Rho small GTPase regulates the stability of individual focal adhesions: a FRET-based visualization of GDP/GTP exchange on small GTPases

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RhoA and Rac1 are small GTPases primarily involved in cytoskeletal remodeling. Many biochemical studies have suggested that they are also key organizers of cell-substrate adhesion. Recently, fluorescence resonance energy transfer (FRET)-based indicators have been developed to visualize RhoA and Rac1 activity in living cells [Yoshizaki et al., J. Cell Biol. 162, 223 (2003); Pertz et al., Nature 440, 1069 (2006)]. These indicators use one of the interactions between RhoA (Rac1) and the RhoA (Rac1)-binding domain of their effector proteins. However, distribution of RhoA activity in single cells has not yet been observed with micrometer-scale resolution. Here, we employed an approach that detects GDP/GTP exchange on small GTPases by using FRET from YFP-fused small GTPases to a fluorescent analogue of GTP, BODIPY(TR)-GTP. This approach allowed us to visualize confined localization of active (GTP-bound forms of) RhoA and Rac1 in individual focal adhesions. Activated RhoA accumulated in immobile and long-lived focal adhesions but was not evident in unstable and temporary adhesions, while activated Rac1 was observed at every adhesion. Our results suggest that RhoA is the major regulator determining the stability of individual cell adhesion structures.

Key words: cell motility, cell-substratum adhesion, Rac

The Rho family encompasses approximately 20 species of small GTPases, which act as molecular switches cycling between an active (GTP-bound) form and an inactive (GDP-bound) form to control a large array of cellular processes, including organization of the actin cytoskeleton, cell polarity, transcriptional activation, cell cycle progression, and cell division¹³. The Rho family is a subset of the Ras super-family of small GTPases which also includes the subfamilies Rac, Cdc42, RhoBTB and Rnd². More particularly among members of the Rho family, RhoA, Rac1 and Cdc42 play important roles in the formation of cell-substratum adhesions.

Among various cell adhesion structures, focal adhesions and focal complexes are small foci-like structures located under the dorsal membrane of cells, which provide integrin- and actin-mediated adhesions with extracellular matrices (ECMs). The dynamics of these adhesions are important to cells. Linking bidirectionally the intracellular cytoskeletons and outside ECMs, these adhesions play significant roles in cell migration, morphogenesis, ECM assembly, and cell proliferation, differentiation, and death⁴. Focal adhesions are known to localize at the termini of stress fibers that provide long-term anchorage, while focal complexes are associated with lamellipodia and filopodia and are thought to support the protrusion of peripheral cell membranes. At the cytoplasmic side of both adhesions, over 50 species of proteins, including vinculin, paxillin, talin, Src, and several guanine nucleotide exchange factors (GEFs) of the Rho family GTPases are localized³.

Understanding the roles of the Rho family in controlling the formation of cell-substratum adhesions greatly improved

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and a fluorescence resonance energy transfer (FRET)-based GTPases by controlling the activities of Rho family is involved in the promotion of cell spreading. Recently, it was thought that RhoA is involved in the development and maintenance of focal adhesions by positively regulating the contractility between myosin II and the actin cytoskeleton. Conversely, it has been reported that the formation of cell-substratum adhesion controls the activities of Rho families: cell adhesions to ECM activate Rac and Cdc42, while newly formed focal adhesions down-regulate the activity of RhoA. Molecules at focal adhesions affect various cell behaviors by controlling the activities of Rho family GTPases. Thus, regulations between the Rho family and focal adhesions are thought to be bilateral.

Particularities of individual focal adhesions and focal complexes are yet to be elucidated. A focal complex can either grow up to a longer-lived focal adhesion or disappear within a minute. This flexible quality of the adhesions leads us to expect excellent mechanisms for regulation that are spatially and temporally restricted at each cell-adhesion apparatus. Accumulation of Rac in focal adhesions has been suggested. In the case of podosome — another integrin-and actin-mediated adhesion with ECMS — Martin and colleagues have shown that active RhoA localizes as doughnut-like "rosettes" around podosomes, which suggests that RhoA regulates podosomes at restricted locations. However, it has not known if there is similar local regulation by Rho in the case of focal adhesions and focal complexes. Localization of p21-activated kinase, a major effector of Rac, at focal complexes and focal adhesions suggests that Rac also locally controls the adhesions. Recently, accumulation of constitutively active mutants of Rac1 in focal adhesions has been reported, suggesting that active Rac1 at focal adhesions is involved in the promotion of cell spreading. Direct observation of the activities of the Rho family at individual adhesion apparatus is indispensable to confirm these suggestions.

Visualization of the activity, not the distribution, of the Rho family is important to elucidate their functions in living cells. Intracellular distribution of proteins can be visualized using GFP fusion probes. Particular localization patterns, which are thought to relate to activity, were observed for the small GTPases Rac1 and 2, Cdc42, RhoB, and TC10 fused with GFP. However, since, in contrast to other GTPases, RhoA remains mainly cytosolic even after stimulation of cells, use of GFP-fusion probes of RhoA is very limited. One way to visualize the active form of RhoA is to use effector proteins of RhoA as the probe. For example, Bement et al. described localization of the GFP-fused Rho binding domain (RBD) of Rhotekin at the cleavage furrow during mitosis, and a fluorescence resonance energy transfer (FRET)-based probe using the association between RhoA-GTP and Rhotekin RBD was designed by Matsuda and his colleagues to visualize up-regulation of Rho activity during mitosis. This FRET probe also demonstrated the activity of RhoA at ruffling membranes. A similar FRET probe has been used by Hahn and his colleagues. However, these probes have not yet successfully visualized the activity of Rho at focal adhesions. It is very likely that Rhotekin RBD cannot demonstrate the localization of Rho-GTP at the adhesion apparatus due to rapid dissociation from Rho and/or competition with other effectors on Rho at the adhesion apparatus. A technique to allow more direct visualization of activated Rho is required.

Here, we have devised a technique that directly demonstrates the GDP/GTP exchange on small GTPases, in which FRET from YFP-fused small GTPases to a fluorescent analogue of GTP, BODIPY(TR)-GTP (BP-GTP). Fluorescence emission spectra of GST-YFP-RhoA (0.24 µM) in the presence of various concentrations of BP-GTP. The excitation wavelength was 488 nm. The dotted line is the emission spectrum from 0.24 µM BP-GTP excited at 488 nm in the absence of GST-YFP-RhoA. All spectra are the averages from three independent experiments.

Figure 1 A FRET-based detection of GDP/GTP exchange on RhoA. (A) The principle for detecting GDP/GTP exchange on RhoA small GTPase based on FRET from YFP fused to RhoA (YFP-RhoA) to a fluorescent analogue of GTP, BODIPY(TR)-GTP (BP-GTP). (B) Fluorescence emission spectra of GST-YFP-RhoA (0.24 µM) in the presence of various concentrations of BP-GTP. The excitation wavelength was 488 nm. The dotted line is the emission spectrum from 0.24 µM BP-GTP excited at 488 nm in the absence of GST-YFP-RhoA. All spectra are the averages from three independent experiments.
Materials and methods

Materials and chemicals
PC12D cell was kindly provided by M. Sano. The cDNAs encoding human Rho GTPases were kindly provided by K. Kaibuchi. The cDNAs encoding human Vav was kindly provided by A. Hall. BODIPY(TR)-GTP was purchased from Molecular Probes (Eugene, OR, USA). All materials used in the plasmid constructions were purchased from Takara (Kyoto, Japan). Other materials and chemicals were obtained from commercial sources. GST or MBP fusion proteins were expressed in *E. coli* and purified according to the manufacturer’s instructions. Dichroic mirrors and filters were purchased from Hamamatsu Photonics (Hamamatsu, Japan) and Omega Optical (Brlattleboro, VT, USA).

Plasmid construction
The cDNA of RhoA was inserted into the BamHI site of pEYFP-C1 (Clontech, Palo Alto, CA). Venus, a fluorescent analogue of GTP, are shown. Similar for EYFP and Venus, only the results using EYFP used instead of YFP. Since the experimental results were measured to detect FRET from YFP to BP.

In vitro binding assay between YFP-RhoA and a fluorescent analogue of GTP
GST-YFP-RhoA was purified and dialyzed in 25 mM HEPES-NaOH (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, and 1 mM dithiothreitol. Dialyzed protein (0.25 μM) was incubated with various concentrations of BODIPY(TR)-GTP in a low magnesium buffer (25 mM HEPES-NaOH pH 7.4, 2.5 mM MgCl₂, 5 mM EDTA, and 1 mM dithiothreitol) at 30°C for 30 minutes. The reaction was stopped by adding MgCl₂ solution to a final concentration of 20 mM. The emission spectra of the mixtures were measured by excitation at 488 nm wavelength at 25°C using a SLM 8000C spectrophorometer (Shimadzu, Kyoto, Japan).

GEF assay
GST-YFP-RhoA was equilibrated with GDP before the measurements. GDP·GST-YFP-RhoA (0.10 μM), BP-GTP (0.25 μM) and various concentrations of non-labelled GTP (0, 0.25, 0.75 μM) were mixed in the presence (0.20 μM) or absence of the DH/PH domain of a Rho GEF, Vav1 in 25 mM HEPES-NaOH (pH 7.4), 1 mM DTT, and 5 mM MgCl₂ at 25°C. At indicated time points after mixing, GST-YFP-RhoA was pull-downed with glutathione (GSH)-beads for the purpose of eliminating non-bound BP-GTP. GST-YFP-RhoA was eluted from the beads with a buffer containing glutathione. The fluorescence emission spectrum was measured to detect FRET from YFP to BP.

In vitro binding assay between YFP-RhoA and Rhotekin
A fusion protein between Rhotekin, an effector protein of Rho, and GST was adsorbed on the GSH-beads. The beads were mixed with a nucleotide-free form of RhoA fused with MBP (MBP-RhoA; 1.0 μM) and incubated with BP-GTP (0, 0.33, 0.67, and 1.0 μM, respectively) in a reaction buffer (25 mM HEPES-NaOH (pH 7.4), 4 mM EDTA, 1 mM DTT) for 3 min. MgCl₂ was added to the final concentration of 15 mM, and the mixture was incubated for 7 min. After washing the GSH-beads 3 times, the MBP-RhoA on the beads was eluted with a buffer containing 500 mM NaCl, 25 mM HEPES-NaOH (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, and 1 mM DTT. Aliquots of the eluent were resolved by SDS-PAGE followed by silver staining.

Cell culture
PC12D cells were plated on 25 mm-φ round cover slips in 35-mm tissue culture dishes and cultured for 36 h in DMEM/F12 medium (GIBCO BRL, Rockville, MD) supplemented with 5% fetal bovine serum, 10% horse serum, and glucose (final concentration: 4500 mg/L) in an atmosphere containing 5% CO₂ at 37°C. The cells were transfected with pEYFP-RhoA/Rac1 using Lipofectamine (GIBCO). After being cultured for further 12 h, the cells were incubated for 2 h in the imaging medium (DMEM/F12, 1% fetal bovine serum, and 4500 mg/L glucose) and used for experiments.

Immunocytochemistry
The cells were fixed with 4% paraformaldehyde and stained with mouse monoclonal anti-vinculin antibody (Sigma-Aldrich, St. Louis, MO) at a 1:200 dilution followed by a secondary antibody conjugated with Alexa Fluors 555 (Molecular Probes) at a 1:500 dilution.

Imaging of FRET
BP-GTP was dissolved in the injection buffer (1 mM BP-GTP, 30 mM HEPES-KOH at pH 7.4, 80 mM KCl, 0.5 mM MgCl₂), filtrated, and microinjected into the cytoplasm of PC12D cells expressing YFP-RhoA. Ten minutes after the injection, the cells were treated with nerve growth factor (NGF; UBI, final concentration of 50 ng ml⁻¹ or LPA (Sigma; final concentration of 100 ng ml⁻¹). Cells expressing YFP-RhoA but not injected with BP-GTP and cells injected with BP-GTP but not expressing YFP-RhoA were used as controls.

Observations were made using an inverted microscope (IX70, Olympus) equipped with a confocal laser scanner unit (CSU-10, Yokogawa Electronic, Tokyo, Japan). Fluorescence signals were separated by a dual-view system (Hamamatsu Photonics) using 550-nm dichroic mirrors and a 515–550 nm band-pass filter for YFP and a 615–645 band-pass filter for BODIPY(TR). To take valance of the fluorescence intensities in both channels, signals from the YFP channel were weakened using a 6%-pass neutral density filter. Images were obtained using a cooled CCD camera (MicroMAX 512BFT, Princeton scientific instruments). The FRET images were reconstructed from images in the YFP- and BODIPY(TR)-channels, according to the method described by Gordon.
et al.\textsuperscript{33}, using MetaMorph software (Universal Imaging Co., Downingtown, PA, USA). The color scale for FRET images is linear to the FRET signal intensity.

Results and discussion

FRET took place between YFP-RhoA and BODIPY(TR)-GTP

Our technique to detect GDP/GTP exchange on small GTPases depends on the FRET from small GTPases fused with YFP to a fluorescent analogue of GTP, BP-GTP (Fig. 1A). Occurrence of FRET was confirmed by \textit{in vitro} experiment. Figure 1B shows the emission spectra of GST-fused YFP-RhoA mixed with BP-GTP, measured using a spectrofluorometer with an excitation wavelength (488±2.5 nm) that selectively excites YFP. GST-YFP-RhoA bound with non-fluorescent GDP or GTP had a similar spectrum to YFP (peak wavelength at 528 nm). In the presence of BP-GTP, the fluorescence intensity increased at around 620 nm and decreased at around 530 nm. The changes of spectra were dose dependent, varying with the concentration of BP-GTP. With the direct excitation of BODIPY(TR)-GTP by 488-nm light, the emission from BP-GTP (peak wavelength at 622 nm) was slight even at the highest concentration of BP-GTP. This result indicates that BP-GTP was bound with YFP-RhoA and that this binding induced FRET from YFP-RhoA to BP-GTP.

GEF stimulated GDP/GTP exchange on YFP-RhoA

Inside living cells, guanine nucleotide exchange factors (GEFs) facilitate the release of GDP from small GTPases, thereby promoting the binding of GTP, of which the concentration is much higher than that of GDP. We examined whether this mechanism takes places between YFP-RhoA and BP-GTP. GST-YFP-RhoA was equilibrated with GDP prior to the experiment. Nucleotide exchange to (BP-)GTP was measured in the presence or absence of the catalytic PH-DH domain of Vav1, a GEF of RhoA\textsuperscript{33}. In this experiment, the concentrations of the GST-YFP-RhoA, BP-GTP, and PH-DH domain were fixed, but the concentration of non-labeled GTP was varied.

As shown in Figure 2, FRET was generated in a GEF-dependent manner. The FRET signal decreased in inverse proportion to the concentration of non-labeled GTP. This result indicates that GDP/GTP exchange was stimulated by GEF and that the binding of BP-GTP after the dissociation of GDP was competitively inhibited by the binding of non-labeled GTP. The inhibition rates inversely proportional to

![Figure 2](image-url)

Figure 2  Competition between BP-GTP and non-labeled GTP in GEF-induced GDP/GTP exchange on YFP-RhoA. (A) Normalized fluorescence emission spectra from the mixtures of YFP-RhoA, BP-GTP, and non-labeled GTP in the presence or absence of PH/DH domain of Vav1, a GEF for RhoA. Spectra at different time points after mixing are shown. The concentration of non-labeled GTP was varied in the presence of Vav1. (B) Changes in YFP fluorescence (530 nm) in the spectra shown in (A) were plotted to show the time course of the GDP/GTP exchange. A decrease in the YFP fluorescence indicates BP-GTP binding on YFP-Rho. Averages of four independent experiments are plotted with SE. Similar results were obtained using PH/DH-domains of LARG\textsuperscript{41}, another GEF for Rho, instead of Vav1 (data not shown).
the concentration of non-labeled GTP at every time point of the binding timecourses (Fig. 2B) indicate that both the affinity and the on-rate of BP-GTP to YFP-RhoA were similar to those of non-labeled GTP. Other GEFs — Db1 and LARG — also increased FRET signal (data not shown). These results lead to the conclusion that YFP-RhoA with BP-GTP is useful as an indicator of GDP/GTP exchange on RhoA.

The complex between RhoA and BP-GTP recognized an effector protein

To examine the activity of RhoA after association with BP-GTP, a pull-down assay of MBP-RhoA-BP-GTP was carried out using an effector protein bound to GSH-beads (Fig. 3). Depending on the dose of BP-GTP, MBP-RhoA bound with the Rho binding domain of Rhotekin fused with GST. This suggests that the FRET indicates the production of active Rho. A BP-GTP dependent interaction of MBP-YFP-Rac1 with the GST fused Rac binding domain of p21-activated kinase (PAK) was also observed (data not shown).

FRET visualized activation of RhoA in PC12D cells

Our technique was used to visualize the correlation between cell-substratum adhesion and activation of RhoA and Rac1 in PC12D cells. PC12D is a flat-shaped variant of the PC12 cell sub-cloned by Sano. PC12D continually changes its shape even in conditions without any stimulation, remodeling the cell-substratum adhesion structures. PC12D cells do not show thick stress fibers (data not shown) and show only sparse vinculin-positive anchoring apparatus, i.e., focal complexes and focal adhesions (Figs. 5 and 7). These characteristics make it easier for us to observe the activities of the Rho family at individual focal complexes or focal adhesions.

BP-GTP was microinjected into the cytoplasm of PC12D cells expressing YFP-RhoA. Next, the cells were observed using a real-time confocal microscope focused on the cell-substrate attachment. Images of the cells were taken in donor (YFP) and FRET channels and the FRET image was reconstructed according to the method described in Gordon et al.

Within 10 minutes of the microinjection of BP-GTP, FRET signals appeared as spot-like distributions focused on the dorsal membrane, independent of any stimulation of cells (Figs. 4 and 6, and Supplement Fig. S1). FRET signals were slight in other regions of the cell. These FRET signals were caused by the spontaneous activation of RhoA in resting cells (Supplement Fig. S1). No FRET signal was observed in cells treated with genistein, an inhibitor of tyrosine kinases, or in cells microinjected with a dominant negative form of RhoA protein (GDP-RhoA<sup>Asn19Gly</sup>; data not shown). LPA is known to induce activation of RhoA in some cell-lines. LPA-stimulation of PC12D cells rapidly increased FRET signals on the dorsal plasma membrane (Fig. 4). Increase of FRET signals after stimulation with LPA (Fig. 4D) was particularly evident in the spot-like distributions observed before the addition of LPA (Fig. 4C). The increase of FRET signals after LPA treatment was suppressed by coinjection of GDP-RhoA<sup>Asn19Gly</sup>, suggesting that these signals had been caused by LPA-stimulated GDP/GTP exchange on Rho. Increase of FRET after more than 10 min of microinjection of BP-GTP was slow in cells without stimulation (Supplement Fig. S1). Prominent FRET signals of RhoA always accumulated in micrometer-scale spots on the dorsal membrane independent of the stimulation (Figs. 4–6 and Supplement Fig. S1). As shown in the following sections, these accumulations of activated RhoA were at the immobile focal adhesions.

Newly activated RhoA was concentrated in focal adhesions

Since RhoA has been known to regulate rearrangement of cell-substratum adhesions, co-localization was examined between spots of strong FRET signals and vinculin, a major

Figure 3 Interaction of BP-GTP-RhoA with an effector protein Rhotekin. (A) A GST fusion of the Rho-binding domain (RBD) of Rhotekin was attached on GSH-beads. MBP-RhoA was incubated with the beads in the presence of various concentrations of BP-GTP. Proteins bound to the beads were dissociated from the beads with a buffer containing 20 mM glutathione and analyzed by SDS-PAGE. Arrowheads represent MBP-RhoA (silver-stained) bound to GST-Rhotekin. Arrows represent GST-Rhotekin (CBB-stained). (B) The amount of MBP-RhoA bound to GST-Rhotekin was plotted as a function of the BP-GTP concentration. The amount of MBP-RhoA was normalized to that in the presence of 1 μM BP-GTP after subtraction of the non-specific binding observed in the absence of BP-GTP. The average and SE of four independent experiments are shown. Similar results were obtained using RBD of Rhino kinase instead of RBD of Rhotekin (data not shown). The difference between the amount of the bound MBP-RhoA with BP-GTP and that with non-labeled GTP is probably due to structural difference between RhoA-GTP and RhoA-BP-GTP. A hydrogen bond network is formed between 2′-OH of GTP and Cys<sup>20</sup> and Pro<sup>31</sup> of RhoA<sup>31</sup>′. BP-GTP is a mixture of 2′- and 3′-isomers and 2′-OH is blocked in the 2′-isomer.
component of both focal adhesions and focal complexes4. PC12D is characterized by prompt neurite formation in response to nerve growth factor (NGF). Membrane ruffling and nondirectional roaming movements of PC12D cells started immediately after the stimulation with NGF, and 10–20 minutes after the stimulation, changes in cell shape with elongation of neurite-like structures were noticeable. These dynamic rearrangements of cell structure helped us observe the correlation between cell-substratum adhesion and activation of the Rho family.

FRET from YFP-RhoA to BP-GTP was observed at the region of cell-substratum adhesions in PC12D cells during cell motion induced by NGF. In spite of dynamic changes of the cell shape, locations of prominent FRET signals on the dorsal membrane were stable during 10 minutes of observation (Fig. 5A). Immunostaining of the same cell using an anti-vinculin antibody after fixation with paraformaldehyde at 20 min of NGF stimulation showed that the spots of prominent FRET signals were co-localized with large vinculin accumulations (Fig. 5B). Since it is known that focal adhesions are large and have an elongated or oval shape while focal complexes are small and have a dot-like shape11, it is highly likely that FRET signals of RhoA were accumulated in the focal adhesions.

Almost all of the FRET spots from RhoA were co-localized with vinculin, and most of the spots were observed before NGF-stimulation and increased their FRET signals after stimulation (arrows in Fig. 5B). Young adhesions in the lamellipodia (focal complexes) that showed a dynamic morphological change within 10 minutes up to the fixation did not show FRET signals from RhoA (arrowheads in Fig. 5B). Similar experiments were done for 5 cells independently. Almost all FRET spots overlapped with vinculin (31 out of 35) and showed slight movement during the 10-minute recording period (34 out of 35). Thus, there was a strong correlation between the stability of the anchoring apparatus and the localization of active RhoA. The areas of stable focal adhesions with accumulations of newly activated RhoA molecules and the areas which showed dynamic morphological changes were mutually exclusive in these cells. These results suggest that the focal adhesions that co-localized with activated RhoA became stable and suppressed local morphological changes of PC12D cells.

To test the correlation between the localization of activated RhoA and the mobility of focal adhesions, displacements of the individual FRET spots were measured using time-lapse imaging during rapid cell movements caused by NGF (Fig. 6). A prominent FRET spot observed in Figure 6 hardly moved for more than 10 minutes. In similar experiments, 58 spots of FRET (from 13 cells) were observed for more than 10 minutes. The majority (48 out of 58) of the spots were almost immobile, that is, the centroids of the spots stayed within a circle of 2.5 µm in diameter during the entire 10 minutes of the recording period. In most cases, the FRET signal increased at the spots during observation, suggesting a correlation between accumulation of RhoA-GTP and stabilization of the anchoring point of cells to the substratum.

Activated YFP-Rac1 accumulated in every vinculin-positive anchoring apparatus

In PC12D cells stimulated with NGF, FRET signals from YFP-Rac1 to BP-GTP were observed in neurites and ruffling membranes (Supplement Fig. S2) as reported previously27,28,36,37, on the other hand, the signals distributed at focal complexes and focal adhesions in resting PC12D cells (Fig. 7). In contrast to RhoA, activated Rac1 localized at all vinculin-positive cell-substratum adhesions, regardless of their motility and lifetimes. In Figure 7A, FRET spots of Rac1 sustained for over 10 minutes are denoted by arrows, and spots that disappeared within 10 min are denoted by arrowheads. The spots denoted by white arrows were immobile, and the spot denoted by the gray arrow was mobile as determined in the above section. The spot denoted by the red arrow grew during the 10 minutes of the imaging period. The FRET spots denoted by red arrowheads disappeared in the same 10 minutes even though their signals were as strong as those of the stable spots. Other FRET spots appeared within 10 min up to fixation. Thus, there was no clear correlation between the amount of active Rac1 molecule and the stability and lifetime of the FRET spots.

The cell was fixed by paraformaldehyde immediately after FRET imaging. Almost all of the FRET spots observed
immediately before the fixation were localized at cell-substratum adhesions stained with an anti-vinculin antibody (Fig. 7B). In similar experiments, 65 spots (from 5 cells) of FRET were observed for 10 min following microinjection. Some (18 out of 65) of the spots were sustained and immobile. Some were sustained but mobile (14 out of 65), and others (33 out of 65) were short-lived or newly formed within 10 min to the fixation.

Rho-GTP, but not Rac-GTP principally regulates the stability of focal adhesions

The localization of FRET signals of Rac1 at focal adhesions observed in this work is consistent with previous research reporting localization of an effector protein of Rac1 and a constitutively active form of Rac at focal adhesions. However, our observation was inconsistent with studies reporting that Rac-GTP promotes dissociation of large focal adhesions into smaller focal complexes. In spite of the accumulation of Rac1-GTP, a significant proportion of the focal adhesion was stable for a long time (Fig. 7). Therefore, localization of the active form of Rac1 is not sufficient to explain motility or disruption of the focal adhesions. Other factors must be involved in the differentiation of the lifetime of adhesion apparatus.

It has been shown that Rho activation induces the formation of actin stress fibers and focal adhesions. Previous techniques have not succeeded in demonstrating localization of active RhoA at focal adhesions, even though RhoA/Rho-kinase complexes have been found on stress fibers. Therefore, there has been debate about whether active RhoA regulates focal adhesions locally at individual adhesion apparatuses and/or globally at every adhesion on a stress fiber. Our technique demonstrates for the first time that the local activity of RhoA correlates with the stability of individual focal adhesions.

The active form of RhoA accumulated only at stable...
adhesions which were immobile, long-lived, and large (Figs. 5 and 6 and Supplemental Fig. 1). It is highly likely that RhoA-GTP plays key roles in stabilizing focal adhesions. FRET signals from RhoA-GTP were increased at stable focal adhesions after stimulation with LPA or NGF (Figs. 4 and 5), suggesting that activation of RhoA took place at the stable focal adhesions and/or the stable adhesions recruited active RhoA molecules after stimulation. It is probable that RhoC regulates focal adhesions in cooperation with RhoA because RhoC has a similar structure to RhoA. The FRET from YFP-Cdc42 was observed in neurites but not observed at cell-substratum adhesions (data not shown), probably because Cdc42 does not function at the adhesion apparatus locally.

A mechanism for stabilizing focal adhesions via activation of RhoA

We propose that there is bilateral regulation between the stabilization of focal adhesions and the activation of RhoA. Recognition between ECM and integrin in cell-substratum adhesions is known to activate a non-receptor type of tyrosin kinase FAK which in turn induces both up- and down-regulation of Rho signaling. In newly-formed focal adhesions, RhoA is down-regulated transiently via activation of FAK. On the other hand, the integrin-FAK signaling recruits ganglioside GM₁-rich lipid rafts where RhoA-mDia signaling is stimulated to stabilize microtubules in that location. In this process, the microtubules are guided by actin filaments and targeted to focal adhesions. Conversely to this stabilization of the microtubules by RhoA, it has been suggested that targeting of microtubules to the focal adhesion stimulates activation of RhoA. Thus, there is a positive feedback loop between activation of RhoA and stabilization of the microtubules at individual focal adhesions. Active RhoA stabilizes focal adhesions via positive regulation of myosin–actin interaction, stimulation of actin polymerization, and

Figure 6  Mobility of the accumulation of YFP-RhoA-BP-GTP. Time-lapse images of FRET signals of a PC12D cell expressing YFP-RhoA are shown. The cell was injected with BP-GTP and stimulated with NGF 10 min after the injection. Time 0 is the start point of the NGF stimulation. FRET signals were slight immediately after the injection (E). YFP-RhoA showed a diffuse distribution in the cytoplasm throughout the observation as shown in (F). A bright spot of FRET was observed on the dorsal membrane when stimulation with NGF was started (arrows in A). The spot was immobile for 10 minutes but increased its FRET intensity (arrows in B–D). The microscope was focused on the dorsal cell surface in images A–F. FRET signals were weak at 2.5 μm above the substratum (G). Bar=10 μm.
Our results suggest that such bilateral regulation between the formation of focal adhesions and activation of RhoA is controlled individually at each cell-substratum adhesion apparatus. The presence of two opposing effects on Rho signaling from the same integrin-FAK pathway will cause differentiation of the accumulation of active Rho at each adhesion apparatus. Inhibition of Rho activation in newly formed focal complexes supports disruption of the complex by Rac. However, once this stage is past, positive feedback between Rho activation and stabilization of the focal adhesions takes place. This mechanism should result in the localization of activated RhoA mostly at long-term focal adhesions, and thus the stability of cell adhesion apparatus is individually determined.

**Conclusion**

In this report, a FRET-based technique to visualize GDP/GTP exchange on small GTPases in living cells is described. This technique allowed us to observe localization of newly activated RhoA and Rac1 in living PC12D cells with micrometer-scale spatial resolution. Independent of stimulation, RhoA-GTP was observed on the long-lived, immobile, vinculin-positive cell-substratum adhesions (focal adhesions) but not observed on the short-lived, mobile, vinculin-positive cell-substratum adhesions (focal complexes), while Rac1-GTP was located on every vinculin-positive cell-substratum adhesion. It has been reported that activation of Rac and Rho disperses or stabilizes the cell-substratum adhesion, respectively. Our findings suggest that the stability of each cell-substratum adhesion is decided individually by controlling Rho but not Rac activity.

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