Focus Article

Optical control of biological processes by light-switchable proteins

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Cellular processes such as proliferation, differentiation, or migration depend on precise spatiotemporal coordination of protein activities. Correspondingly, reaching a quantitative understanding of cellular behavior requires experimental approaches that enable spatial and temporal modulation of protein activity. Recently, a variety of light-sensitive protein domains have been engineered as optogenetic actuators to spatiotemporally control protein activity. In the present review, we discuss the principle of these optical control methods and examples of their applications in modulating signaling pathways. By controlling protein activity with spatiotemporal specificity, tunable dynamics, and quantitative control, light-controllable proteins promise to accelerate our understanding of cellular and organismal biology.

How to cite this article:
WIREs Dev Biol 2015, 4:545–554. doi: 10.1002/wdev.188

INTRODUCTION

Biological processes are regulated in both time and space. A comprehensive knowledge of the spatiotemporal information of proteins is critical for understanding the complex biological systems. Fluorescent proteins (FPs) have given us the power to monitor proteins in a spatiotemporal manner, while our ability to perturb and control proteins has lagged behind. Genetic perturbations such as RNA interference-mediated knockdown or drug-controllable transcription factors can link genes with phenotype, but they are slow relative to post-translational processes as changes to mRNA levels occur over hours.[1,2] Pharmacological perturbations are much faster, but they do not allow spatial control,[3] and specific inhibitors or activitors are not easily designed for all proteins.

Optobiological approaches that use genetically encoded light-sensitive proteins to control protein activities allow both tight temporal and spatial control over biological processes. In general, these methods use two broad strategies to link photoswitching to protein activities. The first strategy is to allosterically link photoactivation to protein activity (Figure 1). The second strategy is to use light to control protein multimerization, localization, or interactions via protein–protein interactions (Figure 2). Here, we present a short summary of recent developments of engineered light-controllable proteins. We focus on the unique aspects and applications of each system.

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Conflict of interest: Michael Z. Lin is an author on a patent application describing the construction of light-controllable proteins by fusion to fluorescent protein domains.

ALLOSTERIC LINKAGE OF PHOTORECEPTION TO PROTEIN ACTIVITY

Rhodopsins

Rhodopsins are seven-transmembrane spanning receptors that bind retinaldehyde as a cofactor. Microbial rhodopsins that are light-gated ion channels or pumps such as ChR and NpHR have been adapted to optically control the electrical excitability of neurons with no or minimal modification.[4,5] For further information on this use of rhodopsins, for which the term optogenetics was originally coined,[6] the reader can refer to several recent comprehensive reviews.[7] In

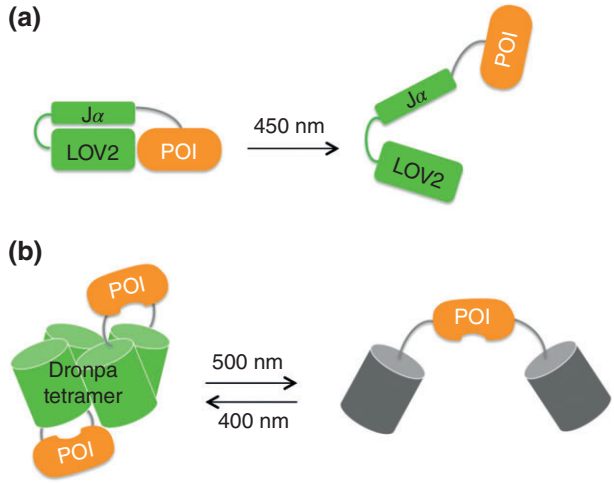

FIGURE 1 | Allosteric linkage of photoreception to protein activity. (a) Tight linkage to the light-oxygen-voltage-sensing (LOV) domain can cage a protein of interest (POI), while light-induced conformational change in the LOV domain results in its uncaging. (b) Fusion of tetramerizing Dronpa to both ends of a protein causes caging, while light-induced Dronpa dissociation results in protein uncaging at the same time as Dronpa off-switching.

animals, visual-system rhodopsins are light-sensitive G-protein-coupled receptors (GPCRs) that activate the specialized heterotrimeric G-protein transducin. Airan et al.[8] replaced the intracellular loops of rhodopsin with those from the Gq-coupled α1a-adrenergic receptor and the Gs-coupled β2-adrenergic receptor. These 'optoXR' rhodopsin-GPCR chimeras, Opto-α1-AR and Opto-β2-AR, were designed to couple via Gq to activate adenylate cyclase and via Gs to activate phospholipase C, respectively. As expected, on green light stimulation, Opto-α1-AR activated Gq and upregulated cAMP levels, while Opto-β2-AR activated Gs and upregulated InsP3. OptoXRs were then used to allow optical control of GPCR pathways in freely moving mice.

LOV Domains

Light-oxygen-voltage-sensing (LOV) domains are found in some light-responsive plant signaling proteins including the phototropin family of light-activated kinases. Light-responsive LOV domains contain a flavin chromophore, either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD).[9,10] Upon excitation by blue light, this flavin is activated from a singlet state to a more active triplet state, which then reacts in microseconds with a conserved cysteine residue within the LOV domain to form a covalent thioether bond.[11] This process reverts spontaneously in the dark over the course of seconds to minutes via hydrolysis of the flavin–cysteine bond.[12] In the best-studied LOV domain, LOV2 from Avena sativa phototropin, light-promoted FMN-cysteine bond formation causes a change in the three-dimensional structure of the domain. In the dark state, the C-terminal α-helix, termed Jα, is packed against the β-sheet structure of the rest of the protein. Light induces partial unfolding of the Jα helix and its separation from the β-sheet.[13]

In 2008, three groups independently used light-dependent changes in LOV domains to control protein activities in an allosteric manner. Lee et al. made a light-activated dihydrofolate reductase (DHFR) by linking the LOV2 domain to a surface loop of DHFR that they hypothesized to be an allosteric site due to its co-evolution with the active site.[14] Strickland et al. demonstrated that the function of a transcription factor could be rendered light dependent.[15] They fused the LOV2 domain to the N-terminus in a manner where helical elements were shared between the Jα helix and the N-terminal α-helix of the transcription factor, but where folding of the transcription factor in an active conformation would not be possible with a well-structured Jα helix bound to the LOV2 domain. Light-induced partial unfolding of Jα then allowed the transcription factor to fold into an active conformation. Moglich et al.[16] used a bacterial LOV domain to render FixL, a bacterial histidine kinase/phosphatase FixL that senses oxygen via a heme-binding PAS domain, to be light-responsive instead. As LOV domains are actually a subset of the PAS superfamily, the researchers replaced the PAS domain of FixL with the FMN-binding LOV domain from the light-responsive transcriptional regulator YtvA. The resulting chimeric protein, YF1, dephosphorylated the transcription factor FixJ in response to light, suppressing transcriptional activity. A light-regulatable gene expression was subsequently created by incorporating into one plasmid YF1, FixJ, and a gene of interest driven by a FixJ-responsive promoter.[17]

In 2009, in perhaps the most widely used example of an engineered light-controllable protein, Wu et al. screened a series of fusions of phototropin LOV2 to the small GTPase Rac1 and obtained a photoactivatable Rac1 (PA-Rac1).[18] A crystal structure revealed that the surface of LOV2 in its closed conformation in darkness engaged in an interaction with Rac1, blocking the binding of effectors to Rac1. Presumably, light-induced release of the Jα helix leads to unbinding of Rac1 from LOV2 and subsequently binding of Rac1 to its effectors. PA-Rac1 enabled optical control of membrane ruffling in mammalian cells, cell migration in live zebrafish,[19,20] and cell movements in *Drosophila*.[21,22] In neurons, researchers have used PA-Rac1 to modulate postsynaptic

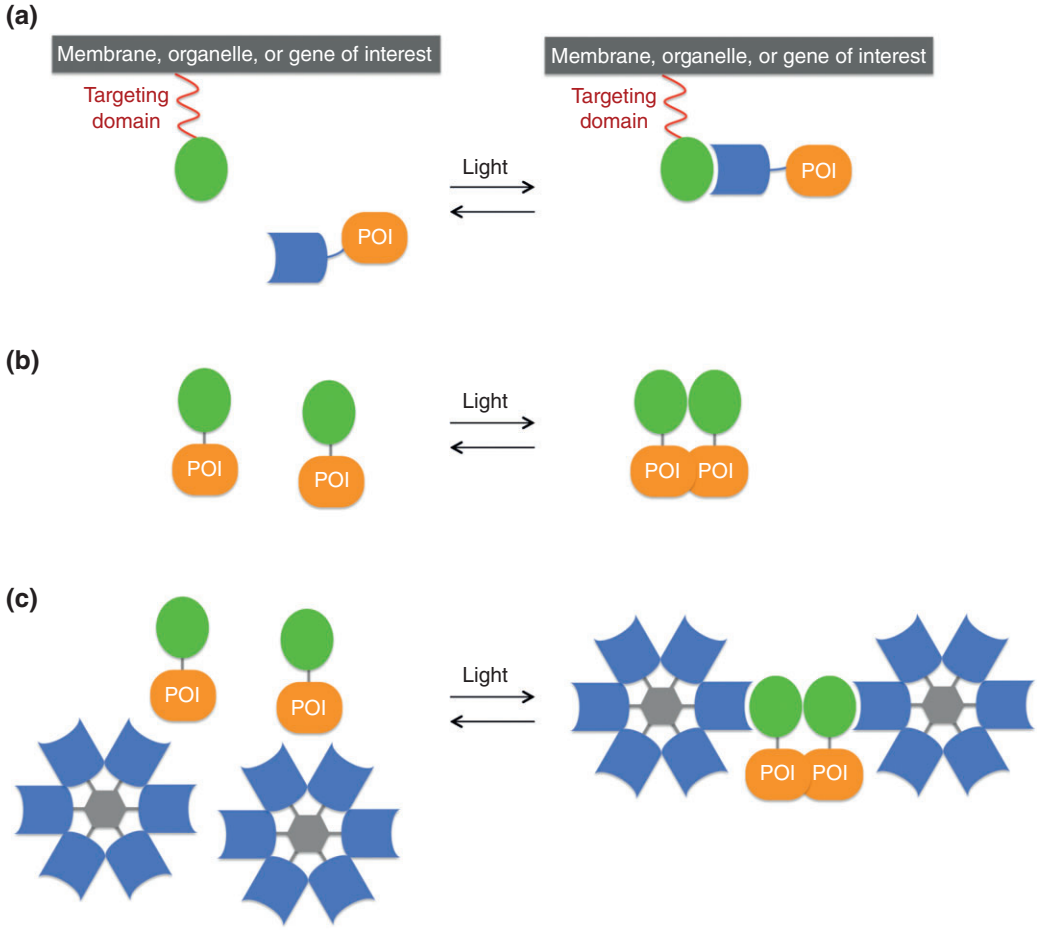

FIGURE 2 | Light-mediated protein–protein interactions can be used for (a) relocalization, (b) oligomerization, or (c) sequestration of a protein of interest (POI). The green domain represents the chromophore-containing domain.

strength[23] and investigate the role of Rac1 in structural and behavioral plasticity induced by cocaine.[24]

One fairly generalizable application of LOV2 appears to be using it as a light-dependent hinge. Nakamura et al. inserted LOV2 between the lever arm and the body of myosin and kinesin motor proteins. The change in the center of gravity of the lever arm upon illumination allowed light to control the speed and directionality of the motors.[25] Fukuda et al. also used the LOV2 as a light-controllable hinge to create a photoactivatable calcium-releasing protein (PACR).[26] They had created a calmodulin-M13 module that showed increased affinity for calcium versus calmodulin alone. They found that insertion of the LOV2 domain within the calmodulin domain of the calmodulin-M13 module allowed blue light to induce calcium release, presumably because opening of LOV2 caused a conformational change in calmodulin that led to M13 dissociation. Using PACR, they were able to increase calcium concentrations at subcellular locations.

One variation of hinge-like photocontrollable proteins are LOV2 fusions with small peptides that become exposed only upon illumination. Renicke et al. and Bonger et al. found that short degrons were inactivated by fusion to the C-terminus of LOV2, but that illumination induced degradation of the fusion protein, presumably by exposing the degrons to allow recognition by ubiquitin ligases.[27,28] Similarly, nuclear localization signals (NLSs) based on a single and small tag can be concealed by fusion to the C-terminus of LOV2, allowing light-inducible nuclear import.[29] Finally, peptide kinase inhibitors can be caged by fusion to the C-terminus of LOV2. Exposure to light results in uncaging of the inhibitors to modulate protein kinase activities in cells.[30] Strickland et al. constructed fusions of a PDZ domain-interacting peptide C-terminal to truncations of the Jα helix that showed light-dependent PDZ domain binding.[31] They found that the affinity and kinetics of the interaction could be tuned by introducing mutations that altered Jα helix docking, making this system adaptable to

signaling pathways with varying sensitivities and response times. Finally, Lungu et al. performed a variation on the idea of caging a peptide by using sequences from bioactive peptides to replace both Jα C-terminal segments and more upstream segments engaged in β sheet interactions. They selected peptides whose sequences suggested some ability to substitute for Jα sequences, then performed computational modeling and mutagenesis to optimize binding to the β sheet in the dark and unbinding after illumination. In this way, they were able to cage peptide sequences mediating protein interactions within the Jα helix, and obtain light-induced protein interactions.[32]

As the above examples show, allosteric regulation by LOV domain fusion allows for the regulation of many different types of protein activities. On the other hand, the small GTPases Ras and RhoA and yeast and metazoan kinases have yet to be rendered light dependent using LOV, despite the precedent of PA-Rac1 and the identity of phototropin itself as a light-activated serine/threonine kinase within the AGC family. It would be interesting to determine if LOV-mediated optical control can be generalized throughout the small GTPase and AGC kinase families, which contain many signaling proteins with highly regulated roles in development and human disease.

Fluorescent Proteins

FPs are typically considered solely as visualization tools, but it has recently been appreciated that they too can be used as optical control elements. In the engineered green FP Dronpa, fluorescence is switched off upon illumination with cyan light and switched on again upon illumination with violet light.[33] The photodissociation is likely due to photoisomerization of the chromophore from a *cis* to a *trans* conformation, with associated flexibility of the β barrel near the phenolate group of the chromophore.[34] As the β barrel near the chromophore is a primary multimerization interface in natural FPs, Zhou et al. hypothesized that a tetrameric mutant of Dronpa would undergo dissociation upon blue light illumination and reassociation upon violet light illumination.[35] After demonstrating this *in vitro*, they then created single-chain light-regulatable proteins by fusing Dronpa domains to each terminus of the Cdc42 activator intersectin. With Dronpa domain attachment sites flanking the active site, the Dronpa tetramer created a steric block of the active site and caged intersectin function. Light-induced dissociation of the tetramer then caused uncaging of intersectin and activation of Cdc42.

A possible advantage of protein caging by tetrameric Dronpa is that it does not rely on a structurally sensitive linkage for uncaging via allosteric control, and thus might be more generalizable. Other desirable features of this method are that the chromophore is created autocatalytically and absorbs at red-shifted wavelengths compared to the flavin chromophores used by LOV2 domains, and that photoswitching of Dronpa fluorescence reports the extent of photouncaging. Interestingly, tetrameric Dronpa may be the first case of an engineered light-controllable protein–protein interaction as light-dependent interactions are not found in natural FPs.

LIGHT-INDUCED PROTEIN MULTIMERIZATION

LOV Domains

The FAD-binding LOV domain of *Neurospora* Vivid, a protein involved in circadian cycle entrainment, homodimerizes in response to blue light. FAD-cysteine adduct formation is believed to induce dimerization at an N-terminal surface far from Jα.[36] Wang et al. fused Vivid to a Gal4 DNA-binding domain that was unable to bind its cognate DNA sequence due to deletion of sequences required for dimerization.[37] Light caused dimerization of the Vivid-Gal4 fusion, induced DNA binding, and activated transcription. This 'LightON' system had impressive dynamic range, with light activating genes in bacteria more than 1000-fold.[38] The LightON system has been used to investigate specific functions for temporal patterns of gene expression in development. Prolonged light-driven transcription of the bHLH gene Asc1 in neuronal progenitor cells induces neuronal differentiation while oscillatory transcription drives proliferation.[39]

The bacterial transcription factor EL222 also engages in light-induced homodimerization. EL222 consists of a LOV domain fused to a helix-turn-helix (HTH) DNA-binding domain. Rather than binding the LOV β sheet, the Jα helix serves as a linker connecting it and the HTH domain.[40] In the dark, the HTH domain binds to the LOV β sheet, occluding a HTH homodimerization interface. FMN-cysteine adduct formation induced by blue light induces a conformational change in the β sheet that releases HTH, allowing it to dimerize and bind DNA. Using EL222, Motta-Mena et al. were able to create an optogenetic gene expression system with rapid kinetics of less than 1 min, low basal gene activity and low toxicity for controlling transcription in several mammalian cells lines and zebrafish embryos.[41]

Cryptochromes

Cryptochromes are FAD-containing proteins that mediate light responses in plants and animals.[42] Bugaj et al. found that *Arabidopsis* cryptochrome 2 (CRY2) formed clusters in cells after strong illumination.[43] Fusion of the Wnt co-receptor LRP6 to CRY2 enabled light-induced LRP6 aggregation and activation of the Wnt–β-catenin pathway. Fusion of Rac1 and RhoA to CRY2 also enabled light to induce their aggregation and the activation of downstream effects. Three other groups also used CRY2 fusions to activate Raf,[44] Trk receptors,[43] and FGF receptor,[45,46] apparently independently. Taslimi et al. isolated mutants of CRY2 with enhanced ability to cluster in response to light that allowed activation of cellular processes with lower light doses.[47]

LIGHT-INDUCED PROTEIN HETERODIMERIZATION

Phytochromes

Phytochromes are photoreceptors found in plants, fungi, and bacteria that contain a covalently bound bilin molecule as the chromophore. The bilin chromophore in the photosensory module of phytochromes exists in red- and far-red-absorbing metastable states, and light induces reversible switching between them. The two states correspond with different conformational states of the photosensory module, which selectively bind proteins or activate linked enzymatic domains. For example, in *Arabidopsis thaliana*, the red-absorbing state is the basal state in the dark, and the light-induced far-red-absorbing state is the signaling state, binding to phytochrome interaction factors (PIFs) that activate transcription of specific genes.[48]

In what was actually the first case of co-opting plant regulatory proteins for optical control of protein function in another organism, Shimizu-Sato et al. in 2002 used plant phytochrome PhyB and PIF3 to control transcription in budding yeast. They fused PhyB to a DNA-binding domain and PIF3 to a transcriptional activation domain so that light would activate transcription. In these experiments, the yeast were fed with phytochromobilin, which can be utilized by PhyB as a cofactor.[49] Yang et al. used the bidirectionally regulated heterodimerization of PhyB and PIF6 to reversibly localize proteins to different subcellular structures in yeast, which they applied to identify distinct functions of the mitotic cyclin Clb2 at different subcellular locations.[50]

Beginning in 2009, the plant phytochrome–PIF interaction has also been used to activate signaling in mammalian cells.[51] Levskaya et al. tested different domains of PIFs to optimize light-induced binding to membrane-localized PhyB in mammalian cells fed with phytochromobilin, obtaining a PhyB–PIF6 pair whose binding and unbinding had fast kinetics of seconds and could be performed over a hundred iterations. They further used the PhyB–PIF6 interaction to control recruitment of the Rac1 activator Tiam to the membrane, inducing local lamellipodia formation upon local illumination. Toettcher et al. subsequently used PhyB–PIF6 to control PI3K membrane localization with light, then coupled automated imaging of fluorescent reporters to computerized control of illumination to enable user-defined signaling outputs to be set.[52] Finally, by controlling the temporal dynamics and strength of Ras signaling, they determined that the strengths of Ras and Erk activation are reliably coupled, but that the dynamics of Ras activation of Erk encode distinct outcomes.[53]

While plant and algal phytochromes use cofactors that are not present in other multicellular organisms, bacterial phytochromes (bacteriophytochromes) utilize biliverdin, which is a natural degradation product of heme in all cells. Thus bacteriophytochrome-based light-activated proteins may be able to function in animal cells without the addition of extra cofactor molecules in some experimental contexts. Gasser et al. noticed that the N-terminal cGMP-binding regulatory module of human phosphodiesterase 2A had similar structural features with the photosensory module of bacteriophytochromes, and thus substituted the former with the latter. This resulted in a light-activated phosphodiesterase (LAPD) that allowed optical control of cAMP and cGMP levels in mammalian cells and zebrafish embryos without adding exogenous cofactor.[54]

LOV Domains

Adding to the variety of photoregulatory mechanisms used by LOV domains, the *Arabidopsis* flavin-binding/Kelch-repeat/F-box 1 (FKF1) protein was found to interact with Gigantea (GI) in response to blue light to control flowering.[55] In 2009, Yazawa et al. fused FKF1 to Rac1 and GI to a membrane-targeting sequence, then used light to recruit Rac1 to the plasma membrane and create cell protrusions locally.[56] They also generated a light-activated transcription factor by fusing GI to the Gal4 DNA-binding domain and FKF to a transactivation domain. Polstein et al. later adapted this transcriptional regulation scheme by replacing Gal4 with a zinc finger protein, which can be modified to target specific DNA sequences. They were able to activate transcription of specific genes in human cells in a

reversible, repeatable, and modulatable manner with light.[57] However, FKF1 and G1 kinetics are not as fast as those of phytochromes and PIFs, with association occurring over minutes and dissociation over hours.[56]

Cryptochromes

CRY2 binds cryptochrome interacting basic-helix-loop-helix protein 1 (CIB1) upon blue light absorption.[42] Kennedy et al. used this light-induced interaction to relocalize a protein to the cell membrane and to induce gene transcription, similarly to how Yazawa et al. had used the FKF1–GI interaction. The CRY2–CIB1 heterodimerization showed subsecond onset and fast reversibility. CRY2 and CIB1 was also used to reconstitute Cre recombinase from two fragments in response to light.[58] Subsequently, the CRY2–CIB1 system was utilized to control membrane phosphoinositide levels by light-induced membrane recruitment of a phosphoinositol phosphatase[59] and to control ERK activation by light-induced membrane recruitment of Raf.[60] Liu et al. demonstrated the utility of the CRY2–CIB1 interaction in controlling transcription in zebrafish.[61] Konermann et al. also used CRY2–CIB1 to control transcription of endogenous genes targeted by TALE domains.[62] This system allowed reversible modulation of genes in the brain of freely behaving mice. Moreover, they achieved targeted epigenetic chromatin modifications in primary mouse neurons by fusing repressive histone effectors such as histone deacetylases to the CRY2 protein and TALEs to CIB1.

CRY2–CIB1 heterodimerization has also been used to sequester and inactivate proteins.[63] Lee et al. fused CIB1 to the dodecameric association domain of CaMKIIa and showed that illumination would induce the formation of large clusters of CRY2 fusions. The aggregates appeared at lower light levels than those used to aggregate CRY2 alone, so the CIB1 multimers appeared to facilitate aggregation. Cell protrusion induced by a Vav2-CRY2 was reversed when CRY2 was induced to aggregate, showing that protein function could be inactivated by sequestration in aggregates. Finally, the authors linked an anti-GFP nanobody to CRY2, and demonstrated that various GFP-tagged proteins could be sequestered into aggregates and inhibited by light.

UVR8

In plants, UV illumination causes the UVR8 protein to bind to the WD40 domain of constitutively morphogenetic 1 (COP1) and activate genes that provide protection from UV light.[64] The chromophores in UVR8 are actually two tryptophan side chains that form cation–π interactions with arginine residues at the dimeric interface. Photoreception reversibly destabilizes the cation–π interactions, leading to dimer dissociation.[65,66] Two groups used the UV-induced UVR8–COP1 interaction to control gene expression in mammalian cells, similar to how PhyB–PIF, FKF1–GI, and Cry2–CIB1 were used.[67,68] In one of these studies, UV regulation of UVR8–COP1 was used together with blue light regulation of VVD and red light regulation of PhyB–PIF, and achieving multi-chromatic multi-gene control.[68]

LIGHT-INDUCED PROTEIN DISSOCIATION

UVR8

The plant photoreceptor UVR8, besides associating with COP1 in response to UV illumination, also dissociates from a baseline homodimeric state. Chen et al. found that light-induced UVR8 dissociation could be used to release proteins from membrane locations,[69] in the reverse of experiments using light to recruit proteins to the membrane using FKF1–GI, CRY2–CIB1, or PhyB–PIF6. Interestingly, the UVR8 dissociation could be accomplished in seconds but was irreversible for hours. The researchers also found that fusion of multiple copies of UVR8 would cause a protein normally secreted via the endoplasmic reticulum (ER) and Golgi apparatus to be retained in the ER, presumably due to the formation of large aggregates, but that light would induce the secretion of the protein.[69] Interestingly, as with tetrameric Dronpa, no chemical cofactors are required for light control.

Fluorescent Proteins

Dronpa FP domains have also been used to release proteins from membrane tethering sites. When a monomeric Dronpa domain was anchored in the plasma membrane and a tetramerizing Dronpa domain was expressed without a localization tag, the tetramerizing Dronpa domain was localized to the membrane, presumably due to some ability to create heterodimers with monomeric Dronpa.[35] The tetramerizing Dronpa could then be released from the membrane by cyan light. However, tetramerizing Dronpa was not completely cleared from the cytosol at baseline, presumably because some protein copies would form tetramers in the cytosol rather than cross-associate with membrane-anchored monomeric Dronpa. Using tetramerizing Dronpa as the membrane-anchored component also resulted in poor membrane localization of cytosolically expressed Dronpa, likely due to tetramers formation at the

membrane again using up most membrane multimerization sites. The complications of this system for creating heterodimers make it less suitable for performing light-induced dissociation. Light-induced dissociation with Dronpa could still potentially be possible if strictly heterodimeric mutants of Dronpa could be found.

CONCLUSION

In general, optical control of protein activity has been achieved by two main strategies. First, allosteric linkages have been engineered to transduce light-induced conformational changes to protein activity changes (LOV2 domains and Dronpa FP domains). Second, light-controlled protein–protein interactions, which include homomultimerization (CRY2, VVD, EL222), heterodimerization (PhyB–PIF, FKF1–GI, CRY2–CIB1, UVR8–COP1), and dissociation (UVR8, Dronpa), have been used to control protein–DNA binding and subcellular localization.

The emerging technology of optical control has the potential to revolutionize multiple fields of biology, but its impact will likely be greatest in quantitative cell biology and developmental biology. For quantitative cell biology, the temporal and dosage specificity of optogenetic activation of proteins can be used to provide insights into pathway kinetics. For example, activation of the Ras–ERK pathway by PhyB–PIF-mediated recruitment of the Ras activator SOS to the membrane was investigated under different light doses, leading to the unexpected discovery that individual cells have a very smooth relationship between SOS activation and ERK nuclear trnslation.[53] Different durations of light stimuli also allowed the identification of signaling pathways that responded specifically to sustained ERK activity.[53]

During development, protein functions are controlled with exquisite temporal and spatial specificity as cells differentiate and migrate to form body plans, tissues, and organs. Genetics alone has provided major insights into how specific proteins control specific events during development. However, tighter control of protein function or cellular phenotypes would allow investigation of protein function with even higher temporal resolution and add spatial resolution to experiments. Light control is an ideal medium for control of protein function during development. Light can be easily controlled to provide temporal specificity with a simple switch, or can be supplied through a microscope lens to provide both temporal and spatial specificity. Optical interrogation of biology has already allowed experimenters to address questions of protein activity dose or duration or of *in vivo* function that had not previously been tractable. CRY2–CIB1 has been used to active endogenous genes in free moving animals, allowing for *in vivo* manipulation of cellular phenotypes with temporal and spatial specificity.[62] PA-Rac1 has enabled the investigation of how movements are coordinated within and between cells during development in flies and zebrafish.[20,22]

In summary, optical control of protein function, mysterious in photosynthetic organisms and seemingly impossible in animals until about a decade ago, has now become a reality. Protein engineering efforts of multiple groups have recently resulted in the development of a variety of methods for controlling protein function with light. No doubt these methods will continue to be refined and improved. Meanwhile, application of these methods has already started, and is likely to accelerate in the coming years. It is possible that optical control methods will soon become routine, just as optical sensing methods rapidly became a standard part of the experimental repertoire of molecular biology laboratories after the cloning of FPs. Thus optically controllable proteins promise to usher in a second optical revolution in biology, this time involving control rather than sensing.

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