Interaction of a novel fluorescent GTP analogue with the small G-protein K-Ras

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A novel fluorescent guanosine 5'-triphosphate (GTP) analogue, 2'(3')-O-{6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoic}-GTP (NBD-GTP), was synthesized and utilized to monitor the effect of mutations in the functional region of mouse K-Ras. The effects of the G12S, A59T and G12S/A59T mutations on GTPase activity, nucleotide exchange rates were compared with normal Ras. Mutation at A59T resulted in reduction of the GTPase activity by 0.6-fold and enhancement of the nucleotide exchange rate by 2-fold compared with normal Ras. On the other hand, mutation at G12S only slightly affected the nucleotide exchange rate and did not affect the GTPase activity. We also used NBD-GTP to study the effect of these mutations on the interaction between Ras and SOS1, a guanine nucleotide exchange factor. The mutation at A59T abolished the interaction with SOS1. The results suggest that the fluorescent GTP analogue, NBD-GTP, is applicable to the kinetic studies for small G-proteins.

Keywords: fluorescence/G-proteins/GEF/nucleotide analogue/Ras.

Abbreviations: DTT, dithiothreitol; GTP, guanosine 5'-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

The small guanine nucleotide-binding protein (G-protein) Ras is a central regulator of cellular signal transduction processes leading to transcription, cell cycle progression, growth, migration, cytoskeletal changes, apoptosis, cell survival and senescence, and functions as a molecular switch (1, 2). The switching mechanism has been well studied at the molecular level. It is known that Ras is activated in the guanosine 5'-triphosphate (GTP) bound state and inactivated in the GDP bound state, *i.e.* it is down regulated by its

own GTPase activity. The conformational change induced by GTP binding enables Ras to transduce a signal downstream through direct interaction with its effectors: Raf kinases, phosphoinositide 3-kinases, Ral guanine nucleotide dissociation stimulator (RalGDS) and phospholipase C ϵ (3). Switching between the active and inactive states is controlled by two groups of regulatory proteins: guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Binding of GEFs to Ras induces the conversion from the GDP-bound form to the GTP-bound form, which sets the switch to 'on'. However, GAPs induce GTP hydrolysis of Ras GTP hydrolysis of Ras, which sets the switch to 'off' (4–6).

The overall structure and functional domains of Ras have been well studied. It has been shown that mutations in the conserved nucleotide-binding region inhibit GTPase and the interaction with nucleotide exchanging factors, resulting in cancer and developmental diseases, neurofibromatosis, Noonan syndrome, Costello syndrome and other disorders. Several oncogenic mutations are known to inhibit GTPase activity (2, 7, 8). For example, a single mutation at codon 12, which codes for a residue in the P-loop found very close to the arginine finger of GAP in the Ras-GAP complex, inhibits GTP hydrolysis. The GTPase cycle of Ras variants with mutations at codon 12 stops at the GAP-mediated step (7, 9, 10). It is important to study the effect of disease-causing mutations of Ras on the GTPase activity and conformational structure of the functional domain.

Fluorescently labelled nucleotide analogues are useful tools for the study of the kinetics of nucleotide-requiring proteins and for analysis of the conformational changes in those proteins. It is known that modifying nucleotides by introducing a fluorophore at the 2' or 3' position of the ribose ring does not reduce their affinity for nucleotide-requiring proteins (11). Therefore, several kinds of 2'(3')-fluorophore nucleotide have been created. For instance, many kinetic studies of myosin and kinesin ATPases use the fluorescent ATP analogue 2'(3')-O-N-methylanthraniloyl-ATP (Mant-ATP), because Mant-ATP is easy to synthesize and changes its fluorescence with high sensitivity upon binding to ATP-requiring proteins (11–13). Fluorescently labelled ATP analogues carrying other fluorophores have also been utilized (14), and Mant-GTP has also been synthesized and utilized for studies on GTPase kinetics and the interaction of G-proteins with their regulatory factors (15–20).

The fluorophores used for the nucleotide analogues each have distinctive fluorescent characteristics. The identity and conformation of the amino acid residues

in the nucleotide binding site have significant effects on the change in fluorescence intensity when the fluorophore binds to the site. Therefore, we need to choose nucleotide analogues that are labelled with a suitable fluorophore for the nucleotide-requiring protein we want to analyse.

The fluorophore 2'(3')-O-{6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) hexanoic} (NBD) is highly sensitive to changes in the hydrophilicity and hydrophobicity of its environment, and it is therefore often used to study membranes (21–23). We have previously synthesized NBD-ATP and utilized it for kinetic studies of myosin ATPase. In contrast to other fluorescent ATP analogues, the fluorescence of NBD-ATP decreased when it bound to the ATPase site of skeletal muscle myosin. However, when NBD-ATP bound to smooth muscle myosin and conventional kinesin, the fluorescence intensity was enhanced. It was demonstrated that NBD-ATP sensitively reflects the conformational difference of the ATP-binding site and is applicable to the kinetic study on ATPases (24–26). Therefore, GTP analogues in which the ribose is modified with the NBD group are expected to work well for the study of G-proteins.

In this study, we synthesized NBD-GTP and studied the interaction of NBD-GTP with a small G-protein. NBD-GTP was a good substrate for Ras, with almost same binding affinity as regular GTP. The fluorescent properties of NBD-GTP were different from those of Mant-GTP, and reflected the conformation of the GTP-binding site in Ras. NBD-GTP is shown to be applicable for kinetic studies of G-proteins.

Materials and Methods

Ligation enzymes were purchased from Toyobo Co., Ltd, unless stated otherwise. Oligonucleotides were obtained from Sigma Genosys. The apparatus for affinity chromatography on Co²⁺-NTA agarose was procured from Clontech. Chemical reagents were purchased from Wako Pure Chemicals unless stated otherwise.

Synthesis of NBD-GTP

Coupling of GTP and NBD acid was carried out by the method of Guillory and Jeng (27). NBD acid (63 mg, 215 µmol) and carbonyl-diimidazole (110 mg, 680 µmol) were stirred for 30 min at room temperature in 0.48 ml of dry N,N-dimethyl formamide. GTP (60 mg, 105 µmol) was dissolved in 0.51 ml of water and then added dropwise to the reaction mixture. The coupling reaction was allowed to proceed for 48 h at room temperature under continuous stirring. The reaction mixture was diluted with acetone, which had been incubated at –30°C, and harvested by centrifugation at 30,000 × g for 10 min at 4°C using a No. 7 rotor (Hitachi Himac RPR20-2). The precipitate was evaporated to dryness and the residue dissolved in 2.5 ml of 0.1% trifluoro acetic acid. The mixture was collected by centrifugation at 280,000 × g for 15 min at 25°C (Hitachi Himac CS 120GX, Japan). The supernatant was removed and purified by high performance liquid chromatography on an ODP50-10E column. Elution was carried out with a linear acetonitrile gradient (0–90%) in 0.1% TFA at a flow rate of 1 ml/min. The elution profile was monitored by the absorbance at 300 nm. Three major peaks were obtained. The first peak eluted was of unreacted GTP, and third was of the unreacted NBD acid. The second peak contained NBD-GTP. The fractions were collected and lyophilized. The purity of the product was analysed by thin-layer chromatography (TLC) on silica gel plates (Silica Gel-70 F254; Wako Pure Chemical, Osaka) using 1-butanol/acetic acid/H₂O (5:2:3, by volume) as the developing solvent, and the *R_f* value was 0.41.

Expression and purification of K-Ras

The cDNA of mouse K-Ras (residues 1–176) in a storage plasmid was kindly provided by Dr Ando (Soka University). The cDNA was amplified by PCR and ligated into the pET15b vector. We generated three oncogenic K-Ras mutants (G12S, A59T and G12S/A59T) based on the normal K-Ras plasmid. Mouse K-Ras expression plasmids were used to transform *Escherichia coli* BL21 (DE3). Mouse K-Ras was purified using a Co-NTA column. Purified mouse K-Ras was dialysed with buffer [30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂ and 0.5 mM dithiothreitol (DTT)] and stored at –80°C until further use.

Expression and purification of H-Ras, RalGDS and c-Raf

A cDNA fragment encoding full-length human H-Ras (Clontech) was fused to the 3'-end of a synthetic sequence encoding a PreScission protease cleavage site using PCR. Human RalGDS cDNA was obtained as previously described (28). Separate cDNA fragments encoding EGFP (Clontech) and a stop codon were fused to the 5'- and 3'-end of the Ras-binding domain (RBD) of RalGDS, respectively. A cDNA fragment encoding the RBD of human c-Raf (Clontech) was fused to the 3'-end of a synthetic sequence encoding a PreScission protease cleavage site. The resulting DNA fragments were cloned into the pET42c expression vector (Novagen) using *Spe*I and *Xho*I restriction sites. GST-tagged H-Ras, GST-GFP-RalGDS(RBD) and GST-cRaf(RBD) were expressed in the *E. coli* strain Rosetta (DE3) (Novagen) and purified using glutathione-Sepharose 4B (GE Healthcare Bio-Science) according to the manufacturer's instructions. Purified GST-tagged H-Ras was then digested by PreScission protease (GE Healthcare Bio-Science), and the GST moiety was removed by glutathione-Sepharose 4B rechromatography. Samples were dialysed against 40 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES)-NaOH, pH 7.4, 1 mM DTT and 5% sucrose.

Expression and purification of SOS-505

The cDNA of the catalytic domain, CDC25 domain and a portion of the Rem domain of mouse SOS1 (SOS-505, residues 584–1088) (29) was amplified by polymerase chain reaction and ligated into the pColdI vector. SOS-505 expression plasmids were used to transform *E. coli* BL21 (DE3). SOS-505 was purified using a Co-NTA column. Purified SOS-505 was dialysed with buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂ and 0.5 mM DTT) and stored at –80°C until further use.

Pull-down assay with effectors: RalGDS and c-Raf

Solutions of 10 µM H-Ras and 1.4 µM GST-GFP-RalGDS (RBD) or c-Raf (RBD) in 100 µl assay buffer (25 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% TritonX-100 and 1 mg/ml BSA) were mixed with 30 µl glutathione-Sepharose 4B beads (GE Healthcare). The mixture was incubated at 30°C for 5 min with 100 µM GTP, GDP, NBD-GTP or NBD-GDP for the nucleotide exchange reaction. The reaction was terminated by adding 10 mM MgCl₂. Then, the mixture was incubated at 4°C for 1.5 h under continuous stirring. After incubation, the resin was washed with 400 µl wash buffer (25 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.05% TritonX-100 and 1 mg/ml BSA) three times. The bound proteins were eluted from the resin by boiling in SDS sample buffer (6.3 mM Tris–HCl, 10% glycerol, 5% β-mercaptoethanol and 0.25 mg/ml bromophenol blue) and were analysed by western blot using anti-His antibodies (Nacalai Tesque) or anti-GST antibodies (Santa Cruz Biotechnology). The bound antibodies were detected using chemiluminescence (ATTO, EzWestLumi plus).

The GTPase activity of Ras

The GTPase activity of Ras was measured at 37°C in the assay buffer (30 mM Tris–HCl, pH 7.5, 120 mM NaCl and 2 mM MgCl₂). Ras was added to the assay buffer to a final concentration of 50 µM. The GTPase reaction was initiated by adding 1 mM GTP and terminated by adding 10% trichloroacetic acid. The released inorganic phosphate (Pi) in the supernatant was measured using the method described by Youngburg and Youngburg (30).

Fig. 1 Structural formula of NBD-GTP.

Results

Synthesis and characterization of NBD-GTP

Fluorescent GTP analogues modified with a fluorophore at the 2' or 3' position of the ribose ring are known to be good substrates for G-proteins. Here, we designed a novel fluorescent GTP analogue, NBD-GTP (Fig. 1). We employed the NBD group because the NBD fluorophore is highly sensitive to its environment and has a longer excitation wavelength than the more commonly utilized Mant group. NBD-GTP was synthesized by coupling of GTP and NBD acid according to the method we previously established for the synthesis of NBD-ATP (27). The UV spectrum of NBD-GTP showed peaks of absorption maxima at 260 and 485 nm due to GTP and the NBD group, respectively (Fig. 2A). The fluorescence spectrum of NBD-GTP in a buffer of 30 mM Tris-HCl, pH 7.5, showed an excitation maximum at 471 nm and an emission maximum at 534 nm (Fig. 2B), which were shifted by ~10–20 nm towards longer wavelengths compared with the spectrum of free NBD acid in methanol. The synthesis was confirmed by TLC and mass spectroscopy, as described in the 'Materials and methods' section.

The biological activity of NBD-GTP as a substrate for signal transduction by Ras was examined using a pull-down assay to monitor the interaction of Ras with its effectors. RalGDS and c-Raf are the primary factors in the downstream signal transduction of Ras. As shown in Fig. 3A (lane 3), NBD-GTP bound Ras interacted with RalGDS and co-precipitated with resin conjugated with RalGDS. In contrast, NBD-GDP bound Ras did not bind to RalGDS (Fig. 3A, lane 4). Similarly, NBD-GTP but not NBD-GDP induced an interaction of Ras with c-Raf (Fig. 3B, lanes 3 and 4). These results suggest that NBD-GTP induces downstream activation of Ras and performs as substrate in a similar manner to regular GTP.

Fluorescent studies on the interaction of NBD-GTP with normal K-Ras

It is known that Mg^{2+} -GDP bound to the catalytic site of Ras exchanges with Mg^{2+} -GTP extremely slowly.

Fig. 2 (A) Absorption spectrum of NBD-GTP. The spectrum was measured in 30 mM Tris-HCl, pH 7.5, using a Shimadzu UV-2200 spectrophotometer. (B) Fluorescence excitation and emission spectra of NBD-GTP. Spectra were measured in 30 mM Tris-HCl, pH 7.5, at 25°C using a HITACHI RF-2500 fluorescence spectrophotometer. The excitation and emission wavelengths were 471 and 534 nm, respectively.

The exchange of GDP and GTP is much faster in the absence of Mg^{2+} (16). We were able to monitor the exchange phenomena by measuring the fluorescence change of NBD-GTP that is interacting with Ras. As shown in Fig. 4A, the addition of Mg^{2+} -GDP bound Ras to the NBD-GTP solution induced a slow increase of 25% in fluorescence.

Subsequent addition of an excess amount of GTP slowly decreased the fluorescence to the basal level of fluorescent intensity before the addition of Ras. The fluorescence changes are likely to represent the displacement of GDP as NBD-GTP binds to the Ras GTPase site, followed by the release of NBD-GTP from the site by exchange with external excess GTP. The extremely slow exchange rate as monitored by the fluorescence change of NBD-GTP is consistent with previously reported results of experiments with regular guanine nucleotide labelled by radio isotope (31). In contrast, in the absence of Mg^{2+} , the fluorescence of NBD-GTP increased much faster and saturated within 60 s (Fig. 5).

Fig. 3 *In vitro* binding of H-Ras to (A) RalGDS or (B) c-Raf. About 10 μ M Ras in the presence of (A) 1 μ M RalGDS or (B) 1 μ M c-Raf was loaded with GTP (lane 1), GDP (lane 2), NBD-GTP (lane 3) or NBD-GDP (lane 4). Proteins absorbed on the resin were dissolved in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (15%) solution. Western blotting analysis was carried out using an anti-His-tag antibody or anti-GST-tag antibody.

The enhancement of NBD-GTP fluorescence was decreased by the addition of increasing concentrations of regular GTP as shown in Fig. 4B. When NBD-GTP and regular GTP are present at equimolar concentrations, the fluorescence of NBD-GTP decreased by 50% from that seen in the absence of regular GTP. This suggests that the K_d of NBD-GTP for Ras is almost identical to that of regular GTP.

The fluorescence emission spectra of NBD-GDP bound to Ras in the presence or absence of Mg^{2+} are shown in Fig. 6. The fluorescence intensity at 534 nm of NBD-GDP in the presence of Mg^{2+} was 16% lower than that in the absence of Mg^{2+} , indicating that the sites that coordinate the NBD fluorophore in the GTPase site are different between Mg^{2+} bound NBD-GDP and Mg^{2+} free NBD-GDP. In the same experimental condition in Fig. 6, the fluorescence intensity of NBD-GDP•RAS exhibited 5.1% lower fluorescence than that of NBD-GTP•RAS in the presence of Mg^{2+} .

Interaction of NBD-GTP with oncogenic mutated Ras

It is well known that *v-k*i-Ras (G12S/A59T) contains two oncogenic mutations (Fig. 7). These mutations affect the GTPase activity of Ras drastically, resulting in incorrect cell signalling. Using NBD-GTP, we examined the effects of the mutations on the characteristics of the nucleotide exchange of Ras. First, we prepared the three Ras oncogenic mutants G12S, A59T and G12S/A59T. The GTPase activities of the Ras mutants

Fig. 4 (A) Fluorescence transients observed upon addition of Ras to NBD-GTP followed by addition of GTP under single-turnover conditions. The buffer conditions were as follows: 30 mM Tris–HCl, pH 7.5, 120 mM NaCl and 2 mM $MgCl_2$ at 25°C. First, 2 μ M Ras was mixed with 1 μ M NBD-GTP, then 100 μ M GTP was added. (B) Fluorescence transients observed upon addition of Ras to NBD-GTP in the presence of GTP. The buffer conditions were as follows: 0.8 μ M Ras was added to a solution of 0.8 μ M NBD-GTP and 0, 0.4, 0.8, 2, 8 and 16 μ M GTP, 30 mM Tris–HCl, pH 7.5, 120 mM NaCl and 20 mM EDTA at 25°C. Changes in NBD-GTP fluorescence were measured at 534 nm (excitation at 475 nm).

Fig. 5 Fluorescence transients observed upon addition of Ras to NBD-GTP under conditions with $MgCl_2$ or EDTA. The buffer conditions were as follows: 30 mM Tris–HCl, pH 7.5, 120 mM NaCl and 2 mM $MgCl_2$ or 10 mM EDTA at 25°C. Spectra were collected after 1 μ M Ras mixed with 0.5 μ M NBD-GTP.

Fig. 6 Emission spectra of NBD-GDP with/without Mg^{2+} . Emission spectra of (1) 2.5 μ M Ras and 2 μ M NBD-GTP were collected in the base buffer conditions, which were as follows: 30 mM Tris-HCl, pH 7.5, 120 mM NaCl and 20 mM EDTA. (2) The emission spectra after addition of 50 mM $MgCl_2$ to (1). Excitation wavelength 471 nm and temperature 25°C.

Fig. 7 Locations of the mutation sites in the crystal structure of Ras. The carbon atoms of the amino acids G12 and A59 are indicated in a black stick model. The three-dimensional structure of Ras was visualized with the molecular graphics program Mol Feat 4.0, using coordinate data from the 3GFT entry in the protein data bank. The GTP analogue and Mg^{2+} are shown as solid spheres.

are summarized in Table I. The GTPase activity of G12S is almost identical to normal Ras. However, A59T and G12S/A59T showed 40–50% lower GTPase activities than normal Ras. This suggests that the mutation at Ala 59 affects the GTPase activity significantly, which is consistent with previously reported results (32).

We subsequently monitored the exchange of NBD-GDP with GTP by the Ras mutants by measuring the fluorescence change of NBD-GDP. As shown in Fig. 8, the addition of excess GTP to the NBD-GDP bound Ras resulted in a decrease of NBD fluorescence. It is well known that in the absence of GEF, the exchange rates of normal Ras are extremely slow. The rates of nucleotide exchange of NBD-GDP with GTP for each Ras mutant are summarized in Table II (in the absence

Table I. The GTPase activity of Ras

GTPase activity (Pi μ M/Ras μ M/h)

Normal	G12S	A59T	G12S/A59T
0.79 ± 0.05	0.79 ± 0.04	0.52 ± 0.06	0.41 ± 0.00

The GTPase activity of 50 μ M Ras was measured at 37°C in the assay buffer (30 mM Tris-HCl, pH 7.5, 120 mM NaCl and 2 mM $MgCl_2$). Data are presented as mean $\pm$ SD.

Fig. 8 Fluorescence transients observed upon addition of GTP to Ras•NBD-GDP under single-turnover conditions. The buffer conditions were as follows: 30 mM Tris-HCl, pH 7.5, 120 mM NaCl and 2 mM $MgCl_2$ at 25°C. Prior to collecting the spectra, 1 μ M Ras premixed with 0.5 μ M NBD-GDP was mixed with 100 μ M GTP.

Table II. The rate of NBD-GDP exchange with GTP in the absence or presence of GEF

	$^a k$ (s^{-1}) ($\times 10^{-3}$)	$^b k$ (s^{-1}) + GEF	$^b k / ^a k$	Kd (Kd of GEF for Ras)
Normal	0.71 ± 0.07	0.22 ± 0.01	310	2.6 ± 0.2
G12S	0.43 ± 0.03	0.15 ± 0.02	349	4.9 ± 0.3
A59T	1.39 ± 0.05	0.23 ± 0.04	165	0.7 ± 0.1
G12S/A59T	0.89 ± 0.02	0.10 ± 0.02	112	0.6 ± 0.2

k (s^{-1}): Exchange rate from GDP to GTP in the ^aabsence or ^bpresence of GEF at 1 μ M Ras and 1 μ M GEF (SOS-505). Data are presented as mean $\pm$ SD.

of GEF). Although the Ras mutants also showed slow exchange rates, significant differences were observed among the mutants. The exchange rate of G12S was 60% slower than that of normal Ras, while the exchange rate of A59T was approximately two times faster than normal Ras, and the exchange rate of G12S/A59T was almost identical to that of normal Ras. These results suggest that the mutation at G12 reduces that exchange rate, whereas the mutation at A59 enhances the exchange rate.

We also monitored the nucleotide exchange rate of Ras in the presence of the GEF SOS-505 using NBD-GDP. In the presence of SOS-505, the exchange rate of NBD-GDP bound to normal Ras with GTP was extremely enhanced as shown in Fig. 9. The Ras mutants also showed a significant enhancement of exchange

Fig. 9 Effect of the bound nucleotide on the stimulated exchange reaction with SOS-505. Prior to collecting the spectra, 1 μ M Ras premixed with 0.5 μ M NBD-GDP and 0, 0.1, 0.2, 1, 5 and 10 μ M SOS-505 was mixed with 100 μ M GTP. The buffer conditions were as follows: 30 mM Tris-HCl, pH 7.5, 120 mM NaCl and 2 mM $MgCl_2$ at 25°C.

rates. The enhanced fluorescence alteration by exchange NBD-GDP/GTP with GEF is due to binding of GTP. GEF (SOS1-505) enhances the exchange GDP to GTP. The GEF did not promote GTP hydrolysis. Therefore, the enhanced fluorescence alteration by exchange is not due to hydrolysis of GTP. The apparent dissociation constants of SOS-505 for normal Ras the three mutants were estimated from the data collected at varying concentrations of SOS-505, as shown in Fig. 9 and summarized in Table II. Interestingly, the K_d of SOS-505 for both A59T and G12S/A59T was approximately four times higher than the K_d for normal Ras. In contrast, SOS-505 showed two times lower affinity for the G12S mutant than for normal Ras. Moreover, it was shown that although the A59T and G12S/A59T bind to SOS-505 with higher affinity than normal Ras, the degree to which SOS-505 enhanced the GDP-GTP exchange rate was smaller for the mutants than for normal Ras. This suggests that the mutation at A59 induces a conformational change in the GEF-binding site that interferes with the transduction of the signal from the GEF-binding site to GDP-binding site.

Discussion

Ras is an essential regulator of cellular signal transduction processes, via its function as a molecular switch. Mutations of Ras are frequently found in cancer and developmental diseases (2). It has been shown that the GTPase activity and concomitant down-regulation of Ras are significantly interrupted by oncogenic mutations. Studies into the mechanisms of these diseases will be advanced by characterizing oncogenic Ras and its interaction with regulatory factors or effectors at the molecular level. Fluorescently labelled GTP analogues are effective tools to monitor the GTPase reaction and the interaction of Ras with regulatory factors. Previously, several GTP analogues that carry a fluorescent dye have been synthesized and utilized for

kinetic studies on nucleotide-requiring proteins (16–20). The degree of fluorescence change induced by binding of these fluorescent nucleotide analogues to proteins depends significantly on the structural environment in the nucleotide binding site. The environment provided by each protein determines how strongly the fluorescent characteristics of the fluorophore are altered. In addition, different fluorescent nucleotide analogues exhibit different fluorescent characteristics when interacting with each target protein. Therefore, the synthesis and characterization of further novel fluorescent GTP analogues are required.

In this study, we focused on the fluorophore NBD. This fluorophore has been widely used for biophysical and cell biological studies on membranes (21–23, 33) because NBD has excellent properties as a probe for spectroscopic and microscopic applications. The NBD group also exhibits high environmental sensitivity. The NBD group has extremely weak fluorescence in hydrophilic aqueous solution, but its fluorescent intensity is drastically enhanced in a hydrophobic medium. Therefore, it is expected that the NBD group covalently linked to GTP will report on conformational changes in the GTP-binding site of Ras, reflecting both the influence of oncogenic mutations and the interaction with regulatory factors. This is supported by our previous studies in which an NBD labelled ATP analogue was applied to monitor the conformational changes at or near the ATP-binding site throughout the ATPase cycle (24, 25).

In this study, we linked the NBD group to the ribose moiety at the 2' or 3' position of GTP, using a hexanoyl alkyl spacer. This resulted in synthesis of NBD-GTP. Previous studies have shown that linking the fluorophore to the ribose of the nucleotide generally has an insignificant effect on the affinity of the resulting nucleotide analogue for its target enzymes. NBD-GTP also showed almost identical affinity for Ras when compared with regular GTP. Moreover, NBD-GTP did not interfere with physiological signal transduction. NBD-GTP but not NBD-GDP induced the binding of Ras to the Ras effectors, RalGDS and c-Raf. NBD-GTP enhanced its fluorescence intensity significantly during its interaction with Ras. This fluorescence change of NBD-GTP was utilized for kinetic studies on Ras. As shown in Fig. 4A, the binding of NBD-GTP to Ras was monitored by measuring the enhancement of the NBD group's fluorescence, and the release of NBD-GTP from the binding site upon adding an excess amount of regular GTP was reflected by decreasing fluorescence. Moreover, NBD-GTP exhibited unique fluorescence characteristics obviously distinguished from Mant-GTP, the other GTP analogue that has been previously used for kinetic studies on G-proteins. NBD-GTP coordinated with Mg^{2+} within the GTPase reaction of Ras showed lower fluorescence intensity than that of NBD-GTP without Mg^{2+} (Fig. 6). In contrast, it is known that Mant-GTP bound to Ras exhibits the opposite fluorescence change in the presence and absence of Mg^{2+} (29). NBD-GTP has a longer hexanoyl alkyl spacer linking NBD fluorophore to ribose moiety than that of Mant-GTP. Therefore, it is suggested that the NBD group

interacts with different amino acid residue from that for the fluorophore of Mant-GTP at near the entrance of the GTP-binding site.

We observed the biphasic of fast phase and slow phase on the time course of fluorescence enhancement with binding of NBD-GTP to Ras. Similarly, the experiments on the time course of fluorescence decrease after GTP addition to exchange NBD-GDP (Fig. 8) also showed two phases. At this stage, we do not have an evidence to explain the phenomena. The possible explanation is that the second slow phase may be caused by the isomers 2'/3'-O-NBD-GTP or the photo bleaching of fluorescence for NBD under the irradiation of excitation light of fluorometer. Indeed, the photo bleaching of Mant-ATP on the kinetic study was reported by Friel and Howard (34).

We demonstrated that the use of NBD-GTP enables us to monitor sensitively the interaction of GTP with Ras and the conformational changes at the GTP-binding site. Therefore, NBD-GTP is a fluorescence labelled GTP analogue that can be used in kinetic studies on G-proteins. Indeed, we successfully monitored the effect of oncogenic mutations on the kinetic properties of Ras and the interaction of Ras with its regulatory factor.

Retroviral Ras contains substitutions at the 12th and 59th amino acids. The mutant A59T prepared in this study showed decreased GTPase activity and an increased nucleotide exchange rate compared with normal Ras (Tables I and II). The mutation of G12S showed almost no effect on the GTPase activity compared with normal Ras (Table I). The second order rate constants of NBD-GTP binding in the absence of Mg^{2+} were also estimated by the rate at various concentrations of Ras mutants. The constants of WT, G12S, A59T and G12S/A59T were 0.13×10^{-6} , 0.34×10^{-6} , 1.20×10^{-6} and $1.27 \times 10^{-6} \text{ M}^{-1}\text{s}^{-1}$, respectively. The mutation at A59 to threonine significantly increased the second rate constant of NBD-GTP binding. These results are consistent with previous reports on G12K, A59T, G12S/A59T and G12R/A59T (32, 35–37).

Crystallographic studies of the mutants also suggest the observation. The formation of a hydrogen bond between the threonine hydroxyl group and the main chain carbonyl oxygen of Pro34 in Switch I region induces conformational shift in the region of the residues 59–61. However, introduction of valine at position 12 causes almost no alteration in the functional regions (38).

In this study, we applied NBD-GDP to monitor the GDP–GTP exchanges for these Ras mutants. In the absence of GEF, the exchange rate is slightly influenced by the mutations as shown in Table II. Mutant G12S showed a decrease in the nucleotide exchange rate, whereas A59T showed a nucleotide exchange rate that was approximately twice as fast as that of normal Ras. In the presence of GEF, although the nucleotide exchange rates of the mutants accelerated significantly, the degree of the acceleration obviously differed for the different mutants (Table II). We also estimated the K_d of GEF for the Ras mutants and suggested that the mutation of A59T significantly increases the affinity of GEF for Ras, whereas the G12S

mutation decreased this affinity. Therefore, the fluorescently labelled GTP analogue NBD-GTP is applicable to studies of Ras kinetics and its interaction with regulators.

In conclusion, the novel fluorescently labelled GTP analogue NBD-GTP was a good substrate for Ras, binding in a manner similar to regular GTP. NBD-GTP enabled us to monitor nucleotide exchange and also the interaction between Ras and GEF. NBD-GTP may be also useful for other G-protein families.

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Conflict of Interest

None declared.

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