Structural basis for intramolecular interaction of posttranslationally modified H-Ras·GTP prepared by protein ligation

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学 位 論 文 の 内 容 要 旨

Structural basis for intramolecular interaction of posttranslationally modified H-Ras•GTP prepared by protein ligation

ペプチドライゲーションによって産生した翻訳後修飾型 H-Ras•GTP に存在する分子内相互作用の構造生物学的研究

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Introduction

The products of the *ras* proto-oncogenes, Ras (H-Ras, K-Ras and N-Ras), function as a molecular switch for controlling cell proliferation, differentiation and survival by interconverting between the GDP-bound "OFF" and GTP-bound "ON" forms (Ras•GDP and Ras•GTP, respectively). They are grossly divided into two parts: the catalytic domain ($Ras^{1\text{-}166}$) corresponding to the amino acid residues 1-166 and the hypervariable region (HVR) corresponding to the residues 167-189 in H-Ras. $Ras^{1\text{-}166}$, whose amino acid sequence is highly conserved among the isoforms, harbors the guanine nucleotides and, in its GTP-bound form, associates with the Ras-binding domains (RBDs) of the effectors including c-Raf-1 through its flexible binding interface composed of two regions, Switch I (residues 32-38) and Switch II (residues 60-75). In addition, the residues 23-31 and 39-50 flanking Switch I are named the "activator region" because they contain various substitutions in a Ras-homologue Rap1, which has an antagonistic effect toward Ras-dependent malignant transformation and because introduction of Rap1-type substitutions into this region indeed abrogated the activity to activate c-Raf-1. On the other hand, the amino acid sequence of HVR is highly divergent except for the C-terminal CAAX motif (A, aliphatic amino acids; X, any amino acids), which is subjected to a series of posttranslational modification reactions, including farnesylation of Cys186 in the CAAX motif, proteolytic removal of the AAX tripeptide, carboxymethylation of the resulting C-terminal Cys, in a sequential manner.

We previously showed that the farnesylation-dependent interaction of Ras with the cysteine-rich domain (CRD, residues 139-184) in the N-terminal regulatory region of c-Raf-1 is necessary for its full activation of c-Raf-1 and is abolished by substitutions, such as N26G and V45E, in the activator region, suggesting that the farnesylated C terminus and the activator region might cooperatively recognize CRD. However, the molecular basis for this interaction remains elusive because of the difficulties in preparing posttranslationally modified Ras in an amount sufficient for structural studies by NMR and X-ray crystallography. In the present study, to address this problem, we make use of a protein ligation reaction catalyzed by a transpeptidase from *Staphylococcus aureus*, Sortase A (SrtA), enabling the preparation of H-Ras carrying various degrees of the modifications in amounts sufficient for structural studies. The structural characterization by using NMR show that HVR makes an intramolecular interaction with a region of the catalytic domain and that this interaction brings the farnesyl moiety to the vicinity of the activator region, which may be responsible for their cooperative recognition of c-Raf-1-CRD. Furthermore, we are able to solve the crystal structure of modified H-Ras for the first time, showing that the existence of modified HVR induces conformational changes

of the local structure.

Methods and Results

In vitro production of H-Ras proteins carrying various degrees of the modifications

We produced three kinds of full-length H-Ras proteins representing various stages of the posttranslational modifications: $H\text{-}Ras^{C(Far)VLS}$, $H\text{-}Ras^{C(Far)}$ and the mature $H\text{-}Ras^{C(Far)OMe}$, and that lacking any modifications, $H\text{-}Ras^{CVLS}$, by the SrtA-catalyzed ligation of bacterially expressed $H\text{-}Ras^{substrate}$, which carries a recognition motif of SrtA, LPXTG, at the C terminus, with the corresponding synthetic HVR peptides. Optimization study of the reaction condition showed that the highest yield of the ligation products was obtained when the reaction was carried out at 30 °C for 24 h at the molecular ratio of 1:8:10 for $H\text{-}Ras^{substrate}$, SrtA and HVR peptides. Typically, 0.3 mg of $H\text{-}Ras^{C(Far)OMe}$ loaded with a non-hydrolyzable GTP analogue, guanosine 5'-(β, γ-imido) triphosphate (GppNHp), was obtained from 24 mg of $H\text{-}Ras^{substrate}$ and 4.4 mg of the C(Far)OMe peptide. The completion of the reactions, the purities and the molecular masses for the ligation products were confirmed by western blotting analyses using an anti-H-Ras C-terminus antibody, SDS-PAGE and MALDI-TOF-MS, respectively.

NMR analyses for the intramolecular interaction between the catalytic domain and HVR and effect of the posttranslational modifications

To investigate the effect of the attachment of the unmodified and modified HVR on the structure of the catalytic domain by NMR, $H\text{-}Ras^{CVLS}$ and $H\text{-}Ras^{C(Far)OMe}$ were prepared by ligating uniformly ^{15}N-labeled $H\text{-}Ras^{substrate}$ with the corresponding HVR peptides, loaded with GppNHp and subjected to the measurements of $^{1}H\text{-}^{15}N$ HSQC spectra, in which NH pairs of the individual residues were observed as individual signals. Spectral comparison between $H\text{-}Ras^{CVLS}$•GppNHp and $H\text{-}Ras^{substrate}$•GppNHp revealed that the residues exhibiting significant signal changes were mainly located in the region encompassing the C terminus (Ile163-Gln165) to the activator region (His27, Val44, Val45 and Glu49) across the central β sheet (Thr2, Lys5 and Leu6 in the β1-strand and Cys51-Leu53 in the β3-strand), suggesting that the unmodified HVR established an intramolecular interaction with these regions of the catalytic domain. The $^{1}H\text{-}^{15}N$ HSQC spectrum of $H\text{-}Ras^{C(Far)OMe}$•GppNHp exhibited similar signal changes to those of $H\text{-}Ras^{CVLS}$•GppNHp. However, it showed additional signal changes for the residues located in the activator region and its proximity, Phe28, Asp54 and Ile55, suggesting that the modified HVR carrying the farnesyl group was positioned in proximity to the distal part of the activator region. The interaction of HVR with the catalytic domain was further proved by

^{1}H-^{15}N HSQC and surface plasmon resonance experiments, where direct binding of the C(Far)OMe HVR peptide to H-Ras$^{1-166}$•GppNHp was observed. Among the residues exhibiting the signal changes in the ^{1}H-^{15}N HSQC spectra of H-Ras^{CVLS}•GppNHp and H-Ras$^{C(Far)OMe}$•GppNHp, Val8, Gly77, Phe78, Leu79, Phe156 and Val160, were buried in the hydrophobic core. Therefore, it was likely that they were incapable of establishing any contacts with HVR and that the observed signal changes were attributed to either a secondary effect of the conformational change in the neighboring residues or the changes in their own conformational dynamics, or both of them.

Crystal structure of modified H-Ras and its structural differences from unmodified H-Ras

We successfully determined the crystal structure of H-Ras$^{C(Far)OMe}$•GppNHp for the first time at the resolution of 2.5 Å. No electron density corresponding to the modified HVR was observed, suggesting that its structure was heavily disordered. The overall structure of the catalytic domain of H-Ras$^{C(Far)OMe}$•GppNHp was similar to that of H-Ras$^{1-166}$•GppNHp (PDB code 3K8Y). Superimposition of the two structures indicated that a large part of the α2-helix in Switch II was unstructured and that the C-terminal part of the α3-helix and the following loop were shifted toward the α2-helix in H-Ras$^{C(Far)OMe}$•GppNHp. The conformational changes agreed well with the NMR data in that the residues in the corresponding region: Ala66, Met67, Arg68, Thr74 and Gly75 in the α2-helix and Lys104 in the α3-helix, showed significant signal changes in ^{1}H-^{15}N HSQC spectrum of H-Ras$^{C(Far)OMe}$•GppNHp. In the crystal structure, Val8, Gly77, Phe78, Leu79 and Val81, which exhibited the HSQC signal perturbations despite their location in the hydrophobic core, were found to be located adjacent to the regions showing the conformational changes, proving that their HSQC signal changes were likely to be ascribable to the secondary effect of the conformational changes in the neighboring regions.

Discussion

Although Ras proteins exert their cellular functions as the posttranslationally modified form, their structural studies had been regularly carried out using the unmodified form often truncated with HVR due to the problem of tremendous difficulties in preparation of modified Ras in an amount sufficient for structural studies by NMR and X-ray crystallography. We overcame this problem by the adoption of the SrtA-catalyzed protein ligation. Moreover, the method enabled us to systematically produce modified Ras proteins representing various stages of the posttranslational modifications, allowing the analyses of the roles of the individual modification steps in future studies.

Previous studies had indicated that Ras directly binds to c-Raf-1-CRD and that this interaction is cooperatively achieved by the farnesylated C terminus and the activator region of Ras. In this study, we have demonstrated the existence of an intramolecular interaction of the modified HVR with the catalytic domain in H-Ras•GppNHp across the central β sheet. This causes positioning the modified HVR carrying the farnesyl group in proximity to the distal part of the activator regions, suggesting that H-Ras$^{C(Far)OMe}$•GppNHp may adopt a compact tertiary structure to form a molecular basis for their cooperative binding to CRD. The farnesyl moiety may contribute to a hydrophobic interaction between Ras and CRD and, in that case, the carboxymethylation of Cys186 may serve to strengthen this interaction as observed for the interaction between farnesylated K-Ras4B and PDEδ, where the binding affinity became approximately two orders of magnitude higher depending on the carboxymethylation. In this line, biochemical and structural characterization of H-RasC(Far)VLS and H-RasC(Far) may provide further insight into the significance of the carboxymethylation as well as the AAX removal.

The crystal structure of H-Ras$^{C(Far)OMe}$•GppNHp revealed that the α2- and α3-helices adopted local structures distinct from those of H-Ras$^{1-166}$•GppNHp. Because the α2- and α3-helices are known to undergo diverse conformational changes in association with a wide range of the physiological functions, the posttranslational modifications may significantly affect the biophysical and biochemical parameters of Ras proteins relevant to their cellular functions. Since such parameters have regularly been determined using unmodified Ras often truncated with HVR, they must be critically examined using the posttranslationally modified form.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲 第2722号	氏　名	柯 浩亮
論文題目 Title of Dissertation	Structural basis for intramolecular interaction of posttranslationally modified H-Ras・GTP prepared by protein ligation ペプチドライゲーションによって産生した 翻訳後修飾型 H-Ras・GTP に存在する分子内相互作用の 構造生物学的研究		
審査委員 Examiner	主　査 Chief Examiner 南　康博 副　査 Vice-examiner 勾坂 敏朗 副　査 Vice-examiner 仁田 亮		

（要旨は１，０００字～２，０００字程度）

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Three kinds of full-length H-Ras proteins representing various stages of the post-translational modifications: $H\text{-}Ras^{C(Far)VLS}$, $H\text{-}Ras^{C(Far)}$ and the mature $H\text{-}Ras^{C(Far)OMe}$, and that lacking any modifications, $H\text{-}Ras^{CVLS}$, were produced by the SrtA-catalyzed ligation of bacterially expressed $H\text{-}Ras^{substrate}$, which carries a recognition motif of SrtA, LPXTG, at the C terminus, with the corresponding synthetic HVR peptides. The purities and the molecular masses of the ligation products were confirmed by SDS-PAGE and MALDI-TOF-MS, respectively.

The effect of the attachment of the unmodified and modified HVR on the structure of the catalytic domain was investigated by measuring the $^{1}H\text{-}^{15}N$ HSQC NMR spectra of $H\text{-}Ras^{CVLS}$ and $H\text{-}Ras^{C(Far)OMe}$, prepared by ligating uniformly ^{15}N-labeled $H\text{-}Ras^{substrate}$ with the corresponding HVR peptides. Spectral comparison between $H\text{-}Ras^{CVLS}$•GppNHp and $H\text{-}Ras^{substrate}$•GppNHp revealed that the residues exhibiting significant signal changes were mainly located in the region encompassing the C terminus (Ile163-Gln165) to the activator region (His27, Val44, Val45 and Glu49) across the central β sheet (Thr2, Lys5 and Leu6 in the β1-strand and Cys51-Leu53 in the β3-strand), suggesting that the unmodified HVR established an intramolecular interaction with these regions of the catalytic domain. The $^{1}H\text{-}^{15}N$ HSQC spectrum of $H\text{-}Ras^{C(Far)OMe}$•GppNHp

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The crystal structure of H-Ras$^{C(Far)OMe}$•GppNHp was determined for the first time at the resolution of 2.5 Å. No electron density corresponding to the modified HVR was observed, suggesting that its structure was heavily disordered. Superimposition of the structures of H-Ras$^{C(Far)OMe}$•GppNHp and H-Ras$^{1\text{-}166}$•GppNHp indicated that a large part of the α2-helix in Switch II was unstructured and that the C-terminal part of the α3-helix and the following loop were shifted toward the α2-helix in H-Ras$^{C(Far)OMe}$•GppNHp. The conformational changes agreed well with the NMR data in that the residues in the corresponding region: Ala66, Met67, Arg68, Thr74 and Gly75 in the α2-helix and Lys104 in the α3-helix, showed significant signal changes in ^{1}H-^{15}N HSQC spectrum of H-Ras$^{C(Far)OMe}$•GppNHp.

Although Ras proteins exert their cellular functions as the post-translationally modified form, their structural studies had been regularly carried out using the unmodified form often truncated with HVR due to the problem of tremendous difficulties in preparation of modified Ras in an amount sufficient for structural studies. In this study, the problem was overcome by adoption of the SrtA-catalyzed protein ligation. Moreover, the method enabled systematic production of modification intermediates, allowing analyses of the roles of the individual modification steps. Previous studies had indicated that Ras directly binds to c-Raf-1-CRD through the farnesylated C terminus and the activator region. This study have demonstrated the existence of an intramolecular interaction of the modified HVR with the catalytic domain in H-Ras•GppNHp. This causes positioning the modified HVR carrying the farnesyl group in proximity to the distal part of the activator region, suggesting that H-Ras$^{C(Far)OMe}$•GppNHp may adopt a compact tertiary structure forming a molecular basis for their cooperative binding to CRD. The crystal structure of H-Ras$^{C(Far)OMe}$•GppNHp revealed that the α2- and α3-helices adopted local structures distinct from those of H-Ras$^{1\text{-}166}$•GppNHp. Because these helices are known to undergo diverse conformational changes in association with a wide range of the physiological functions, the post-translational modifications may significantly affect the biophysical and biochemical parameters of Ras relevant to their cellular functions. Accordingly, it was proposed that such parameters must be critically examined using the post-translationally modified form since they have regularly been determined using unmodified Ras often truncated with HVR.

The candidate, having completed studies on the structural basis for intramolecular interaction of post-translationally modified H-Ras•GTP and having advanced the field of knowledge in the area of Cancer Biochemistry, is hereby recognized as having qualified for the degree of Ph.D. (Medicine).