred to YEP24-ADC1-CYR1, producing YEP24-ADC1-CYR1VN, where N represents the location of the insertion in amino acid position. YEP24-ADC1-CYR1V658 and YEP24-ADC1-CYR1V700 were used for construction of YEP24-ADC1-CYR1( $\Delta$ 659-700) carrying an internal deletion of amino acid 659-700. pAD4-GST-CYR1(*X-Y*) was used for expression of an adenylyl cyclase polypeptide corresponding to amino acid *X-Y* as a GST-fusion under the control of the *ADC1* promoter. Mutants of adenylyl cyclase carrying single amino acid substitution in their LRR domain were described before (11). DNA fragments corresponding to various subfragments of adenylyl cyclase were cloned into pGEX-2T (Amersham Pharmacia Biotech) for expression as GST-fusions in *Escherichia coli*.

*In Vitro Ras-Binding Assay*----The post-translationally modified forms of Ha-Ras and its effector mutants were purified from *Spodoptera frugiperda* Sf9 insect cells infected with baculoviruses expressing them as described before (38). They were preloaded with GTP $\gamma$ S or GDP $\beta$ S and examined for association with GST-CYR1(640-809) and its mutants, which were attached to glutathione-Sepharose beads as described (17). Bound Ha-Ras was detected by immunoblotting with anti-Ha-Ras antibody (F235; Oncogene Science, Inc.).

*Adenylyl Cyclase Assay*----Adenylyl cyclase was solubilized from the crude membrane fraction of the yeast TK36-1 harboring YEP24-ADC1-CYR1 or its mutant with buffer C {50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.2, 0.1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 1 mM  $\beta$ -mercaptoethanol} containing 1% LubrolPX, 0.6 M NaCl, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 100,000  $\times$  g for 1 h at 4 °C, the resulting supernatant was used for measurement of adenylyl cyclase activity in the presence of 2.5 mM MgCl<sub>2</sub> with the addition of various concentrations of purified Ha-Ras, which had been loaded with GTP $\gamma$ S as described previously (17). For measurement of the Mn<sup>2+</sup>-dependent activity, 2.5 mM MnCl<sub>2</sub> replaced MgCl<sub>2</sub> and Ha-Ras. Adenylyl cyclase in the extract was detected by immunoblotting with anti-CYR1CT polyclonal antibody (14). For adenylyl cyclase-inhibition assays, the Ha-Ras-dependent adenylyl cyclase activities were measured in the presence of varying concentrations of purified GST-CYR1(640-809) or its mutant purified from *E. coli* as described previously (35).

*Other Methods*----Survival of yeast cells after heat shock treatment at 55°C for 5 min was examined by a replica plating method as described previously (4, 13). Anti-

CYR1CT antibody, which was raised against C-terminal 15-amino acid synthetic peptide of adenylyl cyclase, was described before (14). The enhanced chemiluminescence immunodetection system (Amersham Pharmacia Biotech) was used for signal development. The RAD-searching program from the ProfileScan Server (<http://www.isrec.isb-sib.ch/software/PFSCAN>) was used for prediction of RADs.

## RESULTS

*Role of the LRR Structure in Interaction with Ras*---The consensus motif of the LRR units is represented by PXX $\alpha$ XXLXXLXXLXLXXNX $\alpha$ XX $\alpha$  (where  $\alpha$  is an aliphatic amino acid and X is any amino acid) (7). Approximately 26-fold repetition of this unit are located in amino acid 674-1300 (Fig. 1). Previously we had shown that the CYR1(606-1380) polypeptide, encompassing the whole LRR domain, possessed the high affinity Ras-binding activity *in vitro* (17, 35). Further, genetic and biochemical studies employing deletion and insertion mutations showed that the LRR domain is required for Ras-dependent adenylyl cyclase activation. This was based on the observations that any deletion or insertion mutations involving the LRR units resulted in abrogation of the *in vitro* activation of adenylyl cyclase by Ras (9, 10). Further, substitution mutations for the consensus residues of the LRR motif; such as P1080L, L1092P and L1099Q mutations, also abolished the *in vitro* response to Ras although similar mutations involving different consensus residues, N1097T and L1086S, had no effect (11). However, the observed loss of the response to Ras might not necessarily reflect the loss of the Ras-binding activity.

In this study, we first examined the effect of the mutations of the LRR domain on its Ras-binding activity, which was indirectly assessed by the ability to suppress the RAS2<sup>Val-19</sup>-dependent heat shock sensitivity of yeast cells. This suppression was caused by competitive sequestration of endogenous RAS2<sup>Val-19</sup> from the endogenous adenylyl cyclase. As reported (11, 39), overexpression of GST-CYR1(606-1380) or GST-CYR1(606-1710), containing the whole LRR domain, suppressed the heat shock sensitivity (Fig. 2A). Interestingly, the P1080L, L1092P and L1099Q mutations, which were known to abolish the *in vitro* response to Ras, did not affect the activity of GST-CYR1(606-1380) to suppress the heat shock sensitivity, implying that they had no effect on the Ras-binding activity. Thus, the effect of these

mutations on Ras-dependent activation appeared indirect: it may be ascribed to a change in conformations of adenylyl cyclase, which may affect the allosteric transmission of the Ras-binding signal to the C-terminal catalytic domain. The results suggested that another structure residing in GST-CYR1(606-1380) than the LRR units might be responsible for the direct association with Ras. While these studies were in progress, scanning by the RAD-searching program unexpectedly predicted an RAD in the N-terminal region of the LRR domain, which was located in amino acid 676-756, overlapping with the LRR units. When polypeptides (amino acid 640-809 and 640-925) containing the predicted RAD were overexpressed in yeast cells, the *RAS2*<sup>Val-19</sup>-dependent heat shock sensitivity was suppressed although their activity of suppression was weaker than that of polypeptides containing both the RAD and the LRR units (Fig. 2B). These results suggested that the RAD may correspond to the Ras-binding site of adenylyl cyclase.

*Direct Association of the RAD with Ras*---We next examined whether the RAD directly associates with Ras *in vitro*. As expected, both GST-CYR1(640-809) and GST-CYR1(640-925), purified from *E. coli*, directly bound to Ha-Ras in a GTP-dependent manner (Fig. 3A). Our previous experiments had shown that mutations D38N and P34G in the effector region of Ha-Ras abolished both the binding activity to GST-CYR1(606-2026) and the ability to activate adenylyl cyclase (38). In particular, P34G mutation affected the interaction with adenylyl cyclase but not with other Ras effectors including human Raf-1, *Schizosaccharomyces pombe* Byr2 and human phospholipase C $\epsilon$  (22, 38). As shown in Fig. 3B, both of the Ha-Ras mutants lost their ability to associate with GST-CYR1(640-809). The association of the RAD with Ras was further analyzed quantitatively by the yeast adenylyl cyclase inhibition assay (35). A dose-dependent inhibition of Ha-Ras-dependent adenylyl cyclase activity was observed by purified GST-CYR1(640-809) added into the reaction mixture (Fig. 3C). GST-CYR1 (640-809) had no effect on the Mn<sup>2+</sup>-dependent adenylyl cyclase activity, indicating that this protein exerted its effect by interacting with Ras but not with adenylyl cyclase (data not shown). We carried out this experiment in the presence of various concentrations of Ha-Ras and GST-CYR1 (640-809) to prove the competitive nature of the inhibition as described (35). At each point of Ha-Ras concentration in the presence of GST-CYR1(640-809), we obtained free Ha-Ras concentration available for adenylyl cyclase activation as that required for giving the same adenylyl cyclase activity in the absence of GST-CYR1(640-809). A difference between the original and the free concentrations of Ha-Ras was



regarded as that bound to GST-CYR1(640-809), and a reciprocal of this value was plotted against a reciprocal of the free Ha-Ras concentration (Fig. 3D). This gave a series of straight lines for each value of GST-CYR1(640-809), which converged on the horizontal axis. The data indicated that GST-CYR1(640-809) bound directly to Ha-Ras and competitively sequestered it from adenylyl cyclase. The  $K_d$  value for Ha-Ras was calculated from the point of intersection with the horizontal axis and determined to be around 80 nM, which was a bit higher than that (10 nM) of CYR1(606-1380) (17, 35). These results imply that the binding affinity of the LRR domain for Ras is mainly accounted for by the RAD.

*Effect of Mutations of the RAD on Adenylyl Cyclase Activation by Ras*-----The RAD was subjected to an insertional mutagenesis to analyse its role in Ras-dependent activation of adenylyl cyclase. We introduced three kinds of 2- or 4-amino acid insertion mutations ( $\nabla$ 658,  $\nabla$ 700 and  $\nabla$ 736) as well as a deletion mutation ( $\Delta$ 659-700) into the RAD and its immediate flanking region. Adenylyl cyclase molecules carrying these mutations were solubilized from the yeast membrane fractions and examined for their activity in the presence of various concentrations of GTP $\gamma$ S-bound Ha-Ras (Fig. 4A). The activities were shown as percentages of the Mn<sup>2+</sup>-dependent activities. All of the mutations  $\nabla$ 700,  $\nabla$ 736 and  $\Delta$ 659-700 involving the RAD completely abolished the Ras-dependent activation. The mutant adenylyl cyclases did not show detectable sign of degradation (Fig. 4B). In contrast, the  $\nabla$ 658 mutation outside of the RA domain did not abolish the activation although the maximal extent of activation attained was significantly lower. The concentration of Ha-Ras giving a half maximal activation was similar between the  $\nabla$ 658 mutant and wild-type adenylyl cyclase, indicating that the affinity for Ha-Ras was also similar. These results agreed well with our previous data that an N-terminal deletion up to amino acid position 657 had no discernable effect on the Ras-dependent activation (10).

We next examined the effect of the same mutations on the Ras-binding activity of the RAD (Fig. 5A). *In vitro* Ras-binding assays were carried out using GST-CYR1(640-809) carrying the mutations. All of the  $\nabla$ 700,  $\nabla$ 736 and  $\Delta$ 659-700 mutations resulted in loss of the Ras-binding activity, whereas the  $\nabla$ 658 mutation had no effect. Consistent with these results, all of the  $\nabla$ 700,  $\nabla$ 736 and  $\Delta$ 659-700 mutations totally impaired the ability of GST-CYR1(640-809) to inhibit the Ras-dependent adenylyl cyclase activation *in vitro* (Fig. 5B). These results unequivocally demonstrated that the RAD comprises a primary Ras-binding site for activation of

adenylyl cyclase by Ras.

## DISCUSSION

Although genetic and biochemical studies by ours and other groups previously indicated the crucial role of the LRR structure in Ras-dependent activation of adenylyl cyclase (9, 10), the present study have clearly demonstrated that the RAD located in amino acid 676-756, overlapping with the LRR structure, is responsible for the direct association with Ras. This was proved by observation of the GTP-dependent association of human Ha-Ras with the RAD polypeptide with an affinity comparable to that with the whole LRR-domain polypeptide. The binding activity of the RAD to yeast RAS2 was demonstrated by its ability to suppress the *RAS2<sup>Val-19</sup>*-dependent heatshock sensitivity when overexpressed in yeast cells. Further, specific mutations of the RAD, which abolished its Ras-binding activity, were shown also to abrogate the *in vitro* response of adenylyl cyclase to Ras protein. These results are in good agreement with our previous data, which indicated that N-terminal deletions down to amino acid 657 had no effect on the response of adenylyl cyclase to Ras (10). The detrimental effect of various mutations affecting the LRR structure on Ras-dependent activation, which we previously observed, appears indirect: it may be ascribed to a change in conformations of adenylyl cyclase, which may affect the allosteric transmission of the Ras-binding signal to the C-terminal catalytic domain. In fact, adenylyl cyclase molecules carrying these mutations were observed to form a high molecular weight complex whose size was considerably greater than that of the wild-type adenylyl cyclase, which may reflect a change in the oligomeric state of adenylyl cyclase (11).

RADs were discovered through a computer algorithm-based analysis by Ponting and Benjamin as domains of about 100 amino acids whose primary structures were conserved in a variety of Ras/Rap1 effectors and effector candidates including mammalian RalGDS and its homologues Rlf and Rgl, afadin/AF-6 and its *Drosophila melanogaster* homologue Canoe, and Rin1 (20). Subsequent studies identified RADs in Ras/Rap1 effector candidates including PLC $\epsilon$  and RA-GEF-1 (21-26). The RADs of these effector and effector candidates exactly corresponded to their GTP-dependent Ras/Rap1-binding sites. The RAD sequences were extremely divergent with one another and required sensitive profile methods for detection. This

extreme sequence divergence suggested possible divergence of their binding partners. In fact, the RADs of the Ras/Rap1 effectors exhibited mutually distinct binding specificities toward various effector region mutants of Ras as well as toward Ras and Rap1. For example, RalGDS RAD binds more strongly to Rap1 than to Ras, whereas Raf-1 RBD exhibits an opposite binding specificity toward Rap1 and Ras (40, 41). Moreover, some RADs that bound other small GTPases than Ras and Rap1 were identified: the RADs of RA-GEF-2 and GFR/MR-GEF were reported to bind specifically to M-Ras (R-Ras3) but not to Ras and Rap1 (42-44). The RAD of AF-6/Afadin was reported to bind to M-Ras as well as to Ras and Rap1(45). On the other hand, the amino acid sequences of the RBDs of Raf-1 and PI3-K $\gamma$  did not score high by the RAD-searching program. However, it was found that the majority of hydrophobic residues of Raf-1 RBD were conserved with the RAD sequences and that the predicted secondary structure of Raf-1 RBD using the fold-recognition algorithm (46) matched to those of RADs (20). In fact, it was demonstrated by a series of X-ray crystallographic studies that the tertiary structures of RBDs of Raf-1 and PI3-K $\gamma$  display a considerable similarity with that of RalGDS RAD despite the quite limited identities in their primary structures (27-33).

The amino acid sequences and the known or predicted secondary structures of the RBDs of Raf-1 and PI3-K $\gamma$  and the RADs of other Ras/Rap1 effectors are shown in Fig. 6. The crystal structures of Raf-1 RBD complexed with Rap1 and RalGDS RAD complexed with Ras revealed that the first two  $\beta$ -strands ( $\beta$ 1 and  $\beta$ 2), the next  $\alpha$ -helix ( $\alpha$ 1) and a following loop are important for the interaction with the small GTPases (27, 29, 31, 41). In general, the interactions are mediated mainly by hydrogen bonds and polar interactions formed by charged residues and both main-chain and side-chain polar groups. Only a few hydrophobic contacts are formed. In case of RalGDS, the major interaction between Ras and its RAD occurs between two antiparallel  $\beta$ -strands;  $\beta$ 2 of Ras including the switch I/effector region and  $\beta$ 2 of the RAD, using hydrogen-bond networks formed between these two  $\beta$ -strands (29, 31). Raf-1 RBD also employs a quite similar mechanism for interaction with Rap1 (27, 28, 30). In particular, complementarity of the electrostatic charge distribution at the interfaces is shown to be important. The effector region of Ras/Rap1 is acidic and generates a negatively charged surface patch that interacts with a positively charged patch generated by basic residues of the RAD/RBDs (27-33, 41). Indeed, previous mutational analyses indicated that some of these charged residues were crucial for binding to Ras/Rap1 (27-33).

However, there exist notable differences in the mechanisms by which Ras and Rap1 recognize RalGDS, Raf-1, and PI3-K $\gamma$ . We shall discuss the differences of the recognition mechanisms in terms of the distribution of the critical basic residues in the  $\beta$ 1-,  $\beta$ 2-strands,  $\alpha$ 1-helix and a loop of the RAD/RBDs and of the interaction mechanism to the various effector region residues of Ras/Rap1. In case of rat RalGDS RAD, three basic residues; R784, K796 and K816 (shown by *bold face* letters in Fig. 6), form a major binding interface with Ras/Rap1. R784 makes an ionic interaction with E37 of Ras/Rap1 (31, 33, 41). K796 and K816 interact with D38 of Ras/Rap1: K796 makes an ionic interaction with D38, while K816 makes a tight hydrogen bond with D38 to form a main interface with D38 (29-31, 33, 41). When the primary structures of RalGDS RAD and two RADs (N-terminal and C-terminal RADs) of afadin/AF-6 were aligned with one another, all of the three basic residues corresponding to R784, K796 and K816 of RalGDS RAD were conserved in both N-terminal and C-terminal RADs (Fig. 6A), suggesting that afadin/AF-6 RADs may be classified into the RalGDS RAD group.

On the other hand, Raf-1 RBD exhibits a different mode of interaction with Ras/Rap1 for the following reasons. It has R59 corresponding to R784 of RalGDS in the  $\beta$ 1-strand, but R59 makes a strong hydrogen bond with E37 of Ras/Rap1 in contrast to the ionic interaction of R784. Strikingly, Raf-1 RBD lacks two Lys residues corresponding to K796 and K816 of RalGDS in its  $\beta$ 2-strand and  $\alpha$ 1-helix, respectively. Instead, R89 forms a hydrogen bond with D38 of Ras/Rap1 and makes a main binding surface in the  $\alpha$ 1-helix, thereby functionally substituting for K816 of RalGDS (27, 33). Although two Lys residues K84 and K87 exist in the  $\alpha$ 1-helix of Raf-1 RBD, they do not contribute to the interaction with D38 of Ras (27, 33). A notable difference exists at the binding surface of the  $\beta$ 2-strand. There exists no basic residue corresponding to K796 of RalGDS, and its function is replaced by T68, whose side-chain hydroxyl group forms a hydrogen bond with D38 of Ras/Rap1 (27, 33).

The crystal structure of PI3-K $\gamma$  RBD complexed with Ras revealed that PI3-K $\gamma$  RBD also exhibits a distinct mode of interaction with Ras/Rap1 from RalGDS and Raf-1 (32, 33). Here again, a notable difference exists at the binding surface of the  $\beta$ 2-strand, where Q231 functionally replaced K796 of RalGDS by forming a hydrogen bond with D38 of Ras. PI3-K $\gamma$  RBD has two Lys residues K251 and K255 in its  $\alpha$ 1-helix, and both of these residues make ionic interactions with the side chain of D38 of Ras in contrast to the hydrogen bonding interaction between K816 of

RalGDS and D38 of Ras. Furthermore, PI3-K $\gamma$  RBD does not have basic residue corresponding to R784 of RalGDS and R59 of Raf-1 RBD, and therefore the interaction with E37 of Ras does not contribute much to its binding to Ras. In addition, the switch II region of Ras is heavily involved in interaction with PI3-K $\gamma$  RBD in contrast to Raf-1 RBD and RalGDS RAD.

Based on the crystal structures of Raf-1 RBD complexed with Rap1(E30D/K31E) mutant and RalGDS RAD complexed with Ras(E31K) mutant, the electrostatic charge of the 31<sup>st</sup> residue of Ras/Rap1 (E31 of Ras and K31 of Rap1) has been proposed to be critical for the differential affinity of RalGDS and Raf-1 for Ras and Rap1 (27, 28, 30, 31). RalGDS RAD has several negatively charged residues at the C-terminal half of the  $\alpha$ 1-helix and the following loop. Among these acidic residues, D820 makes an ionic interaction with K31 of Ras(E31K) but not with E31 of wild-type Ras (31). In contrast, the corresponding residues of Raf-1 RBD are replaced by the positively charged K84. This residue was demonstrated to make a tight salt bridge with E31 of Rap1(E30D/K31E) but not with K31 of wild-type Rap1 (28, 30). Taken together, attractive or repulsive interactions between these charged residues existing in the interfaces are crucial for the binding specificity to the small GTPases.

The primary and predicted secondary structures of yeast adenylyl cyclase RAD are aligned with those of the other Ras effectors (Fig. 6A). Adenylyl cyclase RAD has R681 corresponding to R784 of RalGDS in the  $\beta$ 1-strand but lacks two Lys residues corresponding to K796 and K816 of RalGDS in its  $\beta$ 2-strand and  $\alpha$ 1-helix, respectively. Moreover, the predicted  $\beta$ 2-strand of adenylyl cyclase RAD possessed a couple of Thr, such as T687, T689 and T690, instead of K796 of RalGDS, which is very similar to the case with Raf-1 RBD. Because D38 of Ras was shown to be crucial for interaction with adenylyl cyclase RAD (Fig. 3B) like the case with RalGDS and Raf-1, some of these Thr residues are expected to be involved in interaction with D38 of Ras/Rap1 like T68 of Raf-1 RBD. Further, three residues KRK at positions 708-710 at the C-terminal end of the  $\alpha$ 1-helix of adenylyl cyclase RAD are similar to KVR at the corresponding positions 87-89 of Raf-1 RBD. Some of these three basic residues are also expected to interact with D38 of Ras/Rap1. However, it is difficult to predict how they interact with D38 without any tertiary structure information. Likewise, it is difficult to predict whether they are involved in interaction with E31 of Ras like K84 of Raf-1. Taken together, these observations suggest that adenylyl cyclase RAD bears a highest similarity to the Ras-binding

domain of Raf-1 among various Ras/Rap1 effectors. Further elucidation of the mechanism of the Ras-adenylyl cyclase interaction will await the tertiary structure determination of their complex by X-ray crystallography.

Recently we and others discovered two novel human RAD-containing proteins, PLC $\epsilon$  and RA-GEF-1 (21-26). PLC $\epsilon$  RAD associates with both Ras and Rap1, while RA-GEF-1 RAD associates only with Rap1. The predicted secondary structures of these RADs are quite different from those of RalGDS, Raf-1 and PI3-K $\gamma$  (Fig. 6B). PLC $\epsilon$  RAD does not have any  $\beta$ -strand. RA-GEF-1 RAD exhibits a different arrangement of  $\alpha$ -helix and  $\beta$ -strand. Thus, these RADs may comprise distinct groups of RADs.

So far many RAD-containing proteins have been identified by the computer search, and the number amounts to 148. However, their primary and secondary structures are very poorly conserved, and most of them have not yet been shown to interact with any small GTPases. Presently prediction of a right binding partner for a particular RAD is quite difficult based on its primary structure. This situation is exemplified by our recent observation on the RADs of RA-GEF-2 and GFR/MR-GEF, both of which bind specifically to M-Ras (R-Ras3) (42-44). The primary structure of RA-GEF-2 RAD exhibits only 19.7% identity with GFR/MR-GEF RAD, whereas it exhibits 81.6% identity with RA-GEF-1 RAD, which specifically binds to Rap1. Thus, it is important to determine tertiary structures of as many RADs, exhibiting diverse binding specificities, as possible to extract a basic principle of molecular recognition of small GTPases by a diverse array of RADs/RBDs.

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## FOOTNOTES

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<sup>1</sup>The abbreviations used are: LRR, leucine-rich repeat; CAP, adenylyl cyclase-associated protein; RAD, Ras-associating domain; RalGDS, Ral guanine nucleotide dissociation stimulator; PLC, phospholipase C; RBD, Ras-binding domain; PI3-K, phosphoinositide 3-kinase; GST, glutathione *S*-transferase; GTP $\gamma$ S, guanosine 5'-*O*-(3-thiotriphosphate); GDP $\beta$ S, guanosine 5'-*O*-(2-thiodiphosphate).

## LEGENDS TO FIGURES

Fig. 1. **Schematic representation of the structures of *S. cerevisiae* adenylyl cyclase and constructs used in this study.** The location of 2- (∇658, ∇700) and 4- (∇736) amino acid insertions are indicated by ∇. *RAD*, Ras-associating domain.

Fig. 2. **Heat shock sensitivity of yeast cells expressing various adenylyl cyclase fragments.** Three independent isolates of TK161-R2V yeast cells harboring pAD4-GST (*Vector*) or pAD4-GST-CYR1 carrying the indicated adenylyl cyclase fragments were examined for heat shock sensitivity by the replica plating method. Shown are photographs of the two replica plates, one subjected to 55°C heat shock for 5 min (*left panel*) and the other without the heat shock treatment (*right panel*), after two days of growth at 30°C. The experiments were repeated three times, yielding equivalent results.

Fig. 3. **Measurement of *in vitro* interaction of Ha-Ras with the adenylyl cyclase *RAD*.** *A*, sixty pmol of purified GST-CYR1(640-809) or GST-CYR1(640-809) polypeptide were examined for *in vitro* association with 15 pmol of Ha-Ras loaded with GTP $\gamma$ S (*GTP*) or GDP $\beta$ S (*GDP*). The GST-fusion polypeptides and Ha-Ras in the eluate were fractionated by SDS-polyacrylamide gel electrophoresis (12.5% gel) and detected by staining with Coomassie brilliant blue (*upper panel*) and by immunoblotting with the anti-Ha-Ras antibody (*lower panel*), respectively. The arrows indicate the GST-fusion polypeptides. A representative result of three independent experiments is shown. *B*, wild-type (*WT*) or mutant Ha-Ras was examined for *in vitro* association with GST-CYR1(640-809). Bound Ha-Ras (*upper panel*) and one-tenth (*lower panel*) of the input Ha-Ras were detected by immunoblotting as described above. A representative result of three independent experiments is shown. *C*, adenylyl cyclase activities dependent on various concentrations of GTP $\gamma$ S-bound Ha-Ras were measured in the presence of various concentrations of GST-CYR1(640-809); 0 nM (●), 10 nM (○), 50 nM (△), 100 nM (▲), and 150 nM (□) as described under “*Experimental Procedures*”. One unit of activity is defined as 1 pmol cAMP formed in 1 min of incubation with 1 mg of membrane proteins. The experiments were performed twice, yielding equivalent

results. *D*, determination of the  $K_d$  value for Ha-Ras. A reciprocal of the free Ha-Ras concentration was plotted against a reciprocal of the concentration of the bound Ha-Ras as described in the text. The  $K_d$  value for Ha-Ras was calculated from the point of intersection with the horizontal axis as described before (35).

**Fig. 4. Effect of mutations of the RAD in Ras-dependent adenylyl cyclase activation.** *A*, adenylyl cyclase;  $\nabla$ 658 (■),  $\nabla$ 700 (○),  $\nabla$ 736 (▲),  $\Delta$ 659-700 (×), or wild type (●), solubilized from the crude membrane fractions of yeast cells, was examined for its activity in the presence of various concentrations of GTP $\gamma$ S-bound Ha-Ras. For the precise comparison of the activity levels among different cyclase specimens, the Ras-dependent activity was presented as a % ratio to the Mn<sup>2+</sup>-dependent activity of the same specimen. The Mn<sup>2+</sup>-dependent activity of each sample ranged from 200 to 300 pmol of cAMP formed during 30 min of incubation at 30°C. The experiments were performed twice, yielding equivalent results. *B*, adenylyl cyclase used in *A* was subjected to immunodetection with the anti-CYR1CT antibody. The molecular weight marker was myosin heavy chain (200 k).

**Fig. 5. Effect of mutations of the RAD on *in vitro* association with Ha-Ras.** *A*, *in vitro* Ras-binding assay was performed using 15 pmol of Ha-Ras and 60 pmol of GST-CYR1(640-809) polypeptides carrying the indicated mutations. The GST-CYR1(640-809) polypeptides (*upper panel*) and the bound Ha-Ras (*lower panel*) in the eluate were detected as described in the legends to *Fig. 3A*. A representative result of three independent experiments is shown. *B*, adenylyl cyclase activity dependent on 2 pmol of GTP $\gamma$ S-bound Ha-Ras was measured in the presence of various concentrations of the following polypeptides; GST-CYR1(640-809) $\nabla$ 658 (■), GST-CYR1(640-809) $\nabla$ 700 (○), GST-CYR1(640-809) $\nabla$ 736 (▲), GST-CYR1(640-809) $\Delta$ 659-700 (×), GST-CYR1(640-809) (●), and GST only (◇). The experiments were performed twice, yielding equivalent results.

**Fig. 6. Multiple alignment of the primary and secondary structures of RAD/RBDs of various Ras/Rap1 effectors.** *A*, amino acid sequence alignment of RalGDS RAD, afadin/AF-6 RADs, Raf-1 RBD, PI3-K $\gamma$  RBD and adenylyl cyclase RAD. Secondary structures of two RADs of AF-6 and adenylyl cyclase RAD were predicted by PHD (47) derived from the EMBL database. The known or predicted secondary structures were shown above their sequences. The alignment of the

adenylyl cyclase RAD sequence with those of the other effectors was carried out based on the alignment of hydrophobic residues as described (20). *Bold face letters* represent the three crucial basic residues of RaIGDS and their counterparts in the other effectors. *B*, the secondary structures of the RADs of RA-GEF-1 and PLC $\epsilon$  were predicted by PHD and indicated above their sequences.

$\alpha$  and  $\beta$  represent  $\alpha$ -helix and  $\beta$ -strand, respectively. *i* and *h* beneath the amino acids indicate residues implicated in ionic interaction and hydrogen bond formation with Ras/Rap1, respectively. *AF-6-N*, the N-terminal RAD of afadin/AF-6; *AF-6-C*, the C-terminal RAD of afadin/AF-6; *r*, rat; *hu*, human; *sc*, *S. cerevisiae*.

Fig. 1

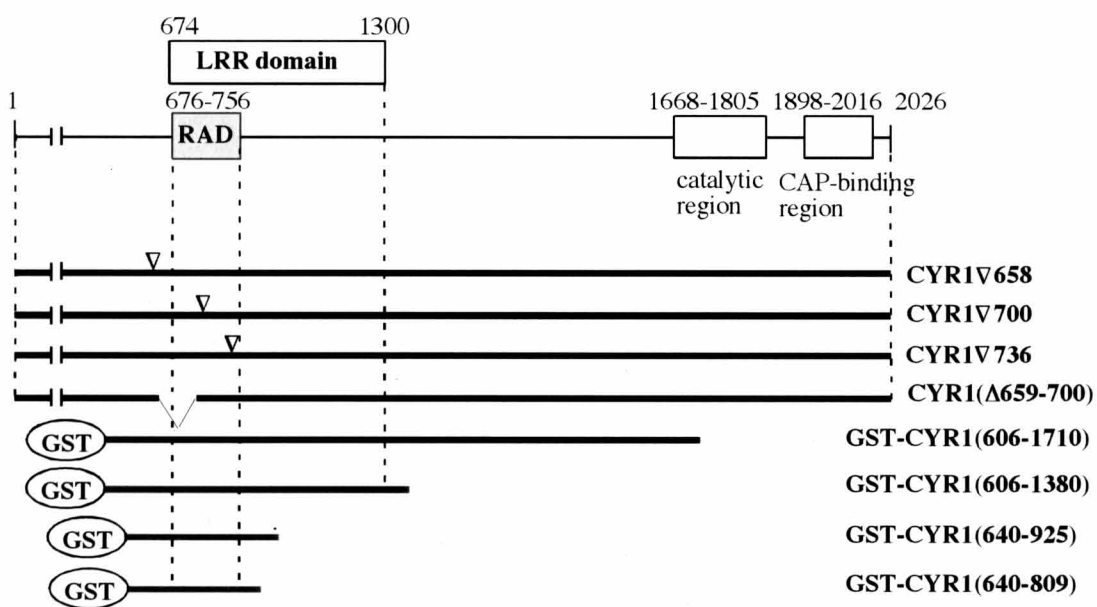
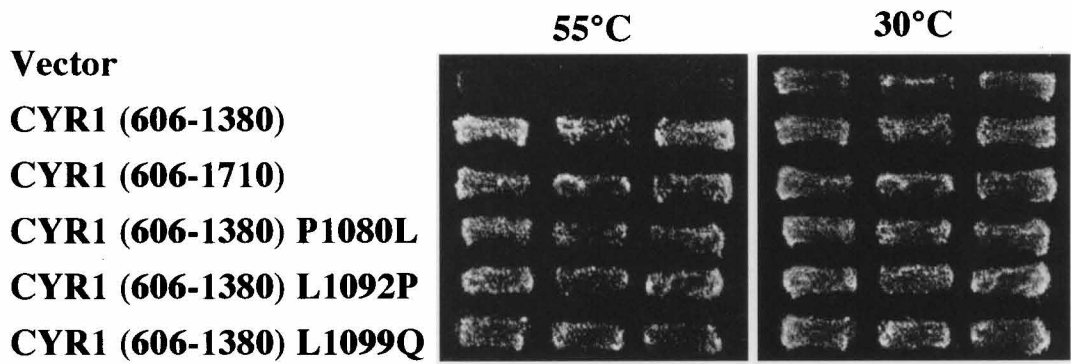


Fig. 2

**A**



**B**

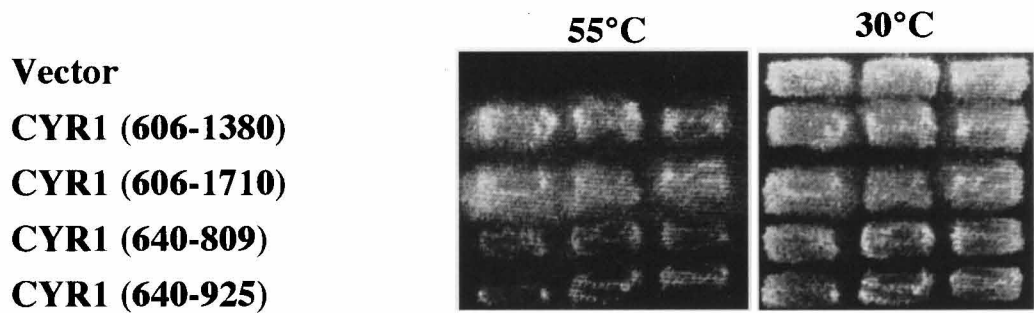
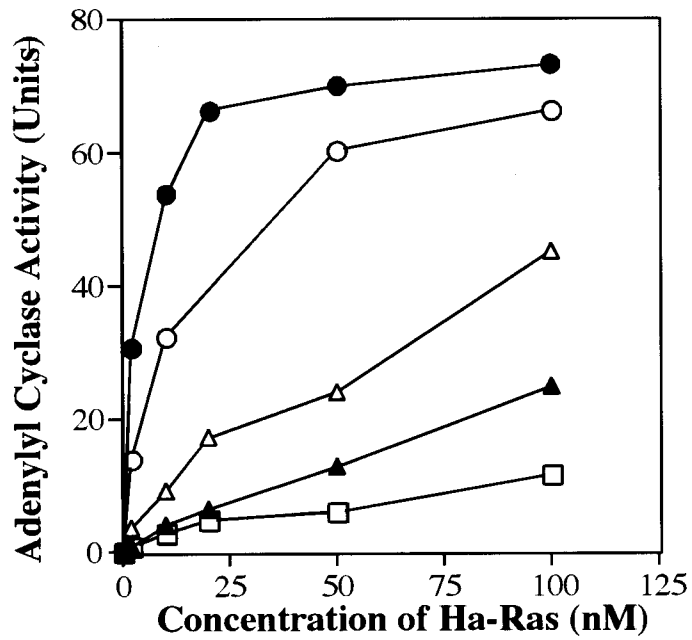




Fig. 3

**C**



**D**

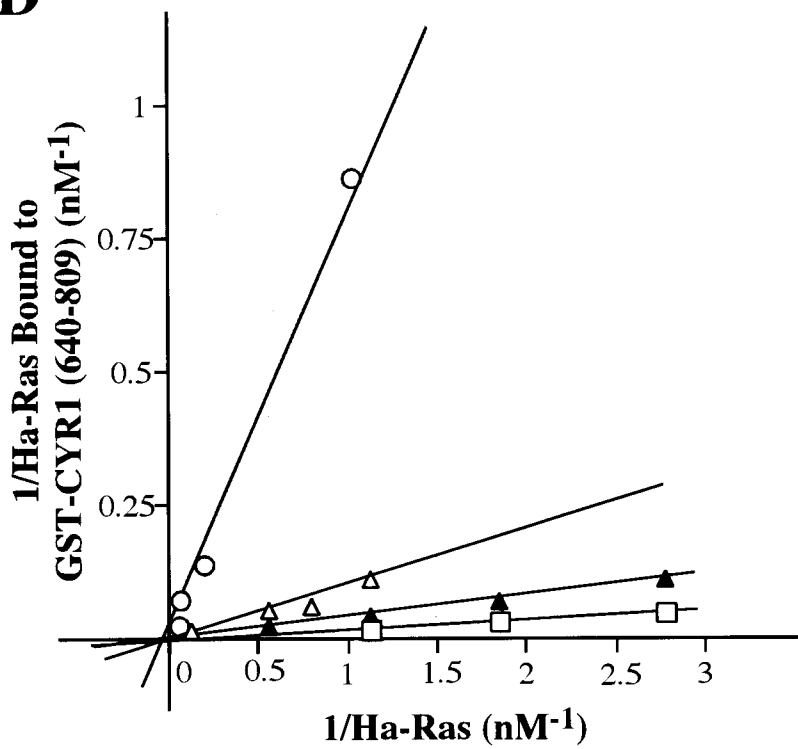
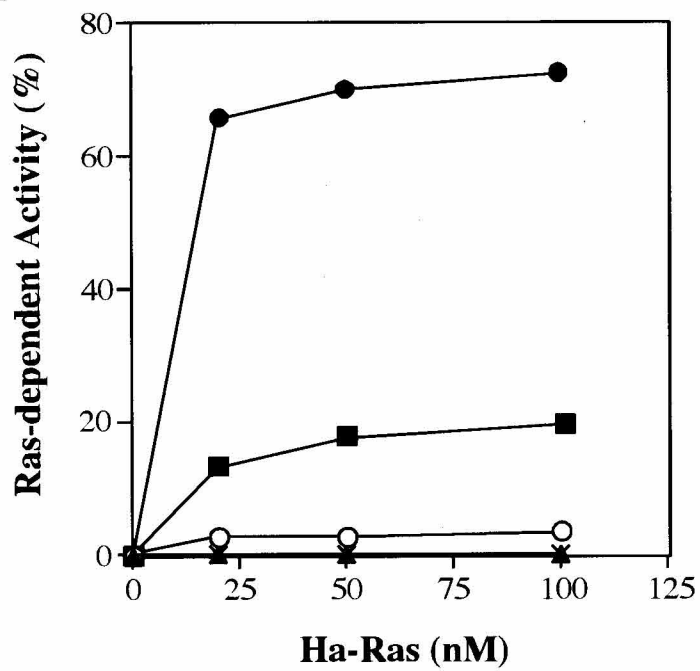




Fig. 4

**A**



**B**

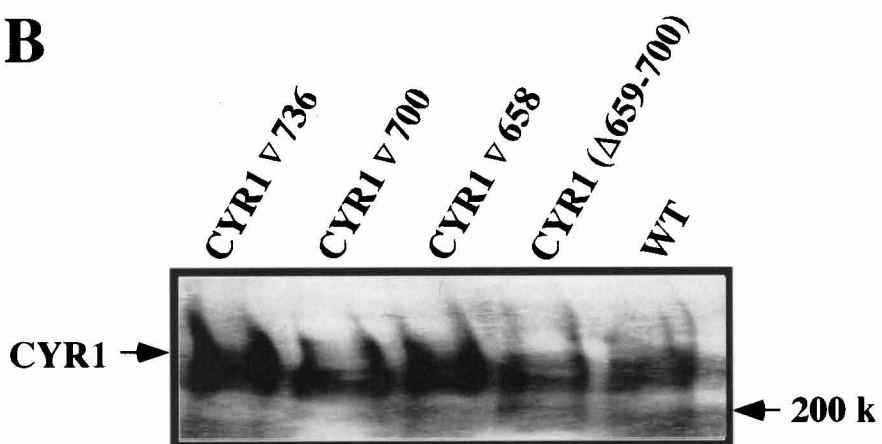
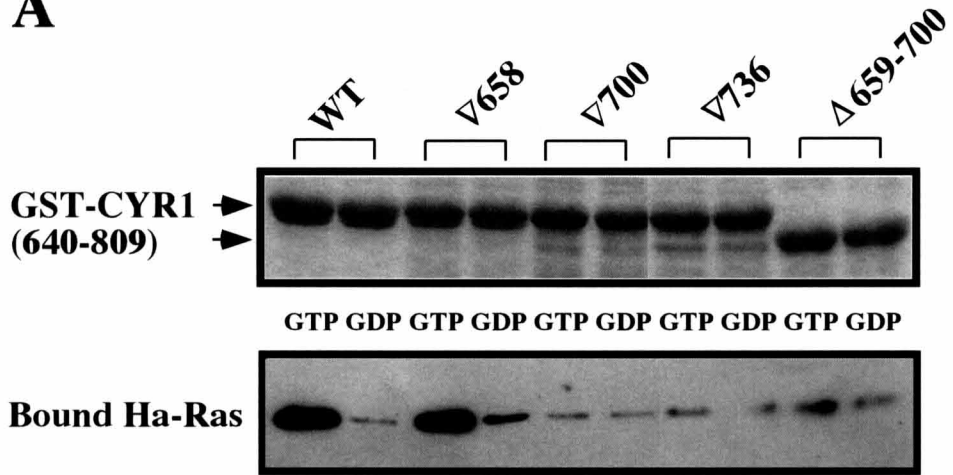


Fig. 5

**A**



**B**

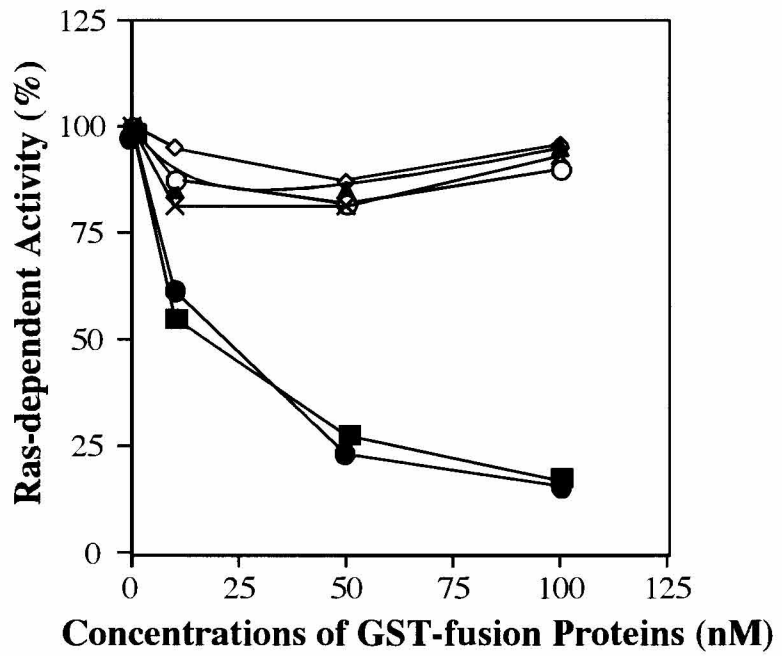
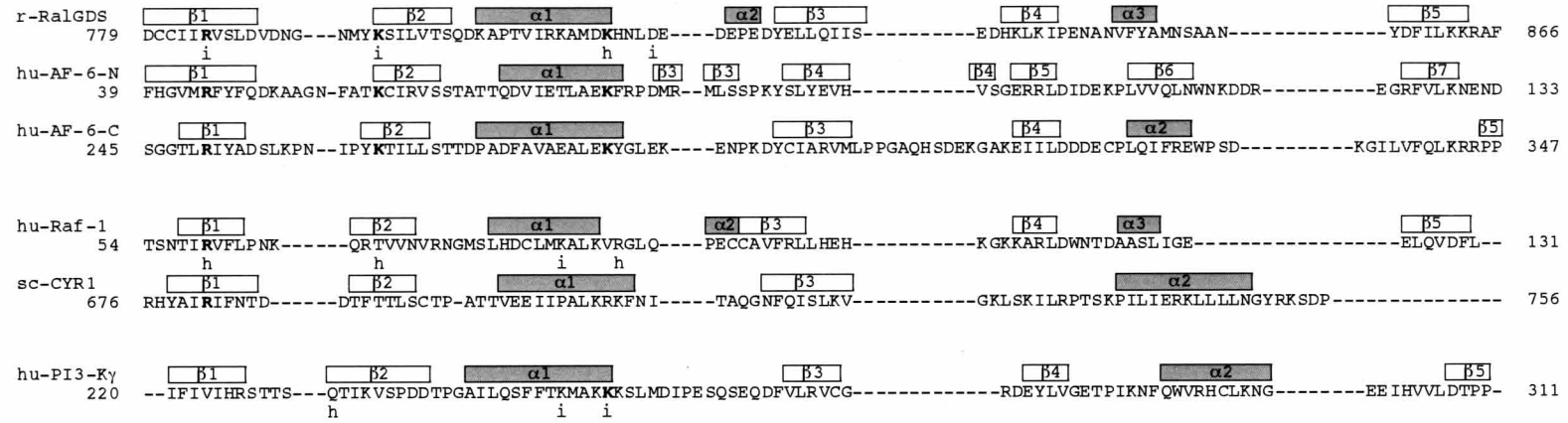


Fig. 6

**A**



**B**

