Double-Mutant Analysis of the Interaction of Ras with the Ras-Binding Domain of RGL†

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Received August 19, 1998; Revised Manuscript Received February 23, 1999

ABSTRACT: RalGDS is a guanine nucleotide dissociation stimulator for Ral, and one of its homologues is RGL (*R*al*G*DS-*l*ike). In this study, the effects of mutations of Ras and the Ras-binding domains (RBDs) of RalGDS and RGL on their binding have been systematically examined. The D33A mutation of Ras reduces the abilities to bind RGL-RBD and RalGDS-RBD. To identify the RGL residue interacting with Asp33 of Ras, double-mutant analyses between Ras and RGL-RBD were conducted. For example, the K685A mutation of RGL-RBD has a much smaller effect on the RGL-RBD binding ability of the D33A mutant than on those of other mutants of Ras. Accordingly, it is indicated that the attractive interaction of Asp33 in Ras with Lys685 in RGL-RBD (Lys816 in RalGDS-RBD) contributes to the Ras·RBD association. This interaction is consistent with the crystal structure of the complex of RalGDS-RBD and the E31K Ras mutant [Huang, L., Hofer, F., Martin, G. S., and Kim, S.-H. (1998) *Nat. Struct. Biol. 5*, 422−426]. This crystal structure exhibits interactions of the mutation-derived Lys31 side chain with three RalGDS residues. Glu31 of Ras discriminates Ras from a Ras-homologue, Rap1, with Lys31, with respect to RalGDS and RGL binding; the E31K mutation of Ras potentiates the abilities to bind RGL-RBD and RalGDS-RBD. To examine the role of Glu31 of the wild-type Ras in the interaction with RGL and RalGDS, double-mutant analyses were conducted. The Ras binding ability of the E689A mutant of RGL-RBD is much stronger than that of the wild-type RGL-RBD, and the E31K mutation of Ras no longer potentiates the Ras binding ability of the E689A mutant. Therefore, the repulsive interaction between Glu31 in Ras and Glu689 in RGL-RBD (Asp820 in RalGDS-RBD) may keep the Ras·RBD association weaker than the Rap1·RBD association, which might be relevant to the regulation of the signaling network.

The Ras proteins (H-Ras, K-Ras, and N-Ras) are small guanine nucleotide-binding proteins that play a critical role in signal transduction (*1*). Nucleotide exchange between GDP[1] and GTP induces major conformational changes in two regions, switch I (residues 30−39) and switch II (residues 57−76) (*2*−*6*). The Ras protein binds and activates the Raf proteins (Raf-1, A-Raf, and B-Raf), which results in the activation of the mitogen-activated protein kinase pathway (*7*−*9*). A region comprising residues 51−131 of Raf-1 can bind Ras in a GTP-dependent manner and has been designated the Ras-binding domain (Raf-RBD) (*10, 11*). The "effector region" of Ras, consisting of residues 32−40, is essential to the interactions with Raf-1 (*7, 8*). The small GTP-binding proteins, Rap1A and Rap1B, exhibit ∼50% amino acid sequence identity with Ras and have the same effector-region sequence as Ras. Actually, the Rap1 proteins bind Raf-1 in a GTP-dependent manner, but much less strongly than Ras does. The structures of Raf-RBD and the complexes of Raf-RBD with Rap1A (the wild type and the Ras-like E30D/K31E mutant) have been determined by NMR spectroscopy and X-ray crystallography, respectively (*12*−*14*). The formation of an antiparallel intermolecular β-sheet characterizes the interaction between the ubiquitin-like fold of Raf-RBD and the GTP-binding fold of Rap1A. Intriguingly, the Glu31 residue, derived from the K31E mutation, and Asp33 are involved in salt bridges with Lys84 of Raf-RBD (*14*).

In addition to the Raf proteins, the GTP-bound Ras can bind RalGDS, RGL (*R*al*G*DS *l*ike), Rlf, and RGL2, which belong to a family of guanine nucleotide dissociation stimulators for Ral (*15*−*19*). The Ras binding abilities of these RalGDS family proteins depend on their C-terminal regions or the Ras-binding domains (RBDs)[2] (Figure 1). The

† This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, a grant from the "Research for the Future" Program of the Japan Society for the Promotion of Science, and a Biodesign Grant from RIKEN.

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[1] Abbreviations: GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; RBD, Ras-binding domain; NMR, nuclear magnetic resonance; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; GTPγS, guanosine 5′-O-(γ-thiophosphate); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GMPPNP, guanosine 5′-*O*-(β,γ-imidotriphosphate).

[2] These domains were designated elsewhere as the Ras-interacting domains (*16*, *20*, *29*).

10.1021/bi9820053 CCC: $18.00
Published on Web 04/02/1999

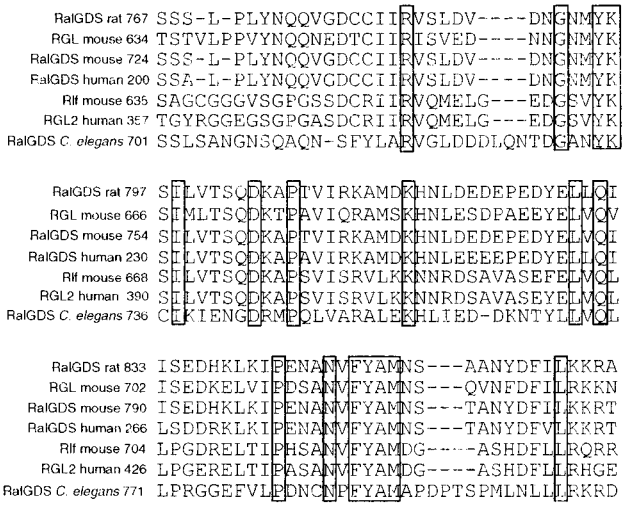

FIGURE 1: Sequence alignment of RalGDS (rat, 767−865; mouse, 724−822; human, 200−298; *C. elegans*, 701−806), RGL (mouse, 634−734), Rlf (mouse, 635−735), and RGL2 (human, 357−457). Conserved amino acids are boxed.

structure of RalGDS-RBD has been determined by X-ray crystallography and NMR spectroscopy (*20*, *21*). Both Raf-RBD and RalGDS-RBD have a ubiquitin-like fold, although the sequence identity between the two Ras-binding domains is as low as 13% (*22*). The effector region of Ras is responsible for RalGDS binding as well as for Raf binding. Some of the effector-region mutations of Ras differentially affect the binding and/or the signaling through the Raf and RalGDS/RGL pathways (*23*−*27*). RalGDS-RBD associates much more strongly with Rap1 than with Ras, and intriguingly, the Rap1-like E31K mutation of Ras impairs Raf-RBD binding and potentiates RalGDS-RBD binding (*28*). Therefore, Glu31 and Lys31of Ras and Rap1, respectively, are the determinants that switch the Ras/Rap1 preferences of Raf and RalGDS (*28*). Recently, the crystal structure of the complex between RalGDS-RBD and the E31K mutant Ras in the GMPPNP-bound form was reported (*29*). The antiparallel intermolecular β-sheet that RalGDS-RBD forms with Ras(E31K) is similar to that of Raf-RBD with Rap1A. On the other hand, the interactions at the amino acid residue level are significantly different between the two complexes (Figure 2). For example, Asp33 in Ras(E31K) forms a salt bridge with Lys816, which is in a different location from that of Lys84 in Raf-RBD on the common ubiquitin-like fold. Furthermore, in the crystal structure of the Ras(E31K)·RalGDS-RBD complex, the side chain of Lys31 in Ras(E31K) interacts with Asp815, Asn818, and Asp820 in RalGDS-RBD (*29*). However, it is still possible that the mutation of Glu31 could affect other interactions between Ras and RalGDS-RBD. Moreover, it remains unclear what role the critical specificity determinant, Glu31, of Ras plays in its interaction with RalGDS.

The RGL protein is most homologous to RalGDS in the RalGDS family; the amino acid sequences of RGL-RBD and RalGDS-RBD are 63% identical (Figure 1). Nevertheless, some of the residues around the Ras-binding interface of RalGDS, including three of the nine residues interacting with the switch I region of Ras, are not conserved in RGL. In the present study, we systematically examined the effects of mutations of Ras, RGL-RBD, and RalGDS-RBD on their binding. By the double-mutant analysis, we found that an attractive interaction between Asp33 in the wild-type Ras and Lys685 in RGL-RBD is actually formed and that the repulsive interaction between Glu31 in the wild-type Ras and Glu689 in RGL-RBD weakens the Ras·RGL-RBD association.

EXPERIMENTAL PROCEDURES

Plasmid Construction. To express RGL-RBD as a GST-fusion protein, the DNA fragment corresponding to amino acid residues 632−734 was amplified by PCR from the mouse gene for RGL (*16*) and was subcloned into pGEX-4T-3 (Amersham Pharmacia Biotech). The GST-fusion forms of human Raf-1, residues 51−131 (GST−Raf-RBD), and rat RalGDS, residues 769−895 (GST−RalGDS-RBD), were prepared as described previously (*28*) for the RalGDS-RBD binding abilities of Ras mutants. For the Ras binding abilities of RalGDS-RBD mutants, a slightly shorter fragment of rat RalGDS, consisting of residues 767−864, was used. Mutations were introduced into a synthetic human c-Ha-*ras* gene (*30*) or into the gene encoding for RGL-RBD or RalGDS-RBD by site-directed mutagenesis, using either a Muta-Gene kit (Bio-Rad) or two subsequent PCR amplifications. All constructs were confirmed by sequencing.

Protein Purification. The wild-type and mutant Ras proteins were expressed in *Escherichia coli* and were purified as described (*31*), except sonication was performed to lyse the *E. coli* cells. The GST−Raf-RBD fusion protein and the wild-type and mutant GST−RGL-RBD and GST−RalGDS-RBD proteins were each expressed in *E. coli* at 30 °C and were purified using glutathione−Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Binding Assay. One microgram of the GST-fusion proteins, in 150 μL of PBS containing 0.05% Triton X-100, was mixed with 10 μL of glutathione−Sepharose 4B beads suspended in PBS. The mixture was incubated at 4 °C for 1 h with various amounts of either the wild-type or mutant Ras protein, which had been complexed with GTPγS as described previously (*28*). After this incubation, the resin was washed with 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ and 150 mM NaCl. The bound proteins were eluted from the resin by boiling in Laemmli's buffer and were fractionated by SDS−PAGE. Immunoblots were probed with the anti-Ras antibody RAS004 (*32*) and were developed using the ECL immunodetection system (Amersham Pharmacia Biotech). The Western blots were scanned, and the relative band intensities were determined with a Bioimage densitometer (MilliGen).

RESULTS AND DISCUSSION

Effects of the Effector-Region Mutations of Ras on RGL-RBD Binding Ability. The Ras protein, in the GTPγS-bound form, was mixed with the GST−RGL-RBD fusion protein and the glutathione−Sepharose, and the amounts of the Ras protein in the precipitates were analyzed by Western blotting (Figure 3A). The most drastic effects were found in the T35S, I36A, D38N, and Y40F mutations of Ras; these mutations abolish the ability to bind RGL-RBD. The D33A and P34A mutations significantly decreased the RGL-RBD binding ability. In addition, the binding ability is slightly reduced by the Y32F and E37G mutations of Ras. For comparison,

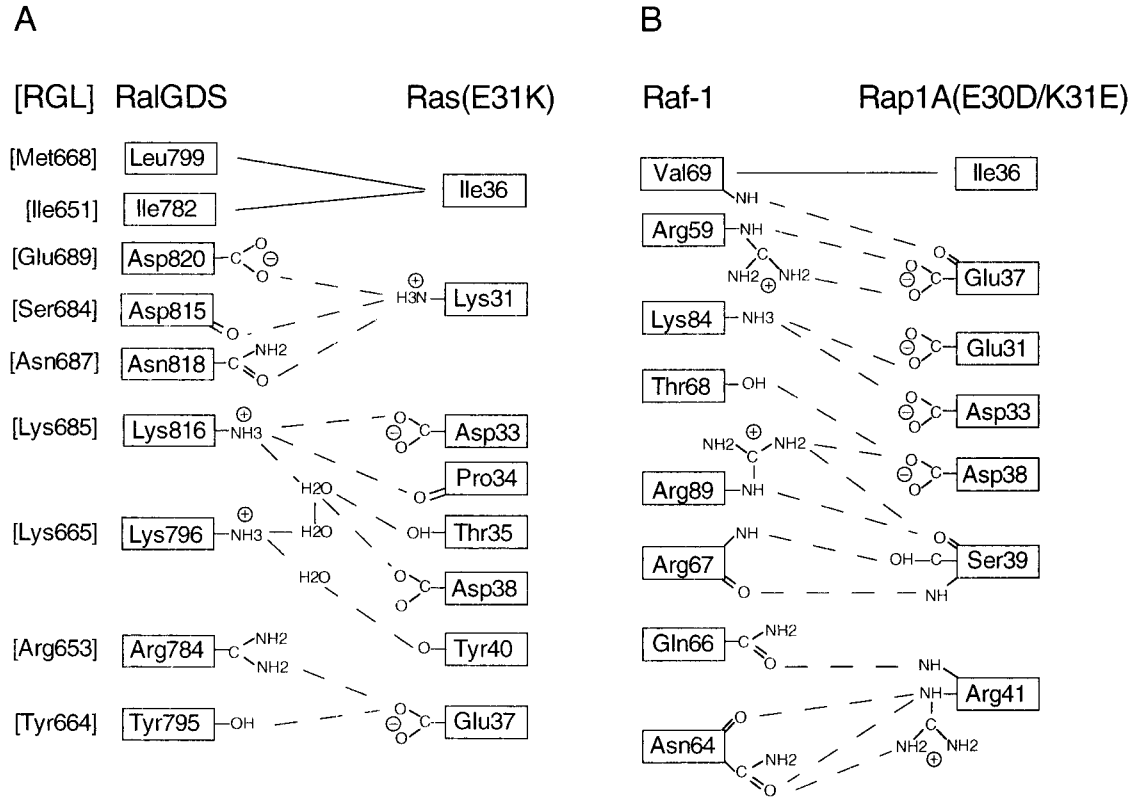

FIGURE 2: (A) Schematic representation of the interaction between the switch I region of Ras(E31K) and RalGDS-RBD. The hydrogen bonds and salt bridges are shown by dashed lines. The hydrophobic interactions are indicated by solid lines. The backbone hydrogen bonds between them are omitted for clarity. RalGDS-RBD constitutes rat RalGDS residues 767−864, which are designated in the paper (*29*) residues 3−100. The RGL-RBD residues corresponding to the RalGDS-RBD residues are shown in square brackets. (B) Schematic representation of the interaction between the switch I region of Rap1A(E30D/K31E) and Raf-RBD (*14*). The hydrogen bonds, salt bridges, and hydrophobic interactions are shown as in (A). Although the side chain of Glu37 in Ras forms hydrogen bonds with Arg784 and Tyr795 in RalGDS, Huang et al. explained that in the Ras·RaGDS-RBD complex, Glu37 of Ras points away from the interface (*29*).

the abilities of these mutant Ras proteins to bind RalGDS-RBD and Raf-RBD were analyzed in the same assay system (parts B and C of Figure 3, respectively). The Raf-RBD binding abilities of these mutants, except for P34A, and the RalGDS·RBD binding abilities of six mutants, other than D33A, P34A, and D38N, had previously been examined by two-hybrid assay and/or by GST pull-down assay (*23*−*26*, *33*). The RGL-RBD binding ability had been examined only for the T35S and E37G mutants by immunoprecipitation assay (*27*). These previous results by precipitation assays are consistent with the corresponding results in the present study. In general, the effects of the Ras mutations on the RGL-RBD binding are similar to those on the RalGDS·RBD binding, but are remarkably different from those on the Raf-RBD binding (Figure 3). The P34A, T35S, I36A, and Y40F mutations of Ras decrease RGL-RBD and RalGDS-RBD binding much more seriously than Raf-RBD binding (Figure 3). Actually, in the crystal structure of the Ras(E31K)·RalGDS-RBD complex (*29*), Pro34, Thr35, Ile36, and Tyr40 of Ras are involved in the interaction with the RBD (Figure 2A). In contrast, Pro34, Thr35, and Tyr40 exhibit no interaction with Raf-RBD in the crystal structure of the Rap1A(E30D/K31E)·Raf-RBD complex (*14*) (Figure 2B). Ile36 of Ras interacts intensively with Leu799 and Ile782 of RalGDS-RBD but interacts only with Val69 of Raf-RBD (Figure 2). Therefore, the effects of these mutations are well explained on the basis of the crystal structures. It should still be noted that the interpretation of the present data is based on the assumption that mutations may affect specific interactions between residues in the binding interface, with a negligible change in the protein structure per se.

Effects of RGL-RBD Mutations on Ras Binding Ability. Next, we prepared various mutants of RGL-RBD and tested them for the ability to bind the wild-type Ras [Ras(wt)] in the GTPγS-bound form (Figure 4A,B). We examined the Ras concentration dependencies for all of the tested RGL-RBD mutants (those for the wild-type and two mutants are shown as examples in Figure 4A) and found that the Ras concentration of 1 ng/μL is suitable for the comparison among them. Thus, the data at the fixed Ras concentration of 1 ng/μL are displayed in Figure 4B. The R681A mutation of RGL-RBD does not affect Ras(wt) binding. The S684A mutation does not reduce, but slightly potentiates, the Ras(wt) binding ability of RGL-RBD. In contrast, the Y664A and K665A mutants of RGL-RBD are completely inactive in Ras(wt) binding. In addition, the K685A and H686A mutations largely impair Ras binding (Figure 4B). On the other hand, it has been reported that the Y795A and H817A mutations (Y664A and H686A, respectively, in RGL) do not affect the interaction of RalGDS-RBD with an activated Ras(Q61L) in the yeast two-hybrid system (*20*). Thus, we prepared mutants of RalGDS-RBD and found that, in our assay system, neither the Y795A (=Y664A in RGL) nor the H817A (=H686A in RGL) mutant of RalGDS-RBD binds Ras(wt) (Figure 4C). Therefore, in our assay system, the effects of the Y795A and H817A mutations of RalGDS on Ras binding are much the same as those of the corresponding mutations of RGL. It is possible that the present pull-down

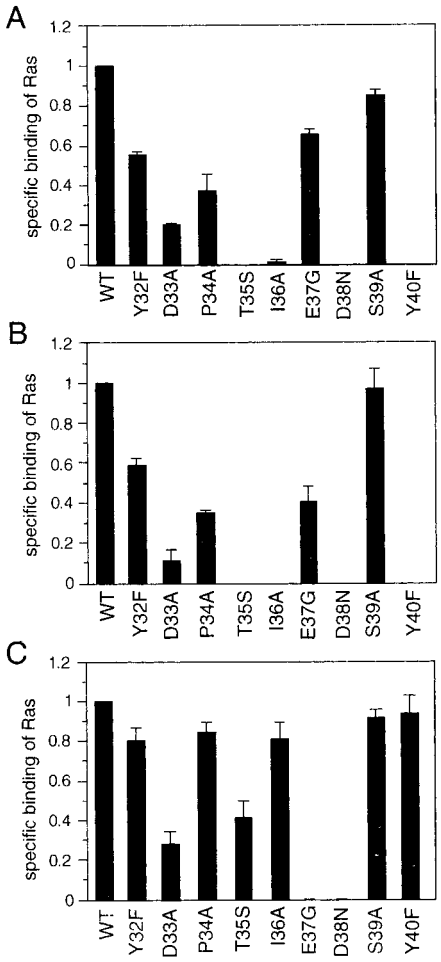

FIGURE 3: Association of the effector-region mutant Ras proteins with RGL-RBD (A), RalGDS-RBD (B), and Raf-RBD (C). The wild-type (WT) and the mutant Ras proteins (0.05, 0.2, and 0.02 μg) were loaded with GTPγS and were tested for GST−RGL-RBD (A), GST−RalGDS-RBD (B), and GST−Raf-RBD binding (C), as described under Experimental Procedures. The intensity of the Ras band was estimated by densitometry. Intensities relative to that pulled down after incubation of the wild-type Ras with RGL-RBD (A), RalGDS-RBD (B), and Raf-RBD (C) are shown. The data represent the average of at least three experiments.

assay system is more sensitive to an increase in the dissociation rate of the complex than the yeast two-hybrid assay system. In the crystal structure of the Ras(E31K)·RalGDS-RBD complex, Tyr795 and Lys796 in RalGDS (Tyr664 and Lys665, respectively, in RGL) interact with Glu37 and Tyr40 in Ras(E31K), respectively. Lys812 in RalGDS (=Arg681 in RGL) is not involved in the interaction with Ras(E31K) (*29*). Therefore, our results for the Y664A, K665A, and R681A mutants of RGL-RBD are consistent with the crystal structure of the Ras(E31K)·RalGDS-RBD complex.

Role of His686 in RGL-RBD. In contrast, although His817 in RalGDS is not involved in the interaction with Ras(E31K) in the crystal structure of the complex (*29*), the mutations of H817A in RalGDS and H686A in RGL both impair the Ras(wt) binding ability. It should be noted here that His817 (=His686 in RGL) is in a hydrophobic interaction with Lys796 (=Lys665 in RGL) in the tertiary structure of RalGDS-RBD (*20*, *21*, *29*). Therefore, we compared the amounts of the various mutant RGL-RBD and RalGDS-RBD proteins in the soluble fraction of the *E. coli* overproducing cell extracts to examine if these His mutations cause any protein aggregation due to misfolding. Actually, the levels of soluble expression of the K665A and H686A mutants of RGL-RBD were significantly less than those of the wild-type and other mutant RGL-RBD proteins (Figure 5A). The corresponding mutation, H817A, of RalGDS causes a more significant decrease in the soluble expression level (Figure 5B) and a more drastic decrease in the Ras(wt) binding ability (Figure 4C). These results suggest that the hydrophobic interactions between His817 and Lys796 in RalGDS and between His686 and Lys665 in RGL are necessary for the proper folding of the proteins and are essential to form their Ras-binding interfaces. In addition, the Ras binding ability and the soluble expression level of RGL-RBD are not decreased by the H686F and H686Y mutations (Figures 4D and 5A), indicating that a hydrophobic ring side chain in this position is important for the formation of the Ras-binding interface.

Does Asp33 in Ras Interact with Lys685 in RGL-RBD? The K685A mutant of RGL-RBD has a quite reduced, but detectable, ability to bind Ras(wt) in the present study (Figure 4B). On the other hand, a more drastic decrease in the Ras(wt) binding ability was observed for the K816A mutation of RalGDS-RBD (Figure 4C). In the crystal structure of the Ras(E31K)·RalGDS-RBD complex, the side chains of Asp33 and Thr35 and the main chain of Pro34 in Ras interact with the side chain of Lys816 in RalGDS (Figure 2A) (*29*). In contrast, the crystal structure of the Rap1A(E30D/K31E)·Raf-RBD complex revealed that Glu31 and Asp33 form salt bridges with Lys84 of Raf-RBD (Figure 2B) (*14*). In RalGDS-RBD, Arg811 and Lys812, but not Lys816, occupy the spatial position corresponding to that of Lys84 in Raf-RBD (*20*). However, RGL has different amino acid residues, Gln680 and Arg681, corresponding to Arg811 and Lys812 of RalGDS (Figure 1). Does Asp33 in Ras interact with Lys685 in RGL-RBD as with Lys816 in RalGDS-RBD?

Double-Mutant Analysis for Asp33 in Ras and Lys685 in RGL-RBD. To explore this interaction, we could conduct a double-mutant analysis, as Ras(D33A) has an impaired but detectable RGL-RBD binding ability, and the K685A mutant of RGL-RBD also has an impaired but detectable Ras binding ability. Thus, for the four RGL-RBD mutants that exhibit Ras(wt) binding abilities weaker than that of the wild-type RGL-RBD, we examined the Ras(D33A) binding abilities (Figure 4E). Three mutations, Y664A, K665A, and H686A, of RGL-RBD reduce the Ras(D33A) binding ability to a negligible level (Figure 4E), probably because of the addition of the two independent effects of the D33A mutation of Ras and the RGL-RBD mutation. In contrast, the K685A mutation of RGL-RBD causes only a 2-fold reduction in the Ras(D33A) binding (Figure 4E), which is even smaller than the 3-fold reduction in the Ras(wt) binding (Figure 4B). This observation indicates that Lys685 in RGL-RBD interacts with Asp33 in Ras and that the K685A mutation of RGL-RBD fails to impair the Ras(D33A) binding ability, because a partner of Lys685 (Asp33 in Ras) has already been removed. Furthermore, we performed the other type of double-mutant survey: how the Y32F, D33A, and E37G mutations of Ras, which reduce the RGL-RBD binding ability, affect the ability to bind the K685A mutant of RGL-RBD (Figure 4F). The ability of Ras(D33A) to bind the wild-type RGL-RBD is lower than those of Ras(Y32F) and Ras(E37G) (Figure 4F).

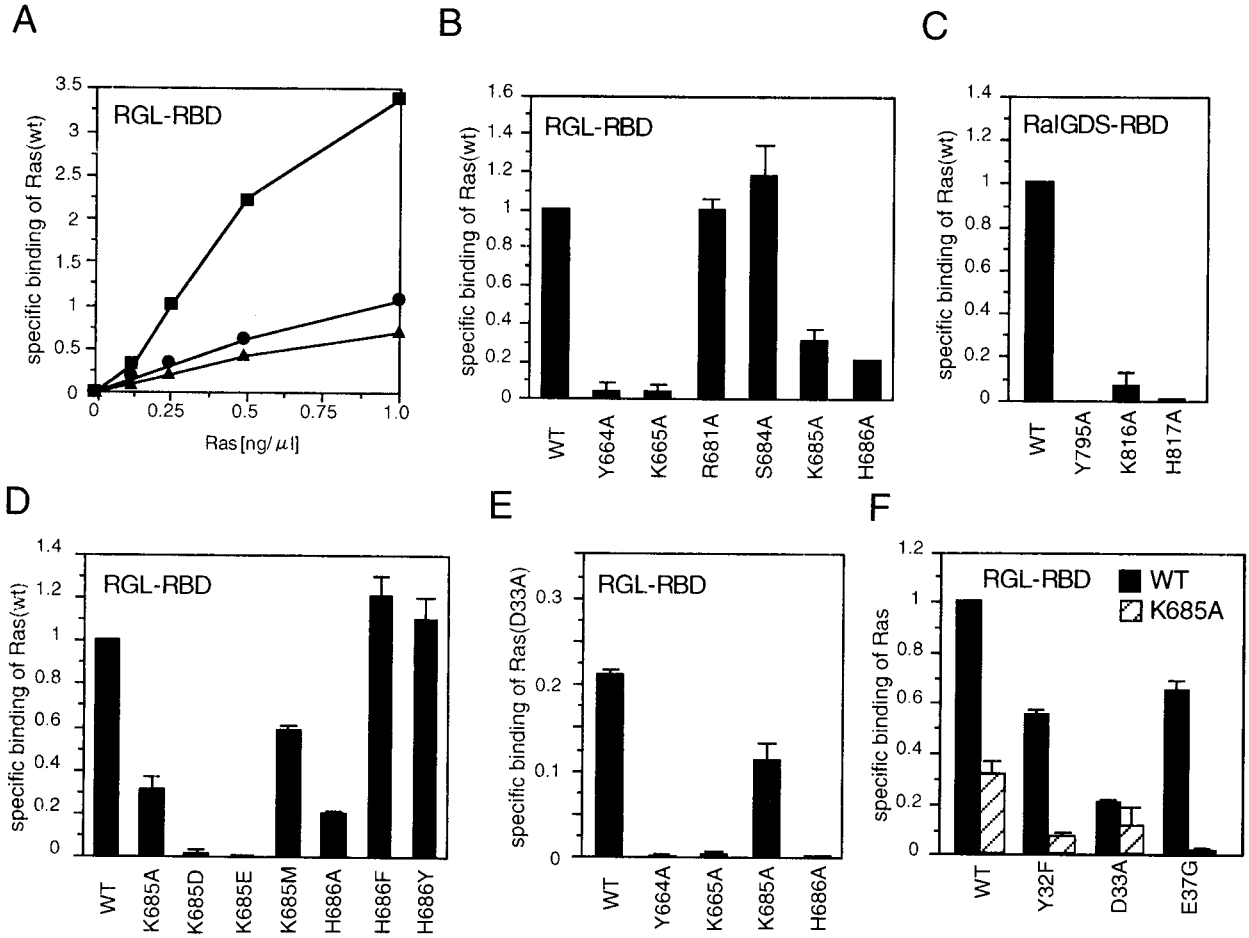

FIGURE 4: Association of various mutant Ras with various RGL-RBD and RalGDS-RBD mutants. (A) Various amounts of the wild-type Ras protein were tested for their abilities to bind the wild-type (■), the K685A (●), and the H686A mutants (▲) of RGL-RBD. The intensities relative to that pulled down after an incubation of the wild-type RGL-RBD and 0.25 ng/μL of the wild-type Ras are plotted. (B, D, and E) The wild-type (B and D) and the D33A mutant Ras proteins (E) (1 ng/μL) were loaded with GTPγS and were tested for the ability to bind the wild-type (WT) and the mutant RGL-RBD with the indicated mutation. Intensities are relative to that pulled down after an incubation of the wild-type Ras with the wild-type RGL-RBD. (C) Association of the wild-type Ras with RalGDS-RBD mutants. The wild-type Ras proteins (1 ng/μL) loaded with GTPγS were tested for the ability to bind the wild-type (WT) and the mutant RalGDS-RBD with the indicated mutation. Intensities are relative to that pulled down after an incubation of the wild-type Ras with the wild-type RalGDS-RBD. (F) Effects of the K685A mutation in RGL-RBD on the ability to bind the mutants of Ras. The wild-type and indicated mutant Ras proteins (0.25 ng/μL) loaded with GTPγS were tested for the ability to bind the wild-type (solid blocks) and the K685A mutant (hatched blocks) of RGL-RBD. Intensities are relative to that pulled down after an incubation of the wild-type Ras with the wild-type RGL-RBD. All of the data represent the average of at least three experiments.

In contrast, Ras(D33A) binds the K685A mutant of RGL-RBD more tightly than Ras(Y32F) and Ras(E37G) (Figure 4F). The K685A mutation of RGL-RBD adds a much smaller effect to the interaction with the D33A mutant of Ras, as compared with the Y32F and E37G mutants All of these results strongly indicate that Asp33 in Ras interacts with Lys685 in RGL-RBD.

Interaction between Asp33 in Ras and Lys685 in RGL-RBD. To examine how Lys685 and Asp33 interact with each other, we made the K685M, K685E, and K685D mutants of RGL-RBD (Figure 4D). The K685M mutation reduces the Ras binding ability. Therefore, the positive charge of Lys685 is important. Actually, the introduction of a negative charge, by the K685E or K685D mutation, completely abolished the Ras binding. The K685M mutant of RGL-RBD binds Ras more tightly than the K685A mutant, indicating that the $-(CH_2)_4-$ moiety of Lys685 is also important. This indicates that Lys685 in RGL-RBD forms a salt bridge with Asp33 in Ras. This feature of the Ras interaction is now found to be well conserved between RalGDS and RGL, despite the differences in the amino acid residues surrounding the Lys residue.

E31K Mutant of Ras. The Lys31 residue of Ras(E31K) has been reported to be located very close to Asp33 and to interact with three residues of RalGDS-RBD in the crystal structure of the Ras(E31K)·RalGDS-RBD complex; the side-chain amino group of Lys31 forms a hydrogen bond (2.6 Å) with the backbone carbonyl group of Asp815, a hydrogen bond (3.4 Å) with the side-chain carbonyl group of Asn818, and a salt bridge (3.2 Å) with the side-chain carboxyl group of Asp820 (Figure 2A) (*29*). These interactions, which are missing in the case of Ras(wt) and RalGDS-RBD, might affect other interactions between RalGDS-RBD and Ras. The E31K mutation of Ras potentiates the RalGDS-RBD binding ability (*28*). Intriguingly, we have found that the E31A mutation also potentiates the RalGDS-RBD binding ability of Ras as much as the E31K mutation (*28*). This result indicates that the potentiation of Ras·RalGDS-RBD binding can be achieved only by the removal of the negatively charged residue at position 31 of Ras and, therefore, shows

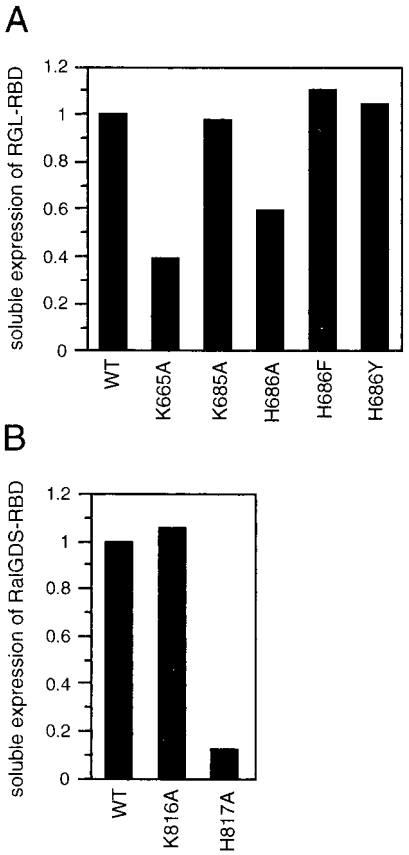

FIGURE 5: Comparison of the expression levels of the wild-type and mutant GST−RGL-RBD (A) or GST−RalGDS-RBD (B) in the soluble fraction. Extracts of sonicated *E. coli* cells overproducing either the GST−RGL-RBD or the GST−RalGDS-RBD fusion proteins were prepared, and the supernatants of these extracts were fractionated by SDS−PAGE. The SDS−polyacrylamide gels were stained with Coomassie Blue and were scanned, and the intensities of the GST−RGL-RBD and GST−RalGDS-RBD bands were determined with a densitometer. The intensities of these bands in the soluble fraction relative to those in whole extracts are normalized by the value of the wild-type GST−RGL-RBD (A) and GST−RalGDS-RBD (B).

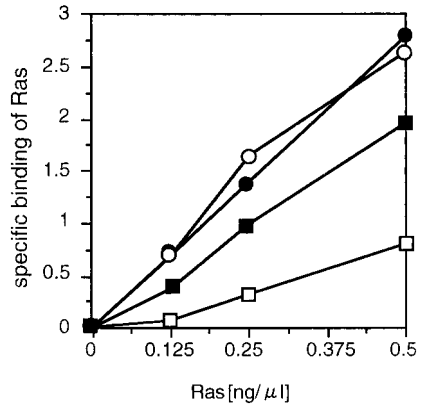

FIGURE 6: Association of the wild-type and E31K mutant Ras with RGL-RBD and RalGDS-RBD. Various amounts of the wild-type (■, □) and E31K mutant (●, ○) Ras loaded with GTPγS were tested for the ability to bind RGL-RBD (■, ●) and RalGDS-RBD (□, ○). The intensity of the Ras band was estimated by densitometry. The intensities relative to that pulled down after an incubation of 0.25 ng/μL of the wild-type Ras and the wild-type RGL-RBD are plotted. The data represent the average of at least three experiments.

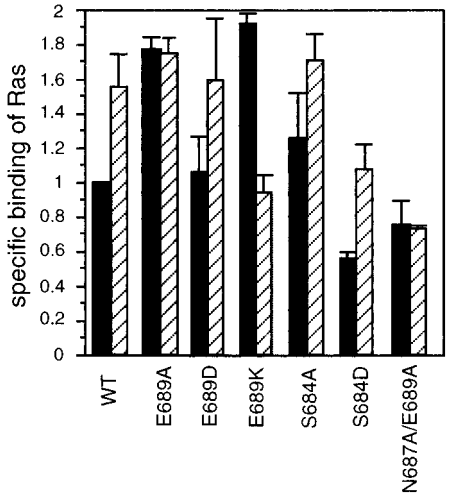

FIGURE 7: Association of the wild-type and E31K mutant Ras with various RGL-RBD mutants. The wild-type (solid blocks) and the E31K mutant (hatched blocks) Ras proteins (0.5 ng/μL) loaded with GTPγS were tested for the ability to bind the wild-type (WT) and the mutant RGL-RBD with the indicated mutation. Intensities are relative to that pulled down after an incubation of the wild-type Ras with the wild-type RGL-RBD. The data represent the average of at least three experiments.

that the negative charge of Asp820 in RalGDS-RBD exerts the putative repulsion against Glu31 in Ras(wt) (*28*). Meanwhile, among the three RalGDS residues that interact with Lys31 in Ras(E31K), Asp815 and Asp820 are replaced by Ser684 and Glu689 in RGL, whereas Asn818 is conserved as Asn687 (Figures 1 and 2). In principle, these three RGL residues might play roles similar to those of the corresponding RalGDS residues, in terms of the interactions with Lys31 in Ras(E31K) in the crystal (Figure 2A); Glu689 in RGL has a negative charge, like Asp820 in RalGDS. Therefore, we examined how RGL-RBD interacts with the amino acid residue at position 31 of Ras [Ras(wt) and Ras(E31K)]. First, the E31K mutation of Ras was shown to potentiate the RGL-RBD binding ability of Ras (Figure 6). RGL-RBD, like RalGDS·RBD, binds Ras(E31A) nearly as strongly as Ras(E31K) (data not shown), indicating that the putative electrostatic repulsion of Glu31 in Ras(wt) with RBD is conserved between RalGDS and RGL, whereas the extent of repulsion is smaller for RGL-RBD than for RalGDS-RBD.

Double-Mutant Analysis for Glu31 in Ras and Glu689 in RGL-RBD. Is Glu689 of RGL-RBD the counterpart of Glu31 of Ras(wt) in the putative electrostatic repulsion? We conducted another type of double-mutant analysis for the combination of the E31K mutation of Ras and the E689A mutation of RGL-RBD. Actually, we found that the Ras(wt) binding ability of the E689A mutant of RGL-RBD is much stronger than that of the wild-type RGL-RBD and that the E31K mutation of Ras no longer potentiates the binding with the E689A mutant (Figure 7). The reason for the failure of the E31K mutation to potentiate the E689A mutant RGL-RBD binding is likely to be that the negative charge due to Glu689 has already been removed. Thus, we conclude that the counterpart of Glu31 in the putative electrostatic repulsion is Glu689 in RGL. In contrast, the E689D mutation of RGL-RBD affects neither Ras(wt) binding nor Ras(E31K) binding (Figure 7), indicating that the negative charge of Asp is as efficient as that of Glu for the electrostatic repulsion. Furthermore, we found that the E689K mutation of RGL-RBD increases the Ras(wt) binding ability as much as the

E689A mutation and decreases the Ras(E31K) binding ability down to the level of the Ras(wt) binding ability of the wild-type RGL-RBD (Figure 7). Accordingly, the pair of positive charges has the same repulsive effect as that of the pair of negative charges on the Ras·RGL-RBD binding.

On the other hand, the E31K mutation of Ras potentiates the binding with the S684A and S684D mutants as well as the wild-type RGL-RBD (Figure 7). Therefore, these mutations of Ser684 have no effect on the putative interactions of the backbone carbonyl group of Ser684 with the side-chain amino group of Lys31 and the electrostatic interaction of the neighboring Glu689 side chain with Glu/Lys31 in Ras. To examine clearly the putative hydrogen-bonding interaction of the side-chain carbonyl group of Asn687 in RGL with the side-chain amino group of Lys31 in Ras(E31K), the N687A mutation was introduced into the E689A mutant of RGL-RBD, which is free from the side effect of the E31K mutation on the neighboring electrostatic interaction between position 689 of RGL and position 31 of Ras. Actually, the N687A/E689A mutant RGL-RBD exhibits appreciably lower Ras(wt) binding and Ras(E31K) binding abilities than those of the parent E689A mutant. Therefore, whether Ras has Glu31 or Lys31, the Asn687 side chain of RGL-RBD can contribute to the Ras binding. Because the E31K mutation in Ras has no effect on the binding with the N687A/E689A mutant RGL-RBD, the hydrogen bond of Lys31 with Ser684 may not be formed, at least in the absence of the two other putative interactions of Lys31 with Asn687 and Glu689.

Electrostatic Interaction of Glu31 of Ras (wt) with Ras-Binding Domains. Our previous observation revealed that the E31K and E31A mutations, but not the E31D mutation, of Ras potentiate the RalGDS binding ability (*28*). In the present study, the E689A and E689K mutations, but not the E689D mutation, of RGL-RBD potentiate the Ras(wt) binding. RalGDS has an acidic residue, Asp820, corresponding to Glu689 in RGL. Consequently, Glu31 of Ras(wt) exerts an electrostatic repulsion against Glu689 in RGL and against Asp820 in RalGDS, which results in the weaker binding of Ras(wt) with RGL and RalGDS than with the Raf kinases. As we described above, the extent of the electrostatic repulsion of Glu31 in Ras(wt) is smaller toward RGL-RBD than toward RalGDS-RBD (Figure 6). The RalGDS-like mutations of RGL-RBD, S684D and E689D, do not reduce the extent of the electrostatic repulsion toward Glu31 of Ras(wt), indicating that the difference in the amino acids at positions 684 and 689 of RGL-RBD alone is not the reason for the smaller extent of the electrostatic repulsion. In this context, the solution structure of RGL-RBD indicates that the spatial location of Glu689 relative to the other Ras-interacting residues in RGL-RBD is appreciably different from that of the corresponding Asp820 in RalGDS-RBD (*34*).

Concluding Remarks. In the present study, we conducted double-mutant analyses with respect to the two Ras residues, Glu31 and Asp33, which are located side by side on the surface of Ras. First, we showed that the attractive interaction between Asp33 in Ras(wt) and Lys685 in RGL-RBD, which corresponds to the interaction between Asp33 in Ras(E31K) and Lys816 in RalGDS-RBD in the crystal (*29*), is actually formed and contributes positively to the Ras·RGL-RBD association. This Lys residue is strictly conserved in the RalGDS family (Figure 1). Second, our double-mutant analysis revealed that the repulsive interaction between Glu31 in Ras(wt) and Glu689 in RGL-RBD, which is artificially replaced by the interactions of Lys31 in Ras(E31K) with three residues in RalGDS-RBD in the crystal (*29*), weakens the Ras·RGL-RBD association. This repulsive interaction of Glu31 in Ras(wt) is likely to be conserved with Asp820 in RalGDS-RBD, and all of the RalGDS family members have either Glu or Asp in this position. We found that the E31K mutation of Ras increases the binding ability more significantly with RalGDS-RBD than with RGL-RBD. The amino acid residues, Glu and Lys, at position 31 of Ras and Rap1, respectively, are one of the determinants that switch their signaling specificities with each other (*14*, *28*, *31*). The binding preferences of the RalGDS family members between Ras and Rap1 may be different from one another. Actually, RalGDS-RBD significantly prefers Rap1 to Ras, whereas Rlf- and RGL2-RBDs only weakly discriminate between Rap1 and Ras (*18*, *19*). Interestingly, we have also reported that the Raf-RBD binding is reduced by the E31K mutation, but not by the E31A or E31D mutation of Ras, probably because of the electrostatic repulsion between Lys84 in Raf-RBD and Lys31 in Ras(E31K) (*28*). Therefore, the mechanisms of the Ras/Rap1 preference in the Raf family are likely to be based on a similar electrostatic repulsion of Lys31 of Rap1 against Raf-RBD (*28*). These refinements of the Rap1/Ras preference within the RalGDS family, as well as those between the RalGDS and Raf families, may be relevant to the proper signaling network. Further understanding of the mechanisms of the Rap1/Ras and RalGDS/Raf preferences may require detailed structural analyses of the Ras-binding domains of the RalGDS family and Raf family proteins complexed with the wild-type Rap1 and Ras proteins.

ACKNOWLEDGMENT

We are particularly grateful to Dr. S. H. Kim for providing us with the coordinates of the Ras(E31K)·RalGDS RID complex, which were used with permission, to generate Figure 2. We also thank Dr. T. Kigawa and T. Terada for helpful comments.

REFERENCES

1. Barbacid, M. (1987) *Annu. Rev. Biochem. 56*, 779−827.
2. Pai, E. F., Kabsch, W., Krengel, U., Holmes, K. C., John, J., and Wittinghofer, A. (1989) *Nature 341*, 209−214.
3. Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., and Wittinghofer, A. (1990) *EMBO J. 9*, 2351−2359.
4. Brünger, A. T., Milburn, M. V., Tong, L., deVos, A. M., Jancarik, J., Yamaizumi, Z., Nishimura, S., Ohtsuka, E., and Kim, S.-H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4849−4853.
5. Milburn, M. V., Tong, L., deVos, A. M., Brünger, A., Yamaizumi, Z., Nishimura, S., and Kim, S.-H. (1990) *Science 247*, 939−945.
6. Ito, Y., Yamasaki, K., Iwahara, J., Terada, T., Kamiya, A., Shirouzu, M., Muto, Y., Kawai, G., Yokoyama, S., Laue, E. D., Wälchli, M., Shibata, T., Nishimura, S., and Miyazawa, T. (1997) *Biochemistry 36*, 9109−9119.
7. Avruch, J., Zhang, X.-F., and Kyriakis, J. M. (1994) *Trends Biochem. Sci. 19*, 279−283.
8. Daum, G., Eisenmann-Tappe, I., Fries, H.-W., Troppmair, J., and Rapp, U. R. (1994) *Trends Biochem. Sci. 19*, 474−480.
9. Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. (1997) *J. Biol. Chem. 272*, 4378−4383.
10. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) *Cell 74*, 205−214.

11. Chuang, E., Barnard, D., Hettich, L., Zhang, X.-F., Avruch, J., and Marshall, M. S. (1994) *Mol. Cell. Biol. 14*, 5318−5325.
12. Emerson, S. D., Madison, V. S., Palermo, R. E., Waugh, D. S., Scheffler, J. E., Tsao, K.-L., Kiefer, S. E., Lui, S. P., and Fry, D. C. (1995) *Biochemistry 34*, 6911−6918.
13. Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995) *Nature 375*, 554−560.
14. Nassar, N., Horn, G., Herrmann, C., Block, C., Janknecht, R., and Wittinghofer, A. (1996) *Nat. Struct. Biol. 3*, 723−729.
15. Hofer, F., Fields, S., Schneider, C., and Martin, G. S. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11089−11093.
16. Kikuchi, A., Demo, S. D., Ye, Z.-H., Chen, Y.-W., and Williams, L. T. (1994) *Mol. Cell. Biol. 14*, 7483−7491.
17. Spaargaren, M., and Bischoff, J. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12609−12613.
18. Peterson, S. N., Trabalzini, L., Brtva, T. R., Fischer, T., Altschuler, D. L., Martelli, P., Lapetina, E. G., Der, C. J., and White II, G. C. (1996) *J. Biol. Chem. 271*, 29903−29908.
19. Wolthuis, R. M. F., Bauer, B., van't Veer, L. J., de Vries-Smits, A. M. M., Cool, R. H., Spaargaren, M., Wittinghofer, A., Burgering, B. M. T., and Bos, J. L. (1996) *Oncogene 13*, 353−362.
20. Huang, L., Weng, X., Hofer, F., Martin, G. S., and Kim, S.-H. (1997) *Nat. Struct. Biol. 4*, 609−615.
21. Geyer, M., Herrmann, C., Wohlgemuth, S., Wittinghofer, A., and Kalbitzer, H. R. (1997) *Nat. Struct. Biol. 4*, 694−699.
22. Ponting, C. P., and Benjamin, D. R. (1996) *Trends Biochem. Sci. 21*, 422−425.
23. Khosravi-Far, R., White, M. A., Westwick, J. K., Solski, P. A., Chrzanowsta-Wodnicka, M., Van Aelst, L., Wigler, M. H., and Der, C. J. (1996) *Mol. Cell. Biol. 16*, 3923−3933.
24. White, M. A., Vale, T., Camonis, J. H., Schaefer, E., and Wigler, M. H. (1996) *J. Biol. Chem. 271*, 16439−16442.
25. Rodriguez-Viciana, P., Warne, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) *Cell 89*, 457−467.
26. Stang, S., Bottorff, D., and Stone, J. C. (1997) *Mol. Cell. Biol. 17*, 3047−3055.
27. Murai, H., Ikeda, M., Kishida, S., Ishida, O., Okazaki-Kishida, M., Matsuura, Y., and Kikuchi, A. (1997) *J. Biol. Chem. 272*, 10483−10490.
28. Shirouzu, M., Morinaka, K., Koyama, S., Hu, C.-D., Hori-Tamura, N., Okada, T., Kariya, K., Kataoka, T., Kikuchi, A., and Yokoyama, S. (1998) *J. Biol. Chem. 273*, 7737−7742.
29. Huang, L., Hofer, F., Martin, G. S., and Kim, S.-H. (1998) *Nat. Struct. Biol. 5*, 422−426.
30. Miura, K., Inoue, Y., Nakamori, H., Iwai, S., Ohtsuka, E., Ikehara, M., Noguchi, S., and Nishimura, S. (1986) *Jpn. J. Cancer Res. (Gann) 77*, 45−51.
31. Shirouzu, M., Fujita-Yoshigaki, J., Ito, Y., Koide, H., Nishimura, S., and Yokoyama, S. (1992) *Oncogene 7*, 475−480.
32. Kanai, T., Hirohashi, S., Noguchi, M., Shimoyama, Y., Shimosato, Y., Noguchi, S., Nishimura, S., and Abe, O. (1987) *Jpn. J. Cancer Res. (Gann) 78*, 1314−1318.
33. Terada, T., Ito, Y., Shirouzu, M., Tateno, M., Hashimoto, K., Kigawa, T., Ebisuzaki, T., Takio, K., Shibata, T., Yokoyama, S., Smith, B. O., Laue, E. D., and Cooper, J. A. (1999) *J. Mol. Biol. 286*, 219−232.
34. Kigawa, T., Endo, M., Ito, Y., Shirouzu, M., Kikuchi, A., and Yokoyama, S. (1998) *FEBS Lett. 441*, 413−418.

BI9820053