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Crystal structures of the state 1 conformations of the GTP-bound H-Ras protein and its oncogenic G12V and Q61L mutants

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ABSTRACT

GTP-bound Ras adopts two interconverting conformations, "inactive" state 1 and "active" state 2. However, the tertiary structure of wild-type (WT) state 1 remains unsolved. Here we solve the state 1 crystal structures of H-Ras WT together with its oncogenic G12V and Q61L mutants. They assume open structures characterized by impaired interactions of both Thr-35 in switch I and Gly-60 in switch II with the γ-phosphate of GTP and possess two surface pockets of mutually different shapes unseen in state 2, a potential target for selective inhibitor development. Furthermore, they provide a structural basis for the low GTPase activity of state 1.

1. Introduction

Small GTPases H-Ras, K-Ras, and N-Ras, the products of the *ras* proto-oncogenes, function as molecular switches by cycling between GTP-bound active and GDP-bound inactive forms in intracellular signaling pathways controlling cell growth, differentiation, and apoptosis [1]. Their interconversion is reciprocally catalyzed by guanine nucleotide exchange factors and GTPase-activating proteins (GAPs) [1]. In particular, GAPs enhance the intrinsic GTPase activity of Ras, leading to its inactivation. The oncogenic potential of Ras is activated by point mutations mainly at codons 12 and 61, the most prevalent of which in human cancers are those yielding G12V and Q61L substitutions. Most of these mutations impair the intrinsic GTPase activity and moreover render Ras insensitive to the GAP action, leading to constitutive activation of downstream effectors such as Raf kinases and phosphoinositide 3-kinases [1,2]. Crystal structures of H-Ras revealed that the exchange of GDP for GTP causes allosteric conformational changes in two adjacent regions, switch I (residues 32–38) and switch II (residues 60–75) [2]. Switch I almost overlaps with the effector region (residues 32–40), which forms a principal interface for effector recognition [2]. Besides, ^{31}P NMR studies revealed that H-Ras in complex with GTP or its non-hydrolyzable analog such as guanosine 5′-[β,γ-imido]triphosphate (GppNHp) exhibits dynamic equilibrium between two 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 GTP [3]. Because association with the various effectors, such as c-Raf-1, shifted the equilibrium toward state 2, state 1 and state 2 are regarded as "inactive" and "active" conformations, respectively [3]. Although crystal structures corresponding to state 2 were solved with wild-type (WT) H-Ras alone or in complex with the effectors [4,5], those corresponding to state 1 have only been solved with its mutants carrying T35S, G60A, and Y32F substitution [6–8] and M-RasWT [9], but not with H-Ras WT. It was revealed that state 1 assumes an open structure distinguishable from state 2 by the loss of the direct and Mg^{2+}-coordinated indirect hydrogen-bonding interactions of Thr-35 in switch I with the γ-phosphate of GppNHp, which causes marked deviation away from the guanine nucleotide and conformational instability of the switch I loop [6]. Further analyses showed the importance of the Gly-60-γ-phosphate direct hydrogen-bonding interaction and

Abbreviations: GAP, GTPase-activating protein; GppNHp, guanosine 5′-(β,γ-imido)triphosphate; RBD, Ras-binding domain; WT, Wild type; rms, root mean square

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the switch II-α3-helix interaction in stabilizing the state 2 conformation [6,10]. In particular, H-RasT35S-GppNHp yielded two distinct state 1 crystal structures, form 1 and form 2, showing the existence of polysterism in switch I and switch II [6]. The solution structure of H-RasT35S-GppNHp also revealed polysterism in the switch regions, prominent in switch I [11].

In the present study, we succeed in solving the state 1 crystal structures of H-RasWT, G12V, and Q61L in complex with GppNHp for the first time. Comparison with the WT state 2 structure provides a structural basis for the lower GTPase activity of state 1.

2. Materials and methods

2.1. Protein purification

Residues 1–166 of human H-RasWT, G12V, T35S, and Q61L, and the Ras-binding domain (RBD, residues 50–131) of human c-Raf-1 were expressed as fusions with glutathione S-transferase in *Escherichia coli* using pGEX-6P-l vector (GE Healthcare), immobilized on glutathione-agarose resin, and eluted by cleavage with PreScission protease (GE Healthcare). H-Ras polypeptides were loaded with GppNHp after further purification by ion exchange chromatography.

2.2. NMR spectroscopy

^{31}P NMR spectra were recorded in the presence or absence of c-Raf-1 RBD on Brucker AVANCE-500 NMR spectrometer [9] and referenced as described before [12].

2.3. Cross-seeding method for crystallization of H-Ras-GppNHp state 1

Crystals of H-RasWT, G12V, and Q61L in complex with Mg^{2+} and GppNHp (16, 6, and 6 mg/ml, respectively) were grown on seeds of a few pieces of finely pulverized crystals of H-RasT35S-GppNHp form 1 [6], which were transferred by using Disposable Crystal Probe manipulators (Hampton Research), at 20 °C by the sitting drop vapor diffusion method using a reservoir solution containing 100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, 200 mM ammonium sulfate, and 30% (w/v) PEG5000 MME. In addition, 1,5-diaminopentane dihydrochloride and β-nicotinamide adenine dinucleotide hydrate were added to the droplets of H-RasWT and Q61L, respectively. A MALDI-TOF mass spectroscopy confirmed that the H-RasWT-GppNHp crystals almost exclusively consisted of the WT protein.

2.4. Data collection and structure determination

The data collections at 100 K were carried out at the BL41XU beamline using MX225HE (Rayonix) CCD detector in SPring-8. The data were processed using the program MOSFLM [13] and scaled with SCALA in the CCP4 program suite [14]. For crystallographic refinement, the crystal structure of H-RasT35S-GppNHp form 1 was used as an initial model for H-RasWT-GppNHp, which was subsequently used for refining the crystal structures of H-RasG12V-GppNHp and H-RasQ61L-GppNHp. The crystal structures of H-RasWT, G12V, and Q61L were refined at 1.90 Å, 1.90 Å, and 2.30 Å resolutions, respectively, with the program REFMAC [15]. After each refinement calculation, the obtained models were corrected with a $2F_o - F_c$ map using COOT [16]. The data collection and refinement statistics are summarized in Supplemental Table I.

2.5. Graphics

Figures were prepared with the programs Raster3D [17] and MOLSCRIPT [18].

2.6. Protein Data Bank (PDB) codes of the coordinates used in this study

H-RasWT-GppNHp (state 1), 4EFL; H-RasG12V-GppNHp (state 1), 4EFM; H-RasQ61L-GppNHp (state 1), 4EFN; H-RasWT-GppNHp (state 2), 5P21; H-RasY32F-GppNHp, 3K9N; H-RasT35S-GppNHp form 1, 3KKN; H-RasT35S-GppNHp form 2, 3KKM; H-RasG60A-GppNHp, 1XCM.

3. Results and discussion

3.1. Crystal structures of H-RasWT, G12V, and Q61L correspond to state 1

The state 1 population occupies 36 ± 2% and 53% for H-RasWT-GppNHp and H-RasG12V-GppNHp, respectively [6,19]. H-RasQ61L-GppNHp also exhibited a comparable state 1 population of 58 ± 2% as shown by ^{31}P NMR (Supplemental Fig. 1). Our multidimensional heteronuclear NMR analysis of H-RasWT-GppNHp had shown that the backbone resonance signals for multiple residues were split into two peaks, one of which coincided well with those of H-RasT35S-GppNHp exclusively adopting state 1 [11]. This prompted us to grow H-RasWT state 1 crystals on the seeds of H-RasT35S-GppNHp crystals. By using the H-RasT35S-GppNHp form 1 crystals with the *I*222 space group as seeds, we obtained crystals of H-RasWT-GppNHp with the same space group. Moreover, subsequent trials for H-RasG12V and Q61L mutants successfully yielded similar crystals. Final $2F_o - F_c$ electron density maps obtained by a crystallographic refinement were clear for all the 166 residues and GppNHp (Supplemental Fig. 2) of WT, G12V, and Q61L with the resolution of 1.90 Å, 1.90 Å, and 2.30 Å, respectively. The structures adopted a Rossmann fold composed of 5 α-helices and 6 β-strands (Fig. 1). Least square fitting showed that the backbone structures of G12V and Q61L, including switch I and switch II, were superimposed very well on that of WT with root-mean-square (rms) deviations of 0.06 and 0.10 Å for 166 Cα atoms, respectively. The backbone structures of the P-loop (residues 10–17) and switch II

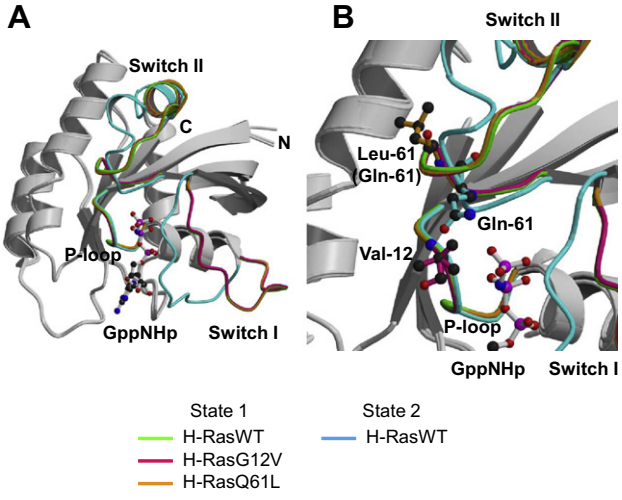

Fig. 1. Comparison of the crystal structures among H-RasWT-GppNHp and its mutants. Superimposition of the backbone structures of the P-loop, switch I, and switch II among WT state 1 (4EFL, *green*), G12V state 1 (4EFM, *deep pink*), Q61L state 1 (4EFN, *orange*), and WT state 2 (5P21, *cyan*) shown in the *ribbon model*. The other regions are overlapped very well and shown in *gray*. GppNHp of WT state 1 is shown in the *ball-and-stick model* (*red*, oxygen; *blue*, nitrogen; *deep pink*, phosphorus). (B) An enlargement of the structures surrounding Val-12 in G12V state 1 (4EFM, *deep pink*), Leu-61 in Q61L state 1 (4EFN, *orange*), and Gln-61 in WT state 2 (5P21, *cyan*), which are highlighted by the *ball-and-stick model*.