低分子量 G タンパク質 Ras の機能ドメイン HVR の特性を利用したRas の人工的制御

Studies on the functional role of HVR domain in the small G-protein H-Ras and its application to Artificial control of Ras

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SYNOPSIS

The small G protein Ras is a central regulator of cellular signal transduction processes, functioning as a molecular switch. Ras have a specific functional domain HVR. HVR determine the important physiological role of Ras. In this study, functional characterization of HVR was studied, and based on the properties, photocontrol of the Ras function was performed by incorporating designed photochromic nanodevices into HVR. Modification of cysteine residues as lipidation sites in HVR with caged compounds induced multimer of Ras. Electron microscopic observation and averaging image analysis of the multimer showed circular ring shape which is consistent with the structure estimated from X-ray scattering. In order to photocontrol Ras , the two different polarity photochromic sulfhydryl-reactive azobenzene derivatives, 4-phenylazophenyl maleimide (PAM) and 4-chloroacetoamido-4'-sulfo-azobenzene (CASAB) were incorporated into HVR. GTPase of the H-Ras modified with CASAB was photocontrolled more effectively than PAM-H-Ras. Interestingly PAM modification induced H-Ras multimerization, but not CASAB. In this study, it has been demonstrated that incorporating photochromic molecules as a regulatory nanodevice into the functional HVR domain enable to control Ras function photoreversibly.

Keywords: Small G protein, H-Ras, caged compound, photochromic molecule, chemical modification,

1. Introduction

Ras is one of the Small G proteins which is known as a molecular switch, is a central regulator of cellular signal transduction processes leading to transcription, cell cycle progression etc. The active ON state and the inactive OFF state of Ras are regulated by the two factors. GTPase activating protein (GAP) induce hydrolysis of GTP to GDP resulting in formation of inactive state of Ras. On the other hand, Guanine nucleotide exchange factor (GEF) replaces GDP with GTP and make Ras ON state. The switching mechanisms utilizing conformational changes in the nucleotide-binding motifs have been well studied at the molecular level. Interestingly, recent studies have shown that G proteins have a common nucleotide-binding motif with the ATP-driven motors, myosin and kinesin. These nucleotide binding proteins might be evolved from a common nucleotide-binding ancestral protein and share a common catalytic core region including switch I, Switch II and P-loop, and molecular mechanism utilizing a nucleotide hydrolysis cycle (1). Previously Studies demonstrated that incorporation artificial regulatory nanodevices such as a photochromic molecule into the functional site of Kinesin enable to control ATPase activity photoreversibly (2). Interestingly, Hyper Variable Region (HVR) is one of the functional parts of the G proteins in which modification induce multimerization and interaction with plasma membrane of Ras. In physiological state Ras forms the nanocluster on the plasma membrane by the lipid modifications of the hypervariable region (HVR) at C-terminal to exhibit physiological function (3). It is believed that this HVR domain might play a crucial role in Ras protein to its cellular function. First in this study, we have demonstrated that chemical modification of cysteines residues in HVR with caged compounds instead of lipidation induces multimerization of H-Ras. Sulfhydryl-reactive caged compound, 2-Nitrobenzyl bromide (NBB) was stoichiometrically incorporated into the cysteine residue of HVR and induced formation of Ras multimer. Light irradiation induced elimination of 2-Nitrobenzyl group, resulting in conversion of multimer to monomer. SEC-HPLC and Small angle X-ray scattering (SAXS) analysis revealed that the H-Ras forms pentamer. Electron microscopic observation of the multimer showed circular ring shape which is consistent with the structure estimated from X-ray scattering. The shape of the multimer may reflect the physiological structural state of Ras. It was suggested that the multimerization and monomerization of H-Ras was controlled by the modification with caged compound at HVR and light irradiation reversibly. However, caged compound exhibit irreversible photo eliminating reaction. Therefore, caged compounds does not work as a reversible photo-switch. In further study, we employed the azobenzene derivative as a photo reversible nano switching device and incorporated into the HVR to control Ras function. We introduced the two highly different polarity photochromic sulfhydryl-reactive azobenzene derivatives, 4-phenylazophenyl maleimide (PAM) and 4-chloroacetoamido-4'-sulfo-azobenzene (CASAB) into cysteine residues in HVR to regulate the GTPase activity by photoirradiation. PAM was stoichiometrically incorporated into the three cysteine residues

in HVR and induced multimerization. The PAM-modified mutants exhibited reversible alterations in GTPase activity accelerated by GEF And GAP, and multimerization accompanied by photoisomerization upon exposure to ultraviolet and visible light irradiation. CASAB was incorporated into two of the three cysteine residues in HVR but not induced multimerization. GTPase of the H-Ras modified with CASAB was photocontrolled more effectively than PAM-H-Ras. Interestingly CASAB modification did not induced H-Ras multimerization. The results suggest that incorporation of photochromic molecules into its functional site enables photoreversible control of the function of the small G protein Ras. Well known photochromic compounds show light sensitivity at a specific wavelength. Upon light irradiation photochromic compounds can change their structure and functions. There are two types of mechanisms observed for returning to their original states. A mechanism that returns by irradiating light with a different wavelength, it's called P-type such as diarylethene and fulgide. Another one, a mechanism that returns by heat, it's called T-type such as spiropyran, azobenzene, and stilbenes. In this study we used small caged compound which may mimic the physiological lipidation and photochromic compound such as azobenzene derivatives (fig 1 & 2).

2. Chemical Modification of H-Ras with caged chemical compound

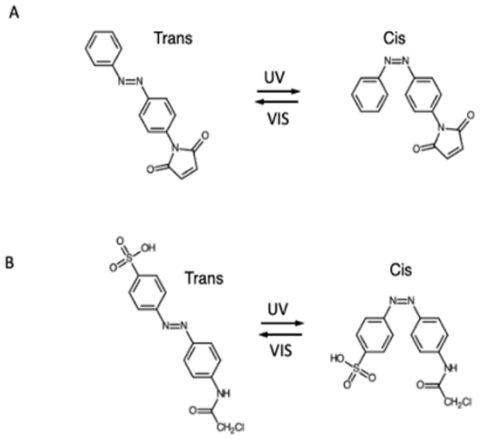

Fig. 1 Chemical structure and photo-isomerization of (A)PAM and (B) CASAB

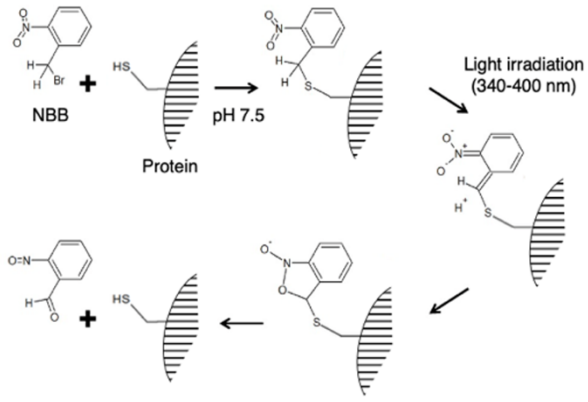

Fig 2: Small Caged compound NBB

We employed a sulfhydryl group reactive caged compound to modify the cysteine residues in HVR. NBB used in this study is one of the well-known cage compounds and can be specifically introduced into the thiol group of the cysteine residue as shown in Then, by light irradiation at 340-400 nm, the nitrobenzyl group is eliminated and the protein reversibly returns to its original state. There are 6 cysteine residues in Human H-Ras. Three of them are in the globular domain and C118 is located on the surface. The remaining three cysteine (C181, C184,C186) which are known as lipidation sites are in the HVR domain. HVR domain is exposed to solvent. NBB is incorporated in to the cysteine residues stoichiometrically. Therefore, it is assumed that the four cysteine residues (C118, C181, C184 and C186) exposed to solvent are modified specifically.

3. Structural analysis of NBB-Ras multimer by X-ray small angle solution scattering and dummy-atom structural modeling

To study the global conformation of the multimer of H-Ras

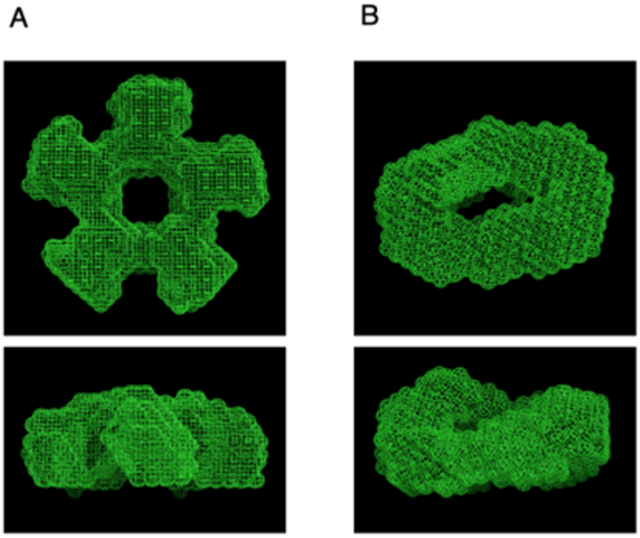

Fig. 3 Small Angle X-ray Scattering

induced by NBB modification at HVR, we utilized a small-angle synchrotron X-ray scattering technique using synchrotron radiation as an intense X-ray source. 2, 4, 6, and 8 mg/ml H-Ras modified with NBB in the buffer of 120 mM NaCl, 30 mM Tris-HCl, pH 7.5, 1 mM MgCl2 were measured at 25 ºC. Intact Ras and NBB-H-Ras irradiated by 400 nm wavelength light for 2h (ΔNBB-H-Ras) were also measured as standard comparison. The radius of gyration, Rg(c), and the intensity at zero scattering angle, I(0,c), for each different concentrations were determined from the Guinier plot of the intensity profile. As shown in Fig. 9A, the plot on the I(0)/c vs c clearly indicated that the estimated molecular weight of NBB-H-Ras is 5 times larger than that of intact H-Ras. Therefore, the multimer composed of NBB-H-Ras is revealed as a pentamer and consistent with the results of size exclusion chromatography. ΔNBB-H-Ras in which 40 % NBB group was eliminated by light irradiation showed much reduced molecular weight suggesting that conversion of pentamer to monomer. The two possible structures were obtained as shown

in Fig. 3. One of them is assumed to have pentagonal symmetry ring structure. Interestingly the symmetric pentamer model of the NBB-H-Ras multimer was similar to one of the model estimated for the cluster of K-Ras by in silico study reported by Sarkar-Banerjee et al. The shape of the other model was shown also as ring structure but not symmetrical structure.

4. Electron Microscopic observation of the Ras multimer

By rotary shadowing and electron microscopy, we observed the configurations of NBB-H-Ras multimer and intact H-Ras monomer. As shown in Fig. 4 A & B, H-Ras modified with NBB showed apparently round shape particle with approximately 20 nm diameter which is consistent of the size of multimer estimated from P(r) function X-ray scattering.
At higher concentration, further larger cluster composed of the round shape particles was also observed. On the other hand, such a particle reflecting formation of multimer was not observed.

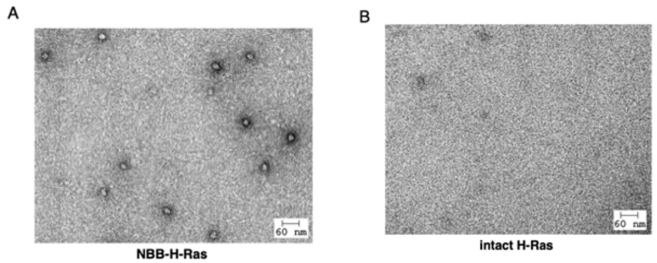

Fig 4: Electron Microscopic Analysis

5. Photoisomerization of the azobenzene derivatives incorporated into HVR of H-Ras.

It is well known that the configurational state (cis or trans) of azobenzene and its derivatives can be monitored by UV/VIS light absorption spectroscopy. The absorption spectrum of PAM and CASAB were similar spectrum and exhibit significant spectral changes upon UV and visible light irradiations reflecting typical azobenzene cis and trans isomerization (Fig. 5). The trans form of free PAM exhibited maximum absorption at 340 nm and the peak significantly reduced by UV irradiation. The alteration of the spectrum was very fast and saturated within 2 min. On the other hand, although PAM modified H-Ras showed similar absorption spectrum, the peak of the spectrum exhibited slower reduction than free PAM with 10 min UV irradiation to be saturation.

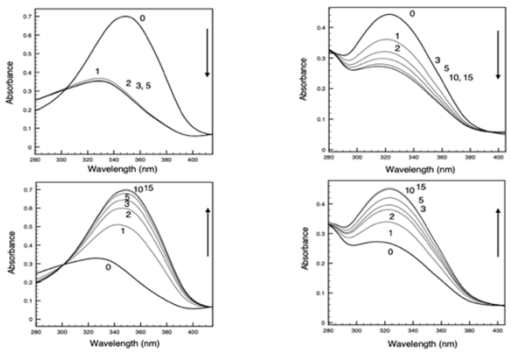

Fig 5: Absorption spectrum of PAM-H-Ras and free PAM

6. Ras GTPase cycle accelerated by GAP and GEF.

Prior to examine photoregulation of Ras GTPase by modification with photochromic compounds, we established the convenient Ras GTPase assay condition. It is known that intrinsic Ras GTPase activity is extremely slow to perform as a switch of cellular signal transduction. GTP bound state Ras behave as switch On and GDP bound state behave switch OFF. As expected, in the presence of GAP and GEF, GTPase of C118S was accelerated by approximately 7.5 times as shown in Fig. 6. Wild type H-Ras showed almost identical GTPase acceleration (data not shown). The GTPase activity was strongly dependent on the concentration of NaCl in the assay buffer. The GTPase cycle accelerated by GAP and GEF produced enough Pi from GTP hydrolysis to quantify using general Pi detecting reagents.

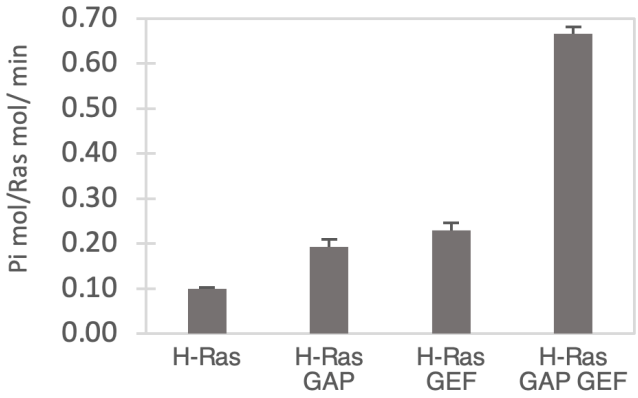

Fig 6: GTPase assay of H-Ras

7. Photocontrol of GTPase cycle of H-Ras modified with PAM and CASAB

The isomerization of PAM-Ras modified with PAM was performed at 0°C in modification buffer using UV light irradiation (Black-ray lamp, 366 nm, 16 W, UVP Inc., San Gabriel, CA, USA) for induction of the cis state for 3 min and using Vis light irradiation of the trans state for 10 min and then repeated. The GTPase activity of PAM-Ras was measured at 25 °C in the GTPase assay buffer. In case of CASAB-Ras photoisomerization was performed with the same condition with PAM. The GTPase activity of CASAB-Ras was measured at 25 °C in the GTPase assay buffer. PAM- C118S exhibited apparent changes in GTPase activity upon UV and Vis light irradiations, as shown in Fig. 7A. The GTPase activities of the Trans-PAM-C118S and Cis-PAM-C118S showed 70% and 80% of intact C118S, respectively. The differnces on the GTPase activity between the two isomerization states was not significant but showed reproducibility of photoreversible alteration (Fig.8). On the other hand, CASAB-C118S changed its GTPase activity more significantly than PAM-C118S accompanied by photoisomerization (Figure 7B). Trans-CASAB-C118S reduced GTPase activity to 62% of intact-C118S. Cis-CASAB-C118S exhibited almost same GTPase activity (95%) with intact C118S. The GTPase activity of CASAB-C118S reversibly photocontrol by UV and visible light irradiations as same as PAM-C118S.

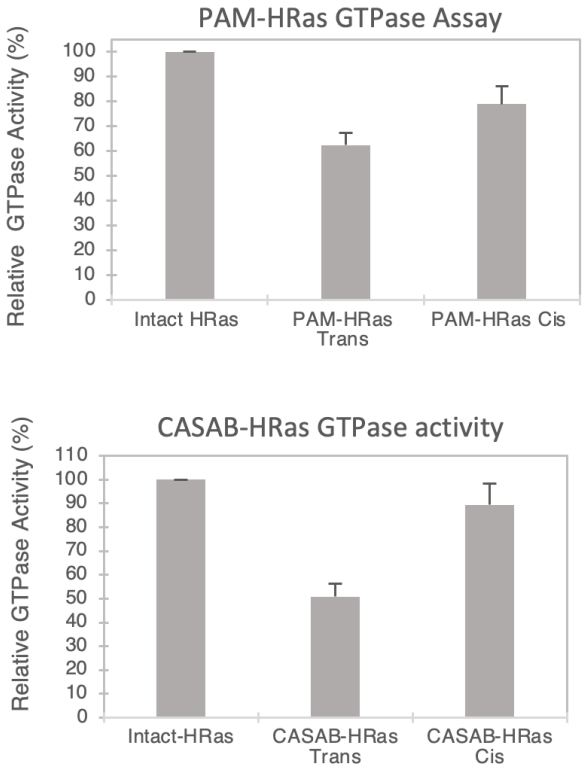

Fig. 7 Photoregulated GTPase assay of PAM and CASAB modified H-Ras

8. Formation of H-Ras multimer by modification with azobenzene derivatives and its photocontrol

Previously we have demonstrated that the modification at the cysteine residues as a lipidation sites in HVR with caged compound induced formation of Ras multimer which may mimic the physiological cluster. Therefore, formations of Ras multimer induced by modification of HVR with PAM and CASAB were also examined by SEC-HPLC according to the methods previously reported. Intact unmodified H-Ras mutant C118S eluted at around 7 min on the SEC-HPLC mentioned in Materials and Methods. This elution time was identical to that of our previously report on the caged modification. On the other hand, PAM C118C showed the elution profile reflecting the formation of Ras multimer as we have previously observed on the caged compound-Ras multimer. The peak at the 5 min is consistent with the elution of Ras multimer modified with Caged compounds. Moreover, cis isomer of PAM-C118S showed slightly lower peak than that of trans isomer. The results may suggest that the cis -trans isomerization of PAM incorporated into the HVR lipidation sites changes the monomer-multimer equilibrium phtoreversibly as shown in Fig.8. On the contrary, although small broad peaks were observed at 5min, Cis and Trans form of CASAB modified H-Ras exhibited

almost no multimer formation. However, apparent differences on the elution profiles around the monomer area were observed between cis and trans CASAB-S118C. The monomer of trans CASAB-S118C exhibited more widely broadened elution profile. This is reflecting the conformational differences of HVR on the globular domain between the two isomerization states.

9. Discussion:

In this study we focused on the regulatory domains HVR of the G protein which regulate physiological roles. Therefore, incorporating the photochromic molecules as a photoswitch into HVR is highly expected to exhibit high efficient photoreversible regulation of Ras function related to physiological roles. Incorporation of thiol reactive caged compound into HVR lipidation sites induced formation of multimer and subsequently the multimer returned to monomer by elimination reaction of the caged group upon light irradiation. However, the caged compounds exhibit irreversible reaction. Therefore, it is not applicable to reversible switching system. We employed azobenzene as a photoswitching molecular device to incorporate into HVR. Azobenzene changes its molecular size and polarity drastically accompanied by cis-trans isomerization, as a result PAM modified H-Ras showed multimer formation and less efficient GTPase regulation. However, CASAB-H-Ras did not formed

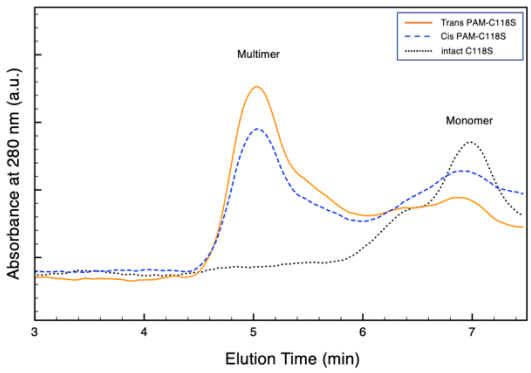

Fig. 8 Elution profile of PAM-Ras on the SEC-HPLC

multimer but higher GTPase regulation. Therefore, it is strongly expected as a reversible photoswitching of the Ras function.

Conclusion:

Structural analysis of H-Ras multimer by Electron microscopic method reveals the disk shape and pentamer formation which is consistent with one of the possible structures estimated from the data of small angle X-ray scattering indicating physiological conformational changes of HVR domain during lipidation. Another approach to regulate the function of Ras using azobenzene derivatives incorporating into the HVR domain showed successful photoregulation. That can be possible application to control the ras function which is involved into the cancer formation.

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