Published in final edited form as:

Curr Opin Chem Biol. 2016 October ; 34: 135–142. doi:10.1016/j.cbpa.2016.08.012.

Molecular Tools for Acute Spatiotemporal Manipulation of Signal Transduction

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Abstract

The biochemical activities involved in signal transduction in cells are under tight spatiotemporal regulation. To study the effects of the spatial patterning and temporal dynamics of biochemical activities on downstream signaling, researchers require methods to manipulate signaling pathways acutely and rapidly. In this review, we summarize recent developments in the design of three broad classes of molecular tools for perturbing signal transduction, classified by their type of input signal: chemically induced, optically induced, and magnetically induced.

Introduction

To adapt to constantly changing environmental conditions, a cell must sense signals from its environment and elicit appropriate functional responses. The information it collects from its environment is transmitted and processed via a complex, interconnected, and highly coordinated network of signaling cascades. The biochemical activities involved in these pathways, including enzymatic activities, recruitment of second messengers, and protein-protein interactions, are under tight regulation both spatially and temporally within the cell. To study this regulation, researchers have developed a wide array of genetically targetable tools to activate or inhibit a particular biochemical activity at specific subcellular locations rapidly and at will. These tools connect an input signal (i.e. inducer) controllable by the researcher such as the addition of a drug, exposure to light, or application of a magnetic field, with the modulation of a particular biochemical activity. The researcher can then use these tools to perturb the temporal dynamics and spatial patterning of particular signaling activities within a pathway and study downstream effects. Temporal control is achieved by manipulating the level of the input signal in time. Spatial control can be achieved by

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targeting these genetically targetable tools to specific subcellular locations or by applying the input signal only to certain regions of the cell.

The development of these tools is guided by certain design considerations, including speed, specificity, sensitivity, tunability, spatial control, reversibility, and modularity, whose relative importance depends on the particular experiment being performed. The perturbation should be induced rapidly upon addition of the inducer and should remain inactive in the absence of that signal. Likewise, the tool should have minimal off-target downstream effects. The tools should be sensitive enough so that there is no need for use of an excessive amount of inducer. In some experimental designs, the amount of modulation of the biochemical activity should scale with the level of input signal, while in other applications, an all-or-nothing response may be more appropriate. Moreover, for studying complex signaling dynamics, it is useful that the tool be reversible—that the associated biochemical activity turns off when the input signal is removed. Lastly, the design of these tools should be modular so that the same approach can be generalized for a wide range of biochemical activities.

Chemically Inducible Tools

Small molecule drugs, such as receptor agonists and pharmacological enzyme inhibitors, have been used extensively to manipulate signal transduction. However, specific activators and inhibitors of certain activities are unavailable, and spatial control of pathway activation is difficult with this approach. On the other hand, genetically targetable molecular components that bind synthetic ligands can be used to design modular tools for manipulating cell signaling in ways impossible with standard pharmacological agents.

Chemically inducible dimerization (CID) systems are a widely used class of chemically induced, genetically targetable tools for acutely perturbing signal transduction in live cells. CID systems are composed of two protein components that only bind in the presence of an exogenous chemical agent. The most widely used CID systems are based on FK506 Binding Protein (FKBP), which binds tightly to the immunosuppressant drugs FK506 and rapamycin. The first CID system was a homodimerization system developed in 1993 by creating a synthetic derivative of FK506 with two FKBP-binding moieties, called FK1012 [1], which could be used to induce the homodimerization of FKBP-tagged proteins. A heterodimerization system was developed a few years later by exploiting the ligand-mediated interaction between FKBP and mTOR [2]. An 89-amino acid fragment from mTOR called FRB was found to be sufficient for binding the FKBP-rapamycin complex but does not bind FKBP in the absence of rapamycin. Rapamycin addition induces the heterodimerization of FRB- and FKBP-fused proteins rapidly, on the order of seconds, yet irreversibly due to the high affinity of the FKBP-FRB interaction. Being able to interact with endogenous mTOR, moreover, the rapamycin input is not truly orthogonal to the cell's native signaling pathways. To address this concern, several rapamycin analogues such as iRap, AP21967, and AP23102 [3,4] have been developed to bind only to engineered FRB but not to endogenous mTOR. Furthermore, the FKBP-FRB design is highly modular, as it can be adapted to control a wide array of signaling activities.

These systems can be used to perturb cell signaling by bringing two proteins into proximity (Figure 1a), recruiting a protein to a particular subcellular location (Figure 1b), or mediating allosteric regulation of an enzyme (Figure 1c). Modulating molecular proximity using CID can be used to mediate the interaction between the two proteins or protein fragments, which can promote downstream signaling (Figure 1a), for example by facilitating the dimerization of receptor tyrosine kinases [5]. It can also be used to mediate the complementation and reconstitution of split protein fragments, as was demonstrated by fusing FKBP and FRB to fragments of the split tobacco etch virus (TEV) protease to modulate caspase activation [6]. In addition to mediating protein interactions, CID can control signal transduction by controlling the translocation of a protein of interest toward or away from its site of action (Figure 1b). One CID component is targeted to a particular subcellular location, while the other is fused to a protein of interest and is diffusible in the cytosol or is hidden at a relatively inert location [7]. Upon addition of the dimerizer, the diffusible component is rapidly recruited by the anchored component to the target site [8]. This approach has been extensively used to control the activity of enzymes involved in phosphoinositide metabolism [9,10] as well as small GTPases [3,11,12]. Another approach, called RapR, is to use the FKBP-FRB heterodimerization system to create allosterically regulated variants of kinases (Figure 1c) [13]. A modified FKBP, called iFKBP, is inserted into the kinase to make it catalytically inactive. When FRB heterodimerizes with the iFKBP domain upon the addition of rapamycin, a change in the conformational rigidity of iFKBP restores the catalytic activity of the kinase. Because the iFKBP is inserted in a highly conserved region of the kinase, the design is highly modular and can be applied to a wide array of kinases. A unimolecular version of RapR, known as uniRapR, was also developed by fusing elements from FRB directly to a modified version of iFKBP and inserting the resulting fusion into the kinase (Figure 1c) [14].

To control multiple activities simultaneously in the same cell using CID, our toolbox has been expanded to include several orthogonal CID systems. Two genetically targetable CID systems using plant hormones as the dimerizer have been developed. The first uses abscisic acid (ABA) to induce the dimerization of the proteins PLY1 and ABI1 [15], while the other uses the membrane-permeable gibberellic acid (GA_3) to rapidly induce the dimerization of the proteins GAI and GID1 [16]. Occurring on the timescale of minutes, ABA-mediated dimerization is slower than FKBP-FRB system; however, unlike the FKBP-FRB system, it can be reversed within 30 minutes by washing out the ABA. The GAI-GID1 system, like FKBP-FRB, functions on the order of seconds but does not reverse upon washout. Combining the FKBP-FRB and the GAI-GID1 systems in different ways allows for Boolean-gated recruitment of signaling activity, including AND, OR, NOR, and NAND logic gates [16, 17]. Furthermore, *Lin et al.* demonstrated that two orthogonal CID systems can be used in conjunction to allow for reversible control of signal transduction. In this approach, downstream signaling is activated by rapamycin-induced translocation of Rac GTPase to the plasma membrane and then inactivated by GA_3 -mediated recruitment to the mitochondrial membrane [18]. In addition to dimerization systems based on plant hormones, other synthetic heterodimerizers have been created by covalently linking two orthogonal ligands, enzyme substrates, or protein-targeting tags [19]. For example, Erhart *et al.* developed a dimerizer of HaloTag and SNAP tag by integrating HaloTag- and SNAP-tag-

binding moieties into a single molecule called HaXS [20]. HaXS-mediated dimerization occurs on the order of 10s of minutes and, unlike the other systems described, results in the two proteins being covalently bound.

Another chemically inducible means of acutely and orthogonally activating a particular signaling pathway is the creation of G-protein-coupled receptors (GPCRs) activated solely by synthetic ligands (RASSLs) [21,22]. These receptors are designed to be insensitive to endogenous ligands but responsive to exogenously added synthetic ligands. First-generation RASSLs have been used to acutely activate GPCR signaling in cardiac tissue *in vivo*. However, the synthetic ligands used in these first-generation RASSLs also had high affinity for endogenous GPCRs. A second-generation RASSL technology, known as Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), was therefore developed. In this approach, GPCRs are activated by clozapine-N-oxide, a pharmacologically inert yet bioavailable synthetic ligand [23]. The DREADD methodology has been applied to a wide array of G-protein signaling pathways, including Gq, Gi, Gs, and β -arrestin signaling [24].

Optically Inducible Tools

Compared to chemically inducible approaches for perturbing signaling pathways, optogenetic approaches offer more precise spatiotemporal control of activation and inactivation. Light, in addition to being non-invasive, can be focused on well-defined subregions of the cell with high spatial resolution. Furthermore, light can be toggled on and off rapidly with high temporal resolution, allowing researchers to achieve more complex and well-tuned dynamics of the desired signaling activities.

Early approaches for optically manipulating signal transduction focused on the development of small molecules that are either photocaged, in which a chemical moiety inactivates the molecule until it is photocleaved, or photochromic, in which the molecule is activated and inactivated through photoisomerization. These tools include a wide range of photoactivatable metal ions, amino acids, second messengers, ligands and other pharmacological agents. Furthermore, photocaged versions of chemical dimerizers, such as photocaged analogues of rapamycin [25], ABA [26], GA₃ [27], and HaXS [28], allow researchers to combine the aforementioned CID systems with light activation (Figure 2a). Likewise, creating photocleavable dimerizers provides a method to engineer rapid reversibility into otherwise irreversible CID systems [29].

Developments in unnatural amino acid incorporation and protein labeling have allowed chemical photocaging approaches to be used to control protein activity in genetically targetable ways. For example, Lemke *et al.* demonstrated the incorporation of a photocaged serine to optically control the phosphorylation of a transcription factor in yeast [30], while Arbely *et al.* developed a photoswitchable mammalian receptor tyrosine kinase through site-specific genetic encoding of photocaged tyrosine [31]. A photogated cysteine was likewise incorporated into the Kir2.1 potassium channel to create a photoinducible inwardly rectifying potassium (PIRK) channel, which can be used to optically suppress neuronal firing [32]. Moreover, photoswitchable click amino acids (PSCaas), which contain an azobenzene photo switch and a click functional group, can also be used to create a covalent

protein bridge between the site of incorporation and a nearby cysteine residue *in situ* [33, 34]. Because the azobenzene moiety undergoes *cis-trans* photoisomerization, a PSCaa-mediated protein bridge can be used to optically control protein conformation.

Azobenzene moieties are also used to create photoswitchable ligands, whose conformation can be modulated using two different wavelengths of light. In the photoswitchable tethered ligand (PTL) approach, these azobenzene-based photochromic ligands are attached to its receptor via maleimide conjugation to an engineered cysteine residue, resulting in a photoactivatable receptor. Broichhagen *et al.* built upon this approach by fusing a GPCR with a SNAP tag coordinated with a photochromic ligand attached to a long flexible linker (Figure 2b) [35]. This method, called photoswitchable orthogonal remotely tethered ligand (PORTL), overcomes the limitations of maleimide-cysteine chemistry, such as nonspecific labeling as well as incompatibility with the intracellular environment. The PORTL approach could also be used in conjunction with PTLs to allow for orthogonal control of two different GPCRs within the same cell.

Photocaging can be carried out not only by small molecule photoswitches, but also by photosensitive protein domains in a completely genetically encodable manner (Figure 2c). Two key strategies have emerged, one using the light-oxygen-voltage-sensing (LOV) domain and the other using the photochromic fluorescent protein Dronpa. LOV domains bind flavin molecules as their chromophore and undergo a conformational change when exposed to blue light (440–473 nm), leading to allosteric control of effector domains. The rigid J α helix in the LOV domain forms an inhibitory surface for its effector and unfolds upon exposure to blue light, allowing for activation of the effector (Figure 2c) [36]. Meanwhile, the fluorescent protein Dronpa, which rapidly converts between a dark state and a bright state upon illumination with 490nm and 400nm light respectively, can also be used to engineer optogenetic control. Dronpa mutants that either dimerize or tetramerize in the bright state but remain monomeric in the dark state are fused to both the N- and C-terminus of a protein of interest, such as a guanine nucleotide exchange factor (GEF) or protease [37]. When in the bright state, the two Dronpa domains form an interface that blocks the protein's activity. However, when exposed to 400 nm light, the interface breaks, allowing for activation of signaling. Both the LOV domain and Dronpa allow for rapid and reversible manipulation of signaling, allowing for multiple cycles of activation and inactivation. However, the modularity of these approaches is limited—proper caging and release of signaling activity may need to be optimized individually for each protein of interest.

In addition to this photocaging approach to regulating signaling activities, there are also fully genetically encodable methods for light-induced dimerization (Figure 2d). Like CID systems, optically inducible dimerization can be used to spatiotemporally activate signal transduction either by inducing protein-protein interactions or by controlling the recruitment of a signaling molecule to its site of action. Moreover, these optical tools are fully reversible and can be repeated for many cycles. An early approach was developed using the plant phytochrome PhyB and the phytochrome interacting factor PIF [38]. PhyB, which binds a phycocyanobilin chromophore, interacts with PIF in the presence of red light and dissociates in far-red light. The requirement for the addition of an exogenous chromophore, however, motivated the development of other light-inducible dimerization systems in which all

components are genetically encoded. Kennedy *et al.* created a completely genetically encodable light-induced dimerization system based on the cryptochrome Cry2 from *Arabidopsis thaliana* and its interacting partner CIB1 [39]. The Cry2-CIB1 interaction is induced rapidly on the subsecond time scale upon stimulation with blue light, and reversible on the minutes time scale when the blue light is removed. In addition to the Cry2-CIB1 system, the LOV domain can also be used for light-inducible dimerization. The LOV domain mediates light-dependent interactions with itself or with a number of naturally occurring or engineered binding partners. Strickland *et al.* engineered an interacting partner for the LOV-domain called PDZ, whose equilibrium binding and kinetic parameters can be tuned by mutation [40]. The LOV-PDZ system, known as tunable light-inducible dimerization tags, or TULIPs, allows researchers to choose tags whose binding affinity and photocycle kinetics are most appropriate for their particular experiment. Recently, both light-induced dimerization using Cry2-CIB1 and the LOV-PDZ has been used to optically control organelle transport by modulating the recruitment of cytoskeletal motor proteins to their cargo [41,42].

Lastly, the optogenetic toolbox also includes naturally occurring as well as nature-inspired light-activated signaling molecules. Microbial rhodopsins, for example, can be exogenously expressed in electrically excitable cells to regulate membrane depolarization (Figure 2e). For example, the blue-absorbing cation channel channelrhodopsin2 (ChR2), as well as the yellow anion channel halorhodopsin, are widely used to activate and suppress membrane depolarization, respectively, both in cultured cells and *in vivo* [43]. Light-induced activation of GPCR signaling pathways, furthermore, is achieved through exogenous expression of naturally light-sensitive GPCRs, such as animal opsins, as well as chimeric GPCRs that combine the light sensitivity of an opsin to activate native G-proteins [44]. Lastly, exogenous overexpression of photoactivated adenylyl cyclases (PACs) [36] as well as guanylyl cyclases [45] allows for acute stimulation of cAMP and cGMP production, respectively, using light.

Magnetically Inducible Tools

Magnetogenetics is an emerging technology to rapidly manipulate cell signaling pathways. Functionalized magnetic nanoparticles offer spatiotemporal control over processes that are less accessible using chemical addition or light, including the ability to apply forces, cluster membrane proteins, and accumulate intracellular proteins without the need for targeting sequences [46].

Magnetic nanoparticles can be used to activate cell signaling by manipulating the mechanics, spatial patterning, and temperature of receptors. Hughes *et al.* demonstrated the possibility of using the forces generated by applying a magnetic field on the nanoparticle to manipulate mechanotransduction in living cells [47]. They added a poly-His sequence to the mechanically sensitive channel TREK1, which was labeled using magnetic nanoparticles functionalized with anti-His antibodies. They then used an external magnetic field to apply forces to the TREK1 channel, resulting in channel activation (Figure 3a). Furthermore, ligand-conjugated magnetic nanoparticles have been used to activate receptors, such as FCεRI and EGFR, by mediating their clustering in the membrane [48,49]. Temperature-sensitive channels, moreover, can be activated using magnetic nanoparticle-mediated thermal

activation. Radiofrequency magnetic stimulation results in heat dissipation from the nanoparticle, resulting in elevated temperatures around the nanoparticles. This localized change in temperature is able to activate temperature-sensitive TRPV1 channels [50].

In addition to membrane receptors, magnetic approaches can be used to manipulate intracellular signaling. Researchers can accumulate magnetic nanoparticles conjugated with a signaling molecule of interest using a magnetic tip, creating highly localized hot spots of activity (Figure 3b). This approach has been demonstrated using nanoparticles conjugated to GEFs in order to locally stimulate GTPase signaling, leading to highly localized cytoskeletal reorganization around the nanoparticle [51,52]. Thus, complex patterns of signaling, such as spatial gradients, can be generated with high spatial and temporal precision [53,54].

The successes in modulating signal transduction using magnetic nanoparticles motivates the development of magnetogenetic tools that are completely genetically encodable. Recent work has demonstrated that overexpressing the protein ferritin allows for the intracellular synthesis of magnetic nanoparticles; when ferritin is fused to a TRPV channel, the resulting particles allow for radio and magnetic activation of that channel [55,56]. In addition, the recent discovery of a new candidate magnetoreceptor MagR, an iron-sulfur cluster-binding protein that creates a rodlike complex with the cryptochrome Cry, motivates the possibility of a new family of fully genetically-encoded magnetogenetic tools [57, 58].

Conclusion

The last decade has seen a dramatic increase in the size of our toolkit of genetically encodable or targetable systems for inducing acute, specific perturbations in signal transduction pathways. These tools allow researchers to activate and inactivate specific biochemical activities with increasingly finer spatiotemporal control in live-cell imaging studies. Future expansion of the toolkit will not only include the development of novel systems and the application of current systems to more and more signaling molecules, but also the fine tuning of key parameters in each system, including kinetics, binding affinities, and sensitivities to input levels. The goal would be to develop a toolkit in which researchers can choose tools with parameters optimized for their particular experiments. Wide application of these tools in live animals will require protein components of minimal size as well as optogenetic tools that offer red-shifted excitation spectra and compatibility with two-photon imaging. Furthermore, the manipulation of many signaling activities in the same cell requires the continued development of systems that are orthogonal to existing tools. Current genetically encodable tools also rely on the overexpression of their protein components, making it difficult to control of the activity of signaling molecules at endogenous levels of expression. Recent advancements in protein labeling through genomic editing with TALEN and CRISPR [59], however, open up the possibility of using these tools to perturb signaling molecules expressed at their endogenous levels. This expanded toolkit will allow researchers to further uncover the mechanisms by which a small number of signaling molecules regulate diverse cellular behaviors.

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Highlights

- Molecular tools to rapidly manipulate signaling cascades enable the study of the spatiotemporal regulation of signal transduction.
- Our toolkit of chemically-induced, light-induced, and magnetically-induced systems molecular tools is growing
- These tools have been applied for a wide range of signaling perturbations, from modulating enzymatic activity, protein-protein interactions, and receptor activation.

Figure 1. Strategies for manipulating signal transduction using chemically inducible dimerization (CID) systems. (a) CID can be used to activate signaling by mediating the interaction between two proteins of interest, for example by inducing the dimerization of receptor tyrosine kinases. (b) CID can also modulate signal transduction by inducing the translocation of a protein of interest to a particular subcellular location. One dimerization domain is fused to a protein of interest, while the other is fused to targeting sequence that anchors it to the site of action of the protein of interest. Addition of the dimerizer results in recruitment of the protein of interest to its site of action, resulting in activation of downstream signal transduction. (c). Chemically inducible kinases, such as in the UniRapR design [14], can be created by using CID to allosterically regulate their catalytic activity. DD: dimerization domain, POI: protein of interest

Figure 2.

Optogenetic approaches for perturbation of signaling pathways. (a) Photocaged dimerizer molecules allow for CID to be combined with light-activation. (b) Light-inducible G-protein-coupled receptors (GPCRs) can be engineered by covalently linking the receptor to photoswitchable tethered ligand (PTL). Shown here is the photoswitchable orthogonal remotely tethered ligand (PORTL) design, in which a photochromic ligand is targeted to the GPCR using a SNAP-tag [31]. The ligand is conjugated to an azobenzene moiety, a flexible polyethylene glycol linker, and benzylguanine for conjugation to the SNAP-tag. Optical control of ligand binding is modulated by photoisomerization of the azobenzene moiety, which affects the ability of the ligand to reach the ligand binding pocket. (c) Optogenetic regulation of enzymatic activity can be accomplished using light-sensitive domains that mediate an intramolecular conformational change in the presence of light. (d) Genetically encodable light-sensitive protein dimerization domains allow for reversible and repetitive induction of protein-protein interactions. (e) Exogenous expression of naturally occurring or

naturally inspired photosensitive proteins, such as microbial opsins, allow for modulation of signal transduction. DD: dimerization domain, POI: protein of interest, $h\nu$: light

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Figure 3. Manipulating signal transduction using magnetic nanoparticles. (a) Functionalized nanoparticles allow for magnetic induction of mechanically sensitive ion channels. (b) Magnetic nanoparticles conjugated to a signaling protein of interest via a targetable protein tag can be accumulated at a particular subcellular location using a magnetic tip, creating local hotspots of activity.