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Using light to control signaling cascades in live neurons

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Abstract

Understanding the complexity of neuronal biology requires the manipulation of cellular processes with high specificity and spatio-temporal precision. The recent development of synthetic photoactivatable proteins designed using the light-oxygen-voltage and phytochrome domains provide a new set of tools for genetically targeted optical control of cell signaling. Their modular design, functional diversity, precisely controlled activity and *in-vivo* applicability offer many advantages for investigating neuronal function. Although designing these proteins is still a considerable challenge, future advances in rational protein design and a deeper understanding of their photoactivation mechanisms will allow the development of the next generation of optogenetic techniques.

Introduction

Investigating neuronal function is a significant challenge that requires tools that allow investigators to manipulate signaling pathways with high temporal and spatial resolution. While this is important in many systems, it is crucial in the nervous system where the timing of signals and the spatial control of signaling cascades are essential for function. Conventional approaches for manipulating signaling pathways such as knockouts, conditional mutants and RNAi lack both temporal and spatial resolution and therefore limit the kinds of questions that can be asked and answered. A number of recent techniques developed around the common theme of photoregulation have shown the potential to achieve high temporal and spatial resolution in live cells.

Photoregulatory techniques rely on artificially introducing a photoactivatable agent into (or around) cells and using illumination to control its activity. Light as a controlling signal offers several advantages: high spatio-temporal control, non-invasiveness, and high specificity due to the scarcity of photo-reactive molecules in mammalian cells. The earliest photoactivatable systems used were photolabile caged molecules, most notably caged neurotransmitters and ions [1,2]. Caged glutamate [3] and caged calcium [4] have been used for more than twenty years to study fundamental questions in cellular and systems neurobiology. More recently, azobenzene containing molecules that undergo reversible light-dependent switching [5] and bind to specific receptors have been used as an alternative

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to caged compounds, as well as for reversible caging of proteins [6,7]. Caging systems, while providing rapid temporal control of activation and inactivation, are limited in the extent of spatial control by the high diffusivity of small molecules. In addition, off-target effects limit their specificity and the difficulty of introducing these molecules into cells and animals poses a technical challenge. Hence, in-vivo applications of these techniques can be cumbersome and limited.

Photosensitive ion-transporting membrane proteins, such as *Chlamydomonas reinhardtii* channelrhodopsin-2 (ChR2, an Na⁺ channel activated at 470 nm) or *Natromonas pharaonis* halorhodopsin (NpHR, a Cl⁻ pump activated at 580 nm) represent the second class of photoregulated systems. These proteins show increased conductance upon illumination, and can therefore be used for light-dependent depolarization or hyperpolarization of neurons [8,9, also see review by Ernst Bamberg in this issue]. Genetic targeting allows cell-type specific regulation, fast gating-kinetics provide millisecond scale temporal resolution, and spatial resolution of up to a single cell level can be obtained. While these opsins have been enormously useful in studying neural circuits in live animals [10,11] they are limited to the control of membrane potential and are poorly suited for controlling signaling pathways in mammalian cells.

Artificial Photoreceptor Proteins

Artificial photoreceptor proteins (APPs) are the latest addition to the photoregulatory toolbox. A typical APP consists of a light-sensing domain (LSD) from a natural photoreceptor and an effector domain (ED) from a target protein of interest. The LSD undergoes reversible conformational changes upon illumination that modulate the activity of the ED through steric occlusion or allosteric changes. APPs provide all the advantages of caging and opsin systems along with high specificity and micrometer-scale spatial precision as low diffusivity and fast deactivation limit their activation to a small zone around the illuminating beam. Most importantly, they are highly versatile and can be used to engineer a variety of light regulated proteins with a diverse set of cellular and molecular functions [12].

Light-Sensing Domains

A number of photoreceptor proteins have been discovered in plants, bacteria as well as fungi [13]. As proteins generally cannot absorb visible light, they depend on cofactors, called chromophores, for light detection. Out of the six major photoreceptor classes [14], LSDs from the phytochromes and light-oxygen-voltage (LOV) domain proteins have been used for designing APPs.

LOV Domain

The LOV domain, a member of the Per-ARNT-Sim (PAS) domain superfamily that contains sensor and interaction domains involved in diverse signaling processes [15], has been found linked to various effector domains [16]. The domain has a typical PAS core structure – a five-stranded antiparallel β -sheet with two α -helices arranged on either side. Conserved residues in the cleft formed by the central β -strand and two α -helices bind the flavin mononucleotide (FMN) chromophore. Absorption of a blue light photon leads to the formation of a thioether bond between a conserved cysteine and the C(4a) atom of the flavin ring to produce the activated state [17,18]. The bond formation occurs in microseconds, while the spontaneous reversion is slower with time constants ranging from 10 seconds to twelve hours depending on the exact sequence of the LOV domain and on the attached ED [18,19].

Formation of the thioether bond in the active state causes small conformational changes in the FMN ring as well as its coordinating residues that lead to the unfolding of a C-terminal helix called J α and the N-terminus helical region of the domain [20]. Notably, a recent FTIR study indicates that the changes in J α may differ in different LOV domains [21]. J α is generally thought to be involved in the regulation of the ED. However, some recent studies dispute this belief [22].

Phytochromes

Phytochromes are red/far-red sensing receptors that use a linear-tetrapyrrole chromophore called bilin. Their photosensory core module (PCM) consists of a PAS, GAF (a family of small-molecule binding domains distantly related to the PAS superfamily) and a phytochrome specific domain (PHY). The three domains share a common core structure, composed of a five-stranded, antiparallel, β -sheet [23,24]. Bilin photoisomerization leads to the interconversion of two major phytochrome states – the red absorbing Pr and far-red absorbing Pfr [25], either of which can be the dark state. A long shared helix between the PHY domain and the ED may play a role in transmitting this structural change to the latter.

Design of Artificial Photoreceptor Proteins

The modular nature of the LOV and PHY domains, the diversity of natural effector domains for both, and the widespread occurrence of their respective chromophores make the design of APPs conceptually simple. Results from some recent studies using APPs based on the LOV and phytochrome domains are described below as examples of the various design strategies that are summarized in figure 1.

An early application of engineered photoreceptors was the development of a light-switchable promoter using the light-dependent interaction of plant phytochromes PhyA and PhyB with phytochrome interaction factor 3 (PIF3) [26,27]. By fusing the phytochrome to a DNA-binding domain, and PIF3 to a transcriptional activation domain, the recruitment of PIF3 by PhyA/B was used for targeted expression of genes in yeast. Interconversion of Pr and Pfr forms by red and far-red light was used to switch expression on and off. Along similar lines, the PhyB-PIF3 interaction was used to create a light-activated protein splicing system by bringing together an artificially split intein [28,29]. Interestingly, weakening the PIF3-PhyB interaction improved the system by reducing background activation. The PIF3-PhyB interaction has also been used to reversibly recruit GDP-locked Cdc42 to its effector Wiskott-Aldrich syndrome protein and initiate actin nucleation by the Arp2/3 complex [30,31].

Levskeya et al [32] used the PCM from a cyanobacterial phytochrome histidine-kinase and the effector domain from a bacterial histidine-kinase EnvZ to create a light-activated histidine-kinase in *E.coli*. In a subsequent study by the same group [32,33], the PIF-PhyB interaction was used to induce translocation of PIF6-fused proteins to the membrane. The recruitment as well as release of PIF6 occurred within seconds. PIF6 fusions to the GEFs (GTP exchange factors) for Cdc42, Rac, and Rho GTPases were used to demonstrate precise control of cell motility using this technique. The study also demonstrated the importance of the tandem PAS repeats in plant phytochromes – the PIF6-PCM interaction was irreversible in the absence of these domains. In all phytochrome-based systems, the bilin chromophore has to be supplied through the media for animal or yeast cells, or through bilin production genes for bacteria [34].

Lee et al [35] used a different approach to design a photoactivatable *E.coli* dihydrofolate reductase (DHFR). Insertion of LOV2-J α into sites statistically identified to be allosterically important was used to achieve a roughly two-fold reversible increase in DHFR activity upon

illumination. Strickland et al [36] designed an allosterically controlled photoactivatable transcription system by joining the LOV2 domain to the bacterial trp repressor (TrpR) through a fusion of the J α helix with the N-terminal helix of TrpR. Due to steric occlusion between the domains, the shared rigid helix could interact with only one of them without bending, and any change (such as LOV photoexcitation) that altered the interaction of the helix with one domain, would also alter the interaction of the helix with the second domain. The fusion protein showed robust light-dependent DNA binding.

In addition to activation of transcription, LOV based proteins have also been used to control the small GTPase Rac1. A photoactivatable Rac [37] was developed by fusing the LOV domain of the plant protein Phot1 to Rac1. Inhibition of the Rac was achieved by steric occlusion of its effector-binding site by the LOV domain in the dark-state. In the lit-state, as J α helix unfolds, the motility of LOV domain increases and the effector site becomes accessible. An interesting aspect of the study was the optimization of the construct using structural modeling. The optimized LOV-Rac1 was used to precisely control the direction and duration of cellular motility and cytoskeletal dynamics, and to investigate the roles of various downstream and upstream factors. In a later study, the LOV-Rac1 construct was used to produce guided movements of epithelial cells in *Drosophila* ovary [38].

A second light regulated Rac1 protein was designed by taking advantage of a general purpose light regulated protein-protein dimerization system [39]. The light-dependent interaction between the LOV-containing protein flavin-binding, kelch-repeat, F-box 1 (FKF1) and its partner GIGANTEA (GI) [40] from *A. thaliana* was used to recruit FKF1-Rac1 to the plasma membrane and achieve directed lamellipodia production. The same interaction was also used to create a light-dependent transcription factor by fusing a nuclear GI to the DNA-binding domain from Gal4 and FKF1 to the transactivator VP16.

The above examples show that both the LOV and the phytochrome domains can be successfully used for designing APPs. Although similar in nature and mode of action, the two domains have some relative advantages and disadvantages. Chief among these is the chromophore requirement – the phytochrome domain requires a bilin chromophore that is not naturally synthesized by mammalian cells. This makes it necessary to externally supply purified bilin, which limits the applications of this system to cells in culture. The FMN chromophore used by the LOV domain is produced by all mammalian cells and is present in sufficient quantities to make external addition unnecessary. Another consideration is the size of the domains - it may generally be easier to engineer a 140 residue LOV domain than a ~650 amino acid phytochrome PCM. However, the robust and reversible PIF3-Phy interaction provides an easy strategy for using the PCM. The set of effector domains that occur naturally with a domain may hint to its suitability for a given class of EDs. Finally, the wavelength of light and its compatibility with the fluorophores being used for imaging the system may also need to be considered, although this is perhaps a minor consideration with the range of fluorophores now available.

Potential Uses of APPs in the Nervous System

Though promising, the APP systems mentioned above have all been used for proof of principle experiments and have not been applied to answer questions in challenging systems like neurons. Many cell biological and systems questions in neurobiology could benefit significantly from the use of existing APPs. For instance Rac proteins play a key role in the development of dendritic arbors, spines and excitatory synapses. The light regulated Rac proteins based on the LOV domains could be used both in neurons in culture and in neurons *in vivo* to control the development of connections between neurons. Similarly, activity-dependent transcription factors are essential for many aspects of development and plasticity

and the LOV based transcriptional activation systems could be used to activate these factors in specific cells in live animals. The LOV systems are well suited for these applications because the FMN chromophore does not need to be introduced into the animal, thus eliminating a major experimental limitation.

The same strategies can be applied to design novel APPs for use in neurons. Photoregulated scaffolding proteins would be useful for studying localized signaling domains such as the postsynaptic density or dendritic spines. Light-activated recruitment of signaling proteins to specific cellular locations, using the FKF1-GI interaction system for example, could be used to investigate the importance of sub-cellular localization in the control and specificity of signaling. Light-dependent translation factors could be used to reveal the function and regulation of local translation. Light-mediated temporal and spatial control of calcium transients at synapses could help understand functionally important phenomena such as synaptic plasticity. The use of APPs to control the temporal and spatial activation of biochemical pathways in neurons both *in vivo* and *in vitro* is thus likely to answer many important questions in neurobiology.

Challenges in Designing and Using APPs

The development of APPs is still in its infancy and many challenges to the development and use of these proteins remain. One of the greatest challenges is the lack of a broadly applicable design strategy for new APPs to control new types of proteins. The development of useful LOV and PCM-based proteins is still a matter of trial and error and is strongly affected by variables such as the linker lengths, exact positions of fusion etc. This makes the search for a light regulated protein uncertain and time consuming. Further, in some cases it is necessary to remove the regulatory domains of EDs from signaling proteins which may limit our ability to design APPs that use effector domains with more complex natural regulatory mechanisms. Finally, even with a well-designed APP, it remains to be shown whether these systems can be activated using fiber-optics and two photon microscopes, technologies that will be required to control these *proteins in vivo*. The design of an illumination system that does not interfere with imaging or cause phototoxic effects, and still provides an acceptable signal-to-noise ratio may not always be straightforward. However, the potential benefits of a well-chosen APP system more than justify the efforts required to overcome these difficulties.

Conclusion and Perspectives

Photoregulation provides a non-invasive way to specifically perturb pathways with high spatio-temporal precision. Artificial photoreceptor proteins have the potential to realize the full power of photoregulation and offer many advantages over traditional methods such as caged molecules. More adventurous selection of target effector domains, reliable rational-design strategies, and a better understanding of LSD function would allow us to move forward from the present studies and create APPs that can dissect neuronal signaling pathways. The next significant milestone would be the development of *in-vivo* optogenetic applications using APPs.

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Figure 1.

Possible strategies to achieve photoregulation of a hypothetical effector domain from an enzyme (E) using a light-sensing domain (L) to alter interactions with substrate (red circles). A) Steric occlusion of the active-site in the dark state, abolished upon illumination due to conformational changes in L and the E-L linker. B) Allosteric modulation of the E active site by light-dependent conformational changes in L. C) L-induced light-dependent dimerization/activator recruitment. D) L-induced light-dependent translocation of E to increase substrate access.