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Optical control of protein activity by fluorescent protein domains

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Abstract

Fluorescent proteins (FPs) are widely used as optical sensors while other light-absorbing domains have been used for optical control of protein localization or activity. Here we describe a previously unknown capability of a mutant of the photochromic FP Dronpa – light-dependent dissociation and association – and use it to control protein activities with light. Fusion of mutant Dronpa domains to both termini of an enzyme domain creates a fluorescent light-inducible protein that is inactive in the dark but can be activated by light. Unlike other methods for optical control of single proteins, Dronpa-based fluorescent light-inducible proteins are simple to generate and have self-reporting abilities. Our findings extend the useful applications for FPs in biological experiments and engineered cellular systems from exclusively sensing functions to also encompass optogenetic control.

The ability to control protein activity by light would be enormously beneficial for studying protein function within physiological contexts, and for controlling synthetic biological systems with high spatial or temporal resolution. (1, 2). Protein domains that naturally exhibit light-dependent conformational changes or interactions have been adapted to control other proteins (3–10). However, existing methods require exogenous cofactors (3), exhibit slow kinetics (10) or obligate dimerization (6, 7), or use toxic blue light (7–10). Furthermore, strategies for optical control within a single polypeptide require extensive screening and optimization for each target (7, 9, 11, 12). Here, we describe the discovery of a protein interaction controlled by less harmful cyan light that requires no cofactors, and apply it to develop a simple generalizable design for light-inducible proteins.

We hypothesized that light could control oligomerization of photochromic fluorescent proteins (FPs). Fluorescence of the monomeric green FP Dronpa switches off under cyan light (~500 nm) and switches on under violet light (~400 nm) (13). With off-photoswitching, β -strand 7 near the chromophore becomes flexible (14). Interestingly, this strand forms part of the cross-dimer interface in the natural tetrameric parent of Dronpa (14). A Dronpa mutant with Lys145 on β -strand 7 changed to Asn (denoted Dronpa145N) is tetrameric at low μ M concentrations, but dilution promotes monomerization and facilitates off-photoswitching (15). This suggests that multimerization inhibits conformation changes associated with off-photoswitching. We hypothesized, conversely, that conformation changes occurring during off-photoswitching might promote monomerization, while on-photoswitching might promote multimerization (Fig. 1A).

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To determine if light could control Dronpa145N multimerization, we performed native polyacrylamide gel electrophoresis (PAGE). Dronpa145N was tetrameric at concentrations from 10 μ M to 100 μ M in the initial bright state, while wild-type Dronpa (Dronpa145K for clarity) was monomeric (Fig. S1A). Cyan illumination of 100 μ M Dronpa145N induced a shift from cyan-absorbing to violet-absorbing species (Fig. 1C) and loss of green fluorescence (Fig. S1B), as previously described (13). Simultaneously, Dronpa145N redistributed from tetrameric toward monomeric species (Fig. 1B, lane 2), implying that off-photoswitched Dronpa145N has a dissociation constant exceeding 100 μ M. Violet light restored cyan absorbance (Fig. 1C) and green fluorescence (Fig. S1B) and also induced retetramerization (Fig. 1B, lane 3), indicating that monomerization had not been due to irreversible protein damage. These results show that the FP domain can engage in light-controlled interactions. To our knowledge, this is the first case of a light-dependent interaction outside of natural light-responsive regulatory proteins.

We next explored whether Dronpa145K and Dronpa145N heterodimerize at high concentrations. To achieve high effective concentrations of Dronpa145K and Dronpa145N without driving Dronpa145N tetramerization, we fused Dronpa145K in tandem to Dronpa145N via a linker (K-N tandem dimer, Fig. 1D). The effective concentration of domains in the same polypeptide has been estimated at $\sim$ 70 μ M (16). The K-N tandem dimer migrated in native PAGE primarily as one species with similar mobility to tdTomato tandem dimer (Fig. 1E). If the Dronpa domains were engaged in light-sensitive intramolecular interaction, illumination should induce a more elongated faster-migrating conformation. Indeed, the tandem dimer migrated faster after cyan illumination, and this was reversed after violet light-induced recovery (Fig. 1E). Expected transitions between cyan- and violet-absorbing forms were again observed (Fig. 1F, S1C). Thus the K-N tandem dimer undergoes reversible light-induced conformational changes consistent with dissociation and reassociation of Dronpa domains.

We then asked whether light-induced Dronpa145N dissociation could occur in mammalian cells. We created N-CAAX, a fusion of Dronpa145N to the K-Ras C-terminal farnesylation motif (CAAX box), and mNeptune-N, a fusion of the far-red FP mNeptune (17) to Dronpa145N (Fig. 2A). Upon 10-fold relative overexpression of N-CAAX to insure an excess of membrane-localized Dronpa, some mNeptune-N was membrane-bound (Fig. 2, C and D). Cyan light switched off Dronpa fluorescence (Fig. 2B) and resulted in release of mNeptune from the membrane (Fig. 2, C and D). Release required prolonged exposures (2 minutes, metal halide lamp at 100% neutral density through a 40x 1.2NA lens) and was only partial, but nevertheless indicated that light could induce Dronpa domain dissociation in cells.

To find conditions for Dronpa domain dissociation that require less light, we explored Dronpa145K-Dronpa145N heterodimerization (Fig. 2E). Dronpa145K-CAAX (K-CAAX) was able to recruit mNeptune-N to the membrane (Fig. 2G). Off-photoswitching of membrane fluorescence was faster than with N-CAAX (Fig. 2F), and release of mNeptune required only 20 s of light (Fig. 2G,H). On-photoswitching of Dronpa by violet light induced membrane relocation of mNeptune-N (Fig. 2G,H). Reversing the positions of Dronpa domains by expressing N-CAAX and mNeptune-Dronpa145K (mNeptune-K) did not result in membrane mNeptune signal (Fig. S2A), perhaps because tetramerization between concentrated N-CAAX molecules outcompeted weaker heterodimerization with mNeptune-K. Use of only monomeric Dronpa domains (K-CAAX and mNeptune-K) also resulted in no membrane mNeptune (Fig. S2B), as expected. These results show that Dronpa145K and Dronpa145N can interact in cells and be bidirectionally controlled by light.

We hypothesized that we could use Dronpa to build light-controllable single-chain proteins. Specifically, we hypothesized that protein functions could be blocked by fusing Dronpa domains to the amino terminus (NT) and the carboxyl terminus (CT) (Fig. S3A). Binding of the two Dronpa domains would “cage” the protein in an inactive state by masking surfaces required for binding interaction partners or substrates, similarly to autoinhibition of many kinases (18), transcription factors (19), and guanine nucleotide exchange factors (GEFs) for monomeric GTPases (20). Protein function can then be induced by light-mediated dissociation of the Dronpa domains (Fig. S3A).

We chose to first control the Cdc42 GEF intersectin, which can be inactivated by terminal circularization (21). We fused Dronpa145K or Dronpa145N at the NT of the intersectin Dbl homology (DH) domain and Dronpa145N at the CT followed by the CAAX sequence, creating K-I-N-CAAX and N-I-N-CAAX (Fig. S3B). We also fused Dronpa145K on only one side of intersectin (K-I-CAAX and I-K-CAAX), which we expected to be catalytically active controls (Fig. S3B). We co-expressed these constructs in fibroblasts with a mNeptune-fascin reporter to mark filopodia and lamellipodia (22). I-K-CAAX or K-I-CAAX robustly induced filopodia and lamellipodia (Figs. S3C,D), as expected for Cdc42 activation inducing filopodia directly and lamellipodia indirectly via Rac (23). Cells expressing N-I-N-CAAX and K-I-N-CAAX produced filopodia or lamellipodia at much lower frequencies than I-K-CAAX or K-I-CAAX (Figs. S3C,D). These experiments were performed by transient transfection, which results in variable expression levels. When designated as low, medium, or high expressers by Dronpa fluorescence (Fig. S4A,C), low expressers, comprising the majority of cells, exhibited basal filopodia or lamellipodia infrequently (0% for N-I-N-CAAX and 8% for K-I-N-CAAX, Fig. S4B,D). Thus, fusion of flanking Dronpa domains cages intersectin activity effectively as long as higher expression levels are avoided, similar to PA-Rac (24).

We next asked whether caged intersectins could mediate filopodia or lamellipodia induction by light (Fig. 3A,E). Illumination with 490/20 nm light for 30 s switched off more than 50% of the fluorescence in both N-I-N-CAAX- and K-I-N-CAAX-transfected fibroblasts (Fig. 3B,F). This light dose induced abundant filopodia formation within 30 minutes in 78% of cells expressing N-I-N-CAAX (Figs. 3C, S5D). This response was light-dependent, as only 10% of cells expressing N-I-N-CAAX formed filopodia in the same time interval without illumination (Fig. S5A,D, $p < 0.0001$ by Pearson chi-squared test). Cells continued to exhibit filopodial mobility throughout 1 h of observation and did not show blebbing that might indicate phototoxicity (Movie S1). Similarly, 90% of cells expressing K-I-N-CAAX formed abundant filopodia within 30 minutes after illumination (Figs. 3G and S5D, Movie S2), compared to 25% not exposed to light (Fig. S5B,D, $p < 0.0001$ by Pearson chi-squared test). Illumination of K-CAAX-expressing cells did not induce filopodia (Fig. S5C,D), confirming the effect is not due to light alone. These results demonstrate that a protein caged by Dronpa fusion can be uncaged by light.

We investigated whether caged intersectin constructs could control filopodia formation with spatial or temporal specificity. First, we performed local illumination (490/20 nm light for 30 s) to portions of cells expressing N-I-N-CAAX or K-I-N-CAAX and observed that filopodia appeared specifically in the illuminated regions (Fig. 3D,H). We next tested whether light could induce filopodia in different locations at different times in one cell. We applied a 30 s uncaging pulse of cyan light at one subcellular region, a 3 s global recaging pulse of violet light, and finally another 30 s uncaging pulse at a different subcellular region. After the first uncaging pulse, filopodia appeared in the first region, while after the global recaging and second uncaging pulse, filopodia appeared in the second region simultaneous with retraction in the first region (Fig. S6).

Whether Cdc42 activation can lengthen existing filopodia has been unclear, as Cdc42 effectors that promote filopodia extension rather than initiation have not been found. Rapid induction of intersectin activity by light allowed us to address this question. We observed that photo-uncaging of intersectin caused lengthening of many pre-existing filopodia (Fig. S7). This suggests that models in which Cdc42 only governs filopodia initiation are incomplete (25), and that, analogous to FMNL2's recently characterized function in lamellipodia extension (26), effectors that promote filopodia extension may also exist.

A potentially unique attribute of our design is generalizability. Other methods for optical control of single polypeptides, fusion to xanthopsin or phototropin, requires extensive screening customized to each target to achieve coupling of light-induced conformational changes to protein activation, and thus have only been applied to a few targets (7, 9, 12). Our caged protein design does not require precise linkages, and thus should be more easily generalizable. Proteases are a class of enzymes for which light activation has not yet been achieved. Unlike GTPases or kinases, proteases are not naturally regulated by membrane recruitment, preventing the use of reversible membrane targeting methods to control them. We thus investigated whether we could create a light-inducible protease by fusion to Dronpa domains. We chose to regulate the hepatitis C virus (HCV) NS3-4A protease because its high sequence specificity and lack of overt toxicity allows assessment of function in mammalian cells (27). Furthermore, it is composed predominantly of beta-strands and loops (28), providing a structural contrast to the completely alpha-helical DH domain (29).

We constructed a Dronpa145N-protease-Dronpa145N fusion (N-protease-N) and, as a protease reporter, a fusion of mCherry, the NS4A/NS4B cleavage site of HCV polypeptide, and the CAAX-box farnesylation signal (mCherry-substrate-CAAX) (Fig. 4A). We expected mCherry fluorescence would be released from the membrane into the cytosol by protease activity. Indeed, mCherry signal was membrane-bound in cells expressing mCherry-substrate-CAAX alone and cytoplasmic in cells co-expressing a positive control Dronpa145K-protease (Fig. 4B). We then used mCherry-substrate-CAAX to report light induction of N-protease-N. After off-switching of Dronpa fluorescence, cells showed an increase in cytosolic mCherry within 10 min and continuing to increase over 60 min (Fig. 4C). This response required illumination (Fig. S8A) and protease (Fig. S8B). Thus, the caged protein design is generalizable, and can be used to control an enzyme domain not easily regulated by relocalization within the cell.

In summary, we have discovered that Dronpa domains are capable of reversible light-dependent dissociation, and have translated this discovery into a simple design for light-controllable single-chain proteins. As these synthetic proteins are fundamentally defined by the use of FP domains, we propose referring to them as FLIPs, for fluorescent light-inducible proteins. The FLIP design has multiple advantages not found together in other protein optogenetic methods. First, the design is generalizable to different protein targets without extensive screening or modification. Second, it can control proteins that cannot be controlled by simple relocalization. Third, chromophore maturation is autocatalytic and independent of exogenous cofactors such as phytochromobilin, or endogenous cofactors such as flavin mononucleotides, whose abundance can vary between cells or physiological states (30). Fourth, bidirectional optical control is possible. Fifth, expression and localization can be visualized by the bright fluorescence of Dronpa using low levels of light. Finally, changes in Dronpa fluorescence report the protein activation state, allowing activation light power to be tuned without using other reporters or biological endpoints, which can introduce variability or delay assessment. We speculate that another advantage of the FLIP strategy may be that additional photochromic FPs can be adapted to enable multiple wavelengths for control.

Since their discovery, FPs have seen widespread use exclusively as sensing tools. We found that photochromic FPs can have dual identities as optical sensors and light-controlled actuators. Our results thus place FPs in an unique central location in the optogenetic toolbox integrating both sensing and controlling functions in a single protein class.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Control of photochromic FP domain association by light. **(A)** Hypothesized bidirectional control of Dronpa145N oligomerization state by 500 nm cyan and 400 nm violet light. **(B)** Native polyacrylamide gel electrophoresis (PAGE) of Dronpa145N (100 μ M) demonstrated 500 nm-induced dissociation and 400 nm-induced tetramerization. mRuby2 (31), tdTomato, and dsRed2 (20 μ M) served as monomeric, dimeric, and tetrameric standards, respectively. All proteins were polyhistidine-tagged at the NT. **(C)** Absorbance spectra confirm reversible photoswitching. **(D)** Hypothesized bidirectional conformational switching by light in a Dronpa145K-Dronpa145N (K-N) tandem dimer. **(E)** Native PAGE of the K-N tandem dimer demonstrated faster migration by the K-N tandem dimer (100 μ M) after 500 nm light, an effect reversed by 400 nm light. The asterisk marks the location expected for tandem dimer migration similar to tdTomato. Some cleavage of the tandem dimer to a monomer in this protein preparation was apparent. **(F)** Absorbance spectra of K-N tandem dimers confirm reversible photoswitching.

Fig. 2.

Control of photochromic FP domain association by light in cells. **(A)** Experimental plan for light-regulated interaction between Dronpa145N-CAAX (N-CAAX) and mNeptune-Dronpa145N (mNeptune-N). **(B)** Quantitation of membrane Dronpa fluorescence during 490/20 nm illumination. **(C)** 490/20 nm light induced off-photoswitching of Dronpa and loss of mNeptune from the plasma membrane. **(D)** Intensity profile between arrows in **(C)**. **(E)** Experimental plan for light-regulated interaction between Dronpa145K-CAAX (K-CAAX) and mNeptune-N. **(F)** Quantitation of membrane Dronpa fluorescence during 490/20 nm illumination. **(G)** 490/20 nm light induced off-photoswitching of Dronpa and loss of mNeptune from the membrane. mNeptune reappeared at the membrane after 3 sec on-photoswitching with 390/15 nm light. **(H)** Intensity profiles between arrows in **(G)**. Scale bars, 20 μm.

Fig. 3.

A light-inducible single-chain GEF. **(A)** Proposed mechanism for photo-uncaging of N-I-N-CAAX activity. **(B)** Off-photoswitching of Dronpa fluorescence in N-I-N-CAAX versus 490/20 nm light dosage during microscopy. Whole-cell fluorescence from 5 cells were quantified and normalized to the initial value. Error bars represent standard deviation. **(C)** In NIH 3T3 cells expressing N-I-N-CAAX, 490/20 nm illumination for 30 s (off-switching) followed by incubation at 37° C for 30 min resulted in robust induction of filopodia as revealed by mNeptune-Fascin. **(D)** Local illumination by 490/20 nm light locally induced filopodia, marked by mNeptune-fascin, in NIH 3T3 cells expressing N-I-N-CAAX. The

dotted circle indicates the area of illumination. **(E)** Proposed mechanism for photo-uncaging of K-I-N-CAAX activity. **(F)** Off-photoswitching of Dronpa fluorescence in K-I-N-CAAX versus 490/20 nm light dosage during microscopy. Experiment was performed as in **(B)**. **(G)** In NIH 3T3 cells expressing K-I-N-CAAX, exposure to 490/20 nm light for 30 s (off-switching) followed by incubation at 37° C for 30 min resulted in robust induction of filopodia. **(H)** Local illumination by 490/20 nm light locally induced filopodia, marked by mNeptune-fascin, in NIH 3T3 cells expressing K-I-N-CAAX. The dotted circle indicates the area of illumination. Scale bars, 20 μ m.

Fig. 4.

A light-inducible single-chain protease. **(A)** Strategy for sensing activity of the N-protease-N protein with mCherry-substrate-CAAX. **(B)** Distribution of mCherry in cells expressing mCherry-substrate-CAAX in the absence (left image) or presence (right image) of co-transfected K-protease. Chart shows fluorescence intensity profile along the line between the arrows of the images. **(C)** As expected from its size (81 kD), N-protease-N was excluded from the nucleus (left). 490/20 nm light for 15 s induced off-photoswitching of Dronpa fluorescence (Dronpa channel) and induced release of mCherry from the membrane (mCherry channel). Intensity profile along the line between arrows (chart) confirmed that

mCherry fluorescence decreases from the membrane and increases in the cytosol and nucleus after illumination. Scale bars, 20 μm .