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Light Activation of Protein Splicing with a Photocaged Fast Intein

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Supporting Information

ABSTRACT: Intein-mediated protein splicing has found broad biotechnological applications. Herein, we describe our recent result in engineering a photoactivatable intein compatible with living mammalian cells. A photocaged cysteine amino acid residue was genetically introduced into a highly efficient *Nostoc punctiforme* (*Npu*) DnaE intein. The resulting photocaged intein was inserted into a red fluorescent protein (RFP) mCherry and a human Src tyrosine kinase to create inactive chimeric proteins. A light-induced photochemical reaction was able to reactivate the intein and trigger protein splicing. Active mCherry and Src were formed as observed by direct fluorescence imaging or imaging of an Src kinase sensor in mammalian cells. The genetically encoded photocaged intein is a general optogenetic tool, allowing effective photocontrol of primary structures and functions of proteins.

Inteins are protein elements that are capable of excising themselves and subsequently splicing adjacent N- and C-terminal extein flanks to form a new truncated peptide.[1] These naturally occurring, self-catalyzing protein-splicing elements have been adapted to achieve efficient protein purification, ligation, labeling, cyclization, cleavage, and patterning.[2,3] In particular, conditional inteins, whose activities are inducible by additional factors, such as small molecules, light, or changes in temperature, pH, or redox states, have previously been utilized to regulate protein activities in vitro and in vivo.[4,5] Photoactivatable inteins are of particular interest because light-based approaches often have sufficient spatial and temporal resolution to meet the need of understanding biology at the cellular and subcellular levels.[6] In a previous work, Noren et al. reported the in vitro preparation of a photoactivatable *Thermococcus litoralis* (*Tli*) Pol-2 intein, using a chemically aminoacylated suppressor tRNA.[7] Furthermore, chemical synthetic methods have also been employed to integrate photocleavable functional groups into the *O*-acyl isomer,[8] the peptide backbone,[9] or the N-terminus[10] of split inteins to achieve photocontrolled protein splicing. Due to the difficulty of directly delivering proteins or peptides into living cells, these studies focused on in vitro applications. In another work, two photoresponsive dimerization domains were each fused to an artificially split intein fragment as a genetically encoded system to control protein splicing in living *Saccharomyces cerevisiae* cells, but the system was not adaptable to mammalian cells.[11] Herein, we report the genetic encoding of a photoactivatable intein and its applications in directly controlling primary structures of proteins and therefore their functions, in living mammalian cells.

The *Nostoc punctiforme* (*Npu*) DnaE intein is among the most well-characterized and efficient inteins, with a splicing reaction half-life of ~60 s at 37 °C.[12,13] The *Npu* DnaE intein is also compatible with a myriad of flanking extein sequences.[14] All these features make the *Npu* DnaE intein an ideal research tool, especially for mammalian studies. Mutagenesis of the first catalytic cysteine residue within the *Npu* DnaE intein to alanine (Cys1Ala) abrogates protein splicing and autocleavage at both intein domain ends.[12,15] This property is different from that of some other recently reported fast inteins, whose Cys1Ala mutants are efficient in undergoing the C-terminal cleavage reaction.[16]

The genetic code expansion technology is capable of introducing site-specific photocaged lysine, tyrosine, serine, and cysteine residues into proteins of interest in living systems, including bacterial, yeast, and mammalian cells.[17−21] Previously, optical control of enzymatic activities,[22−24] ion channels,[25] gene expression and silencing,[26] and protein translocation[27,28] have been demonstrated by replacing critical protein residues with photocaged unnatural amino acids (UAAs). In this study, we show that a genetically encoded photoactivatable intein can be readily derived by replacing the Cys1 residue of *Npu* DnaE intein with a photocaged cysteine, and it is highly effective in directly modulating primary protein structures, thereby rendering a general approach for controlling protein activities in living cells.

Previous efforts have utilized mutant pairs of pyrrolysyl-tRNA synthetase (PylRS)/tRNA[29,30] and *Escherichia coli* leucyl-tRNA synthetase (*Ec*LeuRS)/tRNA[25] in mammalian cells for the genetic encoding of unnatural cysteine derivatives that can be decaged with long-wavelength UVA radiation. In particularly, an orthogonal *Ec*LeuRS/tRNA pair originally engineered for the encoding of a photocaged serine in yeast[19] was found to be capable of encoding a photocaged cysteine (**1** in Figure 1**a**) in mammalian cells.[25] Based on these results, we modified our pMAH mammalian expression plasmid[31] to express the mutant *Ec*LeuRS and tRNA genes. Expression of the full-length GFP protein in Human Embryonic Kidney (HEK) 293T cells bearing EGFP-Tyr39TAG (a gene for enhanced green fluorescent protein with an amber codon at residue 39) was observed to be dependent on **1** (Figure 1**b**). Photolysis of **1** is expected to generate an aldehyde byproduct, which may further react with free cellular amines to inadvertently promote cell toxicity (Figure S1a).[32] Therefore, we also prepared a new UAA, **2** (Figure 1**a**), photolysis of which yields a cysteine and a less reactive ketone byproduct (Figure S1bc). Since **2** is structurally similar to **1**, we also tested **2** for amber suppression

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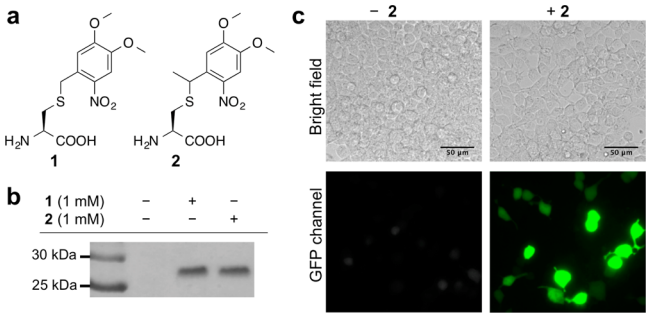

Figure 1. Genetic encoding of photocaged cysteines in HEK 293T cells. (a) Chemical structures of two photocaged cysteines, **1** and **2**. (b) SDS-PAGE analysis of Ni-NTA-purified EGFP, containing **1** or **2**, expressed in HEK 293T cells. (c) Microscopic imaging of EGFP-expressing HEK 293T cells in the absence (left column) or presence (right column) of **2** (scale bar: 50 μm).

in the presence of the mutant *Ec*LeuRS/tRNA pair. We achieved an appreciable yield of full-length GFP from HEK 293T cells, as observed by SDS-PAGE analysis and fluorescence microscopic imaging (Figure 1**b** and **c**). Electrospray ionization mass spectrometry (ESI-MS) further confirmed the genetic incorporation of **2** in the recombinantly expressed EGFP (Figure S2).

To determine whether **2** can be utilized to photocontrol the protein splicing activity of the *Npu* DnaE intein, we inserted a full-length *Npu* DnaE intein sequence into mCherry (Figure 2**a**). The residue 138 on a long loop between the β-strands 6

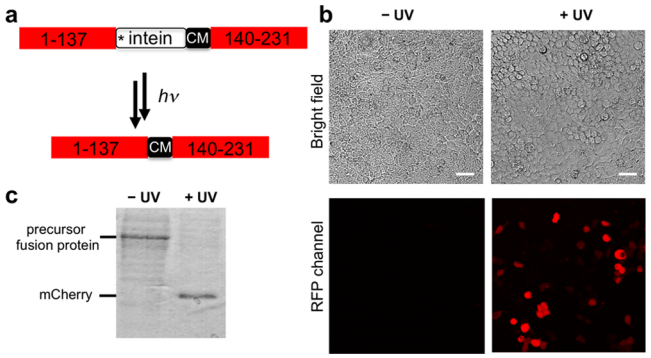

Figure 2. Photoactivation of mCherry. (a) Primary structures of the intein/mCherry chimeric protein and its photoconverted product after UV-induced protein splicing. The red portion of the bar represents the mCherry sequence. The asterisk (*) represents the Cys1 residue for UAA incorporation. The "CM" region are two extein residues (+1 and +2). (b) Microscopic imaging of HEK 293T cells expressing the construct treated with or without UV irradiation (scale bar: 50 μm). (c) SDS-PAGE analysis of the Ni-NTA-purified proteins from HEK 293T cells, with or without UV irradiation.

and 7 of mCherry was chosen as the insertion site (Figure S3).[33] Moreover, the codon of the Cys1 residue of *Npu* DnaE intein was mutated to an amber codon (TAG) for UAA incorporation. The chimeric construct was subsequently expressed in HEK 293T cells, with cell culture media containing **2**. Almost no fluorescence was observed prior to UVA treatment (Figure 2**b**), suggesting that the intein insertion disrupted the fluorescence of mCherry. Next, we used a UVA lamp to directly illuminate cells in cell culture dishes, and strong red fluorescence was observed in 1 h after irradiation (Figure 2**b**). This rate of developing red fluorescence in cells was comparable to the rate of chromophore maturation of mCherry.[34] These results indicate that the caged intein was photoactivated to undergo protein splicing and form a highly fluorescent reconstituted mCherry. Since the construct was 6xHis-tagged at the C-terminal end, Ni-NTA agarose beads were utilized to purify proteins from untreated or UVA-treated cells. SDS-PAGE analysis of the proteins confirmed the highly efficient, light-induced protein splicing: upon UVA-treatment, nearly all of the chimeric protein was converted to the spliced product (Figure 2**c**).

We next explored the use the photocaged intein in controlling enzymatic activities. We inserted the photocaged intein into the catalytic domain of Src, a human tyrosine kinase.[35] The kinase catalytic domain has eight cysteine residues and 12 serine residues. We designed chimeric proteins by randomly and individually inserting the intein into three sites in Src (Figure 3**a** and Figure S4). First, we inserted the

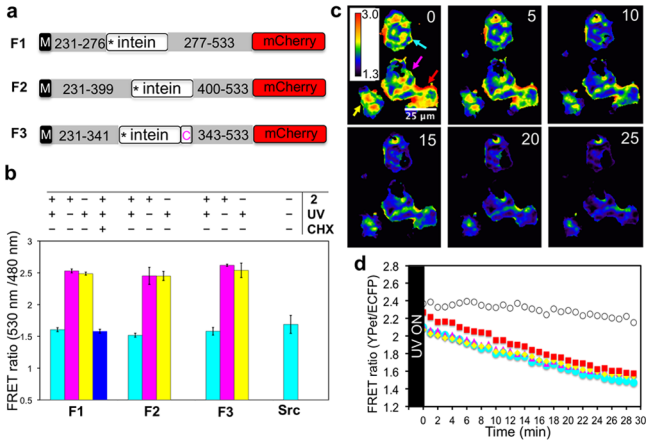

Figure 3. Photoactivation of Src kinase. (a) Primary structures of the chimeric proteins tested in this study. The gray portion of the bars represents the sequence of the human Src kinase between the indicated residues. The asterisk (*) indicates the Cys1 residue for UAA incorporation; "M" is methionine, as the translational start site; and "C" is cysteine, used to replace residue 342 of Src. (b) Activity of the chimeric proteins before and after UVA irradiation, as measured from FRET ratios of a KRas-Src sensor. In the absence of **2**, the full-length proteins were not synthesized and are thus used as negative controls. A wild-type Src was also prepared as a positive control. To block ribosomal protein synthesis during and after UVA irradiation, cycloheximide (CHX) was also added to a control group. (c) Pseudocolored ratio images of representative UVA-treated HEK 293T cells expressing the F1 construct in the presence of **2** at the indicated post-treatment time (in minutes). The color bar represents fluorescence ratio (YPet/ECFP) (scale bar: 25 μm). (d) FRET ratios plotted versus time for HEK 293T cells. Color symbols are for individual cells in panel c, marked at 0 min by arrows in the same colors. The FRET ratios of an identically treated control cell cultured in the absence of **2** (see Figure S5) are shown as open black circles.

intein between Gly276 and Cys277, or Val399 and Cys400 of Src (F1 and F2 in Figure 3**a**). For these two constructs, protein splicing is expected to generate a product identical to the wild-type Src kinase catalytic domain. We also built the third construct, F3, in which the intein was placed downstream of Met341 (Figure 3**a**). Because the *Npu* DnaE intein requires a cysteine residue at the +1 site for efficient protein splicing,[12] we also mutated Ser342 to cysteine, to which appended was the native Src sequence from residue 343 to residue 533. The splicing product of F3 is expected to be different from the wild-

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type protein by a single Ser342Cys mutation. It is worth noting that a serine-to-cysteine mutant is tolerated in many cases without dramatically affecting protein activities.[36] We also fused mCherry at the C-terminal end as an expression indicator of the UAA-containing full-length proteins. Next, we used a KRas-Src sensor,[37] based on Förster resonance energy transfer (FRET) between ECFP and YPet, to evaluate the activities of F1, F2, and F3 in the presence or absence of UVA irradiation. This sensor was well-validated in previous studies, and Src kinase activity is known to decrease the intensity ratio (YPet/ECFP) of the sensitized YPet fluorescence emission to the direct ECFP donor emission.[37] HEK 293T cells containing each of the 3 constructs and the Ras-Src sensor were treated with UVA light and, then, lysed for fluorescence quantification with a plate reader (Figure 3**b**). All of our three constructs were inactive prior to UVA irradiation, while UVA light was able to activate them, leading to the decrease of the FRET ratios of the sensor. A reduced FRET ratio was also observed for cells coexpressing a wild-type Src kinase and the Src sensor. Furthermore, negative control experiments were performed with HEK 293T cells containing each of the three constructs but cultured in the absence of **2**. Cells in the negative groups were also subjected to the identical UVA treatment, so that the partial photobleaching of the Src sensor did not mask the FRET changes caused by the photoactivation of the Src kinase activity. Moreover, we utilized fluorescence microscopy to closely monitor the process (Figure 3**c**). HEK 293T cells coexpressing the Src sensor and the chimeric F1 construct were irradiated on an epi-fluorescence microscope equipped with a DAPI excitation filter. Next, we carried out time-lapse, two-channel FRET imaging of ECFP and YPet. The FRET ratios of the Src sensor gradually decreased in the monitored 30 min period. In contrast, the UVA-treated control cells cultured in the absence of **2** showed no obvious change in FRET ratios during the imaging period (Figure 3**d** and Figure S5). It was noted that considerable Src-induced FRET changes occurred during the 2 min of UVA illumination. Analysis of single cells showed that the average FRET ratio (YPet/ECFP) at 0 min, when time-lapse FRET imaging started, was 2.11 ± 0.08 for cells containing the photoactivated Src. In comparison, negative cells identically treated with UVA radiation had an average FRET ratio of 2.35 ± 0.03. This is not surprising, considering the fast kinetics of the *Npu* DnaE intein. The UVA illumination condition did not affect cell viability[38] but effectively activated the photocaged intein to promote the formation of Src via protein splicing. These data support that the photocaged *Npu* DnaE intein is an effective tool for the control of enzyme activities.

UV radiation may also decage the charged unnatural aminoacyl tRNA, which may be further utilized by cellular ribosomes to synthesize proteins. We added cycloheximide (100 μg/mL) to block ribosomal protein synthesis during and after irradiation, the photoactivation of Src kinase was not affected (Figure 3**b**). In addition, the activation of Src was observed right after UV irradiation (Figure 3**d**), when ribosomal protein synthesis from the decaged aminoacyl tRNA was unlikely to be achieved in this short time frame. These results suggest that the direct decaging of the accumulated chimeric proteins in cells was the major pathway in our experiments.

In summary, we have engineered the first genetically encoded photoactivatable intein compatible with living mammalian cells, in which a photocaged cysteine is used to genetically replace the Cys1 residue of a highly efficient *Npu* DnaE intein. By incorporating the photocaging group, the protein splicing activity of the intein was effectively and efficiently inhibited, and the activity was only observed after a brief exposure to long-wavelength UVA light. The resulting photocaged intein was inserted into other proteins to directly control their primary structures. Because the *Npu* DnaE intein is compatible with a myriad of extein sequences, such manipulation should be quite versatile. A downstream C-extein Cys+1 residue is required for protein splicing, but cysteine can be found in many proteins. In addition, a single cysteine mutation may be tolerated by many proteins. Thus, the approach described here may be applied to a large percentage of proteins. We acknowledge that additional N- and C-terminal extein sequences might affect the kinetics of protein splicing. This issue can be addressed by using evolved inteins that splice with higher efficiency at various splice junctions.[39] One might also prepare several chimeric constructs at different splice sites to screen for variants retaining excellent expression, stability, and postphotoactivation splicing kinetics. The use of the photoactivatable inteins to control protein activity is highly attractive, because it requires little information on the biochemistry or 3D structures of the proteins of interest. The photoactivatable intein reported here is a new and powerful addition to the mammalian optochemical genetic toolbox, permitting the modulation of proteins directly at the amino acid sequence level.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

General methods, protein sequencing information, mass spectrometry, and FRET imaging of control cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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