The Ras and Rap1A proteins can bind to the Raf and RalGDS families. Ras and Rap1A have Glu and Lys, respectively, at position 31. In the present study, we analyzed the effects of mutating the Glu at position 31 of the c-Ha-Ras protein to Asp, Ala, Arg, and Lys on the interactions with Raf-1 and RalGDS. The Ras-binding domain (RBD) of Raf-1 binds the E31R and E31K Ras mutants less tightly than the wild-type, E31A, and E31D Ras proteins; the introduction of the positively charged Lys or Arg residue at position 31 specifically impairs the binding of Ras with the Raf-1 RBD. On the other hand, the ability of the oncogenic Ras protein to activate Raf-1 in HEK293 cells was only partially reduced by the E31A mutation but was drastically impaired by the E31K mutation. Correspondingly, RasG12V(E31K) as well as Rap1A, but not RasG12V(E31R), exhibited abnormally tight binding with the cysteine-rich domain of Raf-1. On the other hand, the E31A, E31R, and E31K mutations, but not the E31D mutation, enhanced the RalGDS RBD-binding activity of Ras, indicating that the negative charge at position 31 of Ras is particularly unfavorable to the interaction with the RalGDS RBD. RasG12V(E31K), RasG12V(E31A), and Rap1A stimulate the RalGDS action more efficiently than the wild-type Ras in the liposome reconstitution assay. All of these results clearly show that the sharp contrast between the characteristics of Ras and Rap1A, with respect to the interactions with Raf-1 and RalGDS, depends on their residues at position 31.

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MAPK/ERKs (1, 2). The Ras-binding domain (RBD) was mapped to amino acid residues 51–131 of Raf-1 (3, 4). In addition to this domain, the cysteine-rich domain (CRD) of Raf-1 was recently found to interact with Ras (5, 6). Both of these interactions of Ras with the two domains of Raf-1 are necessary for transformation by Ras and for the activation of Raf-1 (6, 7). Mutational analyses have shown that the effector region (amino acid residues 32–40) and the activator region (amino acid residues 26–31 and 41–48) of Ras are involved in the interactions with the Raf-1 RBD and the Raf-1 CRD, respectively (1, 6).

The guanine nucleotide exchange factors for the Raf proteins, such as RalGDS and RGL, have been reported to associate with Ras in a GTP-dependent manner; the interaction was abolished by mutations in the effector region of Ras (8–12). It has been shown that Ras enhances the guanine nucleotide-exchange activity of RalGDS in COS cells (13). The Ras-binding domain of RalGDS has been identified (8–12), and there is no apparent sequence homology between the RBDs of the Raf family and those of the RalGDS family. Recently, however, the RalGDS RBD was shown to have a ubiquitin-like fold similar to that of the Raf-1 RBD (14, 15).

The Rap1A (or Krev-1) protein belongs to the Ras superfamily, and has the same amino acid sequence in the effector region as that of Ras (16–18). However, at position 31, Rap1A has Lys, whereas Ras has Glu. The Rap1A RBD prefers Rap1A over Ras, but the Raf-1 RBD prefers Ras over Rap1A (19). The crystal structures of the complexes of the Raf-1 RBD with Rap1A-GMPPNP (the wild-type) and the E30D/K31E mutant have been solved by x-ray crystallography (20, 21). In these two complex structures, Lys44 of the Raf-1 RBD is not involved in the interaction with the wild-type Rap1A but forms strong and weak salt bridges with Glu31 and Asp31, respectively, of the E30D/K31E mutant Rap1A. The replacement of Lys by Glu at position 31 of Rap1A increased the affinity for the Raf-1 RBD and decreased that for the RalGDS RBD (21). It was proposed, therefore, that the negative charge of Glu44 creates a favorable complementary interface for the Ras-Raf interaction (21). We have found that the Rap1A-type E31K and D30E/E31K mutations impair the GAP-induced increase in the Ras GTPase activity and the ability to induce neurite outgrowth of PC12 cells (22). Other groups have also reported that the E31K and D30E/E31K mutations reduce the transforming activity of oncogenic Ras in NIH3T3 cells (23, 24). In the present study, we analyze in more detail how the Glu residue at position 31 of Ras is involved in the interaction with the two different targets, we tested more mutations at position 31 for their effects on various Ras functions.

The Ras and Rap1A proteins can bind to the Raf and RalGDS families. Ras and Rap1A have Glu and Lys, respectively, at position 31. In the present study, we analyzed the effects of mutating the Glu at position 31 of the c-Ha-Ras protein to Asp, Ala, Arg, and Lys on the interactions with Raf-1 and RalGDS. The Ras-binding domain (RBD) of Raf-1 binds the E31R and E31K Ras mutants less tightly than the wild-type, E31A, and E31D Ras proteins; the introduction of the positively charged Lys or Arg residue at position 31 specifically impairs the binding of Ras with the Raf-1 RBD. On the other hand, the ability of the oncogenic Ras protein to activate Raf-1 in HEK293 cells was only partially reduced by the E31A mutation but was drastically impaired by the E31K mutation. Correspondingly, RasG12V(E31K) as well as Rap1A, but not RasG12V(E31R), exhibited abnormally tight binding with the cysteine-rich domain of Raf-1. On the other hand, the E31A, E31R, and E31K mutations, but not the E31D mutation, enhanced the RalGDS RBD-binding activity of Ras, indicating that the negative charge at position 31 of Ras is particularly unfavorable to the interaction with the RalGDS RBD. RasG12V(E31K), RasG12V(E31A), and Rap1A stimulate the RalGDS action more efficiently than the wild-type Ras in the liposome reconstitution assay. All of these results clearly show that the sharp contrast between the characteristics of Ras and Rap1A, with respect to the interactions with Raf-1 and RalGDS, depends on their residues at position 31.

The Ras protein in the GTP-bound form associates with and induces the activation of the Raf-1 serine/threonine kinase (for a review, see Ref. 1). The activated Raf-1 phosphorylates and activates the MAPK/ERKs, which in turn activate the MAPK/ERKs (1, 2). The Ras-binding domain (RBD) was mapped to amino acid residues 51–131 of Raf-1 (3, 4). In addition to this domain, the cysteine-rich domain (CRD) of Raf-1 was recently found to interact with Ras (5, 6). Both of these interactions of Ras with the two domains of Raf-1 are necessary for transformation by Ras and for the activation of Raf-1 (6, 7). Mutational analyses have shown that the effector region (amino acid residues 32–40) and the activator region (amino acid residues 26–31 and 41–48) of Ras are involved in the interactions with the Raf-1 RBD and the Raf-1 CRD, respectively (1, 6).

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EXPERIMENTAL PROCEDURES

Plasmid Construction—Mutations were introduced into a synthetic human c-Ha-ras gene (25) by site-directed mutagenesis with two PCR steps. The ras genes with the mutations in the oncogenic G12V background were subcloned into pMAMneo (CLONTECH) (22) for induced expression in PC12 cells and into pCMV5 (26) for transient expression in HEK293 cells. To prepare the Raf-1 RBD as a GST fusion protein, the DNA fragment corresponding to amino acid residues 51–151 was amplified by PCR from the human full-length ras gene (27, 28) and was subcloned into pGEX-4T-3 (Amershams Pharmacia Biotech). The gene for the rat RalGDS RBD was prepared by PCR with the primers, GCCGCGGATCCGGGCGGAC GCGGCTGC- GACTTAAAGATGCTTGGCAGTTGAG, from a rat brain cDNA library (CLONTECH). To prepare the GST fusion form of the RalGDS RBD PCR product was digested with BamHI and SalI and was then inserted into the BamHISalI sites of pGEX-4T-1.

Protein Purification—The wild-type and mutant Ras proteins were expressed in Escherichia coli and were purified by chromatography on DEAE-Sephaloc and Sephadex G-75 (Amershams Pharmacia Biotech) columns as described (22). To obtain the post-translationally modified forms of Ras, RasG12V(E31A), RasG12V(E31R), and Rap1A, each protein was produced in Sf9 cells infected with baculoviruses containing either the ras, the rasG12V (E31A), the rasG12V(E31R) or the rap1A gene and was purified as described previously (29, 30). The GST fusion forms of the Ras binding domains, GST-Raf-1 (51–131) and GST-RalGDS (769–895), were each expressed in E. coli at 30 °C and were purified using a glutathione-Sepharose 4B (Amershams Pharmacia Biotech) according to the manufacturer instructions. A recombinant Xenopus kinase-negative MAPKK (KN-MAPK) in a GST fusion form and a histidine-tagged Xeno- pus MAPKK were purified from E. coli as described (31, 32). For the liposome reconstitution assay, baculoviruses producing a GST-fused Ras (Gst-Ras), GST-RalB, GST-Rap1A, or GST-RalGDSb (E31K) were generated as described (33, 34). All procedures of passage, infection, and fractionation of S9 cells and the isolation of recombinant baculoviruses were carried out as described (35). The post-translationally modified forms of Ras, RasG12V(E31A), GST-RalGDSb were purified from the membrane fraction of Sf9 cells as described (36). GST-RalGDSb was purified from E. coli as described (33, 34).

In Vitro Binding Assays—One μg of either GST-Raf-1 (51–131) or GST-RalGDS (769–895), in 150 μl of phosphate-buffered saline containing 5 mM MgCl2 and 0.5% Triton X-100, was mixed with 10 μl of glutathione-Sepharose 4B beads suspended in phosphate-buffered saline. The mixture was incubated at 4 °C for 1 h with various amounts of either wild-type or mutant Ras, which had been complexed with GTPγS as described previously (37). After the incubation, the resin was washed with 20 μl of Tris-HCl buffer (pH 7.5) containing 5 mM MgCl2 and 150 mM NaCl. The bound proteins were eluted from the resin by boiling in 10 μl of Laemmli’s buffer and were fractionated by SDS-PAGE. The Raf-1 RBD-GST fusion protein was purified as described previously (38). Briefly, MBP-Raf-1(136–206), immobilized on the amylose resin, was incubated with 12 pmol of the wild-type or mutant Ras or Rap1A bound with GTPγS in 20 μl of Tris-HCl buffer (pH 7.5) containing 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM MgCl2, and 0.1% Lubrol PX. After an incubation at 4 °C for 2 h, the resin was washed, and the bound proteins were eluted and subjected to SDS-PAGE. Immuneblots were probed with the anti-Ras antibody RAS004 (38) or with an anti-Rap1A antibody (Santa Cruz Biotechnology). The kinase reaction with a histidine-tagged MAPKK and 6 μl of reaction mixture (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.67 mM EDTA, 1 mM n-octylglucoside (1/25 of critical micellar concentration), 1 mM DTT, and 0.08 mg/ml bovine serum albumin). The mixture was centrifuged on a discontinuous sucrose density gradient at 100,000 × g for 2 h at 4 °C, and the liposomes were recovered at the 0.15–1.2 mM sucrose interface. GST-RalGDSb was incubated with the liposomes that contained the [3H]GDP-bound form of GST-Ral (0.5 pmol) and the GTPyS-bound form of GST-Ras, GST-Rap1A, GST-RasG12V(E31K), or Ras(E31A) were made, added to the liposomes, and incubated for 10 min on ice in 400 μl of reaction mixture (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.67 mM EDTA, 1 mM n-octylglucoside (1/25 of critical micellar concentration), 1 mM DTT, and 0.08 mg/ml bovine serum albumin). The mixture was centrifuged on a discontinuous sucrose density gradient at 100,000 × g for 2 h at 4 °C, and the liposomes were recovered at the 0.15–1.2 mM sucrose interface. GST-RalGDSb was incubated with the liposomes that contained the [3H]GDP-bound form of GST-Ral (0.5 pmol) and the GTPγS-bound form of GST-Ras, GST-Rap1A, GST-RasG12V(E31K), or Ras(E31A) (1.5–2 pmol) in 80 μl of reaction mixture (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 200 μM GTP, 0.03% CHAPS, and 1 mM DTT) for 30 min at 30 °C. Assays were quantified by rapid filtration on nitrocellulose filters (41).

RESULTS AND DISCUSSION

To determine which properties of the amino acid residue at position 31 of Ras are necessary for the interactions with its targets, Raf-1 and RalGDS, we substituted the Glu31 of the c-Ha-ras protein by Asp, Ala, and Arg and analyzed the effects of these mutations, as well as those of the previously reported Lys mutation.

Binding to the Raf-1 RBD—First, we examined the effects of the E31D, E31A, E31R, and E31K mutations on the interaction of Ras with the Raf-1 RBD (amino acid residues 51–131 of Raf-1). Various concentrations of each mutant Ras protein in the GTPγS-bound form were added to the GST-Raf-1(51–131) fusion protein mixed with glutathione-Sepharose, and the amounts of the Ras protein in the precipitates were analyzed by Western blotting (Fig. 1). The Ras(E31D) and Ras(E31A) mutant proteins were found to bind the Raf-1 RBD as strongly as the wild-type Ras (Fig. 1). In contrast, the E31R and E31K mutations reduced the extent of the Raf-1 RBD binding by about 5-fold (Fig. 1). It has been reported that Glu31 and Arg33 of the Ras-type mutant of the Raf-1 protein (E30D/E31E) make stronger and weaker salt bridges, respectively, with Lys84 of the Raf-1 RBD (21) and that the K84A mutation drastically reduced the affinity of the Raf-1 RBD for Ras(E31D) (43). Therefore, it has been proposed that the salt bridge between Lys84 of Raf-1 and Glu31 of Ras is also important for the Ras-Raf association (21). However, this study showed that the Ras(E31A) mutant can bind to the Raf-1 RBD as efficiently as the wild-type Ras. Therefore, the introduction of a basic residue at position 31 causes significant repulsion between Ras and the Raf-1 RBD, whereas the acidic residue characteristic of Ras does not appear to create any attractive interface for the Ras-Raf interaction. On the
Various amounts of the E31D (E) mutant or the wild-type Ras (○) were loaded with GTPγS and tested for GST-Raf-1(51–131) binding, as described under “Experimental Procedures.” The intensity of the Ras band was estimated by densitometry. When GST was used instead of GST-Raf-1(51–131), no bands were detected. The data represent the average of at least three experiments.

other hand, the D33A mutation of Ras impaired its ability to bind the Raf-1 RBD. 2 Consistently, it was reported that the D33N mutation of Ras causes a drastic reduction in the affinity for a Raf-1 fragment containing the RBD (4). Thus, for Ras binding with the Raf-1 RBD, the salt bridge from the Lys84 of Raf-1 to the Asp33 of Ras is much more important than that to the Ghu31 of Ras. This difference in the interactions of the Raf-1 RBD with Glu31 and Asp33 between the Ras and Rap1A backgrounds may be due to the structural differences at other positions within these proteins; Ras has Ile21 and His27, corresponding to the Rap1A residues Val21 and Ile27, respectively, which are involved in the interface with the Raf-1 RBD in the crystal structure (20).

Activation of Raf-1—Next, to examine the effect of these mutations on the ability of Ras to activate Raf-1, we transfected HEK293 cells with each mutant ras gene in the oncogenic G12V background. The anti-Raf-1 immunoprecipitate was incubated with [γ-32P]ATP in the presence of MAPKK and the KN-MAPK, and the incorporation of 32P into the KN-MAPK was quantitated (Fig. 2A). The amounts of the mutant Ras proteins expressed in HEK293 cells were nearly the same (Fig. 2B). The E31A and E31D mutations had no effects on the stimulation of the MAPKK kinase activity of Raf-1. On the other hand, the Raf-1 activation activity of Ras was partially impaired by the E31R mutation and, furthermore, was drastically decreased by the E31K mutation.

Activities in PC12 Cells—To analyze the effects of these mutations on the activities of Ras in another cell type, pheochromocytoma (PC) 12 cells were stably transfected with pM-AMneo vectors that conditionally express the RasG12V, RasG12V(E31D), RasG12V(E31A), RasG12V(E31R), or RasG12V(E31K) protein. The RasG12V(E31K)-expressing cells did not extend neurites, as reported (22), whereas the RasG12V(E31D)-, RasG12V(E31A)-, and RasG12V(E31R)-expressing cells extended neurites as well as the cells expressing RasG12V (3). The expression levels of RasG12V, RasG12V(E31D), RasG12V(E31A), and RasG12V(E31R) were nearly the same (Fig. 4A). The level of RasG12V(E31K) was slightly lower than those of other mutants, probably because of its autoinhibitory activity (22), but was nevertheless sufficiently higher than that of the endogenous Ras (Fig. 4A). In conclusion, the neurite-inducing activity of RasG12V was abolished by the E31K mutation but not affected by the E31D, E31A, and E31R mutations (Fig. 3). It should be emphasized that the E31K mutation impairs this function of Ras much more drastically than the E31R mutation. Furthermore, the abilities of these RasG12V, RasG12V(E31A), RasG12V(E31D), RasG12V(E31R), and RasG12V(E31K) mutants to induce ERK activation in PC12 cells were examined from delayed mobility of the phosphorylated form on SDS-PAGE (Fig. 4B) and also by an in-gel kinase assay (data not shown). The ERK activities in the RasG12V(E31A)-, RasG12V(E31D)-, and RasG12V(E31R)-expressing cells were elevated as well as in the RasG12V-expressing cells (Fig. 4B). On the other hand, in the cell expressing RasG12V(E31K), the ERK activity was the same as in the control cell. In summary, the abilities of the Ras mutants to activate Raf-1 correlate with their abilities to induce ERK activation and also with the induction of neurite outgrowth in PC12 cells. Therefore, the inability of RasG12V(E31K) to induce neurite outgrowth is probably due to the inability to activate Raf.

Binding to Raf-1 CRD—Ras activation of Raf-1 in both HEK293 and PC12 cells was decreased more severely by the E31K mutation than by the E31R mutation, although Ras(E31R) and Ras(E31K) had nearly the same Raf-1 binding activities. Recently, we found that Ras binds to the Raf-1 CRD (amino acid residues 132–206) in a GTP-independent manner, which is necessary for the activation of Raf-1 (6) while another group reported a weak GTP-dependence of the binding of Ras with the Raf-1 CRD (5). Furthermore, we found that RasG12V(E31K) as well as Rap1A has an abnormally enhanced ability to bind the Raf-1 CRD (30). It seems to be

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through this abnormal interaction of Lys\(^{31}\) with the Raf-1 CRD that Rap1A\(^{G12V}\) and Ras\(^{G12V}(E31K)\) dominantly inhibit the Raf-1 activation by Ras\(^{G12V}\), which is related to the Ras-antagonizing activity of Rap1A (30). In this study, we examined the ability of Ras\(^{G12V}(E31R)\) to bind to the Raf-1 CRD. Since the binding of Ras to the CRD requires post-translational modification of the C terminus of Ras, we purified the modified form of Ras\(^{G12V}(E31R)\) from Sf9 cells, and used it for the Raf-1 CRD binding assay. The Ras\(^{G12V}(E31R)\) was shown to bind the Raf-1 CRD as strongly as Ras (the GDP-bound forms, data not shown). Therefore, it is likely that the difference in the Raf-1 activation ability between the E31K and E31R mutants is due to the difference in the ability to bind the Raf-1 CRD. The reason why the extent of Raf-1 activation by Ras\(^{G12V}(E31K)\) is much lower than that by Ras\(^{G12V}(E31R)\) may be that Ras\(^{G12V}(E31K)\) autoinhibits the Raf-1 activation. The present mutagenesis analyses indicate that the wild-type Glu\(^{31}\) residue of Ras is not required for interaction with either RBD or CRD of Raf-1. In contrast, the Lys residue replacing Glu\(^{31}\) appears to interact with the Raf-1 CRD. It is interesting that the Raf-1 CRD very specifically recognizes the amino acid at position 31 and prefers Lys to Arg.

**Binding to the Raf1-Binding Domain (RBD)**—As in the case of the Raf-1 binding domain (RBD), we tested the Ras\(^{E31D}\), Ras\(^{E31A}\), Ras\(^{E31R}\), and Ras\(^{E31K}\) mutant proteins in the GDP-bound form for their abilities to bind with a GST fusion protein of the rat Raf-1 RBD, the C-terminal 127 residues corresponding to the mouse Raf1-RBD (10). Interestingly, the Ras\(^{E31A}\), Ras\(^{E31R}\), and Ras\(^{E31K}\) mutants bound the RalGDS RBD more tightly than the wild-type Ras (Fig. 6). In contrast, the E31D mutation did not affect the interaction of Ras with the RalGDS RBD. This indicates that the negative charge at position 31 of Ras particularly weakens the interaction with the RalGDS RBD.

**Binding Preference Switching**—The affinities of Ras for the Raf-1 RBD and Rap1A for the RalGDS RBD are 100 times higher than those of Ras for the RalGDS RBD and Rap1A for the Raf-1 RBD, respectively (19). Furthermore, the K31E mutation of Rap1A has been reported to cause a 15-fold increase in the affinity of Rap1A for the Raf-1 RBD and a 20-fold decrease in that for the RalGDS RBD (21). Symmetrically, in this study, the E31K mutation of Ras was found to decrease the Raf-1 RBD-binding (about 5-fold) and to increase the RalGDS RBD-binding (about 5-fold). Therefore, not only in the Rap1A background (21) but also in the Ras background, the residue at position 31 serves as the determinant for the preference of either the Raf-1 RBD or the RalGDS RBD. On the other hand, the modes of Ras binding are similar between the Raf-1 and RalGDS RBDs, in that only one type of charge at position 31 of Ras is required for interaction with either Raf-1 or Rap1A.
Various amounts of the E31D (E31D), Ral with GST-Rap1A; (E31R), Ral alone; then the remaining radioactivity was counted. With the indicated concentrations of RalGDS for 30 min at 30 °C, and was incorporated with the GDP/GTP exchange activity of RalGDS toward Ral in the liposomes, RalGDS stimulated the dissociation of GDP from Ral (Fig. 7). These Ras mutants, as well as Rap1A, exhibited higher RalGDS activation activities than that of the wild-type Ras. Thus, in the reconstitution assay system, the RalGDS-activation activity of Ras/Rap1A depends on the RalGDS-RBD-binding activity.

All of the results of the present study demonstrate that the sharp contrast between Ras and Rap1A, in terms of the Raf-1 and RalGDS interactions, depends on the characteristics of their residues at position 31. Intriguingly, the mechanisms of these interactions appear to be different from the attraction of the charge at this position for the target domain.

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