Designing biosensors for Rho family proteins – deciphering the dynamics of Rho family GTPase activation in living cells

Olivier Pertz and Klaus M. Hahn*

The Scripps Research Institute, Department of Cell Biology, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
*Author for correspondence (e-mail: khahn@scripps.edu)

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Summary

Rho family GTPases are molecular switches that couple changes in the extracellular environment to intracellular signal transduction pathways. Their ability to regulate behaviors such as cell motility suggests very tight kinetic and spatial control of their activity, which is missed in most biochemical assays. Fluorescent probes that non-invasively report the changing subcellular location of Rho GTPase activity in single living cells are now allowing us to examine spatio-temporal regulation of the activity of these proteins, and are providing new biological insights. Several strategies can be used to construct such probes, and there are advantages and disadvantages associated with the diverse probe designs.

Key words: Rho family GTPase, Fluorescence resonance energy transfer, Microscopy, Cell motility, Dye, Ratio-imaging

Introduction

Rho family GTPases are molecular switches that couple changes in the extracellular environment to intracellular signal transduction pathways (Etienne-Manneville and Hall, 2002). By responding to a wide variety of membrane receptors, they orchestrate cellular processes as different as cell migration, cell-cycle progression and cytokinesis, vesicle trafficking, microbial killing (through regulation of phagocytosis and NADPH oxidase activity) and agonist-regulated gene transcription. The ability of Rho proteins to regulate such a wide variety of cellular processes necessitates tight regulation of their activity. The most widely studied example of such tight regulation is in cell motility, which consists of repeated cycles of protrusion at the leading edge and retraction at the trailing end. These cycles must be coordinated with each other and with cell adhesion to allow directed motility. The finding that selective activation of each individual GTPase is coupled to specific cytoskeletal and adhesive structures has led to a model in which distinct Rho GTPases are selectively activated at discrete locations and with precise kinetics to generate motility.

For a long time, the presumed local activity of a given GTPase could be inferred only from the appearance of specific cytoskeletal and adhesive structures. The development of different ‘pull-down’ assays using effector domains that selectively bind to GTP-loaded GTPases enabled measurement of active Rho-family GTPases in cell-lysates. Although quantitation of relative activity became possible, these methods provided no spatial information, and only limited temporal resolution. The clear need for information about the spatio-temporal dynamics of Rho protein activation led to the development of fluorescent probes that allow direct, non-destructive measurement of the nucleotide state of Rho GTPases in individual living cells.

Immunofluorescence experiments or live-cell imaging studies using green fluorescent protein (GFP) fusions of Rho GTPases could not fully elucidate the dynamics of Rho protein signaling. Such studies showed that the pool of Rho proteins sequestered by interaction with guanosine dissociation inhibitor (GDI) in the cytoplasm can translocate to the plasma membrane upon stimulation with growth factors or overexpression of guanosine nucleotide exchange factors (GEF) (Kranenburg et al., 1997; Michaelson et al., 2001). However, they could not reveal whether membrane localization of a GTPase correlates with GTP loading. This was accomplished using GFP fused to domains from Rho effectors, simple practical probes devised to monitor the location of GTP-loaded GTPases in living cells. Bourne and co-workers used a YFP-tagged CRIB (Cdc42- and Rac-interactive binding motif) domain from p21-activated kinase (PAK) to monitor the location of GTP-bound Rac in neutrophils (Srinivasan et al., 2003). Similarly, Kim et al. showed that a GFP-tagged CRIB domain from the Wiskott-Aldrich syndrome protein (WASP) is recruited to nascent adherens junctions containing E-cadherin in a calcium switch experiment, which suggests that GTP-loaded Cdc42 is recruited to this location (Kim et al., 2000). The same GFP-fusion was used to detect activated Cdc42 in fixed cells and showed that active Cdc42 is recruited to the immunological synapse in an antigen-specific manner (Cannon et al., 2001). An important caveat with such probes, however, is their lack of specificity. The GTPase-binding domain of PAK shows only a threefold difference in affinity for Rac versus Cdc42, and so cannot clearly distinguigh between them (Thompson et al., 1998). The WASP domain does not discriminate between Cdc42, TcL (Vignal et al., 2000), Tc10 and RhoT (Abe et al., 2003). In addition, such GFP fusion proteins are diffusely distributed throughout the cell, which leads to a high background and makes it technically challenging to observe accumulation of the probe at discrete locations in some important cases.

Bimolecular FRET-based probe for Rac

To overcome the specificity problem and greatly enhance contrast and sensitivity, investigators have engineered more complex probes to use fluorescence resonance energy transfer (FRET). In FRET, energy is transferred non-radiatively from an excited donor to an acceptor fluorophore, which then emits at its characteristic wavelength. FRET depends on the proper overlap of the donor emission and acceptor excitation spectra, the distance between both fluorophores, and their relative orientation. FRET is effectively limited to fluorophores that are 10-100 Å apart, making it a sensitive measure of molecular proximity. For example, one FRET-based probe that can report Rac activation (Kraynov et al., 2000) consists of two components: a GFP-Rac fusion protein that provides the donor fluorophore; and the CRIB domain of PAK (known as PBD for p21 binding domain, see Fig. 1A), which binds selectively to activated Rac. PBD can be expressed in *E. coli* and specifically labeled with an Alexa-546 acceptor dye on an introduced cysteine residue. When it is microinjected into cells expressing GFP-Rac, FRET occurs only when and where PBD binds to GTP-loaded GFP-Rac, resulting in decreased GFP emission and an increase in Alexa FRET emission.

Since the acceptor and donor fluorophores are on separate proteins that may have different subcellular distributions, quantitation of FRET is complicated because of contributions of donor emission 'bleedthrough' and direct excitation of the acceptor fluorophore to the FRET image. A fraction of these contributions have therefore to be substracted from the raw FRET image, and this will depend on the specific filters used and must be quantitated for each imaging system (Chamberlain et al., 2000). Since this probe does not reveal activation of the endogenous GTPase and requires ectopic expression of GFP-Rac, an obvious experimental restriction is that the amount of probe has to be tightly controlled. Excessive expression of Rac leads to its activation, presumably because there is only a finite amount of GDI to sequester Rac in the cytoplasm. The amount of PBD must also be tightly controlled, since it can exert dominant-negative effects by titrating out the active endogenous GTPase. A similar probe consisting of Rac and PBD fused to GFP variants (CFP and YFP) has also been designed, but the FRET efficiency in this case is lower, because fluorophores embedded in a protein cannot approach each other as closely as free dyes. Although the biosensor based on GFP variants has the important advantage of being genetically encoded, the enhanced sensitivity of the dye-based sensor is valuable for studies of activation of small amounts of Rac in some subcellular locations (Kraynov et al., 2000).

The ability to detect Rac activation with high spatial resolution has led to valuable biological insights. For example, studies of a wound healing model indicate that there is a gradient of Rac activity that is highest near the leading edge and fades away towards the nucleus. This supports a model in which Rac is activated at the leading edge to promote lamellipodial protrusion (Merlot and Firtel, 2003). In polarized cells, additional hot spots of perinuclear activity have been observed, perhaps corresponding to endomembrane compartments in which activated Rac may regulate vesicle trafficking. As expected, activated Rac is also evident in ruffles induced when quiescent Swiss 3T3 fibroblasts are stimulated with serum. An example is shown in Fig. 1 and illustrates the gain in sensitivity and selectivity achievable by FRET compared with the use of labeled PBD alone. Unlike FRET, simple localization of this effector domain must be discerned over a diffuse background of the same probe. Also, the effector domain interacts with multiple targets, but the FRET signal will be observed only at locations where the effector domain binds to GFP-Rac.

Del Pozo et al. observed that FRET between PBD and the constitutively active V12 mutant of GFP-Rac occurs only in protrusive areas, such as lamellipodia, rather than uniformly over the entire cell (Del Pozo et al., 2002). This is despite the fact that both the constitutively active Rac and the Alexa-labeled PBD were uniformly distributed in their experiments. It is known that GTP-loaded Rac binds to Rho GDI, albeit with lower affinity (Sasaki et al., 1993) than GDP-loaded Rac. The observation of FRET in only some locations suggested that the GTP-loaded Rac bound to Rho GDI cannot interact with an effector. Using biochemical techniques, Del Pozo et al. clearly showed that Rho GDI and the effector PAK compete in a mutually exclusive fashion for interaction with GTP-loaded Rac. Indeed, mutation of the Rac CAAX box to disrupt the interaction between Rho-GDI and V12 GFP-Rac produced uniform distribution of FRET throughout the cell. The interaction between GTP-loaded Rac and PAK occured around subcellular locations where integrins engage with the

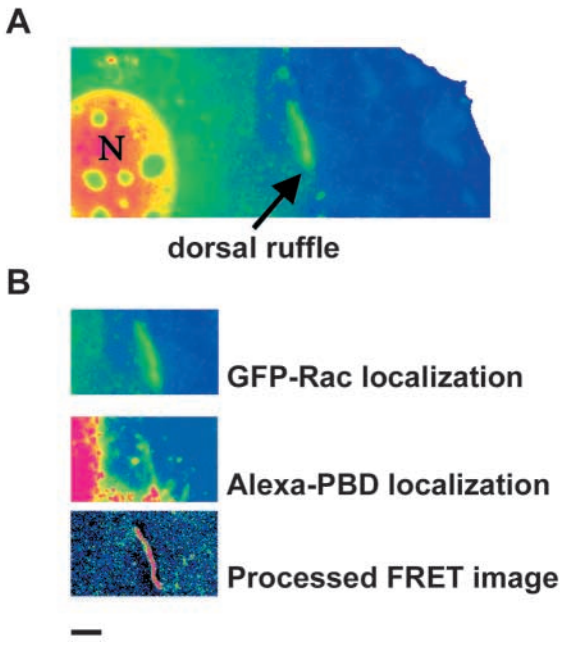

Fig. 1. Example of specificity and sensitivity of FRET in a dorsal membrane ruffle. (A) GFP-Rac localization in a Swiss 3T3 fibroblast stimulated with serum. (B) GFP-Rac, Alexa-PBD and processed FRET images of the same ruffle. GFP-Rac localization illustrates the presence of Rac in the ruffle but does not provide information about its activation status. PBD localization cannot be used to quantify Rac activity because of the high background from unbound PBD in the cytoplasm, and because of lack of specificity. All images are color-coded with warmer colors representing higher concentrations or higher levels of FRET. Bar, 22 μm. N, nucleus. Figure reproduced with permission from the American Association for the Advancement of Science (Kraynov et al., 2000).

extracellular matrix. Integrins therefore appear to activate a signaling pathway that induces dissociation of Rac from Rho-GDI, permitting subsequent membrane translocation of Rac and localized interactions with effectors. Here, use of the Rac FRET probe highlighted a new level of complexity in GTPase signaling, suggesting that GTP loading and membrane recruitment are two events that are controlled separately by distinct signaling pathways. This also showed that the Rac probe does not strictly measure GTP loading but instead reveals the locations where Rac binds to effectors. Note that competition with other molecules that bind to Rac could also eliminate the signal at some subcellular locations.

Another surprise was the difference in the Rac activation patterns of motile fibroblasts in the wound-healing model described above and neutrophils undergoing chemotaxis (Gardiner et al., 2002). When a neutrophil senses a chemoattractant gradient, it extends a lamellipod in which a broad area of Rac activity is observed. Interestingly, at this early time point, additional Rac activity is observed at the retracting tail. As the neutrophil becomes fully polarized, Rac activity at the stably protruding leading edge becomes concentrated into discrete spots that appear and disappear during protrusion. Rac activity in the retracting tail displays a less discrete pattern, but changes in intensity appear to match tail retraction and attachment/detachment to the substratum. The very localized and dynamic changes in Rac activity observed in neutrophils contrast with the broad activity gradient observed in wound healing fibroblasts, possibly owing to the highly motile behaviour of neutrophils. The observation that Rac regulates tail retraction in neutrophils, previously suggested to depend on Rho (Worthylake et al., 2001), illustrates the value of directly measuring GTPase activity in living cells.

Genetically encoded unimolecular FRET probes for Rac, Cdc42 and RhoA

When engineering a FRET-based probe, it is much easier to carry out quantitative measurements of FRET if both the donor and acceptor fluorophores have a fixed stochiometry and are distributed evenly throughout the cell. Physically linking the two fluorophores accomplishes this. The corrections described earlier for FRET biosensors in which the donor and acceptor fluorophores are on separate proteins are no longer critical here. Since both fluorophores are identically distributed throughout the cell, the contributions of donor bleedthrough and direct acceptor excitation are the same wherever the probe is analyzed, and are not corrected. The change in proximity and/or orientation of the fluorophores can then be described as a simple ratio of FRET/donor emission. This concept has been applied successfully for multiple genetically encoded probes and applied to a variety of intracellular signaling events (reviewed by Miyawaki, 2003). These probes typically consist of a single-chain fusion protein in which two protein domains that conditionally interact with one another are sandwiched between two GFP variants capable of undergoing FRET (e.g. CFP and YFP). When the protein domains interact, the fluorophores re-orient, leading to a change in FRET efficiency. With an adequate calibration system, one can calculate the level of interaction from the FRET/donor emission ratio.

Such a probe successfully reveals activation of the small GTPase Ras (Mochizuki et al., 2001). Mochizuki et al. fused Ras to a domain from its effector Raf that selectively binds to GTP-loaded Ras, using a short linker, and sandwiched the two domains between CFP and YFP. We refer to this probe design as ‘GTPase-effector fusion’. High FRET levels are evident when the Raf domain binds to GTP loaded Ras; low FRET levels are evident when Ras is GDP loaded. This probe design imposes significant constraints. Because the C-terminus of Ras is fused to the Raf domain, the CAAX-box that enables farnesylation and subsequent membrane targeting had to be removed from Ras. Farnesylation and plasma membrane attachment was rescued by fusion of the CAAX-box of K-Ras4B to the C-terminus of the whole fusion protein. Since Ras is constitutively membrane anchored, constraining localization to the plasma membrane was acceptable in this case.

Similar probes based on PBD can reveal activation of Rac and Cdc42 (Fig. 2B) (Itoh et al., 2002). Since Rho family GTPases are not constitutively membrane anchored, but can be sequestered in the cytoplasm through their interaction with Rho GDI, moving the native CAAX-box to the C-terminus of the probe produces disadvantages. An intact C-terminus is required on Rho family GTPases, because the interaction between Rho GDI and the GTPase involves both the isoprenoid moiety and the switch I and II domains (Hoffman et al., 2000). These biosensors therefore ignore regulation provided by Rho GDI, measuring the activation of Rac and Cdc42 only at the plasma membrane. They miss activation patterns on endomembranes, which can be observed with the bimolecular Rac probe. This may be especially significant for Cdc42, which is located mostly on the Golgi, the endoplasmic reticulum and the nuclear envelope rather than on the plasma membrane (Michaelson et al., 2001). These biosensors do reveal valuable information about localized activation at the plasma membrane. In motile HT1080 cells, the biosensors showed a gradient of both Rac and Cdc42 activation that gradually increases towards the leading edge, and decreases rapidly when cells change direction. Furthermore, they showed that Cdc42 is most active at the tip of the leading edge, whereas Rac activity is highest behind the leading edge.

In the same paper, Itoh et al. described an additional FRET probe that consists of the PAK CRIB domain flanked by CFP and YFP (Fig. 2C). We refer to this probe design as ‘effector domain only’. For this probe, decrease in FRET efficiency produced by binding was explained by structural data showing that the PAK CRIB domain undergoes a dramatic conformational change upon interaction with GTP-loaded Rac or Cdc42 (Hoffman and Cerione, 2000). This may lead to a change in the orientation and/or the distance between CFP and YFP. The biosensor is not sufficiently sensitive to detect activation until it is targeted to the membrane by the CAAX box from K-Ras 4B, revealing activity at the cell’s leading edge. It also suffers from lack of specificity (e.g. it does not discriminate between Rac and Cdc42), but has the important advantage that it can respond to regulation by Rho GDI.

Seth et al. adopted the ‘effector domain only’ probe design for the CRIB domain of WASP (Fig. 2C). They observed a decrease in FRET efficiency associated with its binding to GTP-loaded Cdc42, which was again explained by structural data showing a drastic conformational change in the WASP CRIB domain upon binding to Cdc42 (Seth et al., 2003). Taking advantage of the modular structure of the WASP protein, Seth

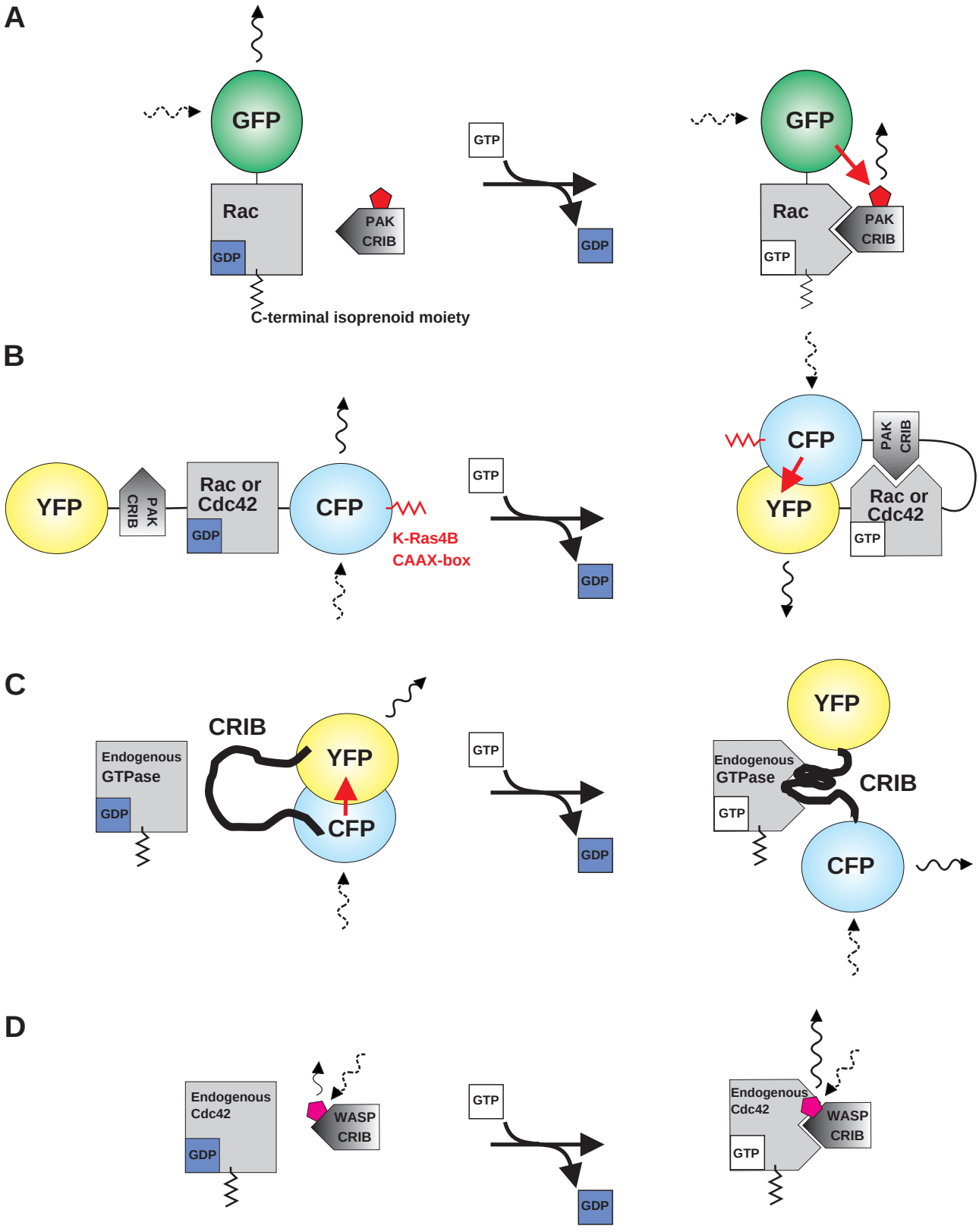

Fig. 2. Design of different fluorescent probes for detection of Rho family GTPase activity in living cells. (A) Bimolecular Rac FRET probe (Kraynov et al., 2000). Red pentagon represents Alexa-546 dye. (B) Unimolecular 'GTPase-effector fusion' Rac and Cdc42 FRET probes (Itoh et al., 2002). Non-natural isoprenoid moiety due to K-Ras4B CAAX-box is depicted in red. (C) 'Effector domain only' probes (Itoh et al., 2002; Seth et al., 2003). (D) Biosensor based on covalently labeling a domain with a solvatochromic dye. Red pentagon represents the dye. Black dashed arrows represent excitation light. Black solid arrows represent emission light. Red arrows represent direction of FRET.

et al. also used Cdc42 and the WASP CRIB domain to construct a biosensor using the 'GTPase-effector fusion' probe design. Using spectroscopic techniques, the deactivation kinetics of this probe in response to incubation with a GTPase-activating protein (GAP) were compared with those of recombinant Cdc42 alone in an in vitro system. This led to the important conclusion that, in such probes, the high local concentration of an effector domain can compete against GAP-mediated GTP hydrolysis. This is consistent with structural studies that have shown that GAPs and effectors interact with similar regions on the GTPase (Mott et al., 1999; Nassar et al., 1998). Although these different Cdc42 probes display a large change in FRET efficiency in vitro, they cannot measure the activity of endogenous Cdc42 in vivo. Activation can only be seen in living cells upon co-expression of Cdc42 with a GEF or a GAP. Changes in FRET efficiency can then be correlated with in vitro measurements, but subcellular localization of activity cannot be discerned. Such probes could nonetheless be valuable for following the kinetics of total cellular activation, or for high-throughput screening in vitro. Furthermore, none of these probes are membrane-targeted. Therefore, engineering-in a localization motif may render them more sensitive.

An important caveat must be considered when one uses these biosensors in wide-field fluorescence microscopy, rather than in techniques that remove out-of-focus light, such as confocal or deconvolution microscopy. Activated GTPases are localized in membranes. Therefore, in the thick parts of cells, activation can be 'diluted' by large volumes of inactive biosensor in the cytoplasm if the probe is soluble. Targeting the probe to the plasma membrane dramatically reduces the amount of cytoplasmic probe, thereby bypassing this 'dilution effect' and enabling a higher sensitivity. The important drawback is that access of the probes to some membranes on which the native GTPase is usually found is eliminated. Furthermore, using alternate targeting motifs may localize these probes to sites where upstream regulators are absent.

Yoshizaki et al. recently devised two FRET-based probes to study Rho activity (Yoshizaki et al., 2003). In an effort to build the most reliable probe using the 'GTPase-effector fusion' design, Yoshizaki et al. constructed a panel of probes using different Rho effector domains and compared each one containing dominant-positive (Q63L) or wild-type Rho. Most of the probes had similar FRET/CFP ratios, suggesting that the effector domain was effectively competing away GAPs that catalyze hydrolysis of bound GTP. Finally the author picked a probe containing the effector domain from protein kinase N. This displayed a significantly different ratio between dominant positive and wild-type probe, presumably because the PKN domain had a weaker affinity for the GTPase. In order to take into account regulation by GDI, Yoshizaki et al. also used the effector domain from rhotekin to produce a FRET probe that takes advantage of the 'effector domain only' design. Again, since the effector domain of rhotekin can bind to GTP-loaded RhoA and RhoC, this probe suffers from the lack of specificity inherent to the design (Reid et al., 1996). Yoshizaki et al., used these two probes to monitor changes in RhoA activity at the plasma membrane during cell division.

A new alternative to FRET

The development of solvatochromic dyes whose fluorescence intensity or wavelength is strongly dependent on the surrounding environment provides a valuable alternative to FRET-based techniques. Recently, new dyes have been developed specifically for use in live-cell biosensors. These exhibit bright fluorescence at long wavelengths, and undergo large changes in fluorescence intensity and/or wavelength in response to solvent polarity or specific hydrogen bonds (Toutchkine et al., 2003). Our group has conjugated such a dye to the WASP CRIB domain (P. Nalbant and K.M.H., unpublished; Fig. 2D). The dye-labeled domain undergoes a strong change in fluorescence intensity upon binding to endogenous Cdc42. This combines the benefits of sensing endogenous proteins with the greatly enhanced sensitivity provided by dyes. The high sensitivity reduces the amount of biosensor required and detects low levels of endogenous activity. Such probes can incorporate different dyes that fluoresce at a wide range of wavelengths, thereby enabling imaging of multiple protein activities in the same cell.

All the probes we have described are based on protein domains that bind to a target with a specific conformation. Owing to competition with endogenous ligands, such domains may not always be able to reach their targets, or they may block normal protein interactions. Therefore attaching environmentally sensitive dyes directly to the GTPase, without the need for a separate domain, is also promising. Site-specific labeling of recombinant proteins with dyes remains technically challenging, but given new technologies that enable site-specific incorporation of unnatural amino acids in proteins (Wang et al., 2001), or total synthesis of proteins (Dawson and Kent, 2000), this is now becoming easier. Taking advantage of the large amount of structural information about Rho family GTPases and their interaction partners, together with the ability to site-specifically label proteins at a desired residue, one can now design probes to sense not only GTP loading but also interactions with specific upstream regulators or downstream effectors.

Concluding remarks

Measuring the activation of Rho family GTPases with high spatial and temporal resolution has greatly enhanced our knowledge of their function and regulation, significantly extending models derived from traditional biochemical and cell biological methods. It will be exciting to use these new tools in probing the many cellular activities requiring spatio-temporal coordination of GTPase activity, including cell motility, neuronal pathfinding, phagocytosis, transformation and gene expression. The high resolution provided by these approaches has the potential to resolve important uncertainties in current models. For example, Rac, Cdc42 and RhoA affect each other's activities to coordinately regulate actin and adhesion dynamics during cell motility and probably in other processes as well. Interestingly, cell biological and biochemical data point to different modes of crosstalk between GTPases. Cdc42 can activate Rac, which in turn stimulates RhoA (Nobes and Hall, 1995), but Rac can also antagonize RhoA (Nimnual et al., 2003), and RhoA has been shown to activate Rac under some conditions (Tsuji et al., 2002). What may initially appear to be contradictory results are probably evidence of spatial and temporal regulation of signaling pathways, which can now be addressed. The biosensors also

promise to decipher the effects of immediate upstream regulators of GTPases, especially GEFs, on spatial patterns of GTPase activity. Finally, observations of GTPases and even GTPase activity on diverse endomembrane compartments in living cells (Kraynov et al., 2000; Michaelson et al., 2001) suggest that localized activation regulates or is regulated by trafficking. In this respect, it will be interesting to understand how Rho family GTPases interact with Arf GTPases, since both are regulators of the actin rearrangements and membrane trafficking that precede cell spreading and migration (Randazzo et al., 2000). Clearly, dissecting the tight regulation of Rho family GTPases with high spatial and temporal resolution will shed light on a diverse range of critical cell behaviors.

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