Site-Directed Mutagenesis, Fluorescence, and Two-Dimensional NMR Studies on Microenvironments of Effector Region Aromatic Residues of Human c-Ha-Ras Protein†

Kazuhiko Yamasaki,‡,§ Mikako Shirouzu,‡ Yutaka Muto,‡ Junko Fujita-Yoshigaki,‡,∥ Hiroshi Koide,‡,⊥ Yutaka Ito,‡,# Gota Kawai,‡,○ Seisuke Hattori,▽ Shigeyuki Yokoyama,*,‡ Susumu Nishimura,●,△ and Tatsuo Miyazawa×,◇

Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan, Division of Biochemistry and Cellular Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Ogawahigashi, Kodaira, Tokyo 187, Japan, Protein Engineering Research Institute, Furuedai, Suita, Osaka 565, Japan, and National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan

Received June 9, 1993; Revised Manuscript Received October 5, 1993®

ABSTRACT: The Tyr residues in positions 32 and 40 of human c-Ha-Ras protein were replaced by site-directed mutagenesis (Y32F, Y32W, Y40K, and Y40W) to examine their roles in the signal-transducing activity and the sensitivity to the GTPase activating protein (GAP). The signal-transducing activity of the oncogenic Ras protein in PC12 cells was lost upon mutations Y32F and Y40K, but retained upon mutations Y32W and Y40W. These results suggest that residues 32 and 40 are both required to have aromatic groups and residue 32 is further required to have a hydrogen donor. On the other hand, three mutations (Y32F, Y32W, and Y40W) caused no appreciable reduction in either GAP-binding affinity or GAP sensitivity. By the Y40K mutation, GAP-binding affinity was slightly lowered, while GAP sensitivity was drastically impaired. Therefore, for residues 32 and 40 of Ras, interactions with GAP appear to be different from those with the target of signal transduction in the PC12 cell. As for the Y32W-Ras protein bound with an unhydrolyzable GTP analogue (GMPPNP), the Trp32 fluorescence is appreciably red-shifted, weaker, and more susceptible to KI quenching as compared to that of the GDP-bound form. Two-dimensional NMR spectroscopy with selectively deuterated Ras proteins revealed fewer and weaker nuclear Overhauser effects on the aromatic protons of Trp32 in the GMPPNP-bound form than in the GDP-bound form. This indicates that the side chain of Trp32 is more exposed to the solvent in the GMPPNP-bound form than in the GDP-bound form. In contrast, fluorescence and NMR analyses for the Y40W-Ras protein indicate that the microenvironment of Trp40 is only slightly affected upon GDP–GMPPNP exchange. Accordingly, the aromatic residue in position 32, rather than that in position 40 of the Ras protein, becomes exposed to the surface of the protein upon GDP→GTP exchange, allowing hydrogen bonding with a target molecule in the signal-transduction pathway.

The human c-Ha-*ras* gene is a member of the *ras* protooncogene family and encodes 189 amino acid residues (Shih et al., 1981; Krontiris & Cooper, 1981; Perucho et al., 1981; Barbacid, 1987; Nishimura & Sekiya, 1987). The Ras protein binds GDP and GTP and hydrolyzes the bound GTP to GDP and inorganic phosphate. Oncogenic *ras* genes found in cancers have a mutation that impairs the guanosine-5′-triphosphatase (GTPase)[1] activity of Ras, for example, in position 12, 13, or 61 (Barbacid, 1987). The GTP-bound form of Ras is active, while the GDP-bound form is inactive in some cellular activities, such as cell proliferation and differentiation (Satoh et al., 1987, 1988; Trahey & McCormick, 1987). The Ras protein is thought to be involved in a signal-transduction pathway; microinjected or overexpressed oncogenic Ras can trigger several growth factor-related cell reactions, including neuronal differentiation of rat phaeochromocytoma (PC12) cells (Bar-Sagi & Feramisco, 1985; Noda et al., 1985) and maturation of Xenopus oocytes (Birchmeier et al., 1985). Furthermore, the ratio of [GTP-bound form]/[GDP-bound form] increases in response to several growth factors (Gibbs et al., 1990; Satoh et al., 1990a,b; Burgering et al., 1991).

The GTPase activating protein (GAP) stimulates the GTPase activity of the Ras protein (Trahey & McCormick,

† This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan. The results of this study were presented in part at the XIV International Conference on Magnetic Resonance in Biological Systems, Warwick, U.K., September 1990.

* To whom correspondence should be addressed. Telephone: +81-3-3812-1805. Telefax: +81-3-5684-2394.

‡ University of Tokyo.

§ Present address: Protein Engineering Research Institute, Furuedai, Suita, Osaka 565, Japan.

∥ Present address: Department of Physiology, Nihon University School of Denistry at Matsudo, Sakae, Matsudo, Chiba 271, Japan.

⊥ Present address: Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuda, Midori-ku, Yokohama, Kanagawa 227, Japan.

Present address: Biodesign Research Group, Institute of Physical and Chemical Research, Hirosawa, Wako, Saitama 351-01, Japan.

○ Present address: Department of Industrial Chemistry, Faculty of Engineering, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan.

▽ National Center of Neurology and Psychiatry.

● National Cancer Center Research Institute.

△ Present address: Banyu Tsukuba Research Institute in Collaboration with Merck Research Laboratories, Banyu Pharmaceutical Co. Ltd., Tsukuba Techno-Park Oho, Ohkubo 3, Tsukuba 300-33, Japan.

× Protein Engineering Research Institute.

◇ This author is deceased.

® Abstract published in *Advance ACS Abstracts*, December 1, 1993.

[1] Abbreviations: GAP, GTPase activating protein; GMPPNP, guanylyl imidodiphosphate; GTPase, guanosine-5′-triphosphatase; HOHAHA, two-dimensional homonuclear Hartmann–Hahn spectroscopy; NOESY, two-dimensional nuclear Overhauser effect and exchange spectroscopy.

0006-2960/94/0433-65$04.50/0

1987; Gibbs et al., 1988; Trahey et al., 1988; Vogel et al., 1988), and is suggested to be one of the target molecules of the Ras protein in signal transduction (Adari et al., 1988; Cales et al., 1988; Yatani et al., 1990; Martin et al., 1992; Duchesne et al., 1993). In addition, GAP binds to the protein tyrosine kinase domains of activated growth factor receptors and is phosphorylated on tyrosine residue(s) (Molloy et al., 1989; Ellis et al., 1990; Kaplan et al., 1990; Kazlauskas et al., 1990; Liu & Pawson, 1991).

Recently, Raf-1 kinase, mitogen-activated protein kinase (MAP kinase), and MAP kinase kinase (MAPKK) have been reported to interact directly with the Ras protein and suggested to be downstream target proteins (Van Aelst et al., 1993; Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). Thus, GAP and these kinases (and other unknown factors) seem to be the targets in different pathways.

The region of the Ras protein that is necessary for interaction with the target molecule (the effector region) consists of the residues from Tyr32 to Tyr40 (Sigal et al., 1986). Some mutations in this region deprive Ras of its signal-transducing activity, GAP-enhanced GTPase activity, and/or interaction with the Raf-1 kinases (Willumsen et al., 1986; Sigal et al., 1986; Clanton et al., 1987; Adari et al., 1988; Cales et al., 1988; Stone et al., 1988; McCormick, 1989; Farnsworth et al., 1991; Stone & Blanchard, 1991; Van Aelst et al., 1993; Moodie et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). However, the roles of each effector region residue in the interaction with the target molecules have yet to be established.

The crystal structures of the GDP-bound and GTP-bound Ras proteins have revealed that the conformation of the effector region is markedly different (Milburn et al., 1990; Schlichting et al., 1990a). However, the details of the effector region conformations of the GTP-bound Ras proteins as reported by the two groups appreciably differ from each other; the effector region residues such as Tyr32 of one molecule are in close contact with those of a neighboring molecule in the crystal (Pai et al., 1990; Milburn et al., 1990; Schlichting et al., 1990a).

NMR studies of the GDP- and GTP-bound Ras proteins in aqueous solution have been reported (Schlichting et al., 1988; Campbell-Burk, 1989; Campbell-Burk et al., 1989, 1992; Ha et al., 1989; Hata-Tanaka et al., 1989; John et al., 1989; Yamasaki et al., 1989, 1992; Schlichting et al., 1990b; Redfield & Papastavros, 1990; Miller et al., 1992; Muto et al., 1993). In our previous study, the proton resonances due to an antiparallel β-sheet structure, including the C-terminal part of the effector region, were assigned, and the conformation of this part was found to be significantly distorted in the GTPγS-bound form (Yamasaki et al., 1989). Such distortion of the β-sheet structure was not found by the X-ray crystallographic studies (Milburn et al., 1990; Schlichting et al., 1990a), but has in fact been confirmed recently by a molecular-dynamics study in search of the solution structure of the Ras protein (Foley et al., 1992). Therefore, more detailed studies of the conformational differences between the GDP- and GTP-bound Ras proteins in solution will be important.

Fluorescence studies of Ras proteins have also been reported (John et al., 1990; Neal et al., 1990; Skelly et al., 1990; Antonny et al., 1991; Rensland et al., 1991). Since the wild-type Ras protein lacks Trp residues, mutations to Trp are useful for elucidating the microenvironment of the mutated sites. Thus, Phe28 was repalced with Trp to study the interaction of the Ras protein with GDP or GTP (Skelly et al., 1990), while Leu56 or Tyr64 was replaced by Trp to monitor the GTPase and GDP/GTP exchange reactions (Antonny et al., 1991).

In the present study, the roles of Tyr32 and Tyr40 in the effector region of the c-Ha-Ras protein were analyzed by site-directed mutagenesis. Since the mutations Y32W and Y40W did not disrupt the signal-transducing activity and the ability to interact with GAP, further fluorescence and NMR analyses were performed using these Trp residues as probes to elucidate the microenvironmental change upon ligand exchange from GDP to GMPPNP (an unhydrolyzable GTP analogue).

MATERIALS AND METHODS

The ras Genes. Chemically synthesized Ha-*ras* genes were expressed under the control of the *trp* promoter (Miura et al., 1986). For assay of the signal-transducing activity, the full-length Ras proteins consisting of 189 amino acid residues were used. For fluorescence and NMR measurements and for GTPase assay, truncated Ras proteins consisting of 171 amino acid residues (Ha et al., 1989) were used, which are as active as the full-length Ras protein with regard to guanine nucleotide binding and GTP hydrolysis (Fujita-Yoshigaki et al., 1992).

Site-Directed Mutagenesis. The techniques used to manipulate genes were as described (Maniatis et al., 1982). The *Cla*I–*Sal*I 0.5-kb fragment carrying the *ras* gene from the expression vector (Miura et al., 1986) was subcloned in the M13mp19 vector with the *Acc*I, *Sal*I, and *Bgl*II sites. Oligonucleotide-directed mutagenesis was carried out with the *in vitro* mutagenesis system Muta-Gene (Bio-Rad). Oligonucleotide primers, 5′GTAGATGAGTTCGACCCGACT for Y32F-Ras, 5′GTAGATGAGTGGGACCCGACT for Y32W-Ras, 5′GAAGACTCTAAACGTAAGCAG for Y40K-Ras, and 5′GAAGACTCTTGGCGTAAGCAG for Y40W-Ras (mismatching nucleotides are underlined), were prepared with a GENET A-II (Zeon) DNA synthesizer and purified by polyacrylamide gel electrophoresis. After the mutagenesis, the *Bss*HII–*Sal*I 0.4-kb fragment from the vector expressing the truncated wild-type Ras protein (Ha et al., 1989) or the full-length Ras protein with the G12V mutation (Miura et al., 1986) was replaced with that from the mutant *ras* genes in the M13mp19 vectors. *Escherichia coli* strains HB101 and DL39 (LeMaster & Richards, 1988) were transformed with the vectors.

Preparation of Ras Proteins Bound with GDP or GMPPNP. The wild-type and mutant Ras proteins bound with GDP were purified from induced *E. coli* cells as described (Miura et al., 1986; Ha et al., 1989). The purified wild-type Ras, Y32W-Ras, and Y40W-Ras proteins (truncated type) showed specific absorbances at 280 nm of 0.56, 0.70, and 0.68 $cm^{-1}\cdot mg^{-1}\cdot mL$, respectively, as determined with the BCA protein assay reagent (Pierce). The Ras proteins bound with GMPPNP were prepared as follows. The GDP-bound Ras protein (0.5 mM) was incubated with 2 mM GMPPNP, 5 mM EDTA, and 10 units/mL apyrase (Sigma) for 15 min at 37 °C (apyrase was used to hydrolyze the free GDP to GMP and inorganic phosphate). After the incubation, the free nucleotide was removed by ultrafiltration using Centricon-10 (Amicon). This procedure of incubation/filtration was repeated 3 times. A small part of the protein was denatured with trichloroacetic acid, and released nucleotides were analyzed by HPLC with a DEAE-2SW column (Shimadzu); more than 93% was GMPPNP.

Assay of Signal-Transducing Activities of Mutant Ras Proteins. Double-mutant G12V/Y32W-, G12V/Y32F-, and G12V/Y40W-Ras proteins (full-length type) were purified and concentrated to 10 mg/mL by ultrafiltration with Centricon-10. Mutant protein solutions were microinjected into PC12 cells, and after 24 h, the responses of the cells were observed.

Table 1: Signal-Transducing Activities and Rate Constants for GTPase and Nucleotide Dissociation

	wild-type	Y32F	Y32W	Y40K	Y40W
neurite outgrowth[a]	+	− (−)[b]	+ (+)[c]	ND (−)[d]	+
dissociation rate constant (min^{-1})					
GDP	4.9×10^{-3} [e]	ND	2.2×10^{-3}	ND	5.6×10^{-3}
GTP	4.4×10^{-3} [e]	ND	5.0×10^{-3}	ND	6.2×10^{-3}
intrinsic GTPase rate constant (min^{-1})	0.018	0.022	0.048	0.010	0.016

[a] Proteins with an additional G12V mutation were used. [b] Stone et al. (1988). [c] Stone and Blanchard (1991). [d] Sigal et al. (1986). [e] Fujita-Yoshigaki et al. (1992).

Measurements of GTP Hydrolysis and Nucleotide Dissociation Rates of Mutant Ras Proteins. Rat GAP was purified from *E. coli* cells expressing the cloned GAP gene (unpublished results) by chromatography on columns of DEAE-Sephacel (Pharmacia) and TSK gel AF-heparin Toyopearl 650 (Tosoh) (to be described elsewhere). GTPase activities of mutant Ras proteins were measured by a modification of the procedure described by Trahey and McCormick (1987). The Ras protein (4 μM) in buffer A [50 mM Tris-HCl (pH 7.5), 1 mM phenylmethanesulfonyl fluoride, and 1 mM dithiothreitol] containing 150 μCi/mL [8,5′-^{3}H]GTP (NEN) and 5 mM EDTA was incubated at 37 °C for 5 min, and free nucleotides were removed using Centricon-10 filtration. The Ras protein bound with ^{3}H-labeled GTP in buffer A containing 5 mM $MgCl_2$ was incubated at 37 °C for up to 20 min. After the incubation, the protein solution was boiled at 80 °C for 3 min, and the denatured protein was removed by centrifugation. Free nucleotides (GTP/GDP) in the supernatant were separated by TLC, and TLC fractions containing GTP or GDP were assayed with a scintillation counter, Model LSC-700 (Aloka). To analyze affinities and sensitivities of mutant Ras proteins to GAP, the amounts of GDP formed in 2 or 10 min in the Ras-bound state were determined at various concentrations of GAP between 0.016 and 0.8 μM. Dissociation rates of GDP and GTP from the Y32W- and Y40W-Ras proteins were measured as described previously (Fujita-Yoshigaki et al., 1992).

Fluorescence Measurements. The Ras protein (0.1 mg/mL) was dissolved in 60 mM phosphate buffer (pH 7.5) containing 10 mM $MgCl_2$ and 150 mM NaCl. Tryptophan fluorescence upon excitation at 290 nm was measured at 25 °C with a Hitachi F-4000 fluorescence spectrophotometer. Fluorescence quenching by KI was measured at 340 nm and analyzed according to the Stern–Volmer equation: $F_0/F = 1 + K_{sv}[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the concentration (in molar) of the quencher (KI), and K_{sv} is the Stern–Volmer quenching constant (Lehrer, 1971).

Deuterium Substitution of Ras Proteins. [1,2,3,4,5-^{2}H]Phenylalanine was synthesized with ^2H_2O and $^2H_2SO_4$ as described (Milstein & Kaufman, 1975). [3,5-^{2}H]Tyrosine was prepared with ^{2}HCl as described (Cohen & Putter, 1970). For efficient biosynthetic incorporation of deuterium-substituted amino acids, transaminase-deficient polyauxotrophic *E. coli* strain DL39 (LeMaster & Richards, 1988), which requires aspartate, isoleucine, leucine, valine, phenylalanine, and tyrosine, was cultured under the conditions described previously (Yamasaki et al., 1992).

NMR Measurements. The Ras protein was dissolved (2.5 mM) in ^2H_2O (99.95% ^{2}H, Commissariat à l'Energie Atomique) containing 20 mM [$^2H_{11}$]Tris (99.4% ^{2}H, MSD), 150 mM NaCl, and 10 mM $MgCl_2$, and the pH was adjusted to 7.5 with ^{2}HCl (99% ^{2}H, Merck) (Yamasaki et al., 1989). The Ras protein was also dissolved (2.5 mM) in ^1H_2O solution (pH 7.5) containing 60 mM sodium phosphate, 150 mM NaCl, 10 mM $MgCl_2$, and 10% ^2H_2O.

400-MHz proton NMR spectra were recorded on a Bruker AM-400 spectrometer at a probe temperature of 37 °C. Chemical shifts were determined relative to methyl proton resonances of the internal standard sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Two-dimensional nuclear Overhauser effect and exchange spectroscopy (NOESY) (Jeener et al., 1979) was carried out at a mixing time of 100 ms (in ^2H_2O) or 150 ms (in ^1H_2O). The two-dimensional homonuclear Hartmann–Hahn experiment (HOHAHA) (Braunschweiler & Ernst, 1983; Davis & Bax, 1985) was performed with a trim pulse of 1.5 ms followed by a mixing time of 50 ms. Free induction decays (64–96 scans) of 6500-Hz sweep width and 2K data points in the t_2 domain were collected for 400 data points in the t_1 domain by time proportional phase incrementation (Marion & Wüthrich, 1983). By zero-filling in the t_1 domain and resolution enhancement with squared sine-bell windows in both the t_1 and t_2 domains, followed by Fourier transformation, the spectra of 2K × 1K data points were obtained.

To interpret NMR data, the crystal structure of the GDP-bound Ras protein (Tong et al., 1991) from the Brookhaven Protein Data Bank was examined with the software Insight II (Biosym Technologies) on a Silicon Graphics IRIS-4D/25TG computer.

RESULTS AND DISCUSSION

Signal-Transducing Activities of Mutant Ras Proteins. Injection of the full-length G12V/Y32W- or G12V/Y40W-Ras proteins caused neurite outgrowth of PC12 cells in 24 h (Table 1). In contrast, the full-length G12V/Y32F-Ras protein did not cause neurite outgrowth upon injection into PC12 cells (Table 1). Therefore, the signal-transducing activity of the oncogenic G12V-Ras protein was not affected by the Y32W and Y40W mutations, but was significantly impaired by the Y32F mutation.

A variety of mutations in the effector region of Ras proteins have been found to reduce the signal-transducing activity (Sigal et al., 1986; Willumsen et al., 1986; Clanton et al., 1987; Stone et al., 1988; McCormick, 1989; Farnsworth et al., 1991; Stone & Blanchard, 1991). In position 32, the mutation of Tyr to Phe impairs the signal-transducing activity of the oncogenic Ras protein in NIH 3T3 cells (Stone et al., 1988) and rat2 cells (Stone & Blanchard, 1991), which is consistent with the present study using PC12 cells. In contrast, the signal-transducing activity is not appreciably affected by the Y32H mutation (Sigal et al., 1986; Stone & Blanchard, 1991). Furthermore, in the present study, the Y32W mutation was found to have no effect on the signal transduction induced by the Ras protein in PC12 cells, which is consistent with the result by Stone and Blanchard (1991) using rat2 cells. For signal transduction, therefore, the side chain of residue 32 of the Ras protein appears to be required to have an aromatic moiety with a hydrogen bond donor, so as to form a hydrogen bond with the target molecule. Unlike the hydrogen donor, it is still unclear if the aromatic moiety is indispensable or not. This should be tested in the future by replacement of Tyr32

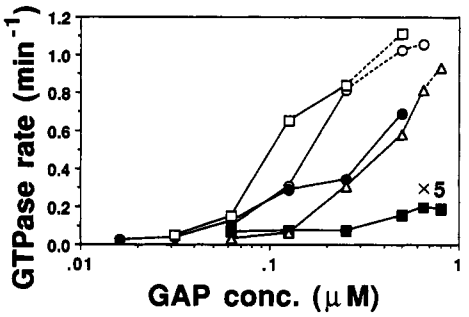

FIGURE 1: GTP hydrolysis rates of wild-type Ras (○), Y32F-Ras (●), Y32W-Ras (□), Y40K-Ras (■), and Y40W-Ras (△) proteins in the presence of GAP. The values for the Y40K-Ras protein were multiplied by 5. The dashed lines indicate that rates higher than 0.8 min^{-1} are possibly underestimated because initial velocities could not be precisely determined.

with a nonaromatic amino acid having a hydrogen donor, *e.g.*, Gln, Asn, Thr, or Ser.

As for position 40, replacement of Tyr by Leu, Ile, Val, Arg, Lys, Ser, or Gly deprives the Ras protein of signal-transducing activity (Sigal et al., 1986; Stone et al., 1988). In contrast, the activity is not affected by the Y40F mutation (Stone et al., 1988). Similarly, the Y40W mutation has no effect on the signal-transducing activity. These results indicate that the side chain of residue 40 is required to have an aromatic moiety, regardless of its hydrogen-bonding ability. Thus, in terms of signal-transducing activity, structural requirements in positions 32 and 40 are both strict but clearly different from each other.

GDP and GTP Dissociation Rates of Mutant Ras Proteins. Rates of dissociation of guanine nucleotides from the Y32W- and Y40W-Ras proteins were measured (Table 1). For both mutant proteins, dissociation rates of GDP and GTP were much the same as those for the wild-type protein (Fujita-Yoshigaki et al., 1992). Therefore, the two mutations Y32W and Y40W are likely to have only negligible effects on the affinities to GDP and GTP.

Intrinsic GTPase Activities of Mutant Ras Proteins. The rate constants of GTP hydrolysis for the wild-type and mutant Ras proteins were determined and are listed in Table 1. The GTPase activity of the Y32W-Ras protein is appreciably higher than those of the wild-type and Y32F-Ras proteins. As for position 40, the Y40K-Ras protein is less active than the Y40W mutant and wild-type Ras proteins with regard to GTP hydrolysis in the absence of GAP.

GTPase Activities of Mutant Ras Proteins in the Presence of GAP. To analyze the interactions of GAP with the mutant Ras proteins, the relative amounts of GDP and GTP after incubation of the GTP-bound form of the Ras protein for 2 min (or 10 min for the Y40K-Ras mutant) were determined at various concentrations of GAP. Thus, apparent GTP hydrolysis rate constants were estimated (Figure 1). For the cases where the obtained rate constants are higher than about 0.8 min^{-1}, we could not precisely determine the initial velocities, and therefore these rate constants are likely to be underestimated. As the GAP concentration was increased, GTPase rates of the wild-type, Y32F-, Y32W-, and Y40W-Ras proteins were remarkably increased, indicating that plateaus of nearly the same level seem to be reached at high GAP concentrations (Figure 1). From the dependencies of the GTPase rate on the GAP concentration, it was found that the GAP-binding affinity of the Y32W-Ras protein is comparable to that of the wild-type Ras protein and those of the Y32F- and Y40W-Ras proteins are only slightly lower (Figure 1). In contrast, the GTPase activity of the Y40K-Ras protein appeared to reach

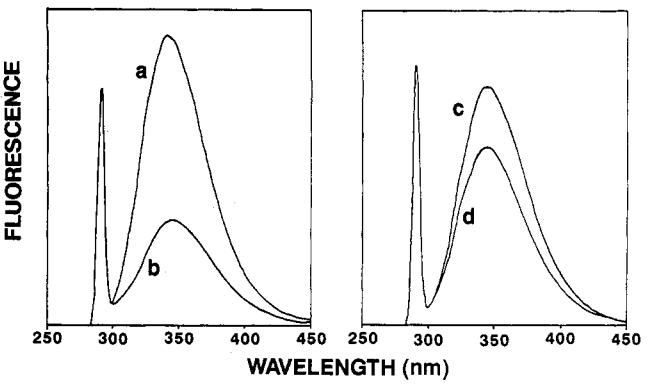

FIGURE 2: Fluorescence spectra of Y32W-Ras (a and b) and Y40W-Ras (c and d) bound with GDP (a and c) or GMPPNP (b and d).

a significantly low plateau (only 4-fold enhancement) at GAP concentrations higher than 0.65 μM (Figure 1 and Table 1). The Y40K mutation drastically reduced the "sensitivity" of Ras GTPase to GAP, while the effect of this mutation on the GAP-binding affinity seems to be much smaller as compared with that on the GAP sensitivity (Figure 1). Therefore, it is likely that the Y40K-Ras protein can bind to GAP with an affinity slightly lower than that of the wild type, but the GTPase activity of Y40K-Ras is not sensitive enough to be largely enhanced. If so, this mutant, Y40K, will be useful for further study on the molecular mechanism of enhancement of the GTPase activity by GAP.

For position 40, it has been reported that the Y40K mutation reduces GTPase activity in the presence of GAP whereas replacement of Tyr with Ile, Phe, or Ser does not affect the activity (Cales et al., 1988; McCormick, 1989). Actually, in the present study, it was further found that the Y40K mutation drastically reduces the GTPase activity of Ras in the GAP-bound state with no appreciable decrease in the GAP-binding affinity. Probably, therefore, the significant enhancement of the GTPase activity of the GAP-bound Ras protein is damaged by a mutation introducing a positive charge in position 40, but not by mutations removing aromaticity, hydrophobicity, or hydrogen-bonding ability of the Tyr residue. As for position 32, we found that the Y32F mutation slightly reduced the GAP-binding affinity but affected only negligibly the GAP sensitivity, while the Y32W mutation affected neither binding affinity nor sensitivity to GAP (there is no other report so far on effects of mutations in position 32 on binding affinity or sensitivity to GAP). These results suggest that the proper interactions with GAP tolerate different mutations in positions 32 and 40. In contrast, structural requirements for several other effector region residues are quite strict with regard to the GAP interaction (Adari et al., 1988; Cales et al., 1988; McCormick, 1989; Farnsworth et al., 1991).

In terms of the signal-transducing activity, however, the structural requirements for the residues in positions 32 and 40 are both strict (present study; McCormick, 1989). Therefore, residues 32 and 40 are involved more critically in the signal-transducing activity than in the GTPase activation by GAP. As for the Y32W-Ras and Y40W-Ras proteins, both the signal-transducing activity and the ability to interact with GAP are retained, thereby allowing the fluorescence and NMR analyses of the microenvironment in the effector region using Trp residues as probes.

Fluorescence Analyses. The fluorescence spectra of the two mutant proteins are shown in Figure 2. For the Y32W-Ras protein, the fluorescence intensity is greatly reduced, to 56%, and the fluorescence maximum is red-shifted by 3 nm upon ligand exchange from GDP to GMPPNP (Figure 2a,b). These results suggest that the microenvironment of the indole

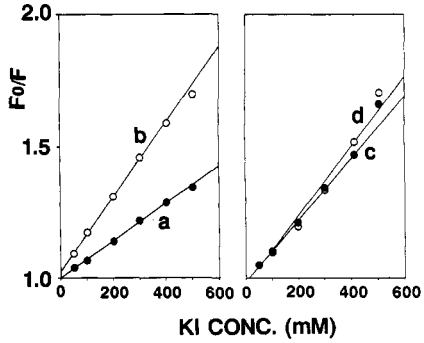

FIGURE 3: Stern–Volmer plots for the fluorescence quenching effects of KI on Y32W-Ras (a and b) and Y40W-Ras (c and d) bound with GDP [a and c (●)] or GMPPNP [b and d (○)].

ring of Trp32 in the GMPPNP-bound form is more hydrophilic than that in the GDP-bound form. On the other hand, for the Y40W-Ras protein, the fluorescence intensity of the GMPPNP-bound form is only slightly lower than that of the GDP-bound form (Figure 2c,d), suggesting that the microenvironment of the indole ring of Trp40 is not greatly affected by ligand exchange from GDP to GMPPNP.

Microenvironmental changes of Trp residues upon ligand exchange were also observed in the Stern–Volmer plots for the quenching effects by KI (Figure 3). The fluorescence of the Y32W-Ras protein in the GMPPNP-bound form (K_{sv} = 1.50 M^{-1}) is more susceptible to quenching than that in the GDP-bound form (K_{sv} = 0.72 M^{-1}). This indicates that Trp32 in the GMPPNP-bound form is more accessible to the solvent than in the GDP-bound form. In contrast, the Stern–Volmer constants of the Y40W-Ras protein in the GMPPNP-bound form (K_{sv} = 1.25 M^{-1}) and the GDP-bound form (K_{sv} = 1.13 M^{-1}) are nearly the same, indicating that the microenvironment of the indole ring of Trp40 in this mutant is only slightly affected by ligand exchange from GDP to GMPPNP.

The present fluorescence analysis revealed that the microenvironment of the Trp residues in the mutant proteins is consistent with those found in previous crystallographic analyses (Milburn et al., 1990; Pai et al., 1990; Schlichting et al., 1990a; Tong et al., 1991). In the crystal of the GDP-bound wild-type Ras protein, the aromatic rings of Tyr32 and Tyr40 form a hydrogen bond with each other (Milburn et al., 1990; Tong et al., 1991). In contrast, Tyr32 of the Ras protein swings out to partly cover the phosphate pocket in the complex with guanylyl 5′-(β,γ-methylenediphosphate) (an unhydrolyzable GTP analogue) (Milburn et al., 1990) or interacts with the γ-phosphate group of a neighboring molecule in the complex with GMPPNP (Pai et al., 1990). In fact, the present fluorescence data on the Y32W-Ras protein indicate an appreciable microenvironmental change of Trp32 from the interior to the exterior of the protein. Furthermore, the small intensity reduction of Trp40 in the Y40W-Ras protein upon ligand exchange from GDP to GMPPNP (Figure 2) is possibly related to the disruption of the hydrogen bond between Tyr32 and Tyr40 found in the crystal.

Two-Dimensional NMR Analyses. For more detailed analyses of the microenvironment of these Trp residues, two-dimensional proton NMR spectra were analyzed. In HOHAHA spectra of the GDP-bound mutant Ras proteins in ^2H_2O solution (data not shown), cross-peaks due to the Trp aromatic protons 4H, 5H, 6H, and 7H are partly overlapped with those due to Phe78, which have already been assigned using site-directed mutagenesis (Schlichting et al., 1990b). [1,2,3,4,5-^{2}H]Phe/[3,5-^{2}H]Tyr-substituted mutant proteins were prepared to eliminate the aromatic proton resonances of the Phe residues and the 3,5-proton resonances of the Tyr

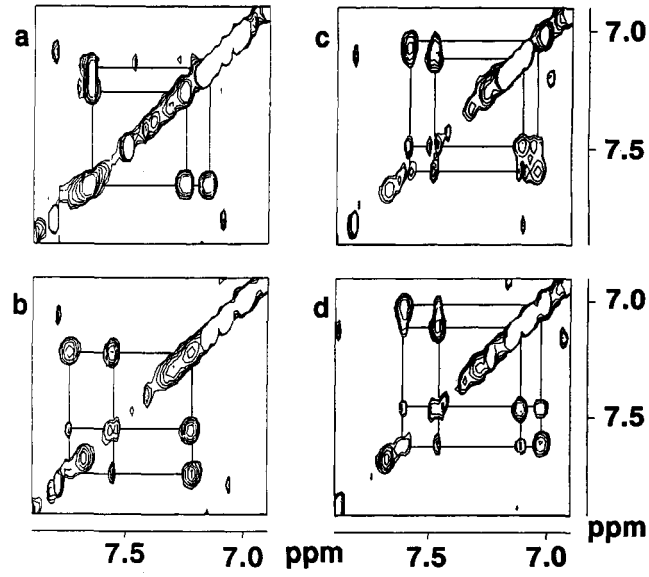

FIGURE 4: HOHAHA spectra of the [1,2,3,4,5-^{2}H]Phe/[3,5-^{2}H]-Tyr-substituted proteins: Y32W-Ras (a and b) and Y40W-Ras (c and d) bound with GDP (a and c) or GMPPNP (b and d) in ^2H_2O solution.

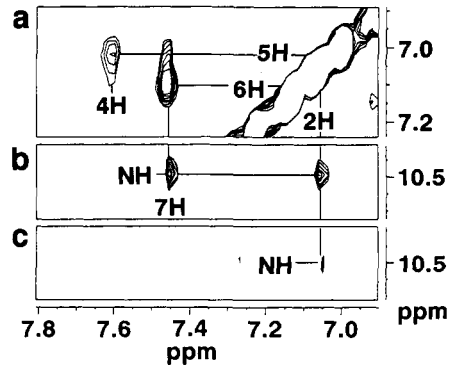

FIGURE 5: HOHAHA (a and c) and NOESY (b) spectra of the Y40W-Ras protein bound with GMPPNP in ^2H_2O (a) or in ^1H_2O (b, c) solution. The protein is substituted with [1,2,3,4,5-^{2}H]Phe/[3,5-^{2}H]Tyr (a) or is not substituted (b and c).

residues. Thus, strong cross-peaks due to the [4H, 5H] and [6H, 7H] of Trp residues are clearly observed in the HOHAHA spectra (Figure 4) and also in the DQF-COSY spectra (data not shown). However, cross-peaks due to [5H, 6H] are not observed, because of the small chemical shift difference between the two resonances.

In the two-dimensional spectra of the GMPPNP-bound Y40W-Ras protein in ^1H_2O solution, cross-peaks due to the indole NH proton (10.5 ppm) are observed, including NOE peaks due to [NH, 7H] and [NH, 2H] (Figure 5b) and a HOHAHA peak due to [NH, 2H] (Figure 5c). From the NOE cross-peak [NH, 7H], the 7H-proton resonance is clearly distinguishable from the 4H-proton resonance, allowing unambiguous assignments of all the aromatic proton resonances of Trp40 in the GDP-bound and GMPPNP-bound forms (Table 2). In contrast, in the GMPPNP-bound Y32W-Ras protein, the indole NH proton resonance of Trp32 was not observed, probably because of rapid exchange with the solvent at pH 7.5. This is consistent with the fluorescence observation indicating the location of Trp32 on the surface of GMPPNP-bound Ras.

The chemical shifts of the aromatic proton resonances of Trp32 differ markedly between the GDP- and GMPPNP-bound forms (Figure 4a,b, Table 2). This indicates a large microenvironmental difference for this residue between the

Table 2: Chemical Shifts (ppm) of Aromatic Protons and NOE Cross-Peaks (ppm)

Y32W-Ras protein in GDP-bound form			Y40W-Ras protein in GDP-bound form		
Trp32	δ	NOE[a]	Trp40	δ	NOE[a]
NH	9.82		NH	9.99	
2H	7.49	3.05, 3.35, 4.28	2H	7.10	0.50, 4.72, 5.53
4H+7H[b]	7.67	−0.52, 0.66, 0.81, 2.90, 3.38, 3.90, 4.56	4H	7.61	0.50, 1.03, 2.79, 3.13, 4.73
			5H	7.04	
5H/6H[c]	7.15		6H	7.11	
6H/5H[c]	7.25		7H	7.49	0.02, 7.00[d]

Y32W-Ras protein in GMPPNP-bound form			Y40W-Ras protein in GMPPNP-bound form		
Trp32	δ	NOE[a]	Trp40	δ	NOE[a]
NH			NH	10.50	
2H			2H	7.06	0.53, 4.60
4H/7H[e]	7.55	3.46, 4.08	4H	7.61	0.53, 1.03, 2.24, 3.12, 4.89
5H+6H[f]	7.22				
7H/4H[e]	7.74	3.48	5H	7.02	
			6H	7.11	
			7H	7.46	

[a] NOE cross-peaks not observed in the wild-type Ras protein are listed, except those among Trp aromatic protons. [b] 4H and 7H resonances are overlapping. [c] This resonance is due to either 5H or 6H. [d] This cross-peak is not observed in the deuterium-substituted protein (Figure 6). [e] This resonance is due to either 4H or 7H. [f] 5H and 6H resonances are overlapping.

two forms. In contrast, the chemical shifts of the aromatic proton resonances of Trp40 in the two forms are similar to each other, except for the indole NH proton resonances (Figure 4c,d, Table 2). This shows that the microenvironment of Trp40 is not greatly affected by ligand exchange.

NOE Cross-Peaks of Y32W-Ras Proteins. The microenvironment of Trp residues may be examined in detail by analysis of the NOE data. The NOESY spectra of [1,2,3,4,5-^{2}H]Phe/[3,5-^{2}H]Tyr-substituted Ras proteins in ^2H_2O solution are shown in Figure 6. The NOE cross-peaks due to Trp32 in the GMPPNP-bound form of the Y32W-Ras protein are fewer and weaker than those in the GDP-bound form (Table 2, Figure 6). The Trp ring proton resonances were clearly observed without appreciable broadening (Figure 4b). It is indicated, therefore, that the aromatic protons of Trp32 in the GDP-bound form are in close proximity to many more protons than those in the GMPPNP-bound form. It is also possible that the tertiary structure involving Trp32 in the GMPPNP-bound form is so dynamically fluctuating to much weaken NOEs.

For the GDP-bound Y32W-Ras protein, an NOE cross-peak is observed at [−0.52 ppm, 7.67 ppm] (Figure 6a). The proton resonance at −0.52 ppm is probably due to an Ile residue, as revealed by the connectivity pattern in the HOHAHA spectrum (data not shown). The location of the HOHAHA cross-peak for the pair of α- and γ-protons of this residue is nearly the same as that of Ile21 of the wild-type Ras protein as assigned by ^{15}N-labeling methods (Muto et al., 1993). The extremely upfield-shifted proton resonance at −0.52 ppm is tentatively assigned to the δ-methyl proton of Ile21, which is probably located upon the indole ring of Trp32.

Recently, large chemical shift differences of Ile21 and Ile36 between the GTP- and GDP-bound forms have been observed using selective ^{15}N-labeling of Ile residues (Miller et al., 1992). Correspondingly, the replacement of Ile21 by Ala abolishes the signal-transducing activity of the Ras protein (unpublished experiments). Therefore, the interaction between the residues in positions 21 and 32 is probably important in the regulation

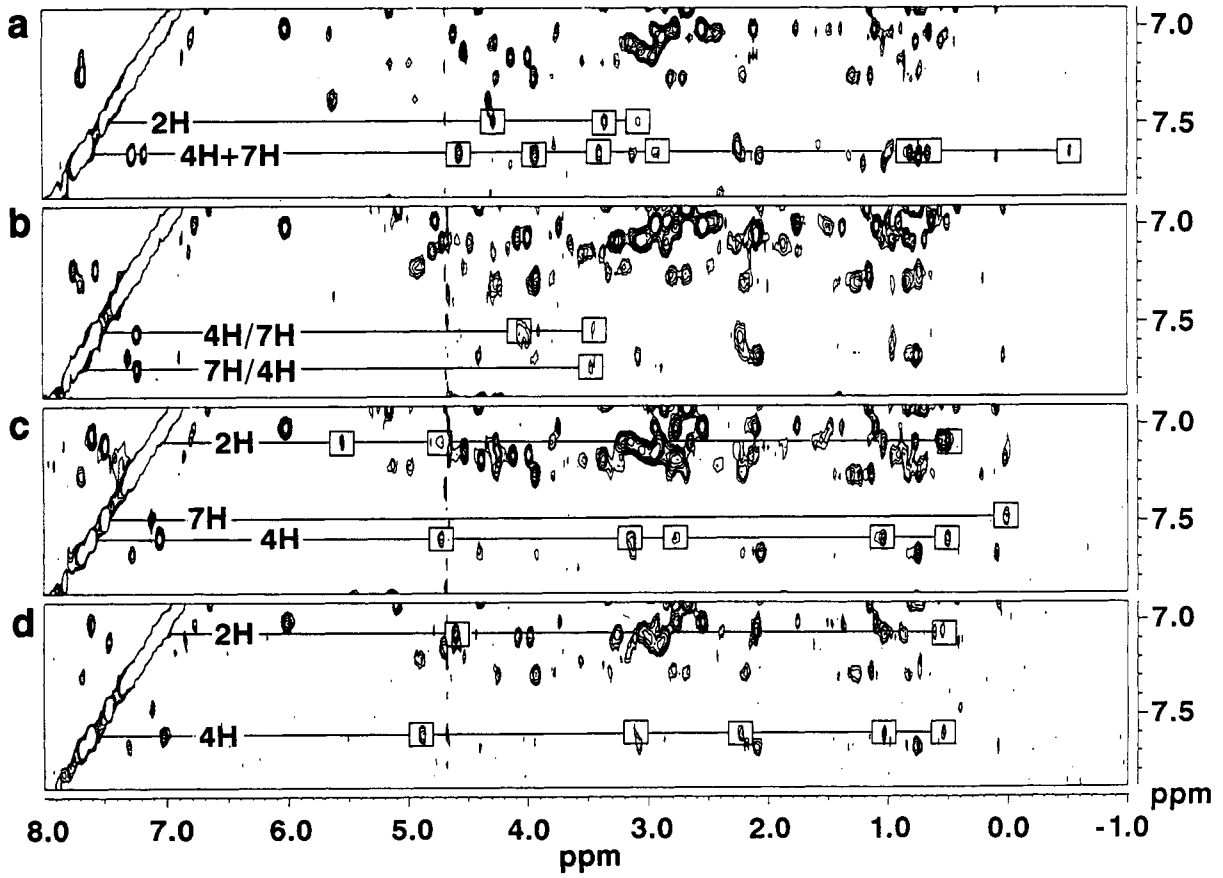

FIGURE 6: NOESY spectra of the [1,2,3,4,5-^{2}H]Phe/[3,5-^{2}H]Tyr-substituted Y32W-Ras (a and b) and Y40W-Ras (c and d) proteins bound with GDP (a and c) or GMPPNP (b and d) in ^2H_2O solution. The NOE cross-peaks due to Trp aromatic protons, which are not observed in the wild-type Ras protein, are boxed.

of the signal-transducing activity. In the crystallographic studies, however, such an interaction has not been described (Milburn et al., 1990; Schlichting et al., 1990a; Tong et al., 1991).

For further analysis of the NOE data, we examined the crystal structure of the GDP-bound Ras protein (Tong et al., 1991). Tyr32 of the structure was replaced with Trp on the computer, and the χ_1 and χ_2 angles of the Trp residue were changed successively by 10° so as to test if the NOE data could be interpreted in any side-chain conformations. Thus, one rotamer [$\chi_1 = -177.3 \pm 7.9°$, $\chi_2 = -95.1 \pm 7.6°$] from the rotamer library (Ponder & Richards, 1987) was found to be suitable as follows. In this rotamer, the δ-methyl proton of Ile21 is located near the 4H proton and just on the indole ring of Trp32, which agrees with the NOE data (Table 2) and the extreme upfield shift of this proton resonance. Further, the 2H proton of Trp32 is in close proximity to the β-protons of Trp32 and Ser17 and the α-proton of Pro34. Thus, resonances at 3.05, 3.35, and 4.28 ppm (Table 2) may be assigned to these protons. One of the methyl groups of Val29 and the γ-methyl group of Ile36 are in close proximity to 4H and 7H, respectively, of Trp32, which leads to tentative assignments of resonances giving NOEs at 0.66 and 0.81 ppm to these methyl protons. Similarly, resonances at 2.90, 3.38, and 3.90 ppm (Table 2) may be assigned to β-protons of Val29, Trp32, and Ile36. A resonance at 4.56 ppm is tentatively assigned to the α-proton of Trp32. In the GMPPNP-bound form, most of NOEs due to these interactions were lost, which suggests significant conformational changes at and around this Trp residue.

NOE Cross-Peaks of Y40W-Ras Proteins. For the Y40W-Ras protein, 10 NOE cross-peaks due to Trp40 are observed for the GDP-bound form, and 7 cross-peaks are seen for the GMPPNP-bound form (Table 2). These seven cross-peaks in the GMPPNP-bound form are also observed at nearly the same chemical shifts in the GDP-bound form (Table 2), suggesting that the microenvironment of Trp40 is similar between the two forms.

The Trp40 aromatic protons of the Y40W-Ras protein exhibit three NOE cross-peaks in the GDP-bound form that are not observed in the GMPPNP-bound form (Table 2, Figure 6c,d), namely, those at 0.02 (A), 5.53 (B), and 7.00 ppm (C).

Resonance B closely corresponds to the α-proton of Ser39 in the GDP-bound wild-type Ras protein (Yamasaki et al., 1989). An NOE cross-peak has been observed between this proton and the α-proton of Leu56 in the GDP-bound form, but not in the GMPPNP-bound form, because of the distortion of the β-sheet structure (Yamasaki et al., 1989). In the present study, the α-proton of Ser39 is shown to be in close proximity to 2H of Trp40 in the GDP-bound form, but not in the GMPPNP-bound form. Thus, the distortion of the β-sheet in the GMPPNP-bound form disrupts the contact between the main chain of residue 39 and the side chain of residue 40.

An NOE cross-peak (A, C) is observed in the GDP-bound Y40W-Ras protein (not deuterium-substituted) (Figure 7) but not in the spectra of the GDP-bound [1,2,3,4,5-^{2}H]Phe/[3,5-^{2}H]Tyr-substituted Y40W-Ras protein (Figure 6c). From the connectivity pattern, resonance C was assigned to the 3,5-protons of Tyr residue(s) rather than to aromatic protons of Phe residue(s). In this context, a hydrogen bond between Tyr32 and Tyr40 was found in the GDP-bound Ras protein in the crystal (Tong et al., 1991). Therefore, resonance C may be assigned to Tyr32; the 7H proton of Trp40 is probably in close proximity to the 3,5-protons of Tyr32 in the GDP-bound form. From an examination of the crystal structure

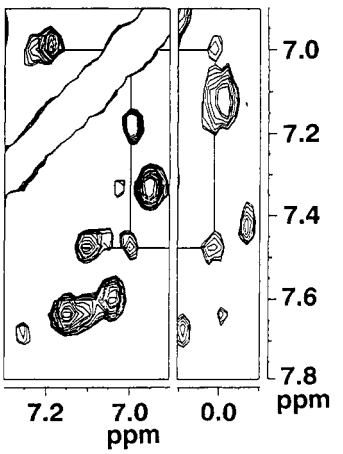

FIGURE 7: NOESY spectra of GDP-bound Y40W-Ras protein in ^2H_2O solution.

(Tong et al., 1991), resonance A was tentatively assigned to the δ-methyl group of Ile21. Note that this proton is also located near the 4H proton of the Trp ring in Y32W-Ras protein. Therefore, it is indicated that there is a hydrophobic cluster consisting of three side chains of residues 21, 32, and 40 in the GDP-bound form. In contrast, NOEs due to this cluster are not observed in the GMPPNP-bound form, which indicates that the cluster is either disrupted or much more dynamic.

These NOE data of the Y40W-Ras protein were similarly examined using the crystal structure. Thus, a stable rotamer [$\chi_1 = -70.4 \pm 7.0°$, $\chi_2 = 100.5 \pm 18.2°$] (Ponder & Richards, 1987) was found to agree well with the NOEs. In this rotamer, the 2H proton of Trp40 is located near the α-proton of Ser39, and the 7H proton of Trp40 is near the δ-proton of Ile21 and the 3,5-protons of Tyr32. As for the resonance at 0.50 ppm, the crystal structure has no corresponding methyl group in close proximity to both the 2H and 4H protons of Trp40 in any allowed conformations. However, by changing the χ_2 angle of Ile24 from –42° to around 150°, the δ-methyl group of this residue is moved into a suitable place. Thus, the resonance at 0.50 ppm may be due to the δ-proton of Ile24. The other NOEs at 4.72, 1.03, 2.79, 3.13, and 4.73 ppm were tentatively assigned to the β-proton of Thr20, the γ-methyl protons of Ile24, and the two β-protons and the α-proton of Trp40, respectively.

Although the microenvironments of Trp40 in the GDP-bound and GMPPNP-bound forms are similar, the chemical shifts of the indole NH resonances are appreciably different between the two forms. Considering the interaction between the 7H proton of Trp40 and the 3,5-protons of Tyr32, the chemical shift difference of the indole NH protons of Trp40 is probably due to a conformational difference in Tyr32; NH of Trp40 is probably located on the aromatic ring of Tyr32 in the GDP-bound form, but not so in the GMPPNP-bound form. This conformational difference is probably responsible for the small but appreciable difference in the fluorescence intensity of Trp40 between the GDP- and GMPPNP-bound forms of the Y40W-Ras protein.

Biological Aspects of the Interactions among Residues 21, 32, and 40. In the present NMR study, a hydrophobic cluster probably consisting of residues 21, 32, and 40 was found for the GDP-bound Ras protein in solution. In contrast, in the GTP-bound Ras protein, such a cluster is not detected. Note that these three residues are biologically important, as described above. Therefore, we propose that the GDP-bound form of the Ras protein is inactive partly because these three residues are buried in the cluster; in the active GTP-bound

form, these residues are released from the mutual interplay and are able to be involved in interaction with the target molecule.

Residue 32 in the GTP-bound form is located on the surface of the protein as mentioned above. Furthermore, this residue is required to have an aromatic moiety with a hydrogen donor as suggested by the present mutational analysis. Thus, residue 32 is likely to interact directly with the target molecule, forming an intermolecular hydrogen bond.

The microenvironment of residue 40 is not greatly affected by nucleotide exchange, although an aromatic residue is strictly required for this site for signal transduction. Thus, the suggested role of this residue is to stabilize the conformation that allows interaction with the target molecule. There is another possibility that, in the GTP·Ras·target ternary complex, some residue of the target molecule occupies the position of residue 32 in the GDP-bound form and interacts directly with residue 40.

Although residue 21 is not an effector region residue, our unpublished mutational study revealed that it has an important role in signal transduction. Therefore, residue 21 in the GTP-bound form may interact with the target molecule. There are some other biologically important residues outside the effector region, such as residues 26, 31, 45, and 48 (Fujita-Yoshigaki et al., 1991; Marshall et al., 1991; Nur-E-Kamal et al., 1992; Shirouzu et al., 1992). It is therefore important to analyze the conformational changes of these residues in solution.

As described above, examination of the NOE data using the crystal structure showed that the hydrophobic cluster possibly includes Ile24, Val29, and Ile36 in addition to Ile21, Tyr32, and Tyr40. Ile36 is an essential residue in the effector region (Sigal et al., 1986), suggesting that the putative disruption of the hydrophobic cluster allows this residue to play its role in the interaction with the target molecule.

Recognition of the Ras Protein by GAP. The conformational change of residue 32 resulting in the disruption of the hydrophobic cluster is probably recognized by the target molecule of signal transduction. As described above, however, mutational studies revealed that residues 32 and 40 are not strictly recognized when GAP enhances the GTPase activity of the Ras protein. Thus, the target molecule and GAP recognize differently at least the two effector region residues of Ras, although there are certain similarities between these two recognition processes (Adari et al., 1988; Cales et al., 1988).

There are some molecules other than the Ras protein that physically interact with GAP, such as growth factor receptors (Molloy et al., 1989; Ellis et al., 1990; Kaplan et al., 1990; Kazlauskas et al., 1990; Serth et al., 1992), GAP-associated proteins (Ellis et al., 1990; Settleman et al., 1992; Wong et al., 1992), and lipids (Tsai et al., 1989, 1991). Therefore, it is possible that they modify the recognition of the Ras protein by GAP and that residues 32 and 40 are recognized by the complex of GAP and those molecules. In this scenario, the complex is possibly the target molecule of signal transduction.

Conclusion. The roles of the aromatic residues in positions 32 and 40 of the Ras protein are elucidated in the present study. The aromatic residue in position 32 is "buried" in the GDP-bound form but is displaced onto the surface of the protein in the GTP-bound form. This microenvironmental difference is probably recognized as a "switch" by the target molecule, so that aromatic residue 32 directly interacts with the target, possibly forming an intermolecular hydrogen bond. In contrast, residue 40 should be important for stabilizing the conformation that allows the interaction with the target molecule.

ACKNOWLEDGMENT

We thank Dr. T. Ohya and Professor Y. Anraku of the University of Tokyo for the synthesis of oligonucleotides and Mr. H. Kasai of the University of Tokyo and Dr. S. Kawakita of the Protein Engineering Research Institute for helpful advice. We are grateful to Dr. B. J. Bachmann of Yale University for providing us auxotrophic *E. coli* strain DL39.

REFERENCES

Adari, H., Lowy, D. R., Willumsen, B. M., Der, C. J., & McCormick, F. (1988) *Science 240*, 518–521.

Antonny, B., Chardin, P., Roux, M., & Chabre, M. (1991) *Biochemistry 30*, 8287–8295.

Barbacid, M. (1987) *Annu. Rev. Biochem. 56*, 779–827.

Bar-Sagi, D., & Feramisco, J. R. (1985) *Cell 42*, 841–848.

Birchmeier, C., Broek, D., & Wigler, M. (1985) *Cell 43*, 615–621.

Braunschweiler, L., & Ernst, R. R. (1983) *J. Magn. Reson. 53*, 521–528.

Burgering, B. M. T., Medema, R. H., Maassen, J. A., van de Wetering, M. L., van der Eb, A. J., McCormick, F., & Bos, J. L. (1991) *EMBO J. 10*, 1103–1109.

Cales, C., Hancock, J. F., Marshall, C. J., & Hall, A. (1988) *Nature (London) 332*, 548–551.

Campbell-Burk, S. (1989) *Biochemistry 28*, 9478–9484.

Campbell-Burk, S., Papastavros, M. Z., McCormick, F., & Redfield, A. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 817–820.

Campbell-Burk, S. L., Domaille, P. J., Starovasnik, M. A., Boucher, W., & Laue, E. D. (1992) *J. Biomol. NMR 2*, 639–646.

Clanton, D. J., Lu, Y., Blair, D. G., & Shih, T. Y. (1987) *Mol. Cell. Biol. 7*, 3092–3097.

Cohen, J. S., & Putter, I. (1970) *Biochim. Biophys. Acta 222*, 515–520.

Davis, D. G., & Bax, A. (1985) *J. Am. Chem. Soc. 108*, 2921–2923.

Duchesne, M., Schweighoffer, F., Parker, F., Clerc, F., Frobert, Y., Thang, M. N., & Tocque, B. (1993) *Science 259*, 525–528.

Ellis, C., Moran, M., McCormick, F., & Pawson, T. (1990) *Nature (London) 343*, 377–381.

Farnsworth, C. L., Marshall, M. S., Gibbs, J. B., Stacey, D. W., & Feig, L. A. (1991) *Cell 64*, 625–633.

Foley, C. K., Pedersen, L. G., Charifson, P. S., Darden, T. A., Wittinghofer, A., Pai, E. F., & Anderson, M. W. (1992) *Biochemistry 31*, 4951–4959.

Fujita-Yoshigaki, J., Shirouzu, M., Koide, H., Nishimura, S., & Yokoyama, S. (1991) *FEBS Lett. 294*, 187–190.

Fujita-Yoshigaki, J., Ito, Y., Yamasaki, K., Muto, Y., Miyazawa, T., Nishimura, S., & Yokoyama, S. (1992) *J. Protein Chem. 11*, 739–747.

Gibbs, J. B., Schaber, M. D., Allard, W. J., Sigal, I. S., & Scolnick, E. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5026–5030.

Gibbs, J. B., Marshall, M. S., Scolnick, E. M., Dixon, R. A. F., & Vogel, U. S. (1990) *J. Biol. Chem. 265*, 20437–20442.

Ha, J.-M., Ito, Y., Kawai, G., Miyazawa, T., Miura, K., Ohtsuka, E., Noguchi, S., Nishimura, S., & Yokoyama, S. (1989) *Biochemistry 28*, 8411–8416.

Hata-Tanaka, A., Kawai, G., Yamasaki, K., Ito, Y., Kajiura, H., Ha, J.-M., Miyazawa, T., Yokoyama, S., & Nishimura, S. (1989) *Biochemistry 28*, 9550–9556.

Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.

John, J., Schlichting, I., Schiltz, E., Rösch, P., & Wittinghofer, A. (1989) *J. Biol. Chem. 264*, 13086–13092.

John, J., Sohmen, R., Feuerstein, J., Linke, R., Wittinghofer, A., & Goody, R. S. (1990) *Biochemistry 29*, 6058–6065.

Kaplan, D. R., Morrison, D. K., Wong, G., McCormick, F., & Williams, L. T. (1990) *Cell 61*, 125–133.

Kazlauskas, A., Ellis, C., Pawson, T., & Cooper, J. (1990) *Science 247*, 1578–1581.

Krontiris, T. G., & Cooper, G. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1181–1184.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.
LeMaster, D. M., & Richards, F. M. (1988) *Biochemistry 27*, 142–150.
Liu, X., & Pawson, T. (1991) *Mol. Cell. Biol. 11*, 2511–2516.
Maniatis, T., Fritsh, E. F., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, 1st ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967–974.
Martin, G. A., Yatani, A., Clark, R., Conroy, L., Polakis, P., Brown, A. M., & McCormick, F. (1992) *Science 255*, 192–194.
Marshall, M. S., Davis, L. J., Keys, R. D., Mosser, S. D., Hill, W. S., Scolnick, E. M., & Gibbs, J. B. (1991) *Mol. Cell. Biol. 11*, 3997–4004.
McCormick, F. (1989) *Cell 56*, 5–8.
Milburn, M. V., Tong, L., de Vos, A. M., Brünger, A., Yamaizumi, Z., Nishimura, S., & Kim, S.-H. (1990) *Science 247*, 939–945.
Miller, A. F., Papastavros, M. Z., & Redfield, A. G. (1992) *Biochemistry 31*, 10208–10216.
Milstein, S., & Kaufman, S. (1975) *J. Biol. Chem. 250*, 4782–4785.
Miura, K., Inoue, Y., Nakamori, H., Iwai, S., Ohtsuka, E., Ikehara, M., Noguchi, S., & Nishimura, S. (1986) *Jpn. J. Cancer Res. 77*, 45–51.
Molloy, C. J., Bottaro, D. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B., & Aaronson, S. A. (1989) *Nature (London) 342*, 711–714.
Moodie, S. A., Willumsen, B. M., Weber, M. J., & Wolfman, A. (1993) *Science 260*, 1658–1661.
Muto, Y., Yamasaki, K., Ito, Y., Yajima, S., Masaki, H., Uozumi, T., Wälchli, M., Nishimura, S., Miyazawa, T., & Yokoyama, S. (1993) *J. Biomol. NMR 3*, 165–184.
Neal, S. E., Eccleston, J. F., & Webb, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3562–3565.
Nishimura, S., & Sekiya, T. (1987) *Biochem. J. 243*, 313–327.
Noda, M., Ko, M., Ogura, A., Liu, D., Amano, T., Takano, T., & Ikawa, Y. (1985) *Nature (London) 318*, 73–75.
Nur-E-Kamal, M. S. A., Sizeland, A., D'Abaco, G., & Maruta, H. (1992) *Mol. Cell. Biol. 267*, 1415–1418.
Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351–2359.
Perucho, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J., & Wigler, M. (1981) *Cell 27*, 467–476.
Ponder, J. W., & Richards, F. M. (1987) *J. Mol. Biol. 193*, 775–791.
Redfield, A. G., & Papastavros, M. Z. (1990) *Biochemistry 29*, 3509–3514.
Rensland, H., Lautwein, A., Wittinghofer, A., & Goody, R. S. (1991) *Biochemistry 30*, 11181–11185.
Satoh, T., Nakamura, S., & Kaziro, Y. (1987) *Mol. Cell. Biol. 7*, 4553–4556.
Satoh, T., Endo, M., Nakamura, S., & Kaziro, Y. (1988) *FEBS Lett. 236*, 185–189.
Satoh, T., Endo, M., Nakafuku, M., Nakamura, S., & Kaziro, Y. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5993–5997.
Satoh, T., Endo, M., Nakafuku, M., Akiyama, T., Yamamoto, T., & Kaziro, Y. (1990b) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7926–7929.
Schlichting, I., Wittinghofer, A., & Rösch, P. (1988) *Biochem. Biophys. Res. Commun. 150*, 444–448.
Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A., & Goody, R. S. (1990a) *Nature (London) 345*, 309–315.
Schlichting, I., John, J., Frech, M., Chardin, P., Wittinghofer, A., Zimmermann, H., & Rösch, P. (1990b) *Biochemistry 29*, 504–511.
Serth, J., Weber, W., Frech, M., Wittinghofer, A., & Pingoud, A. (1992) *Biochemistry 31*, 6361–6365.
Settleman, J., Narasimhan, V., Foster, L. C., & Weinberg, R. A. (1992) *Cell 69*, 539–549.
Shih, C., Padhy, L. C., Murray, M., & Weinberg, R. A. (1981) *Nature (London) 290*, 261–264.
Shirouzu, M., Fujita-Yoshigaki, J., Ito, Y., Koide, H., Nishimura, S., & Yokoyama, S. (1992) *Oncogene 7*, 475–480.
Sigal, I. S., Gibbs, J. B., D'Alonzo, J. S., & Scolnick, E. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4725–4729.
Skelly, J. V., Suter, D. A., Kuroda, R., Neidle, S., Hancock, J. F., & Drake, A. (1990) *FEBS Lett. 262*, 127–130.
Stone, J. C., & Blanchard, R. A. (1991) *Mol. Cell. Biol. 11*, 6158–6165.
Stone, J. C., Vass, W. C., Willumsen, B. M., & Lowy, D. R. (1988) *Mol. Cell. Biol. 8*, 3365–3569.
Tong, L., de Vos, A. M., Milburn, M. V., & Kim, S.-H. (1991) *J. Mol. Biol. 217*, 503–516.
Tsai, M.-H., Yu, C.-L., Wei, F.-S., & Stacey, D. W. (1989) *Science 243*, 522–526.
Tsai, M.-H., Roudebush, M., Dobrowolski, S., Yu, C.-L., Gibbs, J. B., & Stacey, D. W. (1991) *Mol. Cell. Biol. 11*, 2785–2793.
Trahey, M., & McCormick, F. (1987) *Science 238*, 542–545.
Trahey, M., Wong, G., Halenbeck, R., Rubinfeld, B., Martin, G. A., Ladner, M., Long, C. M., Crosier, W. J., Watt, K., Koths, K., & McCormick, F. (1988) *Science 242*, 1697–1700.
Van Aelst, L., Barr, M., Marcus, S., Polverino, A., & Wigler, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6213–6217.
Vogel, U. S., Dixon, R. A. F., Schaber, M. D., Diehl, R. E., Marshall, M. S., Scolnick, E. M., Sigal, I. S., & Gibbs, J. B. (1988) *Nature (London) 335*, 90–93.
Vojtek, A. B., Hollenberg, S. M., & Cooper, J. A. (1993) *Cell 74*, 205–214.
Warne, P. H., Viciana, P. R., & Downward, J. (1993) *Nature (London) 364*, 352–355.
Willumsen, B. M., Papageorge, A. G., Kung, H.-F., Bekesi, E., Robins, T., Johnsen, M., Vass, W., & Lowy, D. R. (1986) *Mol. Cell. Biol. 6*, 2646–2654.
Wong, G., Müller, O., Clark, R., Conroy, L., Moran, M. F., Polakis, P., & McCormick, F. (1992) *Cell 69*, 551–558.
Yamasaki, K., Kawai, G., Ito, Y., Muto, Y., Fujita, J., Miyazawa, T., Nishimura, S., & Yokoyama, S. (1989) *Biochem. Biophys. Res. Commun. 162*, 1054–1062.
Yamasaki, K., Muto, Y., Ito, Y., Wälchli, M., Miyazawa, T., Nishimura, S., & Yokoyama, S. (1992) *J. Biomol. NMR 2*, 71–82.
Yatani, A., Okabe, K., Polakis, P., Halenbeck, R., McCormick, F., & Brown, A. M. (1990) *Cell 61*, 769–776.
Zhang, X., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., & Avruch, J. (1993) *Nature (London) 364*, 308–313.