Genetic Encoding of Photocaged Cysteine Allows Photoactivation of TEV Protease in Live Mammalian Cells

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Supporting Information

ABSTRACT: We demonstrate the evolution of the PylRS/tRNA_{CUA} pair for genetically encoding photocaged cysteine. By characterizing the incorporation in Escherichia coli and mammalian cells, and the photodeprotection process in vitro and in mammalian cells, we establish conditions for rapid, efficient photodeprotection to reveal native proteins in live cells. We demonstrate the utility of this approach by rapidly activating TEV protease following illumination of single cells.

Insight into dynamic processes in cells and organisms would be facilitated by approaches to control protein function with spatial and temporal precision. Rapid control of protein function with light provides a promising route to perturb processes more rapidly than most biological adaptations, providing information that cannot be obtained by gene knockouts or knockdowns. Several exciting strategies have emerged for the photocontrol of protein function, including the control of protein localization or assembly mediated by the genetic fusion of photoresponsive interaction domains to target proteins (or their fragments), and the introduction of a photocage will allow photocontrol; and (ii) following illumination, the structure, function, activity and localization of the resulting protein are entirely native.

Proteins bearing photocaged amino acids have been synthesized by chemical ligation and introduced into cells via microinjection, and more recently synthesized directly in cells via genetic code expansion. Photocaged lysine, tyrosine, and serine have been site specifically incorporated in place of key residues, allowing photodecontrol of nuclear localization sequences, kinases, and other enzymes, and photodecontrol of the sites of post-translational modifications.4,8–11 These approaches have revealed the kinetics, and feedback/feedforward contributions in elementary steps of complex signaling pathways with high spatial and temporal resolution.1,4,8

Key cysteine residues are found in many classes of proteins including: phosphatases, ubiquitin ligases, cysteine proteases (including caspases and deubiquitinas) and inteins. Cysteine residues are also the sites of protein palmitoylation and form disulfide bonds in proteins. The ability to (i) genetically encode the site-specific incorporation of a photocaged cysteine residue into a target protein and (ii) rapidly and cleanly convert the caged cysteine to cysteine upon illumination of living cells would provide an important approach for controlling diverse biological processes.

The incorporation of an ortho-nitrobenzyl (ONB) caged cysteine 1 has been reported in yeast using the Escherichia coli (E. coli) leucyl-tRNA synthetase/tRNA_{CUA} pair.11 However, 1 has not been incorporated in other host organisms. Moreover 70% deprotection of this amino acid in yeast lysate required 20 min illumination with a hand-held UV lamp of unknown spectral distribution and power, and photodecaging of this amino acid has not been extended to living cells.

The pyrrolysyl-tRNA synthetase (PylRS)/tRNA_{CUA} pair from methanogenic archaeal species, including Methanosarcina barkeri (Mb), Methanosarcina mazei (Mm) and their synthetically evolved variants, have been used to direct the site-specific incorporation of unnatural amino acids12 into proteins produced in E. coli, yeast, mammalian cells, Caenorhabditis elegans, Drosophila melanogaster, and Arabidopsis thaliana.13–18 Here we demonstrate the evolution of the PylRS/tRNA_{CUA} pair for genetically encoding photocaged cysteine. By carefully characterizing the incorporation in E. coli and mammalian cells, and the photodeprotection process in vitro and in mammalian cells, we establish conditions for rapid near-quantitative photodeprotection to reveal native proteins in live cells. We demonstrate the utility of this approach by rapidly activating TEV protease following illumination of single cells.

We initially evolved a synthetase/tRNA_{CUA} pair for 1 (Figure 1a). To evolve the orthogonal MbPylRS/tRNA_{CUA} pair for the site-specific incorporation of 1, we created a library of MbPylRS mutants in which five residues in the amino acid binding site (N311, C313, V366, W382, and G386) were randomized to all possible natural amino acids. We performed three rounds of mutagenesis and microinjection, and more recently synthesized directly in cells via chemical ligation and introduced into cells via microinjection, and more recently synthesized directly in cells via genetic code expansion. Photocaged lysine, tyrosine, and serine have been site specifically incorporated in place of key residues, allowing photodecontrol of nuclear localization sequences, kinases, and other enzymes, and photodecontrol of the sites of post-translational modifications.4,8–11 These approaches have revealed the kinetics, and feedback/feedforward contributions in elementary steps of complex signaling pathways with high spatial and temporal resolution.1,4,8

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specific for the unnatural amino acid. The majority of the selected clones had the mutations: N311M, C313Q, V366G, W382N, and one nonprogrammed mutation, R85H. We named the synthetase PCCRS (SI, Table S1). Production of full-length sfGFP from E. coli bearing GFP-N150TAG (A gene for superfolder GFP with an amber codon at position 150) and the PCCRS/sRNA_CUA pair was dependent on the addition of 1 (Figure 1b). Similarly, production of ubiquitin (Ub) from E. coli bearing UbK6TAG and the PCCRS/sRNA_CUA pair was dependent on the addition of 1 (Figure 1b). SFGFP-N150-1 and Ub-K6-1 were produced in good yield (∼5 mg/L of culture). Electrospray ionization mass spectrometry (ESI-MS) confirmed the genetically directed incorporation of 1 in recombinant proteins (Figure 1c and SI, Figure S1a), but also demonstrated that a substantial portion of the protein contained a mass consistent with reduction of the nitro group within the compound to an amine.

Following irradiation of Ub-K6-1 with 365 nm (∼35 mW/cm²) light for 10 min, the ONB caging group on ubiquitin was quantitatively decaged upon irradiation at 365 nm (∼4 mg/L of culture) and dependent on the presence of 2 (Figure 1b). ESI-MS confirmed the incorporation of 2 in ubiquitin and sfGFP (Figure 1c). Ubiquitin containing 2 is quantitatively decaged upon irradiation at 365 nm (∼35 mW/cm²) for 1 min (Figure 1c and SI, Figure S1d). Therefore, 2, unlike 1, is incorporated with minimal reduction in E. coli, and is quantitatively decaged. On the basis of these advances we proceeded to investigate the genetic incorporation of 2 into proteins in mammalian cells.

To demonstrate the genetically directed site-specific incorporation of 2 into a protein in mammalian cells, we introduced the MmpPCCRS (a variant of MmpPyrRS containing the mutations discovered in MmPyrRS that direct the incorporation of 2)/sRNA_CUA pair into a human embryonic kidney 293T (HEK293T) cell line stably expressing mCherry-TAG-eGFP-HA gene (a fusion between the genes encoding a red fluorescent (mCherry) protein and a green fluorescent protein (eGFP) and the human influenza hemagglutinin (HA) tag separated by the amber codon (TAG)). Anti-HA immunoblotting and fluorescence microscopy confirmed that the expression of full-length mCherry-eGFP-HA is dependent upon the addition of 2 (Figure 2a and SI, Figure S2a). The incorporation efficiency of 2 is comparable to that of N-tert-butylcarboxyl-l-lysine 3, a good substrate for the PylRS/sRNA_CUA pair (22,23) (Figure 2a). To demonstrate the generality of this approach for incorporating 2 into proteins we expressed an sfGFP-N150TAG gene in HEK293T cells in the presence of the MmpPCCRS/sRNA_CUA pair and 2 and purified the protein (Figure 2b). ESI-MS demonstrated the genetically directed incorporation of 2 into sfGFP (sfGFP-N150-2) (Figure 2b and SI Figure S2b) in mammalian cells without reduction. Illumination of HEK293T cells expressing sfGFP-2 (1 min, 365 nm, ∼4 mW/cm²), followed by purification of sfGFP and ESI-MS demonstrates the efficient conversion of 2 to cysteine in the protein (∼90% deprotection with 1/10th of the power used for quantitative deprotection in vitro). This demonstrates that genetically encoded 2 can be cleanly and efficiently decaged in proteins expressed in live mammalian cells (Figure 2c and SI, Figure S2bc).

To exemplify the photocentricity of protein function in mammalian cells via the genetically directed incorporation of 2 we replaced the catalytic cysteine (C151S) in Tobacco Etch Virus (TEV) protease. We synthesized TEV protease incorporating 2 (TEV-C151-2) in HEK293T cells, bearing TEV-C151-TAG and the MmpPCCRS/sRNA_CUA pair (Figure 3a,b).

To report on TEV protease activity in cells we used an ECFP-TevS-YFP-V5 fusion protein containing a TEV cleavage site (TevS) at the linker between ECFP (enhanced cyan fluorescent protein), YFP (yellow fluorescent protein), and C-
the appearance of a single YFP-V5 immuno-responsive cleavage product (Figure 3c: lanes 2, 6, 10, 14, and SI, Figure S3). No cleavage of the sensor was observed when 2 or 3 was incorporated into TEV protease in place of the catalytic cysteine, demonstrating that this is sufficient to abrogate TEV protease activity (Figure 3c: lanes 3 and 4). Upon illumination (1 min, 365 nm, 4 mW/cm²) of cells bearing TEV-C151-2, we observe an increase in the YFP-V5 cleavage product resulting from photoactivation of TEV-C151-2 to TEV protease (Figure 3c: lanes 4, 8, 12, 16). Illumination of control cells expressing TEV-C151-3 did not result in cleavage of the reporter, confirming the specificity of the approach (Figure 3c: lanes 3, 7, 11, 15). In these experiments the extent of reporter cleavage following photoactivation is less than with wild-type TEV protease, and this is in part because the experiment is a three-plasmid transient transfection, and not all cells contain the reporter, synthetase, tRNA, and TEV protease genes.

We subsequently monitored TEV protease activity over 4 h by FRET fluorescence-activated cell sorting (FRET-FACS) (Figure 4 and SI, Figure S4a–f). Control FRET-FACS experiments demonstrate that cells bearing the CFP-TevS-YFP reporter show a high FRET signal, resulting from energy transfer from CFP to YFP upon excitation of CFP (SI, Figure S4). The FRET signal of the reporter is unchanged upon activation of TEV-C151-2 or TEV-C151-3 from a construct containing the TEV-C151-TAG gene separated by a self-cleaving T2A sequence from the mCherry gene allowing cells containing the construct to be marked with red fluorescence.

In cells bearing TEV-C151-2, and illuminated for 1 min, FRET-FACS analysis revealed a shift in the population from high FRET (FRET³⁰⁰) to low FRET (FRET⁰), indicating a decrease in FRET (Figure 4a). This shift occurred within 15 min following illumination, as revealed by the drastic decrease in FRET³⁰⁰ population over time (Figure 4b). In contrast, there was no change in the FRET⁰ population over time for cells

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**Figure 2.** Genetically directed incorporation and photodecaging of 2 in mammalian cells. (a) Immunoblotting of proteins from HEK293T cells expressing mCherry-TAG-sfGFP-FLAG/tRNACUA pair in the presence or absence of 2 (5 mM). (b) SDS-PAGE analysis confirmed the expression of sfGFP-N150-2 in HEK293T cells in the presence of the MinPCC2RS/tRNACUA pair and 2. The full-length sfGFP was purified by immunoprecipitation using an anti-GFP antibody. (c) Efficient conversion of SFGFP-N150-2 (A; observed: 27921 Da, expected: 27922 Da) expressed and illuminated in live HEK293T cells to SFGFP-150-C (B; observed: 27728 Da, expected: 27728 Da) (See SI Figure S2b,c, for detailed analyses).

**Figure 3.** Photocontrol of TEV protease activity in mammalian cells. (a) Strategy for photoactivating TEV protease. Caging a catalytic cysteine (C151) in TEV protease deactivates it. TEV protease can be rapidly activated by removing the caging group with light. (b) Site-specific incorporation of 2 in TEV protease. Full-length TEV-C151-2 was expressed in HEK293T cells in the presence of 2 and the MinPCC2RS/tRNACUA pair. (c) TEV protease can be efficiently activated with light in HEK293T cells. Cells were illuminated (1 min, 365 nm, ~4 mW/cm²). All cells contained the reporter of TEV protease activity.

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**Figure 4.** Following TEV protease photoactivation by FRET in mammalian cells. (a) Histograms of FRET/CFP ratio in mCherry positive cells determined by FRET-FACS. (b) Percentage of FRET³⁰⁰ population, for mCherry positive cells, at time points postillumination confirms rapid TEV protease photoactivation. (See SI, Figures 5a–f for detailed FACS analysis). (c) Single-cell analysis of TEV protease photoactivation by live-cell fluorescent imaging. Fluorescent confocal micrographs showing an increase in CFP/YFP fluorescent intensity ratio after illumination in a representative cell expressing FRET sensor and TEV-C151-2. (d) Analysis of TEV protease activation in individual cells expressing the FRET sensor and TEV-C151-2 or cells expressing only the FRET sensor.
containing TEV-C151-3 and the FRET sensor or cells containing only the FRET sensor. These results confirm that TEV protease can be activated and the FRET sensor in most cells was efficiently cleaved within 15 min.

Single-cell fluorescence microscopy allowed us to follow the activity of TEV protease with greater temporal resolution in live cells (Figure 4c,d). Following photoactivation of TEV protease the CFP/YFP ratio increases within 5 min, and approximately 80% of the FRET sensor in cells is cleaved within 15 min. These results further demonstrate the photoactivation of TEV protease in live cells.

In summary, we have demonstrated the evolution of a pyrrolysyl-tRNA synthetase/tRNACUA pair for the efficient site-specific incorporation of 2 into proteins in E. coli and mammalian cells, the efficient decaging of 2 in proteins expressed in E. coli and in mammalian cells and the rapid photoactivation of TEV protease in mammalian cells. By combining the approach reported here with the introduction of TEV sites into proteins of interest it will be possible to create spatially and temporally controlled optically activated protein knockouts. Extending this approach to animals and combining it with emerging genome-editing approaches will allow rapid cell-specific and developmental stage specific photoactivated protein knockouts. We anticipate that this well characterized system for photocontrol of cysteine residues in proteins will form the basis of powerful approaches for rapidly controlling diverse biological processes.

**ASSOCIATED CONTENT**

Supporting Information
Supporting experimental data and procedures. 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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