Ras proteins have multiple effectors of distinct structures that do not share significant structural homology at their Ras interaction sites. To prove possible differences in their recognition mechanisms of Ras, we screened 44 human Ha-Ras proteins carrying mutations in the effector region and its flanking sequences for interaction with human Raf-1, Schizosaccharomyces pombe Byr2, and Saccharomyces cerevisiae adenyl cyclase. The Ras binding specificities were largely shared between Raf-1 and Byr2 although Ras mutants, Y32F, T35S, and A59E, had their affinities for Byr2 selectively reduced. The only exception was Ras(D38N), which lost the ability to bind Raf-1 while retaining the activity to bind Byr2 and complement the Byr2 phenotype of S. pombe. On the other hand, adenyl cyclase had quite distinct requirements for Ras residues; mutations P34G and T58A selectively abolished the ability to bind and activate it without considerably affecting the interaction with Raf-1 and Byr2. Y32F mutant, whereas losing the ability to activate Raf-1 and Byr2, could activate adenyl cyclase efficiently. In addition, V45E mutation was found to impair the ability of Ras to activate both Raf-1 and adenyl cyclase without significantly affecting the binding affinities for them. These results demonstrate that significant differences exist in the recognition mechanisms by which the three effector molecules associate with Ras and suggest that a region of Ras required for activation of the effectors in general may exist separately from that for binding the effectors.

The ras genes are widely conserved in a variety of eukaryotic organisms from yeasts to mammals. Their protein products belong to a family of small guanine nucleotide-binding proteins and operate in key processes of intracellular signal transduction systems that are involved in regulation of cell growth and differentiation. In higher eukaryotes including Caenorhabditis elegans, Drosophila melanogaster, and vertebrates, Ras proteins are involved in intracellular signaling from receptor tyrosine kinases, which results in activation of a phosphorylation cascade comprising Raf proteins, MAP1 kinase kinases (MEKs), and MAP kinases (ERKs) (for reviews, see Refs. 1 and 2). Recent studies demonstrated direct binding of Raf-1 and its homologue B-Raf to Ras (3–9), but the precise mechanism of the Raf activation remains to be clarified. In addition, other mammalian proteins have been shown to associate directly with Ras in a GTP-dependent manner. These include Rap guanine nucleotide dissociation stimulator (10–12), phosphatidylinositol 3-OH-kinase (13), the ε isoform of protein kinase C (14), and Rin1 (15). However, the significance of their interaction with Ras is presently unknown. In the fission yeast, Schizosaccharomyces pombe, its single Ras homologue, Ras1, is involved in signal transduction from mating pheromone receptors, and its function is mediated by a protein kinase cascade that ultimately regulates a member of the MAP kinase family (for a review, see Ref. 16). Protein kinase Byr2 was recently shown to be a direct downstream target of Ras1 (4, 17, 18). On the other hand, in the budding yeast Saccharomyces cerevisiae, adenyl cyclase is an immediate downstream effector of a pair of Ras proteins, Ras1 and Ras2, which are structural, biochemical, and functional homologues of vertebrate Ras (for a review, see Ref. 19).

Mutational studies of the effector molecules identified discrete Ras-binding regions in them. The Ras-binding site of mammalian Raf-1 was mapped to an 81-amino acid segment in its N-terminal regulatory domain (7, 20–22). Recently another Ras-binding site was identified in Raf-1 corresponding to the cysteine-rich region (23, 24). In yeast adenyl cyclase, the leucine-rich repeats domain, which is composed of tandemly repeated 23-amino acid leucine-rich motifs (25), was shown to bind directly to Ras (7, 26). Byr2 contains a Ras-binding site within its N-terminal 206 amino acid residues (18), but further mapping has not been attempted. Comparison of the primary structures of these Ras-binding domains revealed virtual absence of homologous sequences among them.

On the other hand, extensive mutational studies on Ras identified amino acid residues whose substitution abolished the ability of Ras to transform NIH3T3 cells, to induce neurite outgrowth in PC12 cells, to induce germinal vesicle breakdown in Xenopus laevis oocytes, or to associate with Raf-1, without affecting the guanine nucleotide binding properties (20, 27–35). First, many of these residues were concentrated in a region
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corresponding to amino acid residues 32–40 of mammalian Ras, so that this region was designated the "effector region." Actually, determination of the tertiary structure of Ras showed that this region almost matches to "switch I," one of the two regions that take significantly different conformations between the GTP- and GDP-bound forms of Ras (36–38). In contrast, the residues 26, 31, 42, 45, 46, 48, 49, and 53, flanking the effector region, were recently found to be critical for the Ras signaling even though they do not change their conformations upon GTP exchange from GDP to GTP (29–31, 34). Therefore, these residues, which are likely to be involved in activation of the Ras effectors, were designated the "activator region" (34) or "constitutive effector region" (35).

Presently, there exists an assumption based on studies using a very limited number of the effector region mutants of Ras that all three distinct effectors recognize a similar structure located in the effector region and possibly in its immediately flanking regions for their interaction with Ras. However, the observed absence of structural conservation among the effectors suggests that they may interact with Ras in a somewhat different mode. This prompted us to systematically survey possible differences in the recognition mechanisms of Ras by the three effector molecules. We use a series of human Ha-Ras mutants bearing amino acid substitutions in the effector region and its flanking region as molecular probes for dissecting differential requirements of particular Ras residues. In addition to semi-quantitative estimations by a yeast two-hybrid assay and by in vitro binding measurements, we employ a method based on inhibition of the Ras-dependent activity of yeast adenyl cyclase for quantitative analysis of the binding reactions between the Ras mutants and the effectors. Results of these binding studies are compared with those of measuring the biochemical and biological activities of the effector proteins.

EXPERIMENTAL PROCEDURES

Materials and Cell Strains—The anti-Ha-Ras monoclonal antibody F235 was purchased from Oncogene Science Inc. (New York). The polyclonal antibody C12, raised against the C-terminal 12-amino acid residues peptide of human Raf-1, was purchased from Santa Cruz Biotechnology Inc. Escherichia coli expressing GST fusion protein of MEK1 (GST-MEK) or GST fusion protein of a kinase negative mutant of ERK2 (GST-KERK) and a recombinant baculovirus expressing the full-length human Raf-1 (39) were obtained from Dr. L. Williams (University of California, San Francisco). The yeast two-hybrid system vector pGBT10 (13) and MBP-GDH and the YP2B reporter strain (4, 40) were provided by Drs. L. Van Aelst and M. Wigler (Cold Spring Harbor Laboratory, New York). The S. cerevisiae strain TK35–1 (MATa his3 leu2 trp1 ura3 cyr1–2 ras2 LEU2) was described (41). The S. pombe diploid strain J260 (h9) h9 ras1:URA4/leu1:URA4 leu1 adel- MI201/adel-M216) was prepared similarly as described before (42). The S. pombe expression vector pREP3 was described (43). Yeast cells were cultured in yeast synthetic media (0.67% yeast nitrogen base and 2% glucose) with appropriate nutritional supplements.

Expression and Purification of Various Ha-Ras Mutant Proteins—Forty-four kinds of amino acid substitution mutations were introduced into the synthetic human H-ras cDNA bearing the oncogenic valine for glycine substitution at position 12 as described previously (30, 31, 33, 35). The mutants were designated Ha-RasV12(X)(Y), where amino acid Y substituted for X at the position n. The mutant genes were amplified by polymerase chain reactions (44) with a pair of primers, 5'–CGGATCCGATATCCGTAAGGATCCCAAACCTG–3' and 5'–AATGCTGACTGATCAATTACG–3', and, after cleavage with BamH I and Pst I in the primer sequence, inserted into the respective baculovirus transfer vector pBlueBac III (Invitrogen Inc.). Accuracy of the Ha-ras amplification was confirmed by nucleotide sequencing of the cloned genes. Recombinant Autographa californica nuclear polyhedrosis viruses containing the mutant Ha-ras genes were constructed by in vivo homologous recombination as described (45). The post-translationally modified forms of the mutant Ha-Ras proteins were produced in Sf9 insect cells infected with the baculoviruses containing the respective genes and purified to almost homogeneity by anion exchange chromatography as described before (46).

Yeast Two-hybrid Assay—The BamHI-PstI fragments bearing the mutant Ha-ras genes were done into the matching cleavage sites of pGBK10. The resulting pGBK-Ras plasmids were used for expression of the mutant Ras proteins as fusions with the GAL4 DNA-binding domain in yeast. The EcoRI-SalI fragment of the human c-ras-1 cDNA corresponding to amino acids 1–257 of Raf-1 was cloned into the matching cleavage sites of pGAD-GH, creating pGAD-Raf-1(1–257), for expression as a fusion protein with the GAL4 transactivation domain. Similarly, pGAD-Byr2(1–287), which was constructed by doning the BamHI-HindIII fragment encoding the N-terminal 287 amino acid residues of Byr2 (18) into the matching cleavage sites of pGAD-GH, was used for expression of Byr2(1–287) as a fusion with the GAL4 transactivation domain. Combinations of either pGAD-Raf-1(1–257) or pGAD-Byr2(1–287) with each of the pGBT-Ras mutants were co-transformed into the yeast reporter strain YP82 by using lithium acetate (47). Three independent colonies each of the resulting Trp+ Leu+ transformants were patched and grown for 2 days on sheets of Whatman No. 50 paper placed on yeast synthetic medium plates lacking tryptophan and leucine and examined for ß-galactosidase activity by observation of blue color development after incubation for 1 h for pGAD-Raf-1(1–257) or 2 h for pGAD-Byr2(1–287) with X-gal as described (40).

Binding Assays between the Effectors and Ras Mutants in Vitro—MBP-Raf-1(1–257), an MBP-fusion protein of the Raf-1 N-terminal 257 amino acid residues, and GST-BYr2(1–287), a GST-fusion protein of the Byr2 N-terminal 287 residues, were produced in E. coli and purified previously (48). The C-terminal 287-residue segment of the yeast adenyl cyclase gene (CYR1) was over-expressed as a GST-fusion protein in yeast cells harboring pAD-GST-CYR1(606–2026) and purified as described previously (26, 46). MBP-Raf-1(1–257), GST-BYr2(1–287), or GST-CYR1(606–2026) was attached to amyllose (for MBP-fusion)- or glutathione (for GST-fusion)agarose and incubated with buffer A (20 mM Tris/HCl, pH 7.4, 40 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Lubrol PX) by continuous mixing for 8 h. After washing with buffer A, an aliquot of the resin with 0.8–2 μg each of either of the Raf-1, Byr2, or CYR1 fusion proteins attached was incubated with 1 or 10 pmol (quantitated by GST-5’ binding) of each of various mutant Ha-Ras proteins, which had been incubated with GDP or GTP, in 300 μl of buffer A for 10 min at 25°C with continuous mixing, and was subsequently washed three times with buffer A. The bound Ras was eluted from the resin by 20 μl glutathione (glutathione-agarose) or 10 μl maltose (amylose-agarose), separated by SDS-PAGE (12% gel) (50), and detected with Western immunoblotting with the anti-Ha-Ras antibody F235. The ECL immunodetection system (Amersham Corp.) was used for the signal development.

Adenyl Cyclase Activation and Inhibition Assays—Yeast adenyl cyclase, overproduced in yeast TK35–1 harboring a plasmid YEP24–ADCI-CYR1, was solubilized from the crude membrane fraction with buffer C (50 mM MES/NaOH, pH 6.2, 0.1 mM MgCl2, 0.1 mM EGTA, and 1 mM 2-mercaptoethanol) containing 1% Lubrol PX, 0.6 mM NaCl, and 1 mM dithiothreitol. The samples were centrifuged at 100,000 × g for 1 h and the supernatant (10 μg of protein) after centrifugation at 100,000 × g for 1 h was used for the adenyl cyclase assay. Measurements of adenyl cyclase activity dependent on the GST-S5 bound form of Ras and of its inhibition by purified MBP-Raf-1 and GST-Byr2 polypeptides were carried out as described before (18, 26).

Assays of Ras-dependent Activation of Raf—The assays were carried out similarly as described before (39). Monolayers of Sf9 cells (2 × 105 cells) were cocomitted with the recombinant baculovirus expressing the full-length Raf-1 and that expressing either one of the mutant Ha-Ras proteins (1 × 106 plaque-forming units each). After 72 h postinfection, the cells were lysed by sonication in 1 ml of buffer B (20 mM Tris/Cl, pH 7.5, 137 mM NaCl, 1% Nonidet, 1% glycerol, 10 mM dithiothreitol, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium vanadate) and centrifuged at 13,000 × g for 30 min. Raf protein was immunoprecipitated from the supernatant fraction (200 μl) by the anti-Raf-1 antibody C12 (2 μl) and protein A-agarose. The Raf kinase activity was determined by incubating the immunoprecipitates in the GST-KERK (13 μl) digestion mixture (20 μl Tris/HCl, pH 7.5, 10 mM MgCl2, 10 μM MnCl2, 20 μM γ-mercaptoethanol, and 50 μM ATP (4000 cpm/ml)) for 30 min at 25°C. After the incubation, the reaction was stopped by the addition of Laemmli's loading buffer (50), fractionated by SDS-PAGE (10% gel), and subjected to autoradiography to detect phosphorylated proteins.

Complementation of Ras—Phenotypes of S. pombe—The Smal-Sall fragments of Ha-rasV12 genes bearing various mutations (30, 31, 33, 35) were cloned into the Ball-Sall cleavage sites of pReP3 for expression
Table I

Interaction of Ha-Ras mutants with Raf-1, Byr2, and adenyly cyclase

The interactions with Raf-1 and Byr2 were examined by the yeast two-hybrid assay. The $K_a$ values for Ha-Ras mutants and $V_{max}$ values were determined from Fig. 2A data.

| Ha-Ras$^{V12}$ mutants | Raf-1 | Byr2 | Adenyly cyclase |
|------------------------|-------|------|----------------|
|                        |       |      | $K_a$ | $V_{max}$ |
| WT                     | +     | +    | 6    | 100      |
| I21A                   | +     | +    | 3    | 65       |
| L23F                   | +     | +    | 6    | 68       |
| I24V                   | +     | +    | 2    | 57       |
| N26G                   | +     | +    | 3    | 41       |
| H27I                   | +     | +    | 500  | 128      |
| V29A                   | +     | +    | 13   | 140      |
| D30A                   | +     | +    | 10   | 131      |
| D30E                   | +     | +    | 16   | 90       |
| D30N                   | +     | +    | 9    | 22       |
| E31K                   | +     | +    | 16   | 90       |
| Y32W                   | +     | +    | 10   | 131      |
| P34A                   | +     | +    | 16   | 90       |
| P34G                   | +     | +    | 16   | 90       |
| P34H                   | +     | +    | 16   | 90       |
| P34S                   | +     | +    | 16   | 90       |
| T35V                   | +     | +    | 16   | 90       |
| D38N                   | +     | +    | 16   | 90       |
| S39A                   | +     | +    | 16   | 90       |
| S39C                   | +     | +    | 16   | 90       |
| S39P                   | +     | +    | 16   | 90       |
| Y40K                   | +     | +    | 16   | 90       |
| Y40W                   | +     | +    | 16   | 90       |
| R41A                   | +     | +    | 16   | 90       |
| Q43A                   | +     | +    | 16   | 90       |
| Q43H                   | +     | +    | 16   | 90       |
| V44A                   | +     | +    | 16   | 90       |
| V45E                   | +     | +    | 16   | 90       |
| I46A                   | +     | +    | 16   | 90       |
| D47A                   | +     | +    | 16   | 90       |
| L52W/D54E              | +     | +    | 16   | 90       |
| L53A                   | +     | +    | 16   | 90       |
| L56A                   | +     | +    | 16   | 90       |
| L56E                   | +     | +    | 16   | 90       |
| D57A                   | +     | +    | 16   | 90       |
| T58A                   | +     | +    | 16   | 90       |
| A59E                   | +     | +    | 16   | 90       |
| Q61T                   | +     | +    | 16   | 90       |
| E63Q                   | +     | +    | 16   | 90       |
| Y64F                   | +     | +    | 16   | 90       |
| S65T                   | +     | +    | 16   | 90       |

under control of the S. pombe nmt-1 promoter (43). The S. pombe jZ840 was transformed with pREP3-Ras plasmids, and the resulting Leu-+ transformants were cultured on yeast synthetic medium plates for 3 days and subsequently spread on SSA sporulation medium plates (51). Sporulation was observed under microscope after 3 days of culture at 30°C. Transformation of S. pombe cells was performed as described (52).

**RESULTS**

Yeast Two-hybrid Analysis of the Ha-Ras Mutants for Interaction with Various Effectors—We employed the yeast two-hybrid system for examination of the interaction of various Ha-Ras mutants with the Ras-binding domains of Raf-1 and of Byr2. Each of the 44 mutant Ha-Ras carried single or double amino acid substitutions in the effector region and its flanking sequence (amino acids 21-65) in addition to the activating mutation of valine for glycine at position 12 (Table I). They included some bearing amino acid changes from Ha-Ras sequence to Rap1A/Krev-1 sequence (53, 54). The strength of interaction was estimated by the $\beta$-galactosidase activity, which was measured by the intensity of blue color developed in the presence of X-gal, of the cotransformants with pGBT-Ras and either of pGAD-Raf-1-(1–257) or pGAD-Byr2-(1–287). The results on all the mutants are summarized in Table I. Only four mutants showed differential interaction with Raf-1-(1–257) and Byr2-(1–287). The Ha-Ras$^{V12}$ mutants, Y32F, T35S and S39P, Y40K, and D57A, exhibited total loss of the ability to interact with both Raf-1 and Byr2.

Measurement of Direct Binding between Ha-Ras Mutants and either Raf-1 or Byr2 in Vitro—Because measurements of interaction by the yeast two-hybrid system do not necessarily reflect direct and quantitative binding, we tested whether the differential interaction of the Ha-Ras mutants to Raf-1 and Byr2 detected by this assay could be reproduced by in vitro binding assays. The post-translationally modified forms of Ha-Ras mutant proteins were produced by the baculovirus expression system and purified. They were loaded with GTPyS or GDP and incubated at the concentrations of 10 nM with agarose resin on which MBP-Raf-1-(1–257) or GST-Byr2-(1–287) was immobilized. The amounts of Ha-Ras proteins bound to the resin were measured by Western immunoblotting with the anti-Ha-Ras antibody (Fig. 1). Out of the three mutants that exhibited the interaction with Raf-1 but not with Byr2 by the two-hybrid assay, only Ha-Ras$^{V12}$(A59E) was found to have lost the ability to bind Byr2-(1–287) while retaining the ability to bind Raf-1-(1–257). In contrast, Ha-Ras$^{V12}$(Y32F) and Ha-Ras$^{V12}$(T35S) retained the ability to bind Byr2 although the activities were reduced significantly (Fig. 1). This was somewhat contrary to the totally negative two-hybrid assay results. On the other hand, Ha-Ras$^{V12}$(D38N) showed clear distinction between Raf-1-(1–257) and Byr2-(1–287) as observed in the two-hybrid assay. In either case, GTP dependence of the binding was clearly observed. These results indicate that negative results with the yeast two-hybrid assay do not necessarily reflect the absence of association between two proteins.

Measurement of Interaction with Yeast Adenylyl Cyclase—Interaction with yeast adenylyl cyclase was measured by the ability of the purified mutant Ha-Ras proteins to activate it in vitro. The post-translationally modified forms of Ha-Ras were used for the assay because it had been shown that the modification of Ras was essential for the adenylyl cyclase activation (46). Out of the 26 mutant Ha-Ras$^{V12}$ proteins tested, P34G, D38N, T58A, and A59E were found to have totally lost the ability to activate adenylyl cyclase in the concentration range...
examined (Fig. 2A). The activation constant \( K_a \) and the maximally stimulated activity \( V_{\text{max}} \) of adenylyl cyclase were calculated for other Ha-Ras mutants by a double-reciprocal plot of the Fig. 2A data and summarized in Table I. A great variance was observed in the estimated \( K_a \) values and the extents of maximal activation among the Ha-Ras mutants. F28A and I46A mutants had their \( K_a \) values increased by about 100-fold while their \( V_{\text{max}} \) was not significantly altered, indicating that their affinities for adenylyl cyclase were severely impaired. Y40W, R41A, and Q43A exhibited a similar property to a lesser degree. In contrast, mutants, E31K, T35S, V45E, and S65T, had their \( V_{\text{max}} \) reduced to 20–30% of the wild type, whereas their \( K_a \) values were unaffected. N26G and R41A also possessed a similar property to a lesser extent.

To examine whether the loss of activation was ascribable to failure in binding adenylyl cyclase, we analyzed in vitro binding of the Ha-Ras mutant proteins to purified GST-CYR1-(606–2026) (Fig. 2B). As expected, the mutants, E31K, Y32F, and T35S, all of which exhibited low \( K_a \) values, bound to GST-CYR1-(606–2026) in a GTP-dependent manner as efficiently as wild type. In contrast, all of the activation-negative Ha-Ras mutants, P34G, D38N, T58A, and A59E, exhibited no detectable binding, indicating that the loss of adenylyl cyclase activation reflects the absence of the binding activity. F28A exhibited very low binding as predicted from its high \( K_a \) value. The binding activities of the Ha-Ras mutants were generally in parallel with their \( K_a \) values of the cyclase activation. Thus, the Ha-Ras mutants, P34G, T58A, and to a lesser extent F28A, selectively lost the ability to bind adenylyl cyclase while retaining the ability to bind Raf-1 and Byr2. These data indicate that yeast adenylyl cyclase possesses a considerable difference in the binding requirement for Ras from that of Raf-1 or Byr2.

**Measurement of Dissociation Constants by Adenylyl Cyclase**

**Inhibition Assay**—Although the results of the in vitro binding and two-hybrid analyses suggested reduced binding affinities of some of the Ha-Ras mutants against Raf-1 or Byr2, its definitive demonstration required quantitative measurements of the kinetic parameters. Dissociation constants \( (K_a) \) of Raf-1 and Byr2 for Ras could be determined in solution by kinetic analyses of their inhibition patterns of Ras-dependent adenylyl cyclase activity \((18, 26)\). We employed this method to estimate the \( K_a \) values of Raf-1 or Byr2 for the Y32F and T35S mutants, which exhibited differential binding to Raf-1 and Byr2, along with the wild-type Ha-RasF12 (Fig. 3). Adenylyl cyclase activities dependent on various concentrations of each of the Ha-Ras mutant proteins were measured with the addition of varying concentrations of the purified MBP-Raf-1-(1–257) or GST-Byr2-(1–287) to the reaction mixtures. In all the combinations, dose-dependent inhibitions were observed (data not shown). At each point of Ha-Ras concentration in the presence of the competitor, MBP-Raf-1-(1–257) or GST-Byr2-(1–287), 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 the competitor. A difference between the original and the free concentrations of Ha-Ras was regarded as that bound to the competitor, and a reciprocal of this value was plotted against a reciprocal of the free Ha-Ras concentration (Fig. 3, A–F). This gave a series of straight lines for each value of the competitor, which converged on the horizontal axis. The data indicated that the competitor polypeptide bound directly to Ha-Ras protein and competitively sequestered it from adenylyl cyclase. The \( K_a \) value for each of the Ha-Ras mutants and wild type was calculated from the point of intersection with the horizontal axis. The Y32F and T35S mutants bound to Raf-1-(1–257) with the \( K_a \) values of 5 and 10 nm, respectively, which were similar to that of the wild type, 7 nm. However, their affinities for Byr2-(1–287) were considerably reduced; the \( K_a \) values for Y32F and T35S were 36 and 10 nm, respectively, compared with that of the wild type, 2 nm. These results were consistent with those obtained by using the other
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Fig. 4. Measurements of the activities of Ha-Ras mutants to activate Raf-1. SF9 cells were infected with the recombinant baculovirus expressing the full-length Raf-1, alone or in combination with that expressing either Ha-RasV12 (WT) or one of its mutants (A38E, Y32F, D38N, and T35S). The amounts of Raf-1 and Ha-Ras proteins present in Nonidet P-40 extracts of the infected SF9 cells were determined by Western immunoblotting with the corresponding antibodies. Raf-1 was immunoprecipitated from the extracts by the anti-Raf-1 antibody and examined for its activity to induce phosphorylation of GST-KNERK in the presence of GST-MEK as described under “Experimental Procedures.” Shown are an autoradiogram of an SDS-PAGE gel separating the phosphorylated proteins (upper panel) and the immunoblots of the SF9 extracts subjected to detection of Raf-1 (middle panel) or Ha-Ras (lower panel). The Ha-Ras bands formed doublets; the upper represents the post-translationally unmodified form and the lower represents the modified form.

Fig. 3. Determination of the K_d values of Raf-1 and Byr2 for various Ha-Ras mutants by the adenyl cyclase inhibition assay. Adenyl cyclase activities dependent on various concentrations of the GTPyS-bound forms of Ha-RasV12 proteins were measured with the addition of varying amounts of MBP-Raf-1 (1–257) (5 pmol (●), 10 pmol (■), and 20 pmol (▲) shown in panels A, C, and E, respectively) or of GST-Byr2 (1–287) (0.5 pmol (●), 1 pmol (■), 2 pmol (▲), and 4 pmol (▲) shown in panels B, D, and F, respectively). The Ha-RasV12 proteins carried no mutation (WT) (panels A and B), Y32F mutation (panels C and D), or T355 mutation (panels E and F). The amount of free Ha-Ras and that bound to MBP-Raf-1 (1–257) or GST-Byr2 (1–287) were calculated as described in the text, and a reciprocal of the concentration of the free Ha-Ras was plotted against a reciprocal of the concentration of the bound Ha-Ras. The K_d values for Ha-Ras were calculated from the points of intersection with the horizontal axis as described before (26).

Coexpression assay methods. Thus, we concluded that Ha-RasV12(Y32F) and Ha-RasV12(T355) had their Byr2 binding affinities selectively reduced without significantly affecting the binding affinities for Raf-1 and adenyl cyclase (see Fig. 2). In addition, the observed good parallelism of the data obtained by the three distinct assay methods made us believe that the Ha-Ras mutants, D38N and A59E, also possess differential binding affinities for Byr2 and Raf-1 although we could not employ the adenyl cyclase inhibition assay for estimation of their exact K_d values.

Measurements of the Activities of the Ha-Ras Mutants to Activate Raf-1—The activity of stimulating MAP kinase had been examined in PC12 cells for some of the Ha-Ras mutants employed in this study (35). Here, we employed the baculovirus infection assay to examine the activities of the Ha-Ras mutants to stimulate the Raf-1 kinase activity, which was measured by phosphorylation of KNERK through the activation of MEK as described under “Experimental Procedures.” Raf-1 protein immunoprecipitated from the lysate of SF9 cells coexpressing Ha-RasV12 and the full-length Raf-1 exhibited an elevated activity of inducing phosphorylation of KNERK in the presence of GST-MEK compared with that from the cells expressing Raf-1 only (Fig. 4, upper panel). The phosphorylation of KNERK was dependent on the presence of GST-MEK as observed by others (data not shown). Then, we examined the effect of coexpression of each of the Ha-Ras mutants with Raf-1. Ha-RasV12(T355) was found to retain the ability to stimulate the KNERK-phosphorylating activity. However, Ha-RasV12 carrying the mutations Y32F, D38N, and A59E almost completely lost the activities. In all the baculovirus infection assays, the amounts of Raf-1 and Ha-Ras proteins expressed in SF9 cells were almost identical as measured by Western blotting with the corresponding antibodies (Fig. 4, middle and lower panels). These results indicated that the mutations Y32F and A59E abolished the ability of Ha-Ras to activate Raf-1 without significantly affecting the binding affinity for it.

Complementation of Ras1-deficient Phenotypes of S. pombe Cells by the Ha-Ras Mutants—The ability of the Ha-Ras mutants to activate Byr2 in vivo was tested by complementation of the sporulation-defective phenotype of S. pombe ras1/’ ras1− diploid J Z840. S. pombe ras1 is required for sexual differentiation and normal cell shape (42, 55). Cells deficient in Ras1 are defective in conjugation and sporulation and have a rounded morphology rather than the elongated morphology of wild-type cells. Overexpression of Byr2 rescues the sporulation defect but not the other defects of Ras1− cells (17). As reported before (55), expression of the wild-type Ha-RasV12 could induce efficient sporulation of J Z840 (Fig. 5B), which otherwise exhibited essentially no sporulation (Fig. 5A). Ha-RasV12(T355) was found to be as active as the wild type in inducing sporulation (Fig. 5D) although the mutation significantly reduced the affinity for Byr2 (Fig. 3). As expected from their poor Byr2 binding activities (Fig. 1), both Ha-RasV12(Y32F) and Ha-RasV12(A59E) had their sporulation-inducing activities severely attenuated (Fig. 5, C and F). On the other hand, Ha-RasV12(D38N) induced
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Mutational studies of Ras by a number of groups showed that the effector region (Tyr^{32}–Tyr^{40}) is critical for its biological activity as well as for binding to its effector, Raf-1 (20, 27–35). This region roughly corresponds to the switch I (Tyr^{32}–Asp^{38}), whose structure is altered significantly between the GTP- and GDP-bound forms (36–38). Recent determination of the crystal structure of a complex between the Ras-binding domain (residues 51–131) of Raf-1 and the Ras-related protein Rap1A/Krev-1 indicated that the residues in the effector domain constitute a principal binding interface (56). In contrast, for the abilities to interact with other Ras effectors including Byr2 and S. cerevisiae adenyl cyclase, only a limited set of Ras mutants has been studied (4, 18, 27). The present study is the first to carry out a systematic survey of a possible difference in the recognition mechanisms of Ras by its distinct effectors. The results are summarized in Table II only for the Ha-Ras mutants that exhibited differential interactions with the effectors.

Also, available data on the activities of these mutants to transform NIH3T3 or rat2 cells and to induce neurite outgrowth in PC12 cells are presented in the same table.

It is shown clearly that the D38N mutation abolishes the ability of Ha-Ras to associate with both Raf-1 and adenyl cyclase without significantly affecting the ability to bind and activate Byr2. The inability of the D38N mutant to interact with Raf-1 was reported before (5, 33). Mutations at position 38 were shown to abolish the biological activity without significantly altering the overall conformation of Ras, suggesting a direct implication of this residue in the effector activation (57). A nonconservative substitution at this position, D38A, was known to abolish interaction with all of the three effectors (5, 27). Here, use of the conservative substitution, D38N, has helped in identifying a subtle difference in the recognition mechanisms of Ras by the effectors. In addition, our screening has yielded three mutants, Y32F, T35S, and A59E, which, to a lesser degree, can bind discriminatively to Raf-1 and Byr2. Determination of the K_d values indicated that the affinity of the Y32F mutant for Byr2 was about 18-fold weaker than that of wild type, whereas its affinity for Raf-1 was unaffected. Similarly, the T35S mutation selectively reduced the affinity for Byr2 by about 5-fold. However, when the activities of these mutants to stimulate the effectors were examined, they did not discriminate between Raf-1 and Byr2; for both of them, Y32F was inactive, while T35S was active. Y32F had also been reported to lack the activity to induce neurite outgrowth in PC12 cells and to transform NIH3T3 cells (58, 59). The A59E mutant lost the ability to bind Byr2, but its ability to bind Raf-1 was also significantly attenuated. This mutant again exhibited severely attenuated activities to stimulate both Raf-1 and Byr2. Thus, we could not obtain any mutant that selectively lost the ability to interact with and activate Byr2. Presently, we do not know the reason why the Y32F and A59E mutations cause the loss of activation of both Byr2 and Raf-1.

In contrast to the situation with Raf-1 and Byr2, we observed clearer differences in the mode of interaction of Ras with S. cerevisiae adenyl cyclase. The P34G and T58A mutations selectively abolished the association with adenyl cyclase, whereas the association with Raf-1 or Byr2 was not significantly affected. The A59E mutant lost the ability to bind Byr2, but its ability to bind Raf-1 was also attenuated to a lesser degree. The affinity for adenyl cyclase was greatly reduced by the F28A and I46A mutations, whereas that for Raf-1 or Byr2 was not so much affected. The Y32F mutant, lacking the ability to activate both Raf-1 and Byr2, could effectively bind and activate adenyl cyclase. These results raise a possibility that adenyl cyclase may recognize the residues Thr^{58} and Ala^{59}, immediately flanking the switch II region, in addition to the effector region. Thus, we have demonstrated that significant differences exist in the recognition mechanisms of Ras by Raf-1, Byr2, and adenyl cyclase and successfully obtained the Ha-Ras mutants, which interact with and activate only a specific set of the effectors. Especially, the D38N and Y32F mutants could selectively activate a single effector out of the three effectors tested. These mutants may be useful in differentially analyzing the individual function of the distinct effector molecules in cells as already proposed (60).

It has been proposed that some residues of Ras surrounding the switch I region are involved in the effector activation (34). Actually, mutations of residues 26, 31, 42, 45, 46, 48, and 53 were found to deprive Ras of its activities to cause cellular events (29–33, 35), whereas most of these mutations did not affect the ability to associate with Raf-1 (20, 33, 35). In the present study, it was demonstrated that mutants, N26G, E31K, and V45E, retained not only the ability to associate with Raf-1
but also those to bind Byr2 and yeast adenylyl cyclase. However, the $V_{\text{max}}$ values of adenylyl cyclase attained by the E31K, V45E, and, to a lesser degree, N26G mutants were considerably reduced compared with that of wild type, whereas their $K_a$ values were not significantly changed. This suggests that the definition of the "activator region" of Ras may also be valid for another effector, yeast adenylyl cyclase. We have recently shown that the activator region of Ras binds directly to the cysteine-rich region of Raf-1, which is located at the C-terminal side of the Ras-binding domain, in a GTP-independent manner and that post-translational modifications of Ras are required for this interaction (24). Because post-translational modifications of Ras were also critical for activation of adenylyl cyclase (46), these data may suggest the presence in adenylyl cyclase of a separate binding site for the activator region of Ras from that for the effector region.

While this work was in progress, White et al. (60) reported the isolation of Ha-Ras effector region mutants, which interact differentially with Raf-1 and Byr2 by screening of a pool of randomly mutagenized Ha-Ras with the yeast two-hybrid assay. By using the results of this assay as a sole criterion, they identified the T35S mutant as that interacted with Raf-1 but not with Byr2 and the E37G mutant as that interacted with Byr2 but not with Raf-1. Although we confirmed the two-hybrid assay result with the T35S mutant, it was clearly shown here that this mutant retains a considerable ability to association with Raf-1 kinase activity, X-H. Deng for skillful technical assistance, and A. Seki and A. Kawabe for help in preparation of this manuscript.

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