Crystal Structure of M-Ras Reveals a GTP-bound “Off” State Conformation of Ras Family Small GTPases*

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Although some members of Ras family small GTPases, including M-Ras, share the primary structure of their effector regions with Ras, they exhibit vastly different binding properties to Ras effectors such as c-Raf-1. We have solved the crystal structure of M-Ras in the GDP-bound and guanosine 5′-β,γ-imido)triphosphate (Gpp(NH)p)-bound forms. The overall structure of M-Ras resembles those of H-Ras and Rap2A, except that M-Ras-Gpp(NH)p exhibits a distinctive switch I conformation, which is caused by impaired intramolecular interactions between Thr-45 (corresponding to Thr-35 of H-Ras) of the effector region and the γ-phosphate of Gpp(NH)p. Previous 31P NMR studies showed that H-Ras-Gpp(NH)p exists in two interconverting conformations, states 1 and 2. Whereas state 2 is a predominant form of H-Ras and corresponds to the “on” conformation found in the complex with effectors, state 1 is thought to represent the “off” conformation, whose tertiary structure remains unknown. 31P NMR analysis shows that free M-Ras-Gpp(NH)p predominantly assumes the state 1 conformation, which undergoes conformational transition to state 2 upon association with c-Raf-1. These results indicate that the solved structure of M-Ras-Gpp(NH)p corresponds to the state 1 conformation. The predominance of state 1 in M-Ras is likely to account for its weak binding ability to the Ras effectors, suggesting the importance of the tertiary structure factor in small GTPase-effector interaction. Further, the first determination of the state 1 structure provides a molecular basis for developing novel anti-cancer drugs as compounds that hold Ras in the state 1 “off” conformation.

Small GTPases H-, K-, and N-Ras, collectively called Ras, are the products of the ras proto-oncogenes and function as molecular switches by cycling between a GTP-bound “on” state and a GDP-bound “off” state in a variety of intracellular signaling pathways that regulate cell growth, differentiation, and apoptosis (1–3). The conformational change induced by GTP binding enables Ras to execute downstream signaling through direct interaction with its effectors, such as Raf kinases, phosphoinositide 3-kinases, RalGDS family proteins, and phospholipase Cε (4). Conversion from the GDP-bound state to the GTP-bound state is controlled positively or negatively by GEFs and GTPase-activating proteins, respectively (4, 5). In particular, GEFs enhance the formation of the GTP-bound active conformation in response to upstream signals mediated by various cell surface receptors. Impairment of the GTPase activity is the most common biochemical defect associated with oncogenic Ras mutations. Ras comprises the Ras family of small GTPases together with a number of its relatives, including Rap1, Rap2, R-Ras, RaI, Rin, Kit, Ribe, etc. Crystal structure of H-Ras indicated that the exchange of guanine nucleotide results in allosteric conformational changes in two adjacent regions of Ras, termed “switch I” and “switch II” (5). X-ray crystallographic and NMR analyses of H-Ras and Rap1A in complex with the RBDs of c-Raf-1 and phosphoinositide 3-kinase γ or with the Ras-associating domain of RalGDS revealed that switch I (residues 32–38 in H-Ras) almost overlaps with the effector region (residues 32–40), which forms a principal interface for effector recognition (6–9). On the other hand, switch II (residues 60–75) plays a limited role in the effector recognition, whereas it plays a crucial role in interaction with GEFs and GTPase-activating proteins (5).

31P NMR studies showed that H-Ras in complex with Mg2+ and a nonhydrolyzable GTP analogue Gpp(NH)p exhibits an equilibrium between two rapidly interconverting conformations, called state 1 and state 2, which are characterized by different chemical shift values for the resonances of the γ and α-phosphate groups of Gpp(NH)p (10). Association with c-Raf-1 RBD induced transition of state 1 to state 2, suggesting that state 1 and 2 corresponds to the “off” and “on” conformations of H-Ras-GTP, respectively. Whereas state 2 corresponds to the structure found in the crystals of H-Ras as well as in its complex with the effectors (10, 11), the tertiary structure corresponding to state 1 has not yet been determined. M-Ras, also called R-Ras3, consists of 209 amino acid residues and shares 60–75% identity in the amino acid sequence with the N-terminal 110 residues with Ras (Fig. 1A). Like other Ras family members, Rap1, Rap2, R-Ras, and R-Ras2 (TC21), M-Ras share the identical effector region with Ras. Overexpression of these effector forms of M-Ras in cancer cell lines affects Ras signaling pathways and suppresses Ras-induced transformation. Hence, the elucidation of the crystal structure of M-Ras is important for understanding the function of Ras proteins and for the development of novel anti-cancer drugs.
sion of the mutationally activated form of M-RasQ71L causes cellular transformation or inhibition of differentiation as well as H-RasQ61L (12). M-Ras is shown to interact, although poorly, with some of the Ras effectors, including c-Raf-1, A-Raf, B-Raf, phosphoinositide 3-kinase, Raf-1, and Rin1, resulting in weak activation of the downstream cascades (13, 14). On the other hand, M-Ras appears to have its own effectors, such as Rap-specific GEFs, RA(PDZ)-GEF-2, and MR-GEF, which is known about the molecular mechanism underlying such differences in effector recognition between Ras and M-Ras. To address this problem, we have determined the crystal structures of M-Ras-GDP and M-Ras-Gpp(NH)p. The overall structure of M-Ras resembles those of H-Ras and Rap2A, except that M-Ras-Gpp(NH)p exhibits a distinctive switch I conformation, which is caused by impaired intramolecular interactions, in particular the interaction between Thr-45 (corresponding to Thr-35 of H-Ras) and the γ-phosphate group of Gpp(NH)p. From 31P NMR analysis, free M-Ras-Gpp(NH)p is shown to predominantly assume the state 1 conformation, which undergoes conformational transition to state 2 upon association with c-Raf-1 RBD. These results indicate that the solved structure of M-Ras-Gpp(NH)p corresponds to the state 1 conformation. The predominance of state 1 in M-Ras may account for its weak binding ability to the Ras effectors, providing a new insight into the mechanism whereby small GTPases with the identical effector region make differential interactions with their effectors.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**Mouse M-Ras (residues 1–178), human H-Ras (residues 1–166), and c-Raf-1 RBD (residues 50–131) were expressed as fusions with GST using pGEX-6p-1 vector (Amersham Biosciences). After removal of the GST tag by cleavage with Prescission protease (Amersham Biosciences), the proteins were purified by ion exchange chromatography to achieve a final purity of >95% as measured by SDS-polyacrylamide gel electrophoresis and used for the crystallization and NMR spectroscopy.

**Crystal Structure of M-Ras—**Crystals of M-Ras-GDP were grown by hanging drop vapor diffusion at 20 °C using the reservoir solution containing 20% (w/v) polyethylene glycol 8000 and 50 mM potassium phosphate at a protein concentration of 10 mg/ml. Gpp(NH)p loading of M-Ras was achieved with thorough degradation of the bound GDP using resin conjugated with alkaline phosphatase (17). Crystals of M-Ras-Gpp(NH)p were grown by the hanging drop method at 20 °C using the reservoir solution containing 16% (w/v) polyethylene glycol 8000, 80 mM sodium cacodylate, pH 6.5, and 160 mM magnesium acetate at a protein concentration of 18 mg/ml. The guanine nucleotide configuration of the protein was monitored on a fast protein liquid chromatography system (Amersham Biosciences). The crystals of M-Ras-GDP and M-Ras-Gpp(NH)p were grown to the dimensions of ~0.5 × 0.1 × 0.1 and ~0.2 × 0.1 × 0.05 mm3, respectively.

**Data Collection and Structure Determination—**The data collections were carried out at BL40B2 and BL26B1 of SPring-8 (Harima) using an ADSC Quantum 4R CCD detector and Rigaku RAXIS-V image plate detector, respectively. The data were collected at 100 K, processed using the program MOSFLM (18), and scaled with the program SCALA in the CCP4 program suite (19). The crystal structure of M-Ras-GDP was determined by the molecular replacement method with the program AMoRe (20) in the CCP4 program suite (19). The structure of H-Ras-GDP (PDB entry 4Q21) was used as a search model. The obtained model was corrected with 2Fo − Fc map using the program XtalView (23). Water molecules were picked by the water-add routine of XtalView (23). The structure of M-Ras-Gpp(NH)p was determined by the molecular replacement method using the structure of M-Ras-GDP as a search model. The crystal structure was refined at 1.3 Å resolution with the programs CNS (21) and REFMAC (22). After each refinement calculation, the obtained model was corrected with 2Fo − Fc map using the program XtalView (23).

**Table 1**

| Crystal  | M-Ras-GDP | M-Ras-Gpp(NH)p |
|----------|-----------|----------------|
| X-ray source | SPRing-8 (BL26B1) | SPRing-8 (BL40B2) |
| Space group | P2₁2₁2₁ | P622 |
| Unit cell parameters (Å) | a = 64.9, b = 70.7, c = 43.4 | a = b = 109.7, c = 69.4 |
| Wavelength (Å) | 0.90 | 1.00 |
| Resolution range (Å) | 47.7 to 1.30 (1.37 to 1.30) | 43.0 to 2.20 (2.22 to 2.20) |
| No. of unique reflections | 49,027 (6,555) | 13,000 (1,848) |
| Multiplicity | 7.0 (7.2) | 19.8 (21.2) |
| I/σ(I) | 6.8 (2.5) | 7.6 (2.5) |
| Completeness (%) | 98.5 (97.2) | 100 (100) |
| Rmerge (%) | 6.4 (28.0) | 7.2 (30.7) |

Refinement

| Resolution range (Å) | 43.4 to 1.30 (1.33 to 1.30) | 43.0 to 2.20 (2.26 to 2.20) |
| No. of reflections | 46,505 (3,361) | 12,365 (889) |
| R factor (%) | 18.3 (23.9) | 22.3 (24.3) |
| Free R factor (5% set) (%) | 20.0 (23.6) | 25.6 (31.6) |
| No. of residues | 169 | 163 |
| No. of nitrogen atoms | 1377 | 1333 |
| Protein | 28 | 32 |
| Nucleotide | 1 | 1 |
| Solvent | 238 | 98 |
| Average B factor (Å²) | 7.8 | 28.8 |
| Main chain | 10.7 | 30.8 |
| Side chain | 6.9 | 22.1 |
| Nucleotide | 0.3 | 1.0 |
| Magnesium | 5.2 | 22.3 |
| Solvent | 21.8 | 31.9 |
| Root mean square deviation from ideality | 0.009 | 0.010 |

bonds (Å) | 1.29 | 1.17 |
RESULTS

Structure of M-Ras in Complex with GDP or Gpp(NH)p—The crystal structure of M-Ras-GDP was refined to 1.3 Å resolution (Table I). The final electron density is clear for all of the atoms except the N-terminal 9 residues. The structure consists of a central β-sheet and five α-helices like H-Ras (Fig. 1B). The β-sheet contains six strands, of which one is anti-parallel and five are parallel. The switch I and switch II regions, assigned to residues 42–48 and 70–85, respectively, by analogy with H-Ras (Fig. 1A), are well defined. For evaluation of the structural similarity, root mean square deviations were calculated based on a least-squares fitting of the 166 Cα atoms. The values were 2.56 Å between M-Ras-GDP and H-Ras-GDP (Protein Data Bank identification code 4Q21), 2.67 Å between M-Ras-GDP and Rap2A-GDP (Protein Data Bank identification code 1KAO), and 2.73 Å between H-Ras-GDP and Rap2A-GDP, indicating that the overall structure of M-Ras-GDP resembles those of H-Ras-GDP and Rap2A-GDP. The switch II region of M-Ras-GDP, consisting of a 310-helix and the loop connecting strand and 2-helix (switch II loop), has a short insertion of a 310-helix (residues 72–74) (Fig. 1B). Presence of a 310-helix in the switch II region is unprecedented in GDP-bound forms of any Ras family GTPases in the Protein Data Bank data base.

The structure of M-Ras-Gpp(NH)p was refined to 2.2 Å resolution (Table I). There is no interpretable electron density for the N-terminal residues 1–10 and for residues 69–73, which form a part of the switch II loop (Fig. 2A). The overall structures of M-Ras-Gpp(NH)p and M-Ras-GDP superimpose well (root mean square deviation of 161 Cα atoms 1.02 Å) except for the switch regions (Fig. 2A). In the GDP-bound forms, the switch I loop of M-Ras, that connects 1-helix and [αβ]-strand, overlaps well with those of H-Ras and Rap2A (Fig. 2B). In contrast, in the Gpp(NH)p-bound forms, the conformation of the switch I loop of M-Ras exhibited a marked deviation from those of H-Ras and Rap2A (Fig. 2B). This was evidenced by calculation of root mean square deviations of seven Cα atoms of the switch I region. The values were 2.13 Å between M-Ras-Gpp(NH)p and H-Ras-Gpp(NH)p (Protein Data Bank identification code 5P21), 2.19 Å between M-Ras-Gpp(NH)p and Rap2A-GTP (Protein Data Bank identification code 3RAP.
Mg²⁺-binding proteins (26, 27). In addition, Asp-57 binds to the Ser-17. These interactions are conserved among various nucleotide-binding loop (P-loop, residues 10–18) are known to coordinate and direct interactions between the phosphate groups, although the hydroxyl groups of Ser-27 make hydrogen bonding networks to H-Ras-Gpp(NH)p (not shown). In contrast, Thr-45 of M-Ras-Gpp(NH)p (corresponding to Thr-35 of H-Ras) fails to interact with the Mg²⁺ ion and any phosphate groups, although the hydroxyl groups of Ser-27 make hydrogen bonds with Mg²⁺ ion and the β- and γ-phosphates like those of H-Ras Ser-17 (Fig. 3A). The corresponding position where the hydroxyl group of Thr-35 of H-Ras-Gpp(NH)p exists is occupied by Wat-3. Asp-67, like H-Ras Asp-57, binds to the side chain of Ser-27 and Wat-1, making an indirect interaction with the Mg²⁺ ion, but Asp-43 fails to make an indirect interaction with any phosphate groups via Wat-2 (Fig. 3A). Because the 2Fo – Fc map for the residues 69–73 was invisible in the M-Ras-Gpp(NH)p crystal, the association of Gly-70 in switch II with the γ-phosphate could not be examined. Collectively, the most remarkable difference found in the nucleotide-binding interfaces between M-Ras and H-Ras/Rap2A is an impairment of the direct and Mg²⁺-coordinated interactions of Thr-45 with the γ-phosphate group. The interaction between Thr-35 and the γ-phosphate of H-Ras-Gpp(NH)p is thought to stabilize the switch I loop by affecting the hydrogen bonding network involving Thr-35, Ser-17, and Asp-57 and thereby yield a formation of links between switch I loop, α₁-helix, and β₁-strand (Fig. 3B). Therefore, the loss of this interaction in M-Ras may have caused a drastic shift of the switch I loop, yielding an open conformation (Figs. 2B and 3B).

The N-terminal part of switch II (residues 69–73) was invisible in the 2Fo – Fc electron density map of M-Ras-Gpp(NH)p, indicating that this region at least does not assume a fixed secondary structure and may be extremely mobile in the crystals. The orientation of the α₁-helix of M-Ras-Gpp(NH)p shows a remarkable difference from those of H-Ras and Rap2A (Fig. 4). This seems to be a peculiar property of M-Ras switch II, because the orientation in the GTP-bound form is generally conserved among various GTPases, including H-Ras and Rap2A (17).

**Structural Property of M-Ras Switch I and Its Implications**—The solved structure of M-Ras-Gpp(NH)p indicated that its distinctive switch I conformation is mainly caused by impairment of the interaction between Thr-45 and the γ-phosphate group. This interaction is conserved in all of the solved structures of H-Ras and Rap2A alone as well as of the complexes of H-Ras and Rap1A with their effectors c-Raf-1, RasGDS, and phosphoinositide 3-kinase γ. On the other hand, electron paramagnetic resonance and 31P NMR studies of H-Ras-Gpp(NH)p had suggested that the coordination of Thr-35 to the γ-phosphate is transient in solution and that some population of H-Ras exhibits the loss of this interaction (10, 28, 29). Measurements of the chemical shift values of α₁, β₁, and γ-phosphates of Gpp(NH)p in the NMR spectrum had demonstrated that switch I of H-Ras-Gpp(NH)p adopts two different conformations, states 1 and 2 (10). Further, it was shown that association of H-RasGpp(NH)p with c-Raf-1 RBD as well as with Ras-associating domains of RasGDS and AF6 induces conversion of state 1 to state 2 (10, 11, 29, 30). Thus, state 2 represents a conformation capable of interacting with the effectors, and in fact all of the solved H-Ras crystal structures in complex with the effectors correspond to state 2 (8, 9). Since state 2 is a predominant form of H-Ras in solution, the crystal structure of H-Ras alone also corresponds to state 2 (26, 27). On the other hand, state 1 had been assigned to be a conformation incapable of binding to the effectors. An H-Ras mutant, T35S, which exhibited a much lower binding affinity to c-Raf-1 RBD compared with wild type, had been shown to predominantly adopt the state 1 conformation in solution, whereas it was converted to state 2 with the addition of c-Raf-1 RBD (11). In the T35S mutant, the loss of the methyl group of Thr-35 is assumed to impair its interactions with the γ-phosphate. These results led to a hypothesis that the interaction between Thr-35 and the γ-phosphate is impaired in the state 1 conformation of H-Ras. However, this hypothesis remained to be proved, because the switch I region failed to show up in the crystal structure of H-RasT35S (11).
We speculated that the solved structure of M-Ras-Gpp(NH)p might correspond to the state 1 conformation. To prove this, we carried out $^{31}\text{P}$ NMR spectroscopic experiments with M-Ras-Gpp(NH)p. At first, we confirmed that H-Ras-Gpp(NH)p shows two resonances each for the $\alpha$-phosphate ($-11.2$ ppm, $-11.7$ ppm) and the $\gamma$-phosphate ($-2.6$ ppm, $-3.4$ ppm), among which
11.2 and 2.6 ppm signals correspond to state 1, and the 11.7 and 3.4 ppm signals correspond to state 2 (Fig. 5A, upper panel, Table II) (10, 11). For assignments of the resonance signals to the β- and γ-phosphates, we followed a recent report by Iuga et al. (31), which was based on rotational resonance experiments on the crystalline H-Ras instead of past reports with different assignments. The relevant signal intensity ratio of state 1 to state 2 was 1:1.5, indicating the relative abundance of state 2 in H-Ras-Gpp(NH)p in solution. In contrast, M-Ras-Gpp(NH)p showed a single resonance each for the α- (−10.4 ppm), β- (−0.1 ppm), and γ-phosphates (−2.8 ppm) (Fig. 5A, lower panel, Table II), indicating that M-Ras-Gpp(NH)p exhibits only one conformation. In addition, the line width of the resonance signal for the γ-phosphate was

FIG. 5. 31P-NMR spectra of M-Ras-Gpp(NH)p. A, comparison of the spectra between M-Ras and H-Ras. The spectra were recorded using 1 mM proteins. α, β, and γ represent α-, β-, and γ-phosphate resonances, respectively. (1) and (2) represent the conformers in state 1 and state 2, respectively. B, effect of the addition of increasing amounts of c-Raf-1 RBD. The M-Ras-c-Raf-1 RBD ratio, represented at the right of each panel, varies between 0 (bottom panel) and 1 (top panel), starting with a solution of −1 mM M-Ras. The signal at 2.3 ppm originates from free phosphate ions. Chemical shift values for the resonances of α-, β-, and γ-phosphates are summarized in Table II.
narrower than that of H-Ras-Gpp(NH)p. This suggests that the mobility of the regions surrounding it, in particular the region including Thr-45 of M-Ras-Gpp(NH)p, is higher than that of H-Ras-Gpp(NH)p. When c-Raf-1 RBD was added at a half molar ratio to M-Ras-Gpp(NH)p, new resonance signals, −11.4 ppm for the α-phosphate and −3.7 ppm for the γ-phosphate, appeared in the lower magnetic field with a concomitant decrease in the original signals (Fig. 5B, middle panel, Table II). When the amount of c-Raf-1 RBD was increased to the equal molar ratio, the resonances for the α- and γ-phosphates were totally converted to the lower magnetic field signals (Fig. 5B, upper panel, Table II). These results imply that, by analogy with the observation with H-Ras, M-Ras predominantly exists as a state 1 conformation in solution, and its association with c-Raf-1 induces a transition of state 1 to state 2. These results taken together led us to conclude that the solved structure of M-Ras-Gpp(NH)p, where Thr-45 dissociates from the γ-phosphate, corresponds to the state 1 structure.

Implications of the Tertiary Structure of M-Ras in a Binding Affinity to Its Effectors—Although M-Ras was known to exhibit a weak binding ability to some Ras effectors, including c-Raf-1, its binding affinity had not been measured accurately. The dissociation constant \( K_d \) of M-Ras for c-Raf-1 RBD was measured and calculated to be 5.6 μM, which was ~10 times higher than that of H-Ras (0.5 μM) (Fig. 6A). Because M-Ras shares the identical effector region residues with H-Ras, a factor other than the primary structure of this region may account for the difference in their effector binding affinity. Our present results suggest that this affinity difference may be explained by a difference in the switch I conformation. M-Ras-Gpp(NH)p predominantly assumes the state 1 “off” conformation, which must be converted to the state 2 “on” conformation to establish the association with c-Raf-1 RBD. Thus, the forward rate of M-Ras binding to c-Raf-1 RBD is slowed by the cost of an additional energy required for the conversion of state 1 to state 2, resulting in an increase of the \( K_d \) value of M-Ras for c-Raf-1 RBD. This hypothesis is supported in part by the fact that M-Ras also shows lower binding activities toward other Ras effectors tested, including RasGDS and phosphoinsitide 3-kinase, compared with H-Ras (13, 14). Our arguments so far were based on the assumption that state 2 of M-Ras takes a conformation similar to that of H-Ras-Gpp(NH)p, where Thr-45 is coordinated to the γ-phosphate. To support this, we measured the binding affinity of an M-Ras mutant T45S to c-Raf-1 RBD (Fig. 6B). By analogy to the case with H-RasT35S (\( K_d = 25 \) μM), this mutation was expected to decrease the affinity of M-Ras to c-Raf-1 RBD. However, M-Ras-T45S indeed exhibited a much higher \( K_d \) value for c-Raf-1 RBD (30-100 μM) compared with wild-type M-Ras, suggesting that the interaction between Thr-45 and the γ-phosphate is restored in the state 2 conformation.

**DISCUSSION**

Switch regions undergo most drastic conformational changes in the overall structure of Ras upon GDP/GTP exchange. Switch I functions as a structural sensor that ensures the GTP-specific recognition of specific effectors. The primary structure of switch I is fully conserved among H-Ras, Rap1, Rap2, and M-Ras, and the solved backbone structures of the GTP-bound forms of H-Ras, Rap1A, and Rap2A superimpose well with one another (17). In contrast, the solved switch I structure of M-Ras-GTP is distinct from those of the three proteins, characterized by the complete loss of the interaction between Thr-45 and the γ-phosphate and by the drastic deviation of the switch I loop. Past \(^{31}P\) NMR studies had indicated that H-Ras-GTP exists in two interconverting conformations, state 1 and state 2 (10). Whereas state 2 corresponds to the “on” conformation capable of interacting with the effectors, state 1 corresponds to the “off” conformation, lacking the ability to

**TABLE II**

|          | α-Phosphate | β-Phosphate | γ-Phosphate |
|----------|-------------|-------------|-------------|
|          | σ_1 ppm     | σ_2 ppm     | σ_1 ppm     |
| H-Ras-Gpp(NH)p | −11.2       | −11.7       | −0.25       | −2.6 | −3.4 |
| M-Ras-Gpp(NH)p | −10.4       | −10.4       | −0.1        | −2.8 | −3.7 |
| + c-Raf-1 RBD | −10.3       | −11.4       | −0.1        | −2.8 | −3.7 |

**FIG. 6. Determination of the \( K_d \) values of M-Ras and H-Ras for c-Raf-1 RBD.** In vitro binding assays between c-Raf-1 RBD and H-Ras, M-Ras, or their mutants were carried out as described under “Experimental Procedures.” The amounts of free Ras and that bound to c-Raf-1 RBD were calculated, and a reciprocal of the concentration of the free c-Raf-1 RBD was plotted against a reciprocal of the concentration of the bound Ras. The \( K_d \) values were calculated from the points of intersection with the horizontal axis. A, closed diamond, H-Ras; closed square, M-Ras; open triangle, M-RasP40D. B, open diamond, H-RasT35S; open square, M-RasT45S.
responding to residues 69–73, is missing. A part of switch II of M-Ras, corresponding to residues 69–73, is missing. The image was prepared by using the program PyMOL (DeLano Scientific).

interact with the effectors. The state 1 conformation was predicted to be characterized by the loss of the interaction between Thr-35 and the γ-phosphate of GTP (11) in contrast to the state 2 conformation, whose crystal structure had been solved in H-Ras-GTP alone or in its complex with the effectors by x-ray crystallography. This led us to hypothesize that the solved M-Ras-GTP structure might correspond to the state 1 conformation. Our 31P NMR studies indicated that M-Ras-GTP predominantly exists as a state 1 conformation in solution. The assignment of state 1 in M-Ras was made initially from the observation that the gradual addition of c-Raf-1 induced a transition to another state which was most likely to be state 2 by analogy to the case with H-Ras. This was also supported from comparison of the environments surrounding the phosphate groups of GTP between M-Ras and H-Ras. Chemical shift changes are found close to the charged residues and aromatic ring residues. In particular, changes of the order of 1 ppm are often observed close to aromatic ring systems, and they are called “ring current shifts” (10). Previous NMR studies with mutants of H-Ras suggested that the ring current of Tyr-32 in switch I is the major reason for the shift of the γ-phosphate resonance. Because the switch I residues of M-Ras, including the corresponding Tyr-42, are identical to those of H-Ras, M-Ras and H-Ras are likely to share a common environmental background for determining the chemical shifts of the γ-phosphate. This enabled us to use the analogy with H-Ras to assign states 1 and 2 of M-Ras through the changes in chemical shifts.

So far, the solved structures of Rap1A and Rap2A, alone or in complex with the effectors, appear to correspond to state 2, because they show robust interactions between Thr-35 and the γ-phosphate and overlap well with H-Ras state 2 (6, 17). On the other hand, the tertiary structure of switch I corresponding to state 1 of M-Ras has been totally unknown. Although the H-RasT36S mutant predominantly exists as state 1, its crystal structure failed to reveal the switch I conformation (11). Thus, the M-Ras structure solved in this study is the first determined state 1 structure of Ras family small GTPases. The reason why the free M-Ras-GTP predominantly assumes the state 1 conformation remains to be addressed. Because the switch I residues are fully conserved, we turned our attention to the residues flanking it such as Pro-40, Asp-41, and Leu-51 of M-Ras, which are not conserved in H-Ras and Rap2A (Fig. 1A). The corresponding residues in H-Ras and Rap2A, especially residues 30 and 31, interact with the ribose ring and the α-phosphate group, respectively, thereby forming a tight association with GTP (Protein Data Bank identification codes 5P21 and 3RAP). However, both Pro-40 and Asp-41 of M-Ras fail to interact with the α-phosphate group, whereas it is partially compensated by a weak interaction between Asp-41 and ribose ring (data not shown). This observation suggests that Pro-40 may be intimately involved in establishing the distinct conformation of M-Ras switch I. The closed ring in the side chain of Pro makes it the most constrained residue type. The dihedral angles (φ) of the residues 40, 41, and 42 of M-Ras are −51°, 41°, and −105°, respectively, whereas those of the corresponding residues 30, 31, and 32 of H-Ras/Rap2A are −130°/−103°, −157°/−66°, and −82°/−95°, respectively. Subsequently, the backbone gap between Co of M-Ras and those of H-Ras/Rap2A increases gradually from Tyr-42/Tyr-32 and reaches to the maximum at Thr-45/Thr-35, yielding a drastic shift of the M-Ras switch I loop from those of H-Ras/Rap2A (Fig. 2B). The importance of Pro-40 of M-Ras was supported in part by the observation that the P40D mutation of M-Ras caused an increase in the binding affinity to c-Raf-1-RBD (Kd = 2.5 μM) (Fig. 6A), whereas mutations of Asp-41 or Leu-51 failed to affect the binding affinity (data not shown).

Free M-Ras exists predominantly as state 1 in solution. This raises a question about the physiological function of M-Ras; it is very unlikely that the only raison d'être for M-Ras is to weakly activate the state 2-specific effectors such as Raf kinases. There may be a set of proteins that specifically interact with state 1 of M-Ras. It may be possible that specific effectors such as RA(PDZ)-GEF-2 and MR-GEF (15, 16) or unidentified effectors of M-Ras interact preferentially with the state 1 conformation of M-Ras. Also, state 1 may be recognized by regulatory factors of small GTPases such as GEFs and GTPase-activating proteins. Our present result on the interaction of M-Ras with c-Raf-1 showed that the interpretation of the binding affinities of small GTPases to the effectors needs to take account of the state conversion. Thus, the action mechanisms of a wide variety of known interacting proteins of Ras family small GTPases shall be reevaluated in terms of the conformational transition.

The complete loss of the interactions between Thr-45 and the γ-phosphate in M-Ras-GTP resulted in a marked deviation of the switch I loop from GTP, forming a groove around the γ-phosphate (Fig. 7, red arrow). By analogy, the state 1 conformation of H-Ras-GTP is predicted to have a groove of similar size, which is much larger than that of the state 2 conformation of H-Ras-GTP (Fig. 7). Compounds that specifically fit into the groove of H-Ras state 1 may function as H-Ras-specific inhibitors by holding H-Ras in the state 1 “off” conformation. Likewise, compounds that specifically bind to the surface of the state 1 structure to stabilize it may also be used as specific

Fig. 7. Comparison of the switch I surface groove between M-Ras-Gpp(NH)p and H-Ras-Gpp(NH)p. The surface groove formed around the γ-phosphate of M-Ras is highlighted by a red arrow. The switch I and switch II residues are shown in yellow and green, respectively. Gpp(NH)p is shown by a CPK model. A part of switch II of M-Ras, corresponding to residues 69–73, is missing.
inhibitors. Thus, the state 1 conformation may provide a new molecular basis for designing anti-cancer drugs targeted for the ras oncogene products.

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