

Visualization of the Activity of Rac1 Small GTPase in a Cell

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Rho family G proteins including Rac regulate a variety of cellular functions, such as morphology, motility, and gene expression. Here we developed a fluorescence resonance energy transfer-based analysis in which we could monitor the activity of Rac1. To detect fluorescence resonance energy transfer, yellow fluorescent protein fused Rac1 and cyan fluorescent protein fused Cdc42-Rac1-interaction-binding domain of Pak1 protein were used as intermolecular probes of FRET. The fluorophores were separated with linear unmixing method. The fluorescence resonance energy transfer efficiency was measured by acceptor photobleaching assisted assay. With these methods, the Rac1 activity was visualized in a cell. The present findings indicate that this approach is sensitive enough to achieve results similar to those from ratiometric fluorescence resonance energy transfer analysis.

Key words: Rac1, FRET, cell imaging

I. Introduction

Rac protein is a member of Rho family GTPases and plays a central role in cell migration by inducing the extension of lamellipodia [5, 22]. The Rho family GTPases are thought to act as morphological switches by cycling between a GTP-bound (active) and GDP-bound (inactive) state. The activity of these proteins is regulated by the interaction of Rho family GTPases with guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The activated small GTPases bind to their effectors. For example, the GTP-bound Rac1 bind to p21 protein (Cdc42/Rac)-activated kinase 1 (PAK) protein through its Cdc42-Rac-interactive-binding (CRIB) domain [10].

Fluorescence resonance energy transfer (FRET) is a nonradiative transfer of energy between two fluorophores that are placed in close proximity and in a proper relative

angular orientation [6]. Variants of green fluorescent protein (GFP) provide genetically encoded fluorophores that serve as the donor and the acceptor in FRET. Some difficulties exist to measure FRET. One of them is spectral bleed through, i.e. the contribution of donor and acceptor fluorescence emission to the FRET channel [2]. Several researcher groups have developed single-molecule probes to detect the activity of Rac1, in which the monitor peptides are sandwiched with the two GFP variants [7, 13–15, 19]. To visualize the FRET efficiency, the ratio of the intensity of donors and the acceptors are mapped in an image of a cell. However, it has been restricted to qualitative or relative assessments owing to the spectral bleed-through contamination resulting from fluorescence overlap between the donor and the acceptor.

We develop a new method to visualize the Rac1 activity in a cell using a pair of intermolecular probes and spectral unmixing and acceptor photobleaching assisted FRET assay.

II. Materials and Methods

Cell culture

Human glioblastoma cell line U251MG was obtained

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from American Type Culture Collection (ATCC, Rockville, MD, USA) and was maintained in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum. The following antibodies were used: anti-Rac1 (BD Biosciences Pharmingen, San Diego, CA, USA) and, and anti- β -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). PDBF-BB was purchased from Sigma-Aldrich (St Louis, MO, USA)

Plasmids

Rac1 cDNA was cloned in frame with Venus, a monomeric yellow fluorescent protein (YFP) as a C-terminal fusion. Venus was a gift from Dr. A Miyawaki, Riken Brain Science Institute, and cDNAs for Rac1 (wild type, dominant negative form and constitutive active form) were gifts from Dr. Y. Takai, Osaka University. CRIB domain of PAK1 fused to cyan fluorescent protein (CFP) as a C-terminal fusion. Transfections were performed with Lipofectamine 2000 (Invitrogen) as directed by the manufacturer.

Live cell imaging

Cells transfected with YFP or CFP constructs were plated on 35 mm-diameter glass-based Petri dishes (Matsunami Glass Industries Ltd., Tokyo, Japan). Cells were imaged on a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany) equipped with temperature and CO₂ controls, an argon laser, a helium/neon and Plan Achromat 40 \times or 63 \times oil Iris lenses.

Spectral linear unmixing

To separate signals from fluorescent proteins in a cell, we used a linear unmixing method, as described previously [25, 26]. Briefly, images were acquired from five spectral channels simultaneously with a Zeiss LSM 510 META confocal microscope (63 \times NA 1.4 oil-immersion objective), covering important parts of the emission from both fluorophores (477.9–584.9 nm) with a 21.4 nm spectral resolution at an excitation wavelength of 458 nm. The acquired 4D image sequence (x, y, time and spectrum) was first background subtracted and separated to two fluorophores (YFP and CFP) using the linear unmixing method implemented in Mathematica software (Wolfram Research, Inc., Champaign, IL, USA). The fluorescence emission spectrum of a mixed specimen is an addition of the abundance-weighted spectral response of all constructs. In the two element case it is expressed by,

$$F_m(x, y, \lambda) = a(x, y)F_a(\lambda) + b(x, y)F_b(\lambda)$$

where $a(x, y)$ and $b(x, y)$ are unknown abundance factors of the two fluorophores at pixel location (x, y) , whose spectral responses are expressed as $F_a(\lambda)$ and $F_b(\lambda)$, respectively. $F_a(\lambda)$ and $F_b(\lambda)$ are measured using single fluorophore specimens. $a(x, y)$ and $b(x, y)$ are estimated using a least-square fitting. One of the pair of fluorophores was used for a reference spectra of a fluorophore. For example, in the u-adFRET assay of YFP-wtRac1 and CFP-Pak-CRIB

pair, we used YFP-wtRac1 for reference of YFP, and CFP-Pak-CRIB for reference of CFP.

Colocalization analysis

To verify colocalization, time-lapse images were analyzed with the "colocalization" plugin of ImageJ. The random or codependent nature were tested using intensity correlation analysis (ICA) [18] in which the distribution of the intensity value for each pixel of a channel is plotted against the product of the difference of the mean (PDM) of the two channels. The PDM value is expressed as

$$\text{PDM} = (\text{red intensity} - \text{mean red intensity}) \times (\text{green intensity} - \text{mean green intensity})$$

The PDM image where each pixel is equal to the PDM value at that location is pseudocoloured in yellow and the areas in blue represent the areas of positive and negative PDM values, corresponding to the presence and absence of colocalization, respectively.

Rac activation assay (pull-down assay)

pGEX-PAK-CRIB [1] was introduced into the Rosetta2 (DE3) strain of *E. coli*, and GST fusion protein was expressed and purified. Cells were washed with ice-cold PBS and harvested in lysis buffer (20 mM Hepes-NaOH, pH 7.9, 300 mM NaCl, 1 mM EDTA, 10 mM NaF, 15% Glycerol, 0.5% Nonidet P-40, and protease inhibitor mixture). After lysis for 15 min at 4°C, the samples were centrifuged at 14,000 \times g at 4°C. Five hundred μ g of the lysate was mixed with 30 μ g of the PAK-CRIB as a GST fusion protein for 2 hr at 4°C. Then the samples were washed four times. Finally, the pelleted beads were resuspended in 15 μ L of Laemmli's sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (15%). Bound Rac1 were detected by Western blotting using the antibodies against Rac1.

FRET analysis (u-adFRET)

For unmixing-acceptor depletion FRET (u-ad FRET), cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, washed with PBS, and mounted in Mowiol reagent containing 10% Mowiol 4–88 (Calbiochem, Beeston, UK), 25% glycerol, and 2.5% 1,4-diazabicyclo [2, 2, 2] octane (Sigma, Poole, UK) in 50 mM Tris/HCl, pH 8.5. One or two ROIs within a field were acceptor-photobleached. The 458 nm laser line was used for imaging as it can excite both CFP and YFP. The 514 nm laser line was used for the acceptor-photobleaching. The acquired image series were subjected to the linear unmixing method and separated images were processed using Mathematica software according to the algorithm described in the previous report [11] and below.

FRET efficiency is calculated from the unmixed images by the following equation,

$$E = (1 - f_d(x, y) / f_{dph}(x, y)) 100\%$$

where $fd(x, y)$ and $fdph(x, y)$ are the fluorescence of the donor before and after photobleaching. Maps of E were depicted using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

III. Results

Localization of YFP-Rac1 and CFP-Pak-CRIB

We constructed intermolecular probes for Rac1 activity that consisted of full-length Rac1 fused to YFP (YFP-Rac1) and CRIB domain of the Pak1 fused to CFP (CFP-Pak-CRIB). In these probes, we expected that binding CFP-Pak-CRIB to YFP-Rac1 would bring CFP close to YFP, increasing FRET from CFP to YFP (Fig. 1A).

First, we used fluorescent microscopy to detect the subcellular localization of YFP-Rac1 and CFP-Pak-CRIB in human glioblastoma cell line, U251MG cells. In U251MG cells, lamellipodial protrusion was clearly observable.

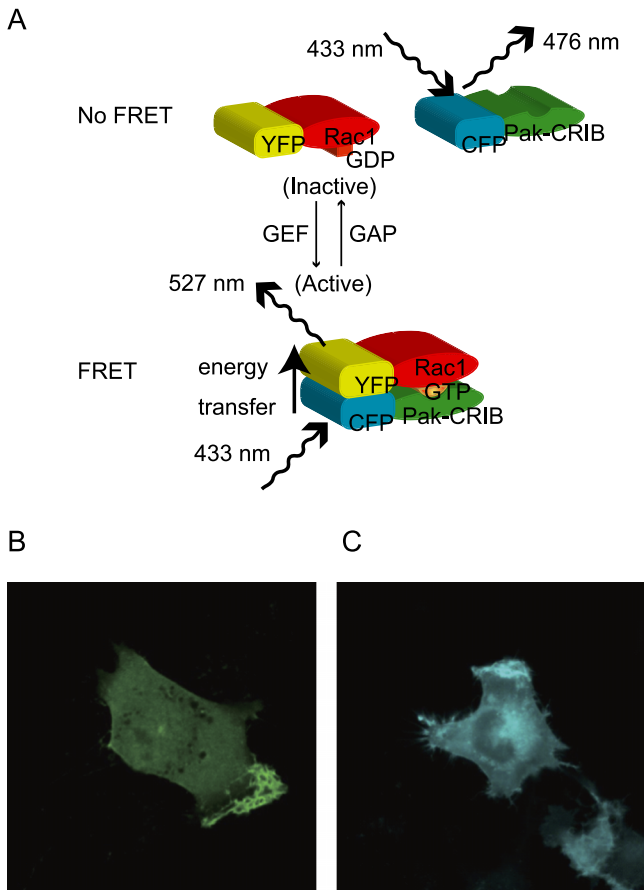


Fig. 1. Distribution of YFP-Rac1 and CFP-Pak-CRIB in U251MG cells. (A) Schematic representations of YFP-Rac1 and CFP-Pak-CRIB bound to GDP or GTP. When Rac1 is bound to GDP, fluorescence of 475 nm emanates from CFP with excitation of 433 nm. When Rac1 is bound to GTP, Pak-CRIB brings CFP into close proximity to YFP, which causes FRET and fluorescence of 527 nm from YFP. (B) Distribution of YFP-wild type Rac1. YFP-wtRac1 localizes in the lamellipodia. (C) Distribution of CFP-Pak-CRIB. CFP-Pak-CRIB localizes in the lamellipodia.

YFP-Rac1 and CFP-Pak-CRIB were localized in the plasma membrane corresponding to lamellipodia and diffused throughout the cytosol (Fig. 1B and C). The subcellular localizations of YFP-Rac1 and CFP-Pak-CRIB were identical to the localization of endogenous Rac1 or full length PAK1, respectively, in previous reports [8, 23]. These results validated the use of YFP-Rac1 and CFP-Pak-CRIB for monitoring Rac activity in a cell.

Intensity correlation analysis

We next examined a time-lapse colocalization analysis between YFP-Rac1 and CFP-Pak-CRIB in U251MG cells in order to detect the interaction *in vivo*. Images were obtained every thirty seconds for five minutes with a confocal laser microscope. After acquisition of the spectral images, YFP and CFP were unmixed using a linear spectral unmixing method to exclude the fluorescence cross-talk of the fluorophores (Fig. 2A and B). The merged images indicated that YFP-Rac1 and CFP-Pak-CRIB colocalized at the lamellipodia (Fig. 2C). To quantify the co-localization of the proteins, we used intensity correlation analysis (ICA) [18]. A pseudocoloured image, where each pixel is equal to the PDM (product from the differences from the means;

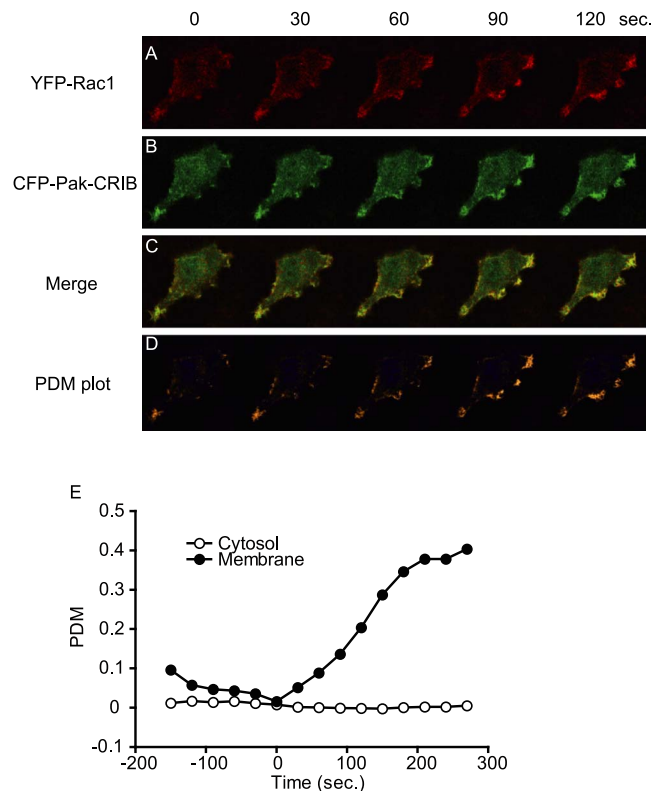


Fig. 2. Intensity correlation analysis of YFP-Rac1 and CFP-Pak-CRIB. (A–C) Time-lapse imaging of YFP-Rac1 (A), CFP-Pak-CRIB (B), merged images (C) upon stimulation of PDGF. (D) Intensity correlation analysis. PDM plot showed a high codependency of Rac1 and Pak-CRIB distribution in lamellipodia. (E) Time sequence of PDM value. After stimuli of PDGF, the PDM value was increased in about 200 seconds.

see Materials and Methods) value at that location (Fig. 2D), showed a high codependency of YFP-Rac1 and CFP-Pak-CRIB in the lamellipodia. Upon the stimulation of platelet derived growth factor (PDGF), the values of PDM within the membrane were increased at two to five minutes (Fig. 2E). These results indicate that the colocalization of YFP-Rac1 and CFP-Pak-CRIB was increased in the lamellipodia and suggest that the CFP-Pak-CRIB binds to the YFP-Pak-CRIB according to the upregulation of the activity of Rac1.

Stimulation of PDGF enhances Rac1 activity

To confirm that the stimulation of PDGF actually elicits the activation of Rac biochemically, we performed a Rac pull-down assay. A GST fusion of Rac/Cdc42 binding (CRIB) motif of PAK was used to affinity precipitate the activated form of Rac [1]. U251MG cells were treated with 20 ng/ml of PDGF and total cell lysates were obtained at the indicated time. Obtained cell lysates were incubated with GST-PAK-CRIB and bound Rac1 were detected with Western blotting using a Rac1 antibody (Fig. 3 upper panel). Amount of total Rac1 was also detected with Western blotting (Fig. 3. lower panel). As shown in Figure 3, upon stimulation of PDGF, cells showed increased Rac activity in two to three minutes after the stimulation (Fig. 3). The results were comparable to that of ICA analysis, and showed the activation of endogenous Rac1 by PDGF stimuli.

Imaging of Rac1 activity in U251MG cells

We next detected the protein interaction between YFP-Rac1 and CFP-Pak-CRIB *in vivo* by a FRET-based assay. U251MG cells co-transfected with YFP-Rac1 and CFP-Pak-CRIB were fixed and used for a quantitative acceptor-depletion-FRET approach combining linear spectral unmixing (u-adFRET) [9, 11]. In this approach, the cross-talk of the fluorophores can be excluded. FRET efficiency (E) is calculated from the unmixed donor and acceptor emission before and after acceptor photobleaching. Figure 4A shows the example of the time sequence of the

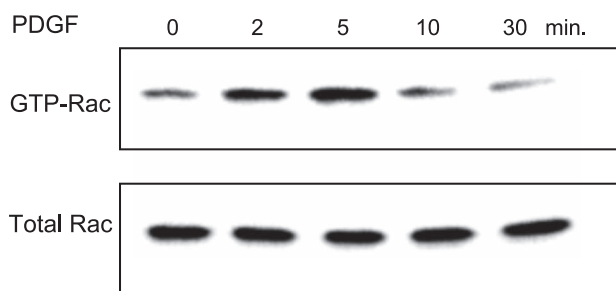


Fig. 3. Rac activation assay. Active Rac1 was pulled down with a GST-Pak-CRIB after stimuli of PDGF. U251MG cells were treated with 20 ng/ml of PDGF. Total cell lysates obtained at the indicated time were incubated with GST-PAK-CRIB and bound Rac1 were detected with Western blotting using a Rac1 antibody (upper panel). Amount of total Rac1 was also detected with Western blotting (lower panel).

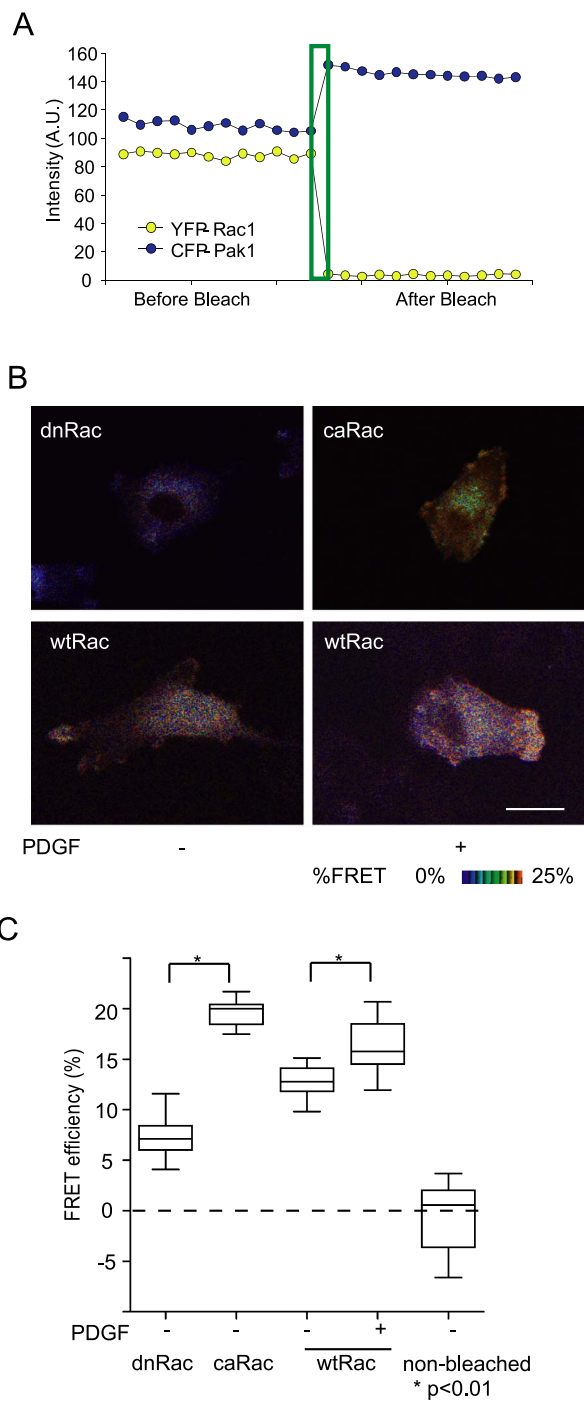


Fig. 4. Imaging of Rac activity in U251MG cells using a u-adFRET assay. U251MG cells co-transfected with YFP-Rac1 and CFP-Pak-CRIB were replated onto glass-bottom dishes. YFP and CFP images were obtained from spectral images using the linear unmixing method. (A) Example profiles of fluorescence of donor (blue) and acceptor (yellow) before and after acceptor-photobleaching. (B) Visualization of the Rac1 activities in cells co-transfected with the combination of CFP-Pak-CRIB and YFP-fused dominant negative Rac1 (upper left panel, as negative control) or wild type Rac1 (lower panels) or constitutive Rac1 (upper right panel, as positive control). (C) Mean FRET. In cells co-transfected with Pak-CRIB and wtRac1, the FRET efficiency is increased with the stimulation of PDGF. * $p < 0.01$

mean fluorescence of the donors and acceptors in the region of interest (ROI). After acceptor photobleaching (Fig. 4A, yellow line), donor emission within ROI was increased (Fig. 4A, blue line), indicating that FRET had occurred. Maps of FRET efficiency indicated that Rac1 was activated in the lamellipodia upon the stimulation of PDGF (Fig. 4B). Statistical analysis showed that FRET efficiency of the cell stimulated with PDGF was higher than that of control cell (Fig. 4C).

IV. Discussion

We describe a FRET-based visualization of Rac1 activity using intermolecular probes as a novel technical assay. FRET is one of the most useful and widely applied tools in use today to measure distances on the molecular scale in cells [16]. A number of trials were done to detect the activity of Rac1 with FRET-based assay. Most of these used intramolecular probes in which FRET pairs consisted of a single molecule [21]. Intramolecular probes have the advantage to detect the activity in a living cell as the probes have equal molar amount of YFP and CFP [16]. However, it requires intensive effort to construct the probes so as to obtain adequate FRET efficiency as small amounts of FRET will occur due to the short distance between the fluorophore pairs in the intramolecular probes. In our system using intermolecular probes, by contrast, the probes can be constructed conveniently and the distance between the fluorophores is enough to suppress the basement FRET. It is also important to place the probes at the proper intracellular localization. In the system using a native Rac1, the subcellular localization of the Rac1 is thought to be identical to the endogenous Rac1 as the CAAX box and the poly-basic region in its C-terminus, which are important to target membrane localization, are intact [14, 24].

To detect FRET rigorously, it is important to increase signal/noise ratio. The major factor to repress signal/noise ratio is the spectral bleed through [2]. Using the emission spectra of specified pure fluorochromes as a reference, the fluorescence intensity of the corresponding fluorochrome within the mixture can therefore be precisely determined from its composite spectrum [26].

Although the acceptor photobleaching method is restricted to the fixed specimen, the assay is applicable to the detection of the FRET in a tissue section if the appropriate fluorescent dyes are selected. FRET analysis using a pair of fluorescent dyes such as FITC and Cy3 [4, 12] or Cy3 and Cy5 have been reported [17]. Recently, Blanco *et al.* reported a way to visualize the RNA splicing variants in cells by a combined method of *in situ* hybridization and FRET analysis using Cy3 and Cy5 labeled probes [3]. The activity of many signaling molecules, such as small GTPases and tyrosine kinases, can be detected as protein-protein interaction [20]. Applications of the assay in the tissue section might improve the ability of immunohistochemistry methods to detect the activity of signaling molecules.

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VI. References

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