Spatiotemporal Regulation of Small GTPases as Revealed by Probes Based on the Principle of Förster Resonance Energy Transfer (FRET): Implications for Signaling and Pharmacology

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Annu. Rev. Pharmacol. Toxicol. 2011. 51:337–58

First published online as a Review in Advance on October 4, 2010

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

This article's doi: 10.1146/annurev-pharmtox-010510-100234

0362-1642/11/0210-0337$20.00

Keywords

Ras, Rho, Rab, GFP, fluorescence imaging, live imaging

Abstract

Low molecular weight ("small") GTPases are key regulators of a number of signaling cascades. Each GTPase is regulated by numerous guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), and each GTPase binds to numerous effector proteins in a GTP-dependent manner. In many instances, individual regulators activate more than one GTPase, and each effector binds to one or more GTPases belonging to the same family. To untangle these complex networks, probes based on the principle of Förster resonance energy transfer (FRET) are widely used. Here, we provide an overview of the probes based on FRET and examples of discoveries achieved with them. In the process, we attempt to delineate the merits, current limitations, and future applications of this technique to pharmacological studies.

INTRODUCTION

GEF: guanine nucleotide exchange factor

GAP: GTPase-activating protein

GDI: guanine nucleotide dissociation inhibitor

FRET: Förster resonance energy transfer

Low molecular weight ("small") GTPases are intracellular molecular switches that activate downstream signaling molecules in response to extracellular and intracellular cues (**Figure 1**). There are five families: Ras, Rho, Arf/Sar1, Rab, and Ran (shown in **Table 1** and reviewed in Reference 1). Biochemical studies have revealed their essential features in terms of synthesis, modification, activation, inactivation, and transmission of signals to downstream effectors. As the research has progressed, the complexity of their regulation has become evident. Each small GTPase is regulated by numerous guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). Furthermore, upon activation, the active (GTP-bound) small GTPases bind to and transmit signals to multiple effector proteins. Conversely, these regulators and effectors are usually associated with multiple GTPases of the same family. Notably, though, most data do not indicate that the signaling of small GTPases is redundant. Signaling molecules within cells often localize to specific locations, and the internal or external cues occur at specific times. Thus a small GTPase may be activated by specific GEFs and transmit signals to specific effectors depending on its subcellular location and cellular context. Therefore, to understand how small GTPases control cellular functions, one must clarify the spatiotemporal changes of their activities within cells (2).

The activity of small GTPases is dictated by the bound guanine nucleotides: GDP in the inactive state and GTP in the active state. Conformational changes that occur upon GDP/GTP exchange can be detected by antibodies specific to the GTP-bound small GTPases, but such conformation-dependent antibodies are not generally available nor can they be used for live cell imaging. Most current techniques detect the activation status of small GTPases by assessing the specific binding of the GTP-bound forms to effector molecules. In this article, we review the techniques used to measure the activity of small GTPases in situ, with a focus on probes based on the principle of Förster resonance energy transfer (FRET); we describe the discoveries achieved

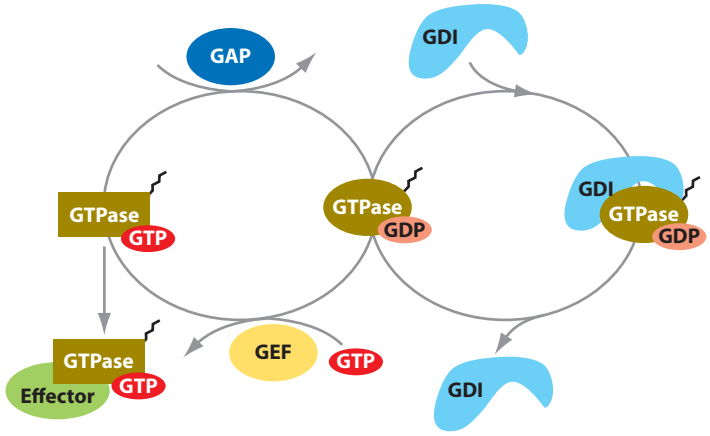

Figure 1

Regulation of low molecular weight ("small") GTPases. Small GTPases cycle between the GDP-bound inactive state and the GTP-bound active state. The activation is mediated by a guanine nucleotide exchange factor (GEF), which replaces GDP with GTP. The GTP-bound small GTPase is inactivated by a GTPase-activating protein (GAP), which strikingly increases the GTPase activity that converts GTP to GDP. GTP-bound, but not GDP-bound, small GTPases are able to bind to and activate effector proteins. Most small GTPases transmit signals on the cytoplasmic phase of the plasma membrane, endoplasmic reticulum (ER), Golgi, or other small vesicles. GDP-bound small GTPases of the Rho and Rab families are sequestered in the cytoplasm by a guanine nucleotide dissociation inhibitor (GDI).

Table 1 Low molecular weight (small) GTPases[a]

Family	Examples	Functions
Ras	H-Ras, K-Ras4A, K-Ras4B, N-Ras, M-Ras, TC21	Gene expression, cell transformation
	Rap1A, Rap1B, R-Ras	Integrin augmentation
	RalA, RalB	Vesicular transport and apoptosis
Rho	RhoA, Rac1, Cdc42, Rnd1	Cytoskeletal reorganization, cell polarity maintenance, gene expression
	TC10 (RhoQ)	Vesicular transport
Arf/Sar1	Arf1-6	Vesicle budding, remodeling of actin cytoskeleton
Rab	Rab1-60	Vesicular transport
Ran	Ran	Nucleocytoplasmic transport of RNAs and proteins

[a]Reviewed in Reference 1.

with such FRET probes; and we discuss the current limitations and future applications of such FRET probes in pharmacological studies.

METHODS FOR DETECTING ACTIVE SMALL GTPases IN SITU

Use of Small Peptides Encompassing the Binding Domain of Small GTPases

One of the research milestones involving small GTPases was the development of the so-called pull-down assay. In the original studies, a short fragment that encompassed the Ras/Rap1-binding domain (RBD) of Raf-1 or RalGDS was used to "pull down" active GTP-bound Ras or Rap1 from cell lysates (3, 4). After the separation of the complex by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the amount of Ras or Rap1 was quantified with antibodies specific to the particular GTPase. Applications for this pull-down method have expanded to the research of virtually all small GTPases and heterotrimeric GTPases [guanine nucleotide binding (G) proteins].

One application of this pull-down method is live cell imaging. For example, the use of green fluorescent protein (GFP)-tagged RBD has shown H-Ras to be activated at the Golgi apparatus (5). This conclusion was based on the observation that GFP-RBD accumulated at the Golgi apparatus in response to external cues. However, whether the affinity of RBD to Ras is sufficiently high for the detection of endogenous Ras-GTP is controversial. In Ras-transformed NIH 3T3 cells, not only the RBD but also the cysteine-rich domain (CRD) adjacent to the RBD are required for the recruitment of GFP-RBD to the active Ras on the plasma membrane (6). In another study, GFP-tagged triadic RBD was used to increase the affinity for the endogenous Ras-GTP (7). Intriguingly, with this high-affinity probe, activation of Ras could not be observed at the endomembrane compartments, indicating that the affinity of the GFP-RBD probes may affect their ability to detect distribution of Ras-GTP. In later studies, small fragments encompassing the binding domain of small GTPases have been used to visualize active small GTPases in fixed cells. For example, the Cdc42/Rac interactive binding (CRIB) domain of Ack1 fused to glutathione S-transferase (GST) is able to detect Cdc42-GTP in paraformaldehyde-fixed HeLa cells (8). The fragment encompassing the binding domain of small GTPases can thus be a powerful tool to detect active small GTPases in situ. It should be kept in mind, however, that these domains are able to bind to multiple GTPases, albeit with different affinities. For example, the RBD of Raf can

RBD: Ras/Rap1-binding domain

GFP: green fluorescent protein

CRD: cysteine-rich domain

CRIB: Cdc42/Rac interactive binding

GST: glutathione S-transferase

bind not only to the archetypal Ras proteins, but also to several Ras-family small GTPases such as Rap1, R-Ras, and M-Ras. Thus without overexpression of small GTPases, there is ambiguity in the interpretation of data obtained with these probes.

YFP: yellow fluorescent protein

Single-Molecule Imaging of Ras-GTP

Use of total internal reflection fluorescence (TIRF) microscopy has facilitated single-molecule imaging of the GFP-tagged RBD of Raf and has clarified the lifetime of Ras-GTP at the plasma membrane (9). Interestingly, the results revealed that the lifetime of GFP-RBD on the membrane ruffles is substantially longer than that on other plasma membrane regions, suggesting two different modes of interaction on the plasma membrane. Furthermore, active Ras has also been detected at the single-molecule level by the use of fluorescence dye-labeled GTP (10). In this work, the binding of this fluorescent GTP to yellow fluorescent protein (YFP)-tagged Ras was detected by FRET.

THE FRET PROBES FOR SMALL GTPases

Overview of the FRET Probes

FRET is a process by which a donor fluorophore in an excited state transfers its energy to a neighboring acceptor molecule (11–14). In most currently used donor-acceptor pairs, the excited acceptor molecule emits fluorescence at its characteristic wavelength, and thus FRET now sometimes connotes fluorescence (rather than Förster) resonance energy transfer. FRET depends on a proper spectral overlap of the donor and the acceptor, the distance between them, and their relative orientation. Many FRET probes have been developed (reviewed in Reference 15); they are classified into two types, intermolecular and intramolecular (**Figure 2**). Intermolecular FRET probes are often used to detect protein-protein interactions (**Figure 2*a***). Each pair of donor and acceptor fluorophores is conjugated with each of the proteins that form a complex. As the distance between the two fluorophores decreases to approximately the Förster distance (i.e., on the order of several nanometers), the efficiency of FRET increases in an inversely proportional manner to the sixth power of this distance.

Supplemental Material

Intramolecular FRET probes are designed to detect the conformational change of proteins and have been increasingly used (see **Supplemental Table 1**; follow the **Supplemental Materials link** from the Annual Reviews home page at **http://www.annualreviews.org**). Such conformational changes can be directly monitored by conjugating the donor and acceptor fluorophores at appropriate locations in the proteins of interest. In the example shown in **Figure 2*b***, the conformational change of the protein to be explored increases the distance between the donor and acceptor and also changes the topology of the donor and acceptor fluorophores, both of which contribute to the increase in FRET efficiency. This design has been successfully applied to single-domain probes of serine/threonine kinases, which often adopt either a closed inactive or an open active conformation (**Supplemental Table 1**). The most serious problem in the development of this type of probe is the lack of information on the structural changes of the molecules of interest. Without the structural information of both the ON and OFF states, researchers are forced to insert the donor and acceptor fluorophores in a trial-and-error manner until optimal positions for the detection of conformational change by FRET are identified. In addition, the structural changes of the signaling molecules in response to external cues are sometimes limited to small regions within the proteins. In this case, it is extremely difficult to find optimal positions for labeling the proteins with donor and acceptor fluorophores.

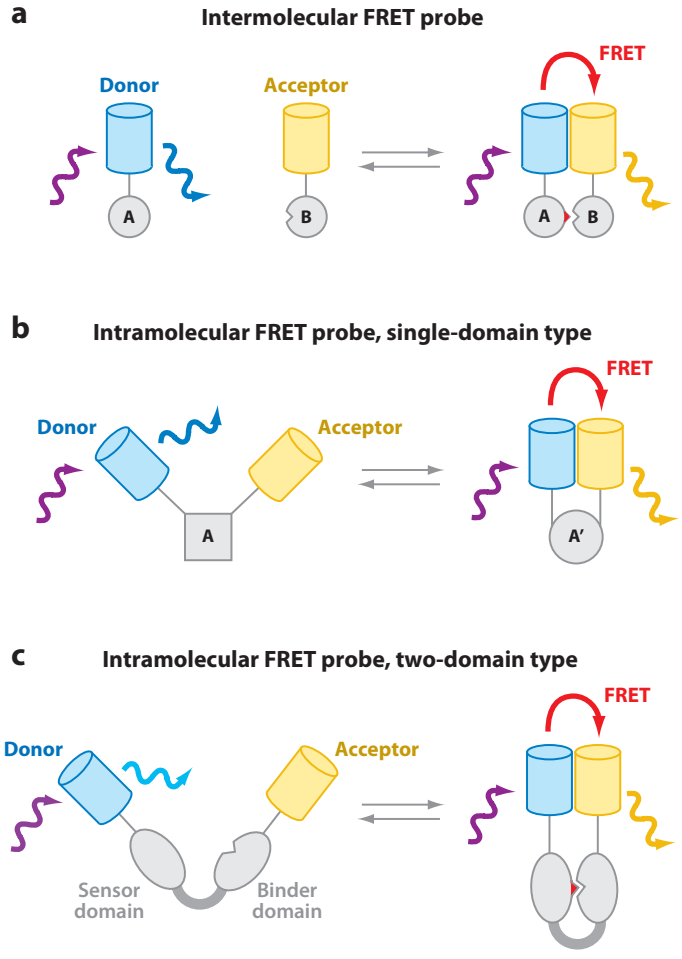

Figure 2

Structure of genetically encoded Förster resonance energy transfer (FRET) probes. (*a*) Intermolecular FRET probes are designed to detect the protein-protein interaction. The donor and acceptor fluorophores are fused to the pair of proteins. When they form a complex, the distance between the donor and acceptor becomes close enough to cause FRET. (*b*) FRET can be used to detect the conformational change of proteins. In the simplest design of the intramolecular FRET probe, donor and acceptor fluorophores are fused to the proper position of the proteins of interest so that conformational change of the proteins alters the distance and/or topology of the donor and acceptor fluorophores, thereby resulting in an increase or a decrease in FRET efficiency. (*c*) The conformational change of the proteins of interest is often minute. Typical examples are the GTP-GDP exchange of small GTPases and phosphorylation of proteins. The domain that displays minimal conformational change upon signal perception is termed the sensor domain. This sensor domain is linked to the binder domain, which binds only when the sensor domain exhibits conformational change. The donor and acceptor sandwich the sensor domain–linker–binder domain complex so that the binding increases FRET.

To overcome this problem, many intramolecular FRET probes adopt a two-domain structure, composed of sensor and binder domains sandwiched with donor and acceptor proteins (**Figure 2*c***). In the case of probes for protein kinases, a substrate peptide and a phosphoamino acid recognition domain such as FHA1 or WW can act as a sensor domain and a binder domain, respectively. Phosphorylation of the substrate domain increases FRET efficiency by the intramolecular binding

of the phosphoamino acid recognition domain to the substrate domain. This general principle of two-domain FRET probes is applicable to the development of probes of many kinases, GTPases, ions, lipid, proteases, and so on. Because of space limitations, we show only some examples of such probes (see **Supplemental Table 1**).

CFP: cyan fluorescent protein

SGBD: small GTPase-binding domain

Supplemental Material

Design of FRET Probes for Small GTPases

The first FRET probe for the measurement of intracellular small GTPase activity was developed by Hahn and colleagues (16). This probe, named FLAIR, assesses intermolecular FRET and consists of the GFP-tagged Rac1 and Alexa-546-labeled CRIB domain of Pak1, an effector of Rac1. The use of this probe requires microinjection of the fluorophore-tagged CRIB domain into the cells before imaging, thus limiting its widespread use. Our laboratory reported the first genetically encoded intramolecular FRET probe for a small GTPase (17). This FRET probe, which we named the Ras and interacting protein chimeric unit (Raichu), was designed to detect changes in the activity of Ras-superfamily GTPases. From the N terminus, the archetype Raichu-Ras consists of a YFP as an acceptor, Ras, RBD of Raf-1, a cyan fluorescent protein (CFP), and a plasma membrane–targeting signal (**Figure 3*a***). The domains are linked with short peptide linkers. Although they have minor differences, many probes based on this backbone have been developed for small GTPases (**Table 2**.)

Unlike Ras-family GTPases, Rho-family and Rab-family GTPases are regulated not only by GEFs and GAPs but also by GDIs, which sequester the inert GDP-bound GTPases in the cytoplasm. The C-terminal region of Rho and Rab necessary for the interaction with RhoGDI is missing in Raichu-type probes, rendering these probes insensitive to RhoGDIs. To overcome this flaw, Hahn and colleagues developed a RhoA probe named RhoA biosensor, which consists of, from the N terminus, small GTPase-binding domain (SGBD), CFP, YFP, and RhoA (**Figure 3*b***) (18). Similarly, we developed a Raichu-Rab5 probe and placed Rab5 in the C terminus, making the probe sensitive to GDIs (**Figure 3*c***) (19). The design of another probe for Rho-family GTPases, GEF sensor, is unique (**Figure 3*d***). In addition to SGBD, the probe contains a VCA domain, which masks the SGBD in the inactive conformation (20).

Notably, the probes described above monitor the activities of regulators of small GTPases—i.e., GEFs, GAPs, and GDIs—but not the small GTPases themselves. Although it is reasonable to speculate that the local balance of the activities of regulators correlates with the activities of endogenous small GTPases at that location, ambiguity remains regarding the correlation between the FRET efficiency of the probe and the activity of the endogenous GTPases. Thus probes composed of RBD sandwiched by donor and acceptor fluorophores have been developed to detect the activities of these GTPases (**Figure 3*e***). This type of probe takes advantage of the tendency of fluorescent proteins to dimerize. In this probe design, in the absence of active small GTPases, FRET is high because of the dimerization of donor and acceptor fluorescent proteins (21). Upon stimulation, the active small GTPases bind to and displace fluorescent proteins, leading to a decrease in FRET. One drawback is that these probes competitively inhibit signaling from GTPases to the effectors. In addition, considering the concentration of GTPase molecules (which is usually between 0.1 to 1 μM), the concentration of probes that would bind to the active small GTPases is much lower than 0.1 μM. At this concentration, fluorescent proteins are not generally detectable by conventional microscopy, unless they accumulate at specific organelles.

Improvement of Fluorescent Proteins

Because of their ease of use, genetically encoded intramolecular probes are the most widely used among several types of probes for small GTPases. Here, the term genetically encoded usually

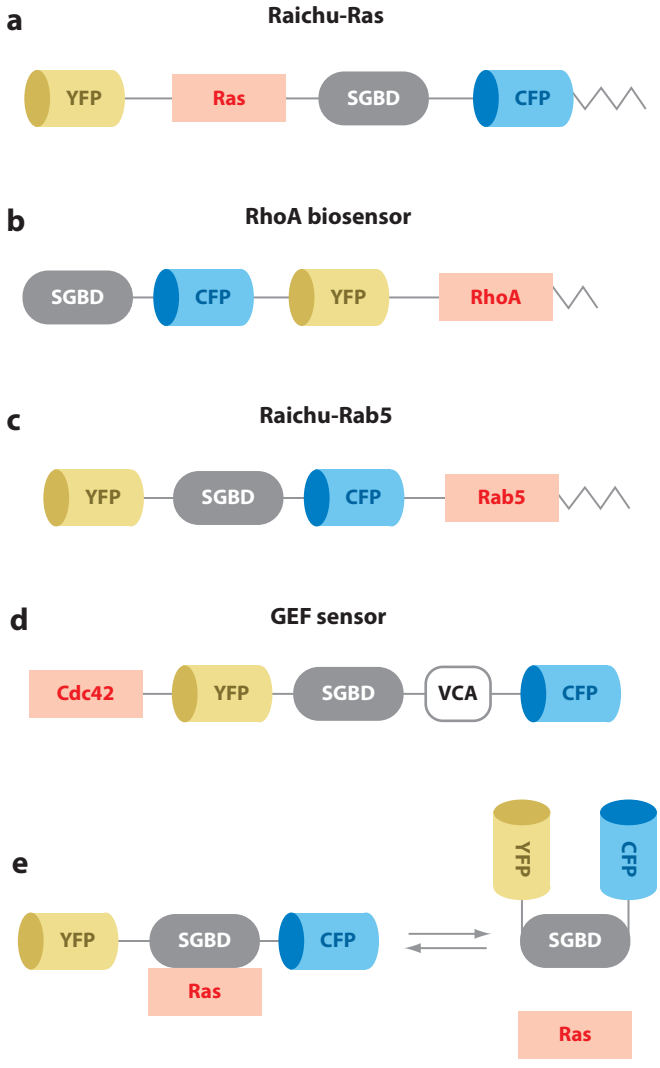

Figure 3

Structure of Förster resonance energy transfer (FRET) probes for small GTPases. (*a–d*) In the design of probes for small GTPases, small GTPases serve as the sensor domain. The binder domain is derived from the effectors of small GTPases. The small GTPase-binding domains are known by various names; here, we collectively refer to them as small GTPase-binding domains (SGBDs). The order of the donor (CFP), acceptor (YFP), small GTPase, and SGBD has been determined only empirically. VCA denotes the C-terminal region of WASP. (*e*) In the design shown here, the binding of active GTP-bound Ras to SGBD dissociates the donor and acceptor so that the FRET decreases. Abbreviations: CFP, cyan fluorescent protein; SGBD, small GTPase-binding domain; VCA, verprolin homology domain, cofilin homology domain, acidic region; WASP, Wiskott-Aldrich syndrome protein; YFP, yellow fluorescent protein.

means that fluorescent proteins serve as both the donor and acceptor fluorophores. In the first genetically encoded FRET probe, which was named cameleon (22), blue fluorescent protein (BFP) and GFP were used as the donor and acceptor, respectively. However, soon after, they were replaced with CFP and YFP because the use of near ultraviolet light for the excitation of BFP evokes phototoxicity and autofluorescence and because the quantum efficiency of BFP is lower

BFP: blue fluorescent protein

Table 2 Genetically encoded intramolecular Förster resonance energy transfer (FRET) probes for small GTPases

Small GTPase(s)	Structure	Name	Reference(s)
Ras, Rap1	YFP-Ras-Raf-CFP	Raichu-Ras, Rap1	17
Ral	YFP-Ral-RalBP1-CFP	Raichu-RalA, RalB	57
R-Ras	YFP-RRas-RalGDS-CFP	Raichu-RRas	60
RhoA	YFP-PKN-RhoA-CFP	Raichu-RhoA	94
RhoA	YFP-Rhotekin-CFP	Raichu-RBD	94
RhoA	RBD-CFP-YFP-RhoA	RhoA biosensor	18
Rac, Cdc42	YFP-PAK-Rac1/Cdc42-CFP	Raichu-Rac1, Cdc42	24, 63
Rac, Cdc42	YFP-PAK-CFP	Raichu-CRIB	63
Cdc42	Cdc42-YFP-N-WASP-VCA-CFP	GEF sensor	20
N-WASP/Cdc42	CFP-N-WASP-YFP	N-WASP-BS	105
TC10	YFP-POSH-TC10-CFP	Raichu-TC10	106
Rab5	YFP-EEA1-CFP-Rab5	Raichu-Rab5	19

Abbreviations: POSH, plenty of SH3s; VCA, verprolin homology domain, cofilin homology domain, acidic region.

Supplemental Material

than that of CFP. Another disadvantage of BFP is its vulnerability to photobleaching. Thus most genetically encoded FRET probes, including those for small GTPases, adopt CFP and YFP as the donor-acceptor pair (**Table 2** and **Supplemental Table 1**).

An important technical breakthrough in the development of FRET probes was the invention of CyPet and YPet (23), which are, respectively, CFP and YFP variants optimized for FRET. In fact, in some instances, replacement of the original CFP and YFP pair with a CyPet and YPet pair can enormously increase the dynamic range of the probe (24), probably due to the increase in the efficiency of dimerization (25). A search for better CFP variants led to the development of mTurquoise (26), the brightest CFP variant currently available. On the other hand, in terms of the disadvantages of the CFP-YFP FRET pair, homologous recombination is not inevitable when retroviral or lentiviral vectors are used to deliver the genes of the probes into the genomes of the cells. This is because both CFP and YFP are derived from the jellyfish GFP and consequently exhibit a high level of sequence identity. It has been shown that teal fluorescent protein (TFP) can replace CFP in the CFP-YFP FRET pair (27, 28). Moreover, we found that Raichu probes containing TFP instead of CFP can be stably introduced into the cells by retrovirus-mediated transfer (29), although the dynamic ranges of the probes are usually lower than those of the probes carrying CFP as the donor.

Beyond CFP-YFP FRET, the GFP and red fluorescent protein (RFP) pair has been the most frequently used, particularly with two-photon excitation microscopy (30). However, in our experience, the dynamic range of the probe is usually larger in the CFP-YFP pair than in the GFP-RFP pair. The reason may be that GFP and RFP do not dimerize with each other, because the tendency to dimerize weakly may be key for the increase in the dynamic range (21). In the following sections, we review discoveries made with FRET probes.

TFP: teal fluorescent protein

RFP: red fluorescent protein

SPATIOTEMPORAL REGULATION OF RAS-FAMILY GTPases

Ras

The prototypical Ras-GTPases are H-Ras, N-Ras, and K-Ras. Many kinds of growth factors rapidly activate these Ras proteins to disseminate signals to many effector proteins. The primary

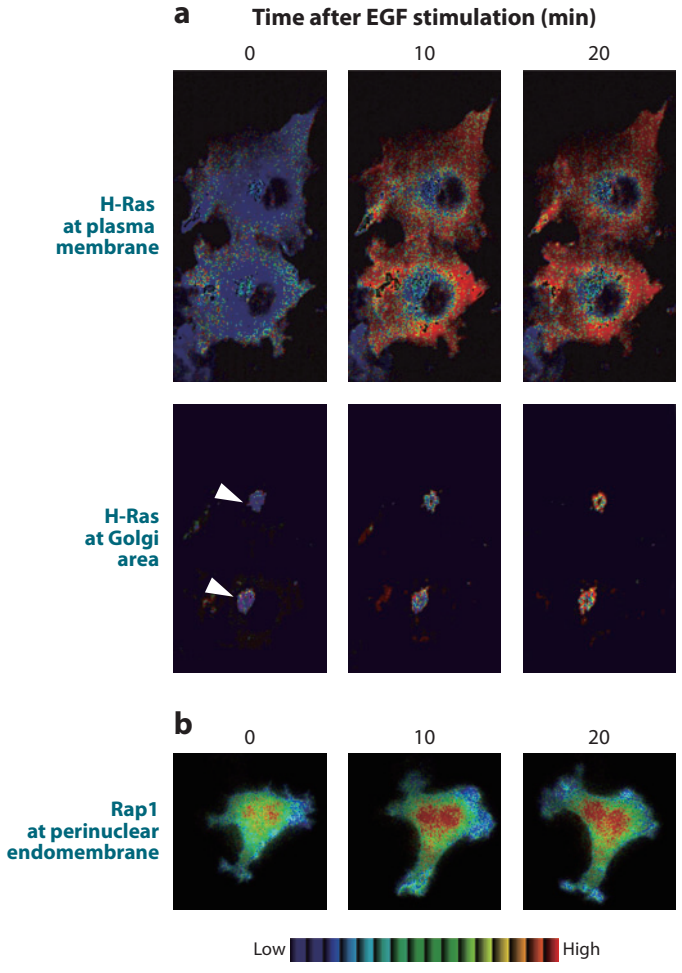

Figure 4

Activation of Ras and Rap1 at different subcellular regions (34). (*a*) COS cells expressing the H-Ras probe were stimulated with epidermal growth factor (EGF) and time-lapse imaged. The H-Ras activity at the plasma membrane reached a zenith at 10 min, whereas the activity at the Golgi area kept increasing up to 60 min. White arrowheads indicate Golgi. (*b*) Rap1 activation in the endosomes (41). PC12 cells expressing a Rap1 probe were stimulated with nerve growth factor (NGF). Rap1 activity was increased mostly at the early and late endosomes.

site of activation of Ras-GTPases is the plasma membrane; however, different effectors can be activated by Ras proteins at different subcellular locations (31). This property may depend on the subtype of Ras; for example, H-Ras is activated at both the plasma membrane and Golgi apparatus (32, 33). This was originally found using GFP-RBD (5) and later confirmed with the Raichu-Ras probe (34) (**Figures 4*a*** and **5**). Upon growth factor simulation, H-Ras is rapidly activated at the plasma membrane and subsequently at the Golgi apparatus. The difference in the timing and location of activation reflects the difference in GEFs; the early activation at the plasma membrane is mediated by Son of sevenless (SOS), whereas the later activation at the Golgi apparatus is mediated by RasGRP1/CalDAG-GEFII (32). Similar to H-Ras, K-Ras is also initially activated at the plasma membrane but then later at a different site, in this case the late endosomes (35).

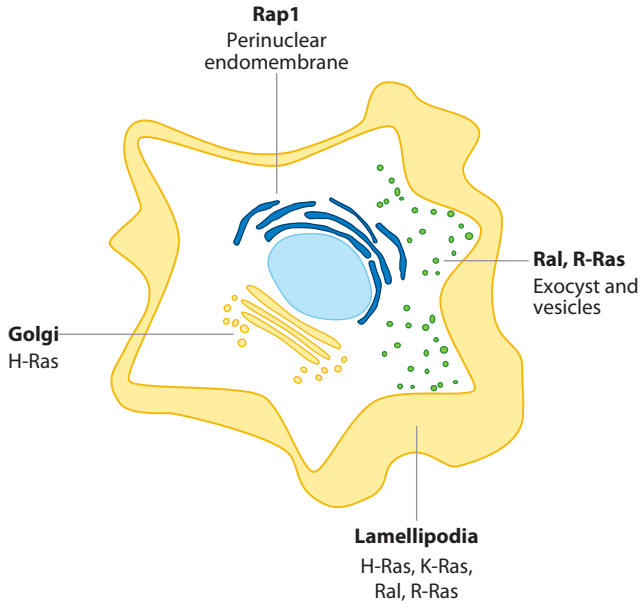

Figure 5

Activation of Ras-family GTPases at different subcellular locations.

Thus although the plasma membrane is the primary site of Ras activation upon growth factor stimulation, each Ras protein is activated in different membrane compartments in the later phase, likely activating different sets of effector molecules. Moreover, the plasma membrane consists of microdomains, within which each Ras protein is differentially distributed (36). For example, H-Ras is in a dynamic equilibrium between lipid rafts and nonraft sites, whereas K-Ras is localized outside lipid rafts. Unfortunately, the size of such microdomains, on the order of tens to hundreds of nanometers, is below the resolution of conventional light microscopy and therefore beyond the scope of current FRET probes.

Rap1

Rap1 has been studied for many years but remains one of the most mysterious GTPases. Early studies indicated that, unlike Ras, Rap1 localized primarily in the endomembrane compartments (37–39). In agreement with these observations, we found that Rap1 is activated mostly at the perinuclear compartment (**Figures 4*b*** and **5**) (17, 40, 41). Rap1 was originally identified as a protein that could revert K-Ras-induced oncogenesis and thus was named K-Ras-revertant 1 (KRev1) (42). A subsequent study suggested that this anti-Ras activity of Rap1 is caused by its competition with Ras for binding to Raf (43). Thus it is plausible that Rap1 sequesters Raf in endomembrane compartments, where Raf cannot be activated. However, later studies suggested that, at least in neuronal cells, Rap1 may activate B-Raf, one of three Raf isoforms (44). In such neuronal cells, Rap1 seems to be activated at the plasma membrane (45, 46). More recently, Rap1 activation at the late endosome has been shown to activate extracellular signal-regulated kinase (ERK) in PC12 cells (41). A neuron-specific factor thus seems to modulate the signaling from Rap1 to Raf at the plasma membrane or the late endosomes.

Apart from the dispute as to whether Rap1 activates or inhibits Raf activity, substantial evidence indicates that Rap1 regulates cell-to-cell and cell-to–extracellular matrix interaction (47).

LDA (leukocyte adhesion deficiency) III, a rare genetic disease associated with immunosuppression, is associated with a defect in the activation of integrin by Rap1 (48). In agreement with this observation, Rap1 activation induces lymphocyte adhesion (reviewed in References 49–51). Further evidence in support of Rap1 function in integrin activation has been provided by studies of a Rap1 GEF, Epac (Exchange protein directly activated by cAMP). Epac, also known as cAMP-GEF/RapGEF3, was identified as a target protein for cAMP (cyclic adenosine monophosphate), in addition to protein kinase A (PKA) and cyclin-nucleotide-gated channels (52, 53). cAMP enhances cell-to-cell contact of endothelial cells via both PKA and Epac (47). In agreement with these observations, studies using a FRET probe have revealed Rap1 activation at the peripheral plasma membrane in endothelial cells (54, 55).

Thus the function of Rap1 is controversial, but this controversy may reflect the pleiotropic function of Rap1 in different subcellular compartments or the timing of its activation. The demonstration of Rap1 activity at specific subcellular compartments will be essential for studies of this GTPase, and FRET probes will be useful tools for such studies.

Other Ras-Family GTPases

Ras has three canonical effectors: Raf, phosphatidylinositol 3-kinase (PI3K), and Ral GEFs. Ral GEFs activate the Ras-family GTPase Ral, which has two isoforms, RalA and RalB. RalA and RalB regulate metastasis and apoptosis, respectively, of human cancer cells (56). Interestingly, stimulation by epidermal growth factor (EGF) activates Ras diffusely in the plasma membrane but RalA only at nascent lamellipodia (**Figures 5** and ***6a***) (57). This restricted activation of RalA depends on Rac1, which is activated in nascent lamellipodia. Thus RalA seems to be regulated temporally by Ras and spatially by Rac1. Because RalA is a component of the exocyst complex (58, 59) and inhibition of RalA abrogates EGF-induced lamellipodial induction (57), Rac1 and RalA likely cooperate to extend lamellipodia, with Rac1 triggering actin polymerization and RalA regulating vesicular components.

Although RalA activation is mostly observed at the nascent plasma membrane in EGF-stimulated cells before stimulation, high RalA activity is also evident in vesicles (**Figures 5** and ***6b***) and seems to be mediated by R-Ras, which is also active at vesicles (**Figures 5** and ***6c***) (60). Thus prior to EGF stimulation, a small fraction of RalA is active in the endomembrane; upon stimulation, a substantial portion of RalA is activated at the plasma membrane. Although it is known that RalA regulates exocytosis by regulation of its binding to Sec5 and Exo70 (reviewed in Reference 56), the spatial information obtained by FRET probes has not been integrated into the model of RalA function. In this context, the recent discovery of Ral GAP as a component of the exocyst complex is likely quite relevant (61). It is tempting to speculate that Ral GAP on the exocyst is activated at the plasma membrane to suppress RalA activity and thus unload the cargo of the exocyst complex.

RHO-FAMILY GTPases

Cdc42

Rho-family GTPases regulate cell morphology by regulating the actin cytoskeleton. One of the Rho-family GTPases, Cdc42, induces filopodia, which are highly dynamic protrusions made of actin filament aligned in tight bundles and which sense environmental cues to guide cell migration (reviewed in Reference 62). In agreement with this activity, FRET probes have detected high Cdc42 activity at the tips of migrating cells (63) and lamellipodia induced by EGF (64). Moreover, high-resolution FRET imaging has revealed that the high Cdc42 activity is restricted to the

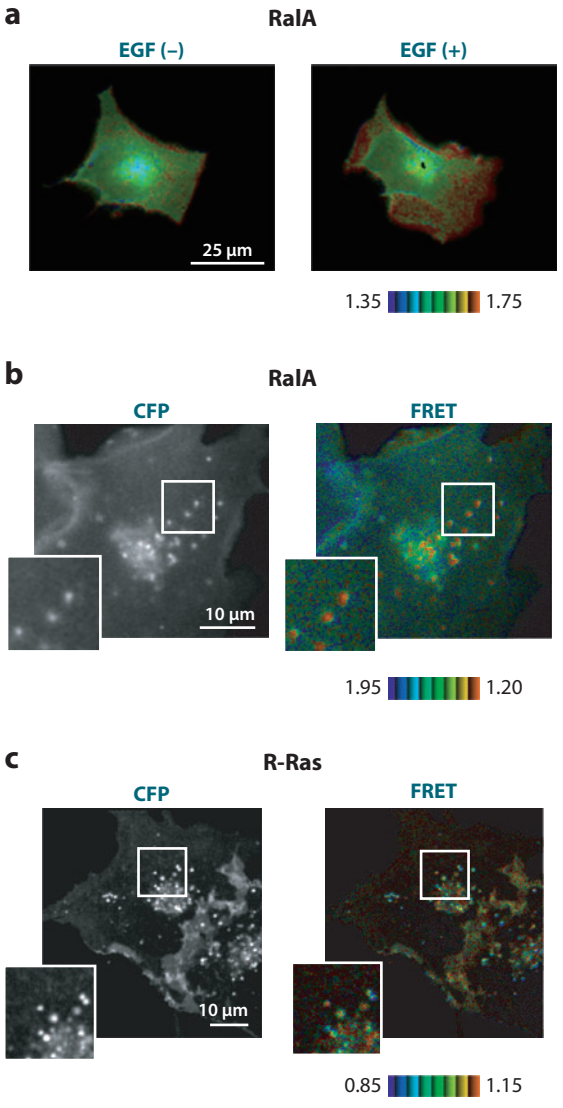

Figure 6

High activity of RalA and R-Ras at endosomes. (*a*) COS cells expressing the RalA probe were stimulated with EGF for 10 min. The FRET image shows localized RalA activation at nascent lamellipodia (57). (*b*, *c*) COS cells expressing the RalA probe (*b*) and R-Ras probe (*c*). The higher magnification shows high RalA and R-Ras activities at the vesicles (60). Abbreviations: CFP, cyan fluorescent protein; EGF, epidermal growth factor; FRET, Förster resonance energy transfer.

root of filopodia (65). This is an important observation in terms of the as-yet poorly understood contribution of Cdc42 to the induction of filopodia.

Rac1

Rac1 is responsible for lamellipodia, sheet-like structures consisting of a cross-linked meshwork of actin filaments (reviewed in Reference 66). The difference in actin filament alignment upon

activation of Cdc42 and Rac1 yielded distinct microscopic morphologies of filamentous filopodia and sheet-like lamellipodia, respectively. Use of FRET probes localized high Rac1 activity in the lamellipodia of randomly migrating cells (16, 24, 63), during wound healing of directionally migrating cells (57, 67), and with CXCR2-mediated chemotaxis (68). In the latter study, there was no overall difference in Rac1 activity between migrating and nonmigrating cells. Thus the localized increase in Rac1 activity seems to be more important than the net Rac1 activity for the chemotactic migration of the cells.

Numerous examples demonstrate that Rac1 activity is regulated by cell-to-cell adhesion and cell-to-substrate binding. The Ajuba/Zyxin family of LIM proteins is a component of cellular adhesive complexes (LIM is an acronym for LIN-11, Isl1, and MEC-3). In the FRET images of Ajuba-null cells, Rac1 activity at lamellipodia is selectively suppressed, suggesting that the cell adhesion signal at focal adhesion sites is essential for the activation of Rac1 in lamellipodia (69). Although the low spatial resolution of FRET images makes it difficult to show high Rac1 activity at focal adhesions, high Rac1 activity at the narrow islands of extracellular fibronectin has been elegantly shown by a micropatterning technique (70). Interestingly, the focal adhesion signals are required not only for Rac1 activation at lamellipodia but also for Rac1 suppression at cell-to-cell adhesion sites (71). FRET images have shown that Rac1 activity is suppressed at cell-to-cell contact areas (71). This suppression of Rac1 at cell-to-cell contact areas is mediated by paxillin and FAK (focal adhesion kinase) and is important for the formation and maintenance of N-cadherin-based cell-to-cell adhesions in the migration of HeLa cells. It should be noted, however, that at least in the case of E-cadherin, the cadherin's intercellular homotypic interaction induces transient Rac1 activation (72).

For directional migration, Rac1 activity must localize toward the direction of migration. In addition to signaling by integrins, engagement of the transmembrane proteoglycan Syndecan 4 with the substrate is critical for this directional migration. Syndecan 4–null fibroblasts migrate randomly due to diffuse, rather than localized, activation of Rac1 (73). The role of Syndecan 4 in the suppression of Rac1 is also demonstrated in the neural crest cells of *Xenopus* and zebrafish embryos (74). Knockdown of Syndecan 4 causes random migration of neural crest cells (in which Rac1 activity is high) in a large region of the peripheral plasma membrane. This regulation of Rac1 by Syndecan 4 involves many signaling molecules (75), including two families of GEF, the Dbl family and the DOCK family, which share little amino acid sequence similarity (76). Accumulating evidence suggests that the high Rac1 activity in lamellipodial protrusion seems to primarily depend on DOCK-family GTPases in fibroblasts (77) and hematopoietic cells (78).

Neurites are another structure in which the contribution of Rac1 has been studied using the FRET technique. Use of Raichu-Rac1 shows localized activation of Rac1 in the neurites of nerve growth factor (NGF)-stimulated PC12 pheochromocytoma cells (79). FRET images also show Rac1 localization in a more physiological state: In hippocampal neurons, higher Rac1 activity occurs in the dendritic spines in comparison with the dendritic shafts (80).

RhoA

RhoA, the archetypal small GTPase of the Rho family, is responsible for actin stress fiber bundling and actomyosin contraction (reviewed in Reference 66). RhoA and Rac1 often antagonize each other (81, 82). RhoA activation increases the rigidity, whereas Rac1 activation relaxes cellular tension. Thus it was assumed that, in migrating cells, Rac1 activity would be high in the front to extend lamellipodia, whereas RhoA activity would be high in the rear to retract the tail (82). Indeed, FRET probes reveal high RhoA activity at the rear end of the cells (83, 84); unexpectedly, however, high RhoA activity was also found at the front of migrating cells (**Figure 7**)

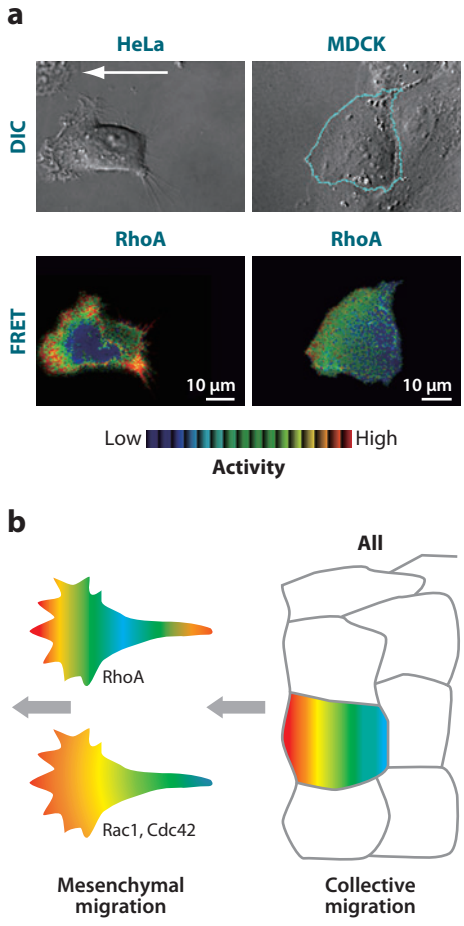

Figure 7

RhoA activity is high at the leading edge of migrating cells. (*a*) In HeLa cells migrating stochastically, high RhoA activity is observed both at the leading edge at the front of the cells and at the uropod (rear). In Madin-Darby canine kidney (MDCK) cells migrating at the edge of the wound, high RhoA activity was observed only at the leading edge at the front of the cells (83). (*b*) Activity map of Rho-family GTPases in mesenchymal and collective migrations. In collective migration, RhoA, Rac1, and Cdc42 show high activity at the front of the cells. Abbreviations: DIC, differential interference contrast; FRET, Förster resonance energy transfer.

(18, 67, 83, 85). This seemingly contradictory observation can be understood as follows (reviewed in Reference 86): There are two major effectors of RhoA, Rho-associated coiled-coil forming kinase (ROCK) and mammalian homolog of *Drosophila* diaphanous (mDia1). The RhoA-activated ROCK, a serine/threonine kinase, induces actomyosin contraction via phosphorylation of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) at the rear of the cells. Meanwhile, RhoA-activated mDia1, a formin homology protein, promotes cell migration in the front of the cells by inducing assembly of actin filament bundles and stabilizing microtubules. More extensive FRET analysis of lamellipodia has revealed interesting relationships among the spatiotemporal activities of Rho-family GTPases (87, 88). RhoA is activated at the cell edge synchronously with edge advancement, whereas Cdc42 and Rac1 are activated with a delay of 40 s

to 6 min depending on the cell type. Rac1 and RhoA may thus be antagonistic on the small scale of lamellipodia, even though they appear to be activated at the same time and same place from a more global point of view. Thus RhoA exemplifies the advantages of FRET imaging for integrating spatial and biochemical information to clarify the function of small GTPases.

The most prominent morphological change of cells is observed during cytokinesis, which is regulated by actomyosin contraction at the cleavage furrow. The spatiotemporal activity change of RhoA has been studied during cytokinesis by different methods (reviewed in Reference 89). RhoA is mostly sequestered in the cytoplasm in its inactive GDP-bound form. Thus recruitment of RhoA to the plasma membrane is regarded as a manifestation of RhoA activation. In agreement with the role of RhoA in actomyosin contraction, accumulation of RhoA at the cleavage furrow has been observed in many cell types (90, 91). In a recent study of budding yeast, GEFs were shown to play a major role in recruiting Rho1, a yeast homolog of RhoA, to the cytokinetic contraction ring (92). Further evidence that RhoA is activated at the cleavage furrow has been obtained with the RhoA-binding domain of rhotekin, which accumulates at the cleavage furrow in four echinoderm species and the vertebrate *Xenopus laevis* (93). FRET imaging, however, reveals that RhoA activity is high not only at the cleavage furrow but also within a diffuse area of the plasma membrane, and that initiation of cytokinesis decreases RhoA activity at the cleavage furrows (94). This discrepant result may reflect that both GEFs and GAPs are recruited to the cleavage furrow and that they both play essential roles in cytokinesis (89). Thus RhoA may rapidly cycle between a GDP-bound inactive and GTP-bound active conformation at the cleavage furrow. Nevertheless, this observation underscores the need for caution when interpreting the results of FRET probe–based measurement of the activity of GTPases and shows that other methods should be used to corroborate such results.

Rab-Family GTPases

The Rab family of GTPases contains the largest number of small GTPases. Most, if not all, of the Rab-family small GTPases regulate intracellular vesicle trafficking (95). Rab5 is one of the most extensively studied Rab-family GTPases. As occurs with Rho-family GTPases, the localization of Rab5 is regulated by GDIs; therefore, its subcellular activity can be visualized by the accumulation of Rab5. For example, this approach has shown that a change in Rab5 activity accompanies the maturation of early endosomes to late endosomes (96). Unfortunately, the spatial resolution of the current FRET probe for Rab5, Raichu-Rab5, is not sufficient to observe the change in Rab5 activity during the maturation of early endosomes. However, the combination of Raichu-Rab5 and Raichu-Rac1 has been used to visualize spatiotemporal changes in the activity of Rab5 and Rac1 during the engulfment of apoptotic bodies into phagosomes, which are much larger than endosomes (19, 97). The requirement of Rac1 for phagocytosis of apoptotic cells is well known (98), and Rac1 induces lamellipodial protrusion, which resembles the phagocytic cup produced at the contact site of apoptotic cells. This gives rise to the expectation that Rac1 is activated at the site of phagocytic cup formation. However, this is not exactly the case because the contact of apoptotic cells with phagocytic cells does not necessarily induce Rac1 activation; however, apoptotic cells are primarily engulfed at sites that exhibit high Rac1 activity and prominent membrane ruffling (**Figure 8**). Upon closure of the phagocytic cup, Rac1 is rapidly inactivated, resulting in disassembly of the actin mesh around the phagosomes (97). This disassembly of the actin mesh allows access to microtubules, to which a GEF for Rab5, Gapex-5, is attached, resulting in a rapid increase in Rab5 activity. Rab5 activation continues for 10 to 20 min, during which the pH in the phagosomes gradually decreases, probably due to fusion with lysosomes (**Figure 8**) (19). Thus with the help of FRET probes, the coordinated and sequential turning ON and OFF of small GTPases has been vividly visualized.

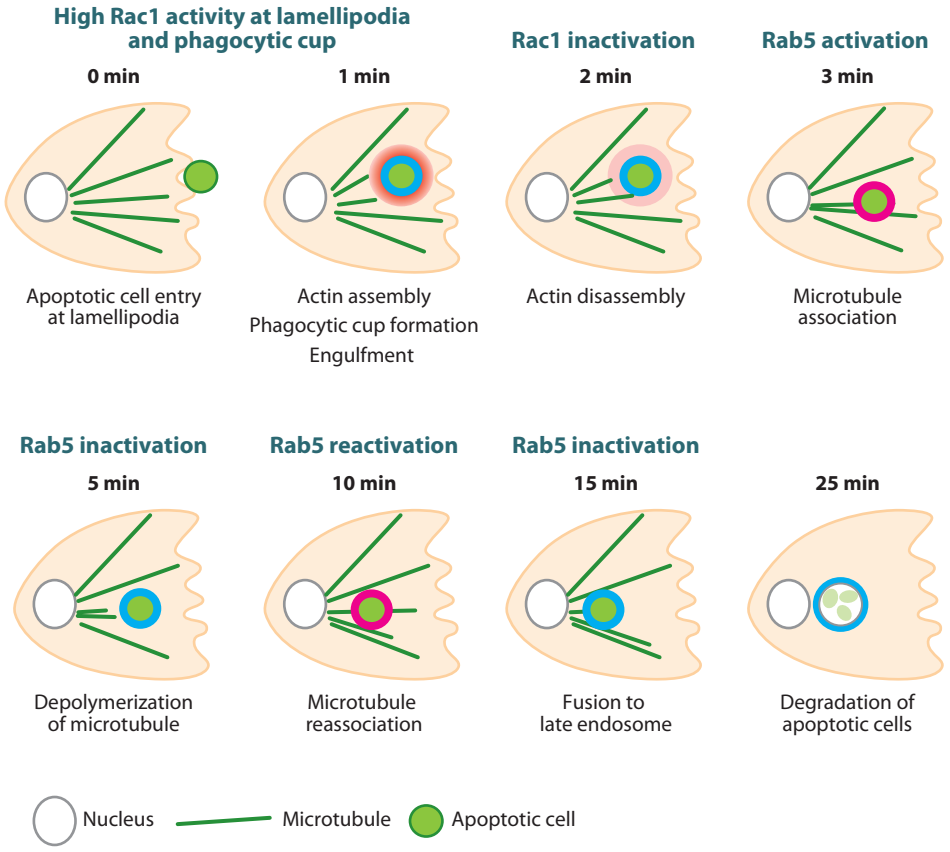

Figure 8

Schematic view of Rac1 and Rab5 activity change during phagocytosis of apoptotic cells. Apoptotic cells are engulfed at the peripheral area, which shows high Rac1 activity (97). Then, Rab5 is transiently activated on the phagosomes containing apoptotic cells (19).

FUTURE CHALLENGES TOWARD PHARMOCOLOGICAL APPLICATIONS

Disadvantages to Overcome

The most serious flaw of FRET probes had been the difficulty in establishing stable mammalian cells lines expressing FRET probes, although this problem is not explicitly described in the literature. The primary reason for this flaw is that both CFP and YFP are mutants of GFP and, as such, they have extremely high DNA sequence homology. Thus when retroviruses are used to deliver the genome of a FRET probe, recombination between *CFP* and *YFP* genes happens during reverse transcription in the virion (99). When linearized expression vectors are used to deliver a FRET probe genome to the mammalian cells, recombination frequently happens between the genomes of CFP and YFP.

The second problem of FRET probes may be their low sensitivity. Using conventional fluorescence microscopic settings, a FRET probe of 1 μM or more is required for detection in tissue culture cells. This concentration is roughly the same as that of many small GTPases. Therefore,

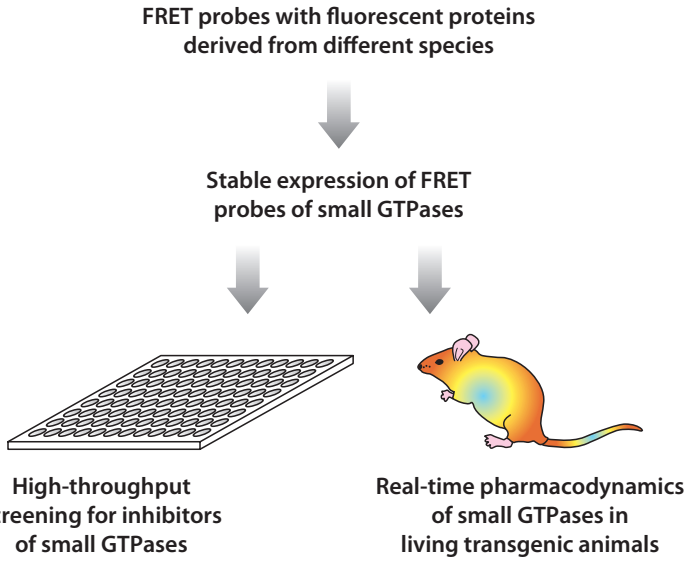

Figure 9

Pharmacological applications of the Förster resonance energy transfer (FRET) probe for small GTPases.

expression of FRET probes can affect physiological signaling cascades. In interpreting data, users must thus consider the effect of such expression.

A third problem is the difficulty of simultaneous use of multiple FRET probes. In addition to a FRET probe with CFP and YFP, a second FRET probe with Orange and Cherry might be used (100), but one cannot use a third FRET probe in the visible light region.

FRET Probes of Small GTPases for the Drug Screening of Inhibitors of Small GTPases

The Ras oncogene product has been a target of drug screening for many years. Even though farnesyl inhibitors can suppress tumor growth by inhibiting plasma membrane targeting of Ras proteins (101), there has been limited success in identifying inhibitors that specifically interact with small GTPases (102, 103). This lack of success is probably ascribable to the difficulty in setting up an in vitro screening system with recombinant small GTPases, GEFs, and/or GAPs. Recent success in the stable expression of FRET probes in cells may provide an opportunity to screen drugs with high-throughput FRET imaging systems (29) (**Figure 9**).

FRET Probes of Small GTPases for Pharmacodynamic Studies

Another promising application of FRET probes for small GTPases is the use of transgenic animals. For example, with a FRET probe for Cdc42, Kamiyama & Chiba produced an activity map of Cdc42 in *Drosophila* (104). Furthermore, transgenic mice harboring FRET probes should become available in the future and will aid in defining the activity changes of small GTPases upon administration of drugs. Availability of such transgenic mice have the potential to change pharmacodynamic studies by facilitating real-time, lengthy monitoring of the activity of small GTPases.

CONCLUDING REMARKS

We have reviewed numerous recent discoveries made possible by FRET probes of low molecular weight G proteins. In addition to this success in imaging by FRET are many attempts that ended in vain. Because the precise quantification of the fluorescence is critical for FRET imaging, photobleaching has to be minimized as much as possible. Consequently, due to low signals, both the spatial and temporal resolutions of FRET images are often less than required. There is much room for improvement in fluorescent proteins. In addition, the dynamic ranges of many FRET probes could be improved by optimization of the site of fusion, linker sequences, and so on. New mathematical approaches may also allow investigators to deduce information from low-resolution images. Thus multidisciplinary efforts are eagerly awaited for the further improvement of FRET imaging. Even so, recent progress in FRET imaging promises widespread application in pharmacological studies in the near future.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank Drs. Asako Sakaue-Sawano, Atsushi Miyawaki, Toshiaki Sakisaka, and Yoshimi Takai for FRET images. This work was supported by a Grant-in-Aid for Scientific Research on Scientific Research on Innovative Areas, "Fluorescence Live Imaging" (No. 22113002), from the Ministry of Education, Culture, Sports, and Science of Japan, and by the Kyoto University Global COE Program Center for Frontier Medicine.

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Annual Review of Pharmacology and Toxicology

Volume 51, 2011

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