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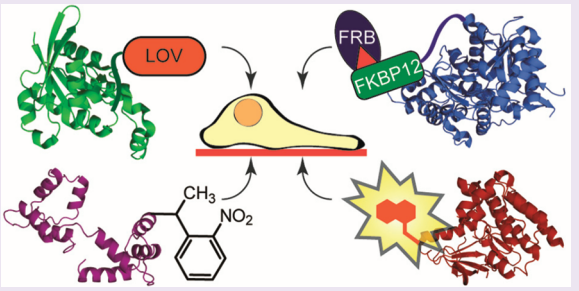
Chemical Tools for Studying Directed Cell Migration

Brenda N. Goguen and Barbara Imperiali*

Departments of Biology and Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, United States

ABSTRACT: Cell migration is required for many physiological processes, including wound repair and embryogenesis, and relies on precisely orchestrated events that are regulated in a spatially and temporally controlled manner. Most traditional approaches for studying migration, such as genetic methods or the use of chemical inhibitors, do not offer insight into these important components of protein function. However, chemical tools, which respond on a more rapid time scale and in localized regions of the cell, are capable of providing more detailed, real-time information. This Review describes these recent approaches to investigate cell migration and focuses on proteins that are activated by light or small molecules, as well as fluorescent sensors of protein activity.



Cell migration is a complex process essential for many fundamental functions including wound repair, the immune response, and embryogenesis. In addition to supporting normal physiology, cell migration contributes to pathological processes including cancer metastasis, vascular disease, and inflammatory conditions such as rheumatoid arthritis.[1] Migration is a highly dynamic process, governed by precisely coordinated protein interactions in specific regions of the cell. In addition, the timing of gene transcription and protein activation and deactivation is tightly regulated at defined points of the migration cycle.[2] Techniques to study migration, including genetic approaches such as gene deletions, siRNA-knockdown of gene expression, and site-directed mutagenesis, have revealed many details about the mechanisms involved.[3] However, migration and associated processes occur on the seconds to minutes time scale, and genetic techniques, which act on the order of hours to days, preclude detailed study of the temporal component of protein activity. The application of small molecule inhibitors has also been used to provide information about protein function,[4] but effects from such inhibitors are global and cannot be localized to a specific region of the cell. In contrast to these more conventional techniques, approaches derived from chemical biology can be applied more rapidly and with tunable effects.[5] These technologies have the potential to provide precise control over the location and timing of protein activation, enabling elucidation of the spatial and temporal roles of specific proteins within a complex network of interactions. Thus, methods drawing on chemical biology are poised to make substantial contributions to the study of cell migration. This Review highlights approaches that have been developed to dissect the functions and interactions of proteins integral to cell migration. Techniques that will be addressed include light-activated proteins, chemical genetics approaches, and fluorescent sensors of protein activities.

CELL MIGRATION

Cell migration is an iterative process, characterized through five primary stages.[1,6,7] First, upon exposure to a migration-inducing signal, the cell becomes polarized with a distinct front (leading edge) and rear (trailing edge) (1). The cell then extends protrusions in the direction of movement (2) and forms transient adhesions with the extracellular matrix (3) through interactions between integrins and the actin network. The adhesions provide traction as the cell body translocates forward (4). Finally, the adhesions at the rear of the cell are disassembled, allowing the cell to advance forward (5).

In the development of tools to investigate migration, particular focus has been devoted to examining the Rho-family GTPases, including RhoA, Rac1, and Cdc42, because of the fundamental role that they play in orchestrating the process.[8] Cycling between an inactive GDP-bound state and an active GTP-bound state, these proteins function as molecular switches and together regulate many aspects of the actin cytoskeleton.[9] All three GTPases can be localized to the plasma membrane through prenylation of the cysteine residue within the *C*-terminal CAAX sequence, where C is cysteine, A is an aliphatic residue, and X is any residue. Although these GTPases can also be localized to the cytosol and to other organelles, in migration they are activated primarily at the plasma membrane.[10] In addition to this spatially controlled regulation, the activities of these GTPases are highly coordinated through the action of many accessory proteins. Guanine nucleotide exchange factors (GEFs) activate the GTPases by promoting exchange of GDP for GTP, while GTPase activating proteins (GAPs) accelerate the rate of intrinsic GTP hydrolysis to deactivate them. The Rho GTPases

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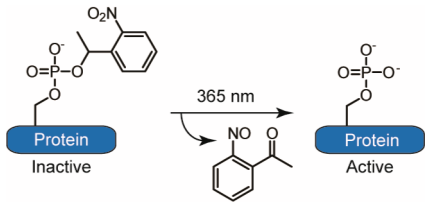

Figure 1. General caging strategy. Caging of an important functionality, in this case a phosphate group of a protein, renders the protein inactive within the biological system. Irradiation at 365 nm releases the caging group to generate the active protein that can exert native effects within the system.

mediate cellular functions through interactions with over 60 known effector proteins, including kinases and scaffold proteins.[11] Activated Cdc42 induces the formation of filopodial, or fingerlike, protrusions, whereas Rac1 activation causes broad lamellopodial protrusions.[9] RhoA activation is involved in membrane protrusions[12] but also leads to the formation of stress fibers and induces cell contractility.[9,10]

LIGHT-ACTIVATED PROTEINS

Approaches in chemical biology have proven to be particularly valuable for gaining insight into the transient protein−protein interactions that mediate migration. Many of these techniques rely on the application of light-sensitive compounds to systematically control or perturb cellular processes. The use of light activation provides unique advantages for investigating complex signaling networks because it is noninvasive and affects only the photoactivatable molecule in the cell. The light intensity and wavelength can be readily controlled to enable dose-dependent activation and fine-tuning of the light-mediated event. The localization of irradiation can be defined through the use of lasers or directed light sources to produce activation at a particular region of a cell, allowing investigation of the spatial component of protein function.[13] However, it must be noted that, in some instances, the effects of localized photoactivation may not be apparent due to rapid diffusion and dilution of the activated molecule.[14] Thus, in these cases, anchoring the light-sensitive protein at the plasma membrane or at an organelle can better reveal the consequences of spatially defined uncaging. Light-activated proteins can be chemically derived by appending an organic photolabile protecting group onto an essential functionality of the protein,[15] or the protein can be fused to a recombinant photoreceptor domain.[13] Both techniques have been successfully applied to interrogate processes in cell migration.

Caged Peptides and Proteins. Caged peptides and proteins exploit the versatility of chemical synthesis for the study of biological processes. With the caging technique, the functional portion of a signaling molecule or the catalytic residue of a protein, for instance, is prepared with a covalently bound, photolabile protecting group. This caging group masks the essential element and renders the molecule inactive within the biological system. However, upon irradiation, the mask is removed and the native active species is immediately released, enabling the downstream effects of the molecule to be observed in real time.[16] Verification that the caged protein is phenotypically silent must be performed in each case, particularly when multiple residues are responsible for activity. Figure 1 depicts an example of caging in which a phosphoserine is the key determinant for function,[17] but this technique can be applied to other functionalities, such as the side chain of cysteine,[18] serine,[19] or lysine,[20] or the protein backbone.[21]

The most commonly used caging groups are based on the *o*-nitrobenzyl family of photolabile protecting groups. Due to synthetic tractability, many analogues with variable photophysical properties have been described,[22] but the 1-(2-nitrophenyl)-ethyl (NPE) group is particularly useful because it is released at wavelengths that are compatible with cellular studies (365 nm). In addition, the photobyproduct, nitrosoacetophenone, is less reactive than the nitrosobenzaldehyde released upon photolysis of nitrobenzyl groups.[23] More recently, caging groups from the coumarinyl family[24−26] and derivatives such as the nitrodibenzofuran chromophore[27] for two-photon uncaging have been developed for cellular applications. Caged peptides have been successfully applied for the study of myosin light chain kinase (MLCK)[28] and FAK,[29] while caged proteins, including cofilin,[18] PKA,[30] myosin regulatory light chain,[31] and paxillin,[32] have been developed to probe the cellular functions of these proteins.

Caged Proteins by Cysteine Labeling. Although caged peptides can be readily generated through solid-phase methodologies,[33] the use of caged proteins provides greater specificity within the cellular environment and enables the protein to be directly activated through irradiation to induce native downstream effects. Caging of full-length proteins has been achieved through site-specific cysteine labeling, though a more general approach exploits protein semisynthesis. Cysteine-targeted caging has been successfully employed to cage the actin-regulating protein cofilin, which is involved in the reorganization of actin networks.[18] Since the protein is deactivated by phosphorylation at Ser3, a photoactivated variant of this protein was generated by selectively labeling a Ser3Cys mutant with α-bromo-(2-nitrophenyl)acetic acid (Figure 2a). While the negatively charged caging group mimicked the phosphorylated residue and rendered the protein inactive, irradiation released the constitutively active Ser3Cys protein. Studies with this caged protein revealed additional roles for cofilin beyond mediating actin depolymerization. Specifically, activated cofilin was shown to induce actin polymerization and protrusions and govern the direction of migration.

The cysteine labeling methodology has more recently been extended to cAMP-dependent protein kinase (PKA), which contains multiple reactive residues.[30] This kinase is involved in signaling cascades governing many aspects of migration, including actin rearrangements, Rho GTPases, and mRLC phosphorylation.[34] The photoactivatable PKA was modified with a multifunctional caging group, designed to liberate the active kinase upon irradiation and to concomitantly generate a fluorescent signal to report successful photolysis (Figure 2b). With this strategy, a short inhibitory peptide sequence targeted to PKA was elaborated with a caged linker, a fluorophore−quencher pair, and an electrophilic maleimide. Binding of the peptide to the enzyme positioned the maleimide near reactive Cys343 to promote alkylation with the caging agent, thereby covalently tethering the peptide inhibitor to the protein. Microinjection of caged PKA into cells and uncaging generated a fluorescent signal, released the inhibitor, and consistent with the role of PKA in cytoskeletal rearrangements, induced stress fiber degradation.

Caged Proteins by Semisynthesis. Although cysteine labeling represents an efficient and direct approach for the attachment of caging groups onto a protein, this strategy is not feasible when

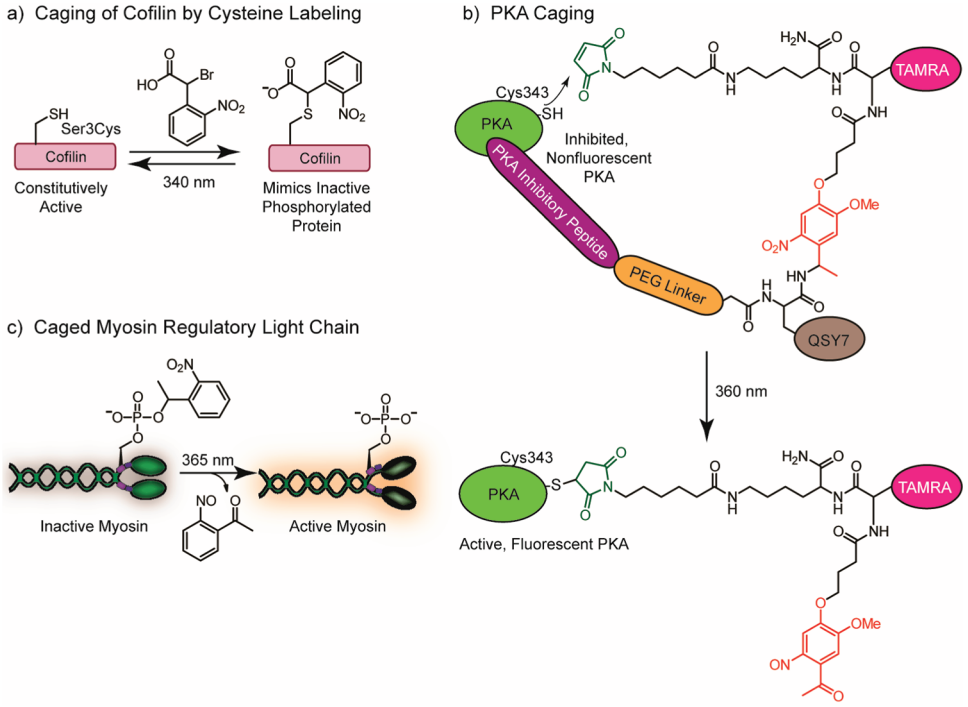

Figure 2. Caged proteins for studying cell migration. (a) Constitutively active cofilin can be caged by reaction with α-bromo-(2-nitrophenyl)acetic acid. The caged protein mimics the inactive phosphorylated protein. (b) The multifunctional reagent for caging PKA. The PKA binding peptide targets the reagent to PKA and positions the maleimide near Cys343 for labeling. The QSY7 chromophore quenches fluorescence of the TAMRA fluorophore. Uncaging releases the PKA inhibitor peptide and the quencher to restore PKA activity and to generate a fluorescence signal. (c) Incorporation of the caged phosphorylated mRLC into myosin eliminates activity. Uncaging releases the native phosphoprotein to restore myosin function.

the essential residue cannot be selectively targeted for derivatization. In these cases, protein semisynthesis or expressed protein ligation, which combines peptide synthesis with recombinant protein expression, can by exploited for the site-specific incorporation of unnatural residues into full-length proteins.[35] However, because efficient peptide synthesis is practically most useful for the synthesis of peptides up to about 50 residues, this approach is most convenient when the unnatural residue is desired near the *N*- or *C*-terminus of the protein.

This semisynthetic technique has been applied to prepare a photoactivated myosin (Figure 2c). Myosin II is an ATPase motor protein responsible for generating the contractile forces necessary to initiate focal complex formation at the leading edge of a migrating cell, as well as to release adhesions at the rear.[36,37] The protein complex is activated upon phosphorylation of the associated myosin regulatory light chain (mRLC) protein at Ser19. To directly probe the effects of myosin on cellular actin dynamics, a photoactivated variant of the mRLC was generated through protein semisynthesis to install NPE-caged phosphoserine at position 19.[31] Following incorporation of this caged protein into the myosin complex, myosin was inactive in the caged state, but activity was restored through irradiation and release of the native phospho-mRLC (Figure 2c). When microinjected into a cell, this protein tool can be used to activate myosin to probe the localized effects of myosin contractility in real time.

Another attractive target for caging is paxillin, which is a multidomain protein that functions as a scaffold within focal adhesions to recruit proteins to control migration, cell survival, and proliferation. Phosphorylation occurs throughout the molecule and creates docking sites for other adaptor and regulatory proteins. For instance, phosphorylation at Tyr31 stimulates the Rho GTPases Cdc42 and Rac1, which in turn promote the formation of membrane protrusions and control focal adhesion turnover.[38] To interrogate the role of this protein in migration and particularly the effect of Tyr31 phosphorylation, paxillin was synthesized by expressed protein ligation to incorporate NPE-caged phosphotyrosine at position 31.[32] The semisynthetic protein was tested to ensure that binding to native protein partners was intact and that it remained a substrate for known kinases. Irradiation released phospho-paxillin, validating the potential of the tool for unraveling signaling pathways at focal adhesions.

While protein semisynthesis has efficiently yielded caged proteins for probing various aspects of migration, an alternative to this approach relies on *in vivo* amber suppression methods using evolved orthogonal tRNA/tRNA synthetase pairs specific for the unnatural amino acid.[39] Recent studies have shown that this system can be applied in mammalian cells to produce caged proteins *in situ*.[40,41] While caged derivatives of lysine have been successfully incorporated in HEK293 cells, the tRNA/tRNA synthetase pairs for caged serine[19] and caged tyrosine[42] have been established for use in bacterial systems. Although application of this technique to any new unnatural amino acid requires evolution of a new tRNA/tRNA synthestase pair that is compatible with mammalian expression,[39] this technology complements semisynthetic approaches by enabling incorporation of the unnatural residue at any position of the protein and by eliminating the need for external delivery approaches. Thus, in combination with semisynthetic approaches, this method offers flexibility for the preparation of protein tools for investigating cell migration.

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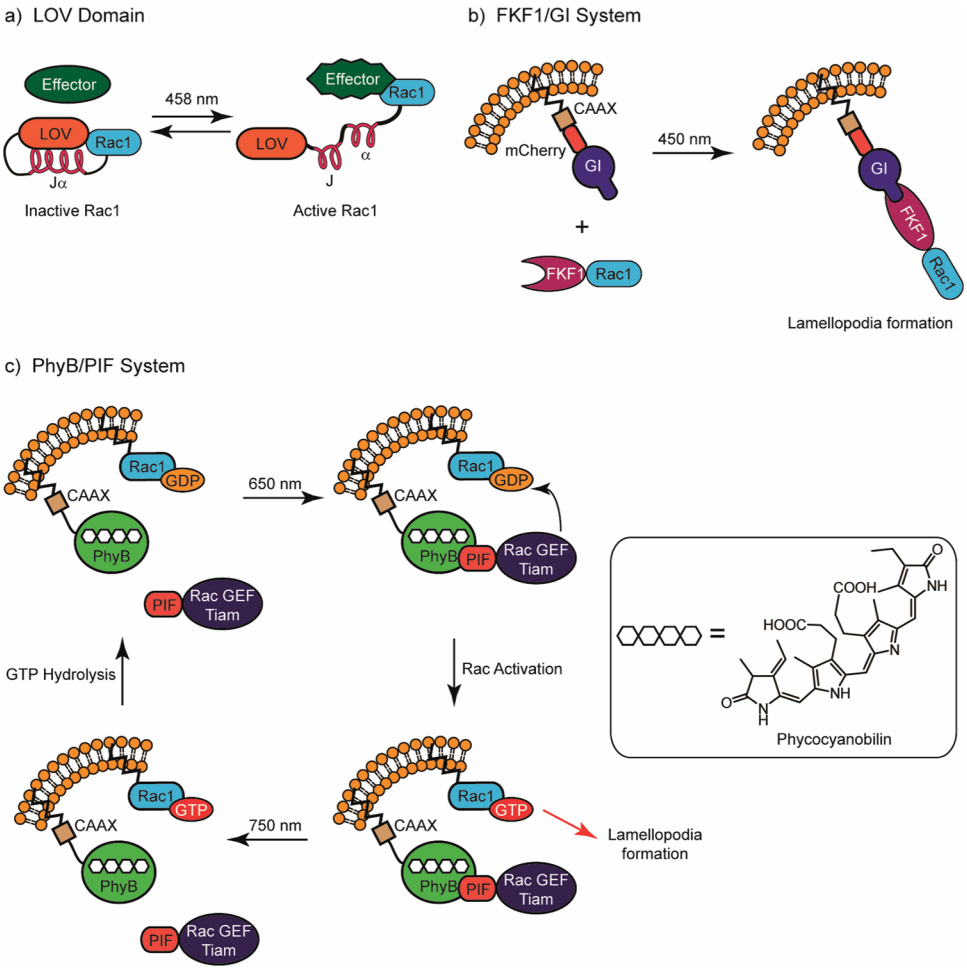

Figure 3. Light-activated Rac1 based on plant photoreceptors. (a) Photoactivated Rac1 was generated by fusing constitutively active Rac1 to the LOV domain. Irradiation causes the Jα helix to unravel and allows effectors to bind Rac1. (b) In the FKF1/GI system, irradiation at 450 nm recruits the FKF1-Rac1 construct to the membrane-bound GI protein. Because Rac1 is active at the membrane, irradiation induces lamellopodia formation. (c) The PhyB/PIF system enables reversible activation of endogenous Rac1. Upon irradiation, the PIF-Tiam construct binds to membrane-bound PhyB. This recruitment leads to Rac1 activation. Irradiation at 750 nm reverses the PhyB/PIF interaction.

Photoreceptor Fusions. As a complementary approach to caging, plant photoreceptors have been adapted to provide genetically encoded proteins that can be activated by light.[13] Proteins from the phytochrome and phototropin classes respond to light through bound chromophores and undergo reversible conformational changes upon irradiation. In plants, these proteins are involved in mediating phototropism, the process of directional growth in response to light. While phytochromes respond to red light through a bilin chromophore, phototropins bind flavins and absorb blue light.[43] Both families of protein domains have been engineered to enable photoactivation of the Rho GTPases.

LOV Domain Fusions. Phototropins react to light through light-oxygen-voltage (LOV) domains, which bind flavin mononucleotide (FMN) and undergo a characteristic photocycle in which the protein forms a noncovalent complex with FMN in the dark. Irradiation with light at 450 nm triggers the formation of a covalent adduct through a cysteine residue of the LOV domain and the FMN molecule. The bond formation induces a concomitant conformational change, in which an associated α-helix, termed the Jα helix, is released and unwinds.[44] Fusion of the LOV domain to a protein at an appropriate position generates photodependent allosteric inhibition and can render protein—protein interactions or activity sensitive to light. This approach has been successfully applied for the generation of a photoactivated Rac1.

The photoactivated Rac1 was created by fusing a LOV domain to a constitutively active mutant of Rac1 (Figure 3a).[45] In the dark state, the LOV domain sterically inhibits interactions between Rac1 and effector proteins. However, upon irradiation at 458 or 473 nm, unwinding of the Jα helix relieves inhibition and enables the active Rac1 to interact with native effectors. When expressed in mammalian cells, brief laser pulses localized to specific regions at the edge of the cell initiated protrusions and ruffling events, which were reversible and dependent on the intensity of irradiation. In subsequent studies, this tool was applied to examine the role of Rac1 during migration in more complex systems. In zebrafish neutrophils, localized Rac1 activation enabled interactions with regulatory proteins to be examined,[46] while application of the protein in the *Drosophila* egg chamber allowed interrogation of the influence of the GTPase on coordinated migration.[47] These studies together demonstrate that this tool can be exploited to extract detailed information about Rac1 in live cells. Further investigation of the

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Table 1. Comparison of Methods for Light-Mediated Protein Function

approach	molecular weight	reversible?
caging group	150−300 Da	no
LOV domain	16,000 Da	yes
FKF1/GI	FKF1: 68,000 Da; GI: 129,000 Da	slowly
PhyB/PIF	PhyB: 100,000 Da; PIF: 11,000 Da	immediately with infrared light

light-dependent dynamics of the LOV domains has revealed mutations that can modulate the properties of the domains for specific applications.[48]

In addition to the LOV domain fusion, two other photoreceptor systems, the flavin binding, Kelch repeat, F-box1 (FKF1)/GIGANTEA (GI) system[49] and the phytochrome B (PhyB)/phytochrome interaction factor 3 (PIF3) system,[50] have been engineered for applications to control protein activity through irradiation-induced dimerization. In the case of FKF1 and GI from *Arabidopsis thaliana*, exposure to blue light (450 nm) induces dimerization of these two proteins (Figure 3b).[49] FKF1 contains a LOV domain, and when irradiated, Cys91 forms a covalent bond with FMN. The resulting conformational change enables the protein to bind the nuclear protein GI. This system has been adapted for investigating the function of the GTPase Rac1 at the cell membrane. In these studies, the photoreceptor domain is expressed with a *C*-terminal CAAX sequence for prenylation and subsequent processing to anchor the module to the membrane, and the GTPase or a GTPase activating protein is fused to the corresponding photoreceptor-binding partner. This construct remains cytosolic until irradiation promotes binding between the light-sensitive proteins and localizes the GTPase or activator to the membrane. Because Rac1 is active primarily at the plasma membrane, global or localized light-mediated recruitment stimulates lamellopodia formation. Due to the long half-life of the FMN-FKF1 complex, sustained interactions were achieved, though extended irradiation for 5 min was necessary. This strategy is promising, but further improvements in the association and dissociation kinetics of the two binding partners will create a system that can be more generally applied.[51]

Phytochromes. In contrast to the LOV domains, phytochromes respond to red light when the associated bilin chromophore undergoes a *cis*-to-*trans* isomerization. PhyB from *A. thaliana* covalently binds the tetrapyrrole chromophore phycocyanobilin (PCB), which undergoes reversible photoisomerization upon irradiation with red or near-infared light and mediates the transition of PhyB between the red-absorbing (Pr) state and the far-red-absorbing (Pfr) state.[50] The PIF3 protein binds PhyB only after it has absorbed 650 nm light and exists in the Pfr state, and in plants, this heterodimer translocates from the cytoplasm to the nucleus to mediate gene transcription. Spontaneous dissociation occurs over hours, but irradiation with infrared light (750 nm) immediately reverses binding.[43] The PhyB/PIF system was adapted for application in mammalian cells and was optimized for reversible association on the time scale of seconds.[50] Like the FKF1/GI system, these photoreceptors have been adapted to enable light-mediated protein dimerization and study of Rac1 *in vitro*[52] and at the cell membrane (Figure 3c).

To overcome the effects of diffusion on localized activation in cellular studies with the PhyB/PIF constructs, irradiation was performed with a patterned light source, which transmitted the deactivating infrared light to the entire sample while focusing the activating red laser to a localized area ($\sim$3 μm) of the cell. This patterning ensured that the activated protein was present only in the desired region by deactivating molecules that diffused away from that area. The 650-nm red laser initiated the PhyB(Prf)-PIF6 interaction to recruit the Rac1 GEF to the membrane, thereby activating endogenous Rac1 to induce lamellipodia formation. While this system offers the advantages of rapid interaction kinetics and reversibility, the PCB cofactor, which is not produced in mammalian cells, must be isolated from natural sources and added to the culture.

Table 1 summarizes the approaches that have been described in the preceding sections. Compared to small molecule caging techniques, the light-sensitive protein domains are advantageous because they are genetically encoded and do not require complex delivery methods. Second, unlike small molecule caged compounds that are irreversibly activated upon irradiation, the activities of these proteins are generally reversible, and in the case of the phytochromes, reversibility can even be controlled through application of far-red wavelength (750 nm) light. Finally, the wavelengths of photoreceptor activation are longer than those used for photolysis of organic caging groups and generate less photodamage after repeated irradiation. However, despite these advantages, the light-activated domains suffer from some drawbacks. In particular, these proteins are large and, in some cases, even larger than the proteins being studied. This additional steric bulk can disrupt native interactions or produce artificial effects. In this regard, small molecule caging groups are superior, as they introduce far fewer structural perturbations. Uncaging releases the native protein, which then maintains proper interactions and functions. Nonetheless, both families of probes demonstrate great promise for exploring complex pathways. As the technology expands, other migration-related targets beyond the small GTPases must be addressed.

CHEMICAL GENETICS

The aforementioned methods exploit light to control protein−protein interactions and activity. A complementary, small molecule-based approach that offers precise temporal control over specific protein interactions is based on chemical inducers of dimerization (CID).[53−55] This technique exploits the natural product rapamycin, or chemically derived analogues, for dimerization of two protein modules.[56] Rapamycin is a macrolide natural product isolated from the bacterium *Streptomyces hygroscopicus* and, with two binding domains, mediates interactions between the mammalian protein FKBP12 and the FKBP-rapamycin-binding domain of mTor (FRB).[57] Rapamycin binds the 12 kDa FKBP with a K_D of 0.2 nM,[58] and this complex in turn binds the 11 kDa FRB fragment with a K_D of 4.2 nM.[59] When these two protein fragments are fused to two proteins of interest, addition of rapamycin leads to dimerization. This approach can be used to recruit a protein to a particular region of the cell, or the technique can be exploited to control protein activity.[60] Second-generation modifications of the CID technology improve spatial control over protein dimerization through caging of rapamycin.[61,62]

Dimerization. The FKBP12-FRB system has been exploited to control the localization and activity of small GTPases, including Rac1, Cdc42, and RhoA, by recruiting them to the cell membrane where they induce lamellipodia, filopodia, or cell contraction,

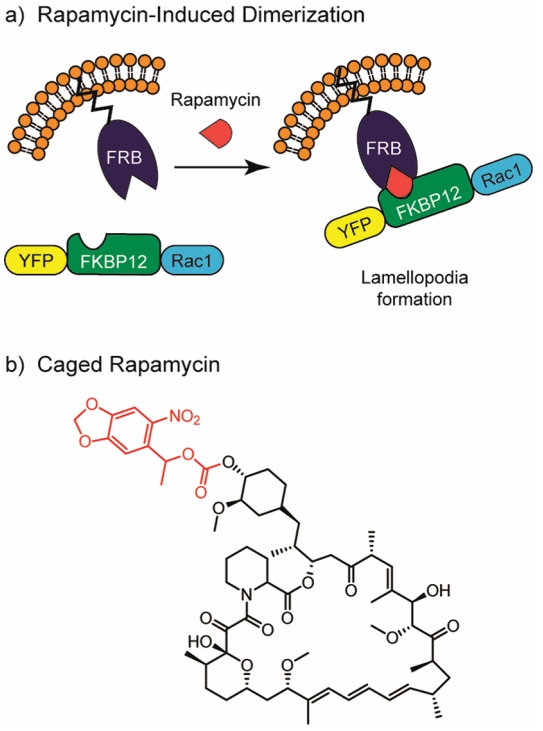

Figure 4. Chemical inducers of dimerization. (a) Rapamycin mediates recruitment of Rac1-FKBP12 to membrane-bound FRB. (b) Introduction of a caging group onto position C-40 of rapamycin enables light-mediated dimerization.

respectively.[63−65] In a study by Inoue, et al., a membrane targeting sequence was fused to the FRB domain, and a constitutively active form of Rac1 was expressed as a *C*-terminal fusion to a YFP-FKBP12 construct (Figure 4a). Addition of a rapamycin analogue, iRap, containing an indole modification, recruited the YFP-FKBP12-Rac1 chimera to the FRB protein at the plasma membrane and induced lamellipodia formation within minutes of iRap addition. In addition to recruitment of the exogenous GTPase to the plasma membrane, activators of the GTPases could be fused to the FKBP construct and translocated to the membrane to stimulate endogenous GTPases. A more recent study expanded the technique to enable protein recruitment to subcellular organelles, including the Golgi, mitochondria, endoplasmic reticulum, and lysosomes through the fusion of FRB or FKBP12 to an organelle-targeted sequence and attachment of the CID binding partner to the protein of interest.[66] This approach was used to recruit GTPase GEFs to the Golgi to examine localized GTPase activation.

Control of Activity. Another application of the FKBP12-FRB system exploits rapamycin to directly initiate kinase activity. Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase localized to focal adhesions, which connect the cell to the extracellular matrix and enable signaling between external factors and cytoplasmic proteins.[67] FAK-mediated signal transduction not only governs migratory processes but also influences cell growth and apoptosis.[68] A genetically encoded, chemically regulated FAK was developed and applied for studies of this kinase in regulating membrane dynamics.[60] A truncated form of the FKBP12 domain, iFKBP, was inserted into the conserved catalytic domain of FAK, and with the increased conformational dynamics of the domain, kinase activity was significantly reduced. Addition of rapamycin induced FRB binding to impart greater structural rigidity and restore activity to near wildtype levels. This allosterically regulated protein was utilized for investigating mechanisms by which FAK promotes increased motility in tumor cells. The general approach was also shown to be suitable for the study of other protein kinases.

While the CID technology has proven useful in studies of FAK and other protein interactions, spatial control over rapamycin release is not possible. In more recent studies, precise temporal and spatial control over the FKBP12-FRB system has been conferred through the development of caged rapamycin analogues.[61,62] In two recent studies, a caging group has been attached at position C-40 of the rapamycin molecule (Figure 4b). The caged molecule does not induce binding of FKBP12 and FRB, but upon irradiation and uncaging, the molecule can trigger dimerization. In the case of rapamycin-induced membrane recruitment, localized irradiation generated isolated membrane protrusions.[62]

A recent chemical genetics technique relies on the ability of cell-permeable small molecules to disrupt the binding between the Bcl-xL and BH3 proteins, which are involved in apoptotic pathways.[69] This property was exploited to control the activity of Intersectin, a GEF for the GTPase Cdc42. The autoinhibitory and localization domains of Intersectin were replaced with the 25-kDa Bcl-xL protein and a short BH3 peptide, and binding between these two small molecule-sensitive domains rendered the catalytic domain of Intersectin inactive. Addition of compounds known to disrupt Bcl-xL/BH3 binding significantly increased GEF activity to generate active GTP-bound Cdc42. This approach is promising for cellular studies and could also be applied to proteins with distinct autoregulatory domains.

With the recent developments of caged rapamycin, the CID technology represents a robust alternative to the light-activated protein systems. While the protein targets of the CID methods extend beyond those of the photoreceptors, further development will be required for the examination of more diverse events in migration. For example, these methodologies are well-suited for the systematic study of signal transduction at focal adhesions because protein recruitment to scaffolds such as paxillin is responsible for initiating signaling cascades. These chemical genetics methods provide a convenient avenue for dictating these protein interactions.

FLUORESCENT SENSORS OF PROTEIN ACTIVITY

In addition to enabling methods to precisely manipulate processes involved in cell migration, chemical biology has also produced technologies that allow researchers to detect the activity of a protein or process in real time through fluorescence outputs. Monitoring the activity of proteins within the cell is particularly important for deciphering localized and transient activity that may not be apparent through traditional biochemical methods, such as Western blotting, which average responses over a population of cells from a single time point.[70] In fact, more subtle aspects of protein signaling often vary between cells and even at the subcellular level.[71] The most common platform for visualizing protein activity within live cells is through the use of genetically encoded fluorescence resonance energy transfer (FRET)-based sensors. However, more recently, environment-sensitive fluorophores have been demonstrated to be particularly valuable in these applications. Like the light-activated proteins, the most common targets of these sensors are the Rho GTPases.

FRET-Based Sensors. Sensors utilizing FRET have been developed for over 30 proteins involved in migration.[70] These sensors are based primarily on two general strategies. Intermolecular, or bimolecular, FRET relies on a protein of interest and an effector domain partner that are each fused to a fluorescent protein. Upon activation of the protein of interest, interactions with the binding partner occur, thereby bringing the two fluorescent proteins closer together and increasing the FRET signal. In contrast, with intramolecular or unimolecular FRET, the protein of interest and an effector binding domain are sandwiched between a pair of fluorescent proteins. Activation of the sensor protein by endogenous signals within the cell promotes binding with the tethered effector and alters the relative proximity of the two fluorescent proteins to modulate the FRET signal. More detailed reviews of this technology appear elsewhere.[70,72−74]

A variation on intramolecular FRET has been applied to create a sensor to monitor the tension across vinculin at focal adhesions.[75] Vinculin is a multidomain protein that interacts with integrins via the talin protein at the head domain and actin filaments through the tail domain. It is recruited to focal adhesions and is subject to varying forces as a focal adhesion forms, matures, and disassembles.[76] The sensor was constructed by introducing a flexible elastic domain between a FRET pair, which was fused between the head and tail domains of the protein. When the protein is under low tension, FRET is high, but as forces across the sensor increase, the elastic domain is pulled apart, and FRET decreases. The sensor was calibrated to quantify cellular forces on the pico-newton scale and was used to map local changes in tension during migration. The forces on vinculin were high during focal adhesion assembly at the leading edge of the cell, but low tension on vinculin correlated with focal adhesion disassembly at the trailing edge.[75]

Environment-Sensitive Fluorophores. Although FRET-based sensors have been successfully applied to probe processes in migration, the use of environment-sensitive fluorophores represents a complementary strategy for monitoring protein−protein interactions. Environment-sensitive fluorophores have emissive properties that are sensitive to the polarity of the solvent environment. In an aqueous-solvated state, fluorescence is low, but in a nonpolar environment, such as that at a protein−protein binding interface, fluorescence intensities can increase significantly. Thus, when appropriately positioned within a protein, these fluorophores can signal a binding interaction or conformational rearrangement through a change in fluorescence.[77]

The solvatochromic fluorophore strategy has been successfully applied to generate a sensor for Cdc42. A merocyanine fluorophore, which undergoes a 12-fold increase in fluorescence in DMF compared to methanol,[78] was attached to the Cdc42/Rac Interactive Binding (CRIB) domain of the Wiskott-Aldrich Syndrome Protein (WASP) effector (Figure 5). The CRIB domain selectively binds the active, GTP-bound form of Cdc42 over the inactive GDP-bound protein. In the presence of activated Cdc42, binding of the sensor positioned the fluorophore in a hydrophobic pocket and increased the emission intensity of the fluorophore. Studies *in vitro* revealed a 3-fold increase in fluorescence in the presence of the activated GTP-bound form of Cdc42 compared to the inactive GDP-bound form. The sensor was then microinjected and used to examine Cdc42 activation during cell adhesion and spreading and at the Golgi.[79]

FRET-based sensors for RhoA[80] and Rac1[81] have been combined with the merocyanine-modified fluorogenic Cdc42 sensor to analyze the coordinated activities of these three GTPases during cell protrusions.[12] These studies revealed the timing and localization of GTPase activation relative to membrane protrusions and showed that RhoA activity was directly correlated with protrusions, whereas Cdc42 and Rac1 were active 40 s later and 2 μm behind the leading edge. This multiplexed approach demonstrates the power of fluorescent biosensors to provide exquisitely detailed information about cellular processes.

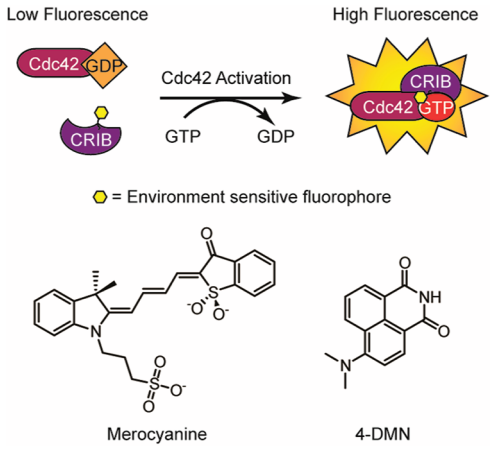

Figure 5. Fluorogenic sensor for Cdc42. A sensor for Cdc42 activation can be constructed by labeling the CRIB binding domain with an environment-sensitive fluorophore. Fluorescence is low when Cdc42 is bound to GDP but increases when Cdc42 is activated. The structures of the merocyanine and 4-DMN fluorophores are shown.

The utility of the fluorogenic Cdc42 sensor was recently expanded through incorporation of the 4-*N*,*N*-dimethylamino-1,8-naphthalimide (4-DMN) fluorophore into the WASP fragment (Figure 5). Compared to commercially available solvatochromic fluorophores, 4-DMN demonstrates much more dramatic increases in fluorescence intensity when positioned in a hydrophobic binding pocket.[82,83] A panel of five Cdc42 sensor variants was generated through cysteine labeling using 4-DMN α-bromoacetamide and maleimide reagents and through protein semisynthesis to incorporate the 4-DMN amino acid.[84] These studies revealed a derivative that exhibits a 32-fold increase in fluorescence in the presence of active GTP-bound Cdc42 compared to incubation with the inactive GDP-bound protein. An advantage of this fluorophore over other dyes is that the sensor effectively acts as a "turn-on" switch for the activated GTPase because background fluorescence is virtually nonexistent. This property is particularly promising for *in vitro* studies of Cdc42 or associated regulatory proteins as well as for high-throughput screens of Cdc42 inhibitors.

While the synthesis and delivery of probes based on solvatochromic dyes are often more challenging than methods for FRET-based sensors, the use of organic fluorophores offers many advantages. FRET-based probes often suffer from the presence of high background fluorescence and only modest signal increases upon a binding event or protein activation. In contrast, sensors based on solvatochromic fluorophores exhibit high sensitivity to the solvent environment and have the potential to yield fluorescence responses with a greater dynamic range. Additionally, whereas the fluorescent proteins that are fused to the FRET-based sensors are large and can disrupt native functions or introduce aberrant interactions, environment-sensitive fluorophores introduce minimal structural perturbations and

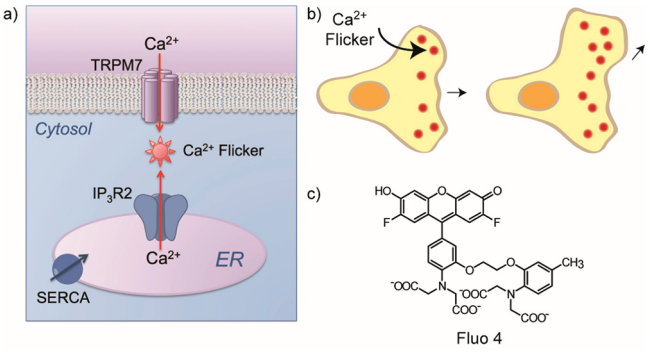

Figure 6. Calcium flickers in cell migration. (a) Calcium flickers are the result of PI(1,4,5)P_3-induced Ca^{2+} release from the endoplasmic reticulum coupled with Ca^{2+} influx from the transient receptor potential ion channel TRPM7. (b) Ca^{2+} flickers are active at the leading edge of the cell and induce cell turning. (c) The structure of the commercially available Fluo 4, a dye with Ca^{2+}-dependent fluorescence properties that was used to study Ca^{2+} flickers, is shown.

require only one chromophore. Thus, further studies must be performed to expand the applications of these solvatochromic dyes to exploit the unique properties they offer.

Sensors for Cellular Calcium. Gradients of ions and metabolites, including calcium and phosphatidylinositol 3,4,5-triphosphate (PI[3,4,5]P_3), play essential roles during cell migration. PI(3,4,5)P_3, which is generated through phosphorylation of phosphatidylinositol by PI 3-kinases, is involved in the establishment and control of cell polarity, in part through the activation of GEFs, which stimulate Rac1 and lamellopodia formation.[8] Calcium also plays a critical role in regulating aspects of migration through involvement in directional sensing,[85] reorganization of the actin network, force generation, and disassembly of rear adhesions.[86] A wide range of fluorescent calcium sensors have been developed to examine the spatial and temporal dynamics of intracellular calcium gradients.[87] One of the first sensors, termed "cameleon," relies on FRET between two fluorescent proteins flanking a fusion of calmodulin (CaM) and the M13 peptide. CaM undergoes a significant conformational change in the presence of Ca^{2+}. The CaM-Ca^{2+} complex in turn tightly binds a number of peptide motifs, including the M13 peptide, and in the sensor, this binding interaction leads to an increase in the FRET signal.[88] More recently, improvements in the sensitivity and fluorescent properties of the sensor have been achieved.[89]

As an alternative to genetically encoded sensors, many sensors have been derived from small molecule dyes that exhibit changes in fluorescence in the presence of Ca^{2+}.[90] A number of these are commercially available and have been utilized to investigate the role of Ca^{2+} in cell migration. In one example, Fluo 4, a dye whose fluorescence emission increases upon Ca^{2+} chelation, was used to study the role of localized high-calcium microdomains, known as calcium flickers, in a migrating cell (Figure 6).[91] These bursts of Ca^{2+} were present in the lamellopodia in the leading edge of the cell and demonstrated polarized distributions during migration.

CONCLUSIONS

The field of chemical biology offers biologists a multitude of technologies for interrogating processes and proteins involved in cell migration. Many of these tools overcome the drawbacks of traditional genetic approaches and the use of small molecule inhibitors by increasing the spatial and temporal resolution with which migration can be observed. Both the light-activated proteins and the systems based on CID enable protein activation to be rapidly and directly initiated, and these methods can also specify the subcellular region of investigation. The fluorescent sensors of protein activation complement light- and chemical-mediated protein regulation by allowing the real-time activity of these proteins to be directly visualized. The properties of the chemical tools make them ideal for the study of cell migration, and the studies described herein demonstrate the value of the approaches for gaining time-resolved information about these dynamic interactions.

Future improvements and investigations will benefit from the application of these tools to examine the existing targets in new systems and extend these technologies to new targets. For instance, application of these tools in three-dimensional matrices, which better mimic the mechanical stimuli of the *in vivo* environment,[92] should provide new understanding of the migration process. Additionally, these tools are poised to be used in combination to examine the interactions among multiple proteins. The fluorescent tools provide a convenient readout of proteins that are activated by light or with small molecules. However, to fully realize the advantages that these tools offer, biologists must be willing to apply these new technologies. Likewise, organic chemists must work to further improve the photophysical properties of caging groups and fluorophores. Together, new insights into cell migration will be revealed, and as a result of the importance of migration in disease states, these discoveries will accelerate the development of novel therapeutics.

AUTHOR INFORMATION

Corresponding Author
*E-mail: imper@mit.edu.

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KEYWORDS

Cell migration: the spatially and temporally controlled process whereby cells move from one part of the body to another. It is involved in normal physiological functions, such as wound repair and the immune response, but it is also responsible for pathological conditions, including metastasis and inflammatory diseases.; Rho GTPases: a family of GTP-binding proteins that function as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state. Rho GTPases, including Rac1, Cdc42, and RhoA, are responsible for many processes involved in cell migration.; Caging group: a photolabile, organic protecting group that can be covalently appended to an essential functionality of a peptide or protein to render the biomolecule inactive within a biological system. Irradiation at an appropriate wavelength releases the caging group and liberates the active peptide or protein.; Semisynthesis: a method to incorporate unnatural amino acid residues into full-length proteins by generating the portion of the protein containing the unnatural element through chemical synthesis (solid-phase peptide synthesis) and

obtaining the remainder of the protein through recombinant approaches. The two peptide fragments are then ligated together to yield the full-length protein.; LOV domain: a protein domain in the light-oxygen-voltage superfamily that noncovalently binds flavin mononucleotide (FMN) in the dark but forms a covalent complex with FMN and undergoes a conformational change upon irradiation at 450 nm.; FKF1/GI: FKF1 contains a LOV domain and upon irradiation at 450 nm binds the protein GI. When these domains are fused to two proteins of interest, irradiation leads to dimerization.; PhyB/PIF: proteins from the phytochrome family that undergo reversible binding upon exposure to 650 nm light. Irradiation at 750 nm reverses binding.; Chemical inducer of dimerization: a small molecule, such as rapamycin, that binds two different protein domains, such as FKBP12 and FRB. When these domains are each fused to two proteins of interest, addition of the small molecule leads to dimerization of the two proteins.; Environment-sensitive fluorophore: a small molecule-based fluorophore whose emissive properties, such as fluorescence intensity, are dependent upon solvent polarity. They are useful as sensors because a change in protein conformation or protein binding interactions, which involve the transfer of the fluorophore from a solvent-exposed state to a hydrophobic binding pocket or vice versa, is indicated through a change in fluorescence intensity.

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