Photoactivatable Proteins

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A Ligation and Photorelease Strategy for the Temporal and Spatial Control of Protein Function in Living Cells**

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The temporal and spatial regulation of protein function is central to biological processes. Hence, the ability to artificially trigger molecular events in a biologically relevant context is useful for the study of living organisms. By chemically modifying a protein with groups that can respond to an external input such as light, control of protein function inside live cells can in principle be achieved. For instance, peptides or proteins that contain photolabile protecting groups appended to functionalities required for biological activity can be delivered into live cells and activated by UV irradiation at a desired time or location, and a cellular response can subsequently be measured.[1] This can be accomplished within single cells or in the context of whole organisms.[2] Although protein photocaging is a powerful tool to investigate biological systems, this approach has been limited by the difficulties associated with the preparation of the caged protein reagents.

To facilitate the semisynthesis of caged proteins and expand the repertoire of suitable chemical reagents, we report the design, synthesis, and use of a ligation and photorelease (LPR) molecule suitable for the introduction of a light-activatable cleavage site within a semisynthetic protein (Scheme 1). In contrast to previously reported strategies relying on site-specific photochemical cleavage,[3] our LPR approach is based on the ligation of a photocleavable synthetic moiety to the C terminus of a recombinant protein. In principle, the chemical and protein components may influence the properties of one another in the ligated state. Irradiation with light and subsequent photorelease of each element may then lead to the recovery of their respective function. We demonstrate that such chimeric semisynthetic proteins can be efficiently prepared and photocleaved in vitro. We also demonstrate that photocleavage of protein constructs microinjected in live cells can be triggered in a

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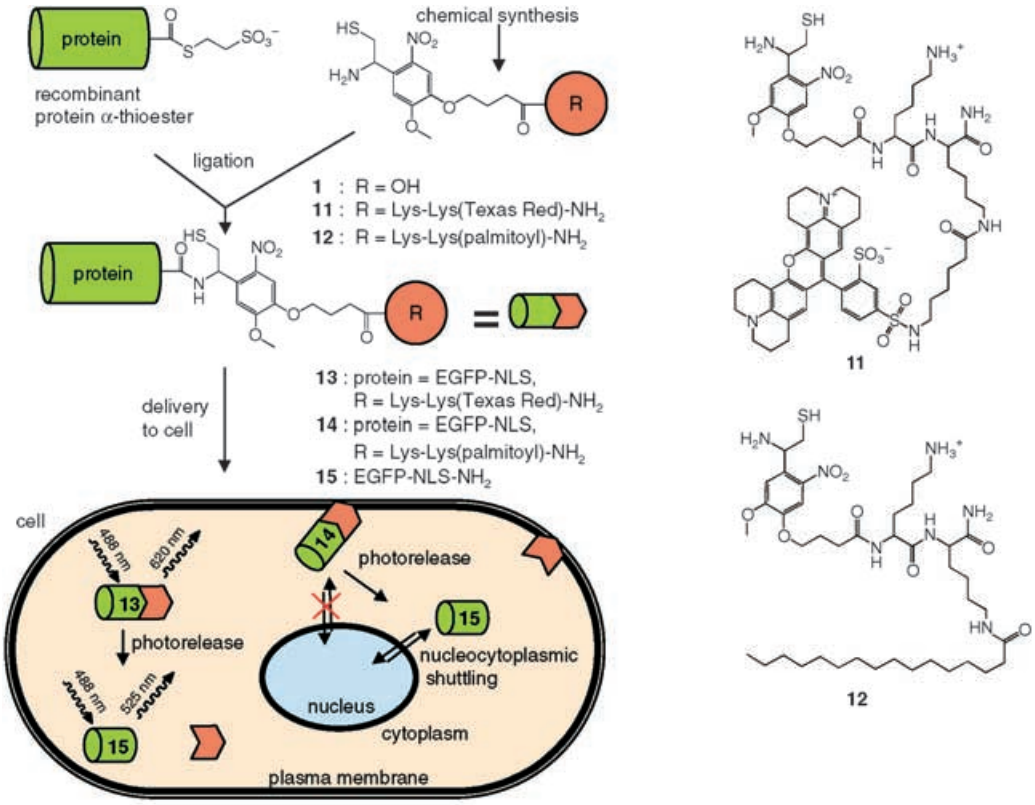

Scheme 1. Schematic representation of the semisynthesis and applications of protein constructs in which a photoactivatable cleavage site is introduced. A synthetic moiety (e.g. peptide, small molecule) is ligated to a recombinant protein through molecule **1**. The semisynthetic protein is then delivered into living cells where the protein and chemical components can be photoreleased from one another with temporal and spatial control.

dosable manner and that the subcellular localization of a protein can be controlled with light.

The LPR molecule **1** was designed to combine a 2-aminoethanethiol group required for native chemical ligation, an *o*-nitrobenzyl chromophore required for bond cleavage,[4] and a carboxylic acid tether for facile conjugation to amine-containing compounds (Scheme 1).[5] For synthetic purposes, the thiol and amine groups were protected as *S*-*tert*-butyl disulfide and *tert*-butyl carbamate, respectively. The protected molecule was synthesized in seven steps from vanillin (24% overall yield), essentially according to the route developed by Dawson and co-workers for the synthesis of a native chemical ligation auxiliary.[6,7] The product is suitable for solid-phase peptide synthesis (SPPS) using Boc (*tert*-butyloxycarbonyl) or Fmoc (9-fluorenylmethoxycarbonyl) protecting groups. Once deprotected, the 2-aminoethanethiol moiety should be compatible with native chemical ligation and expressed protein ligation (EPL).[8]

The native chemical ligation and photocleavage reactions were characterized using a model α-thioester peptide, Gly-Lys(TAMRA)-Gly-$SCH_2CH_2SO_3Na$ (**7**; TAMRA = 5-carboxytetramethylrhodamine), and a peptide synthesized with **1** at its N terminus, **1**-Lys-Lys(Fl)-NH_2 (**8**; Fl = 6-carboxyfluorescein).[7] The two peptides were incubated in phosphate-buffered saline (PBS), 100 mM 2-mercaptoethanesulfonic acid sodium salt (MESNa; pH 7.5), and the expected product, Gly-Lys(TAMRA)-Gly-**1**-Lys-Lys(Fl)-NH_2 (**9**), was obtained in near-quantitative yield in less than 1 h. Subsequent irradiation of **9** (in 1 mM in PBS, pH 7.5) at 325 nm with a He–Cd laser for 2 s was sufficient for quantitative photolysis and led to the formation of a single N-terminal product, Gly-Lys(TAMRA)-Gly-NH_2 (**10**). The LPR molecule was thus shown to react rapidly with thioesters and to efficiently generate the corresponding amide upon photolysis.[7]

The applicability of our caging strategy was next tested in the context of EPL. The enhanced green fluorescent protein (EGFP) containing the SV40 T-antigen Lys-Lys-Lys-Arg-Lys-Val nuclear localization signal (NLS) at its C terminus was used as a model protein and obtained as the recombinant protein α-thioester (from transthioesterification of an EGFP-NLS-GyrA intein fusion expressed in *E. coli*, see ref. [11]).[9,10] The peptides **1**-Lys-Lys(TR) (**11**) and **1**-Lys-Lys(Palm) (**12**; TR = Texas Red-X, Palm = palmitoyl) were synthesized by SPPS and used as model small molecules. The ligation reactions between the EGFP-NLS α-thioester and peptides **11** and **12** were monitored by reverse-phase HPLC, electrospray mass spectrometry (ES-MS), and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and were complete in less than 12 h (Figure 1a). The products, EGFP-NLS-**1**-Lys-Lys(TR) (**13**) and EGFP-NLS-**1**-Lys-Lys-(Palm) (**14**), were purified and their identity confirmed by ES-MS. Protein **13** is labeled with a fluorophore that can undergo intramolecular fluorescence resonant energy transfer (FRET) with EGFP.[12] Protein **14** is modified with a non-hydrolyzable

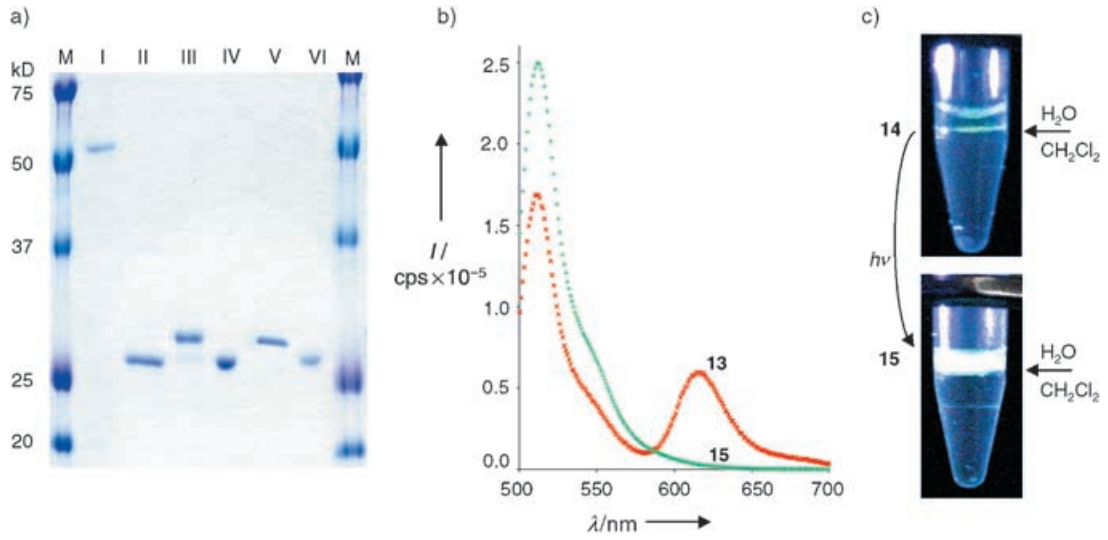

Figure 1. In vitro characterization of the semisynthetic proteins and their photocleaved products. a) SDS-PAGE analysis, Lanes M = marker; I = EGFP-NLS-GyrA-CBD; II = EGFP-NLS α-thioester; III = EGFP-NLS-**1**-Lys-Lys(TR) (**13**); IV = **13** + UV irradiation (**15**); V = EGFP-NLS-**1**-Lys-Lys-(Palm) (**14**); VI = **14** + UV irradiation (**15**). For all samples, UV irradiation was performed with a He–Cd laser at 325 nm for 2 s. Proteins were detected by coomassie staining. b) Fluorescence spectra of protein **13** before and after (→**15**) UV irradiation. Fluorescence excitation was performed at 488 nm. c) Images of test tubes containing protein **14**, before and after UV irradiation, in a two-phase system of water and dichloromethane. An arrow indicates the interface of the two solvents.

lipid, thereby mimicking a widespread post-translational phenomenon found in eukaryotic organisms.[13]

The photocleavage of **13** and **14** was first tested in vitro. Both constructs were irradiated with UV light, and the photolysis products were analyzed by HPLC, ES-MS, and SDS-PAGE. Under the conditions tested, the photolytic conversion was found to be quantitative, with the EGFP-NLS amide **15** (from **13** + UV or **14** + UV) identified as the only proteinogenic product (Figure 1a). The fluorescence spectrum of **13** showed FRET ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 620$ nm) between EGFP and TR with an efficiency of 33%. Protein **13** was used to determine the kinetics and quantum yield of photolysis by using the decrease in FRET intensity as a direct measure of photocleavage between the FRET pair (Figure 1b).[14] Using irradiation at 365 nm and 1 mW cm^{-2}, the kinetics of photolysis was first-order with a half-life of $t_{1/2} = 178$ s and an observed quantum yield of 0.11. Photolysis was quantitative in less than 2 s when a high-intensity laser was used. The behavior of **14** before and after photorelease of the lipid moiety was tested by monitoring the affinity of the protein for a hydrophobic environment. When dichloromethane was added to **14** dissolved in PBS containing 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS, pH 7.5), the protein localized exclusively at the interface of the two solvents (Figure 1c). Upon irradiation with UV light, the EGFP-NLS moiety was released from the interface and diffused in the aqueous phase.

Protein **13** was microinjected into Hela cells cultured on glass slides and live-cell imaging was performed on a confocal microscope. A FRET signal ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 620$ nm) with an efficiency of 30% was observed accompanied with perfect colocalization of the signals from the green ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 525$ nm) and red ($\lambda_{ex} = 565$ nm, $\lambda_{em} = 620$ nm) channels (Figure 2a). Upon

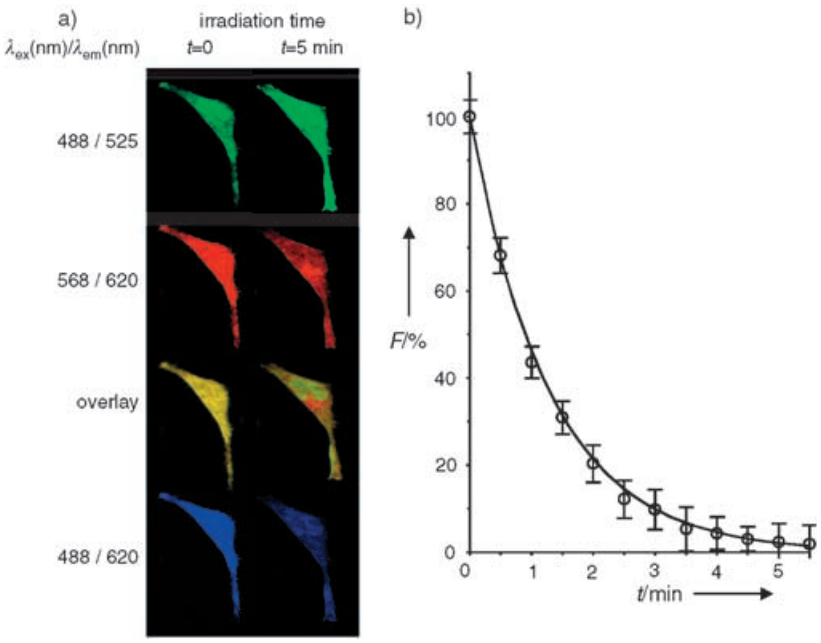

Figure 2. FRET assay. Hela cells were microinjected with **13** and exposed to low-intensity UV light (360 nm) at varying irradiation times. a) Pseudo-color images from confocal microscopy. The fluorescence signal from EGFP ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 525$ nm), Texas Red ($\lambda_{ex} = 568$, $\lambda_{em} = 620$ nm), and FRET between EGFP and Texas Red ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 620$ nm) were recorded before and immediately following UV irradiation. b) Time course of photocleavage. The FRET intensity F (averaged over the cytoplasmic area and normalized at t(irradiation) = 0) is plotted versus time of irradiation, along with an exponential curve of best fit, which yielded a first-order rate constant for photolysis of 1.3×10^{-2} s^{-1}. Error bars represent the standard deviation obtained from the averaging of two experiments.

irradiation of the whole cell at 360 nm (5 μW), the FRET intensity diminished and EGFP fluorescence inversely increased. The observed kinetics of photolysis was first-order with a half-life of $t_{1/2} = 53$ s (Figure 2b). This demonstrates that photolysis of a protein in a cell can be achieved in a dosable manner with low-intensity UV light.[15] This is especially relevant if different concentrations of a photocleaved species would be expected to trigger different responses. Interestingly, the red and green signals accumulated in different subcellular locations after UV irradiation (Figure 2). These results demonstrate that a protein and a small molecule can be photoreleased from one another, thereby triggering changes in their respective function or localization.

The possibility of photocontrolled cellular localization was further explored with protein **14**. It was expected that the modification of EGFP with a lipid would target the protein to cellular membranes.[16] Indeed, when microinjected in Hela cells, **14** localized with great specificity at the plasma membrane, with less than 6 and 2.5% of the total signal present in the cytoplasm and nucleus, respectively (Figure 3).

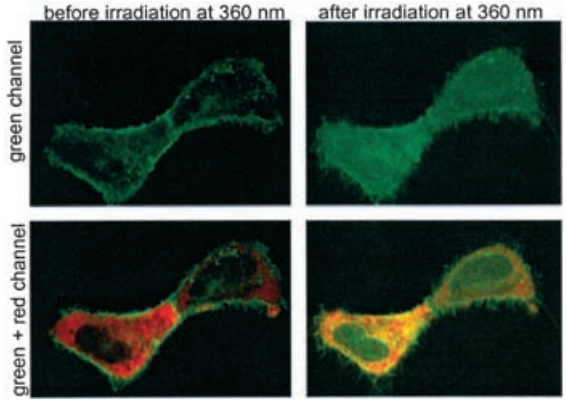

Figure 3. Representative images illustrating the photocontrol of the subcellular localization of a protein. Hela cells were microinjected with **14** and Dextran-TMR (70 kDa). Dextran-TMR was used as a red fluorescent marker that is excluded from the nucleus when injected in the cytoplasm. The images were acquired on a confocal microscope with green ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 525$ nm) and red ($\lambda_{ex} = 568$, $\lambda_{em} = 620$ nm) channels and pseudo-colored. The fluorescence signals were recorded before and immediately following UV irradiation (360 nm).

When the cells were irradiated with UV light, the cleaved product **15** diffused in the cytoplasm and nucleus with respective 11- and 15-fold increases in signal. Even though an NLS was present in the construct, the protein did not accumulate in the nucleus presumably because its low molecular weight (28 kDa) allowed it to diffuse through the nuclear pore complex (NPC).[17] It is therefore interesting to note that the photocaging of a functional lysine present in the NLS sequence might not be sufficient to achieve photocontrol of the nuclear import of a small protein such as EGFP.[18] Indeed, a protein with a molecular weight below the NPC diffusion limit (≈ 40 kDa) might diffuse in and out of the nucleus in both its caged-NLS and uncaged-NLS states. In contrast, using a non-hydrolyzable but photocleavable lipid, EGFP was excluded from the nucleus in the absence of light but underwent free nucleocytoplasmic shuttling after irradiation. Hence, these results raise the possibility of the photocontrol of a protein's activity not by the caging of a critical group required for function but rather by control of its location.[19]

In summary, we have reported the synthesis of a LPR molecule and demonstrated its utility in the synthesis of caged proteins. We demonstrated that a photoactivatable cleavage site can be introduced in a semisynthetic protein containing complex chemical modifications. Furthermore, we showed that photocleavage can be achieved in a dosable manner to generate a controlled amount of cleaved product inside a living cell. Finally, we demonstrated that the localization of an EGFP-NLS construct can be controlled by photochemically releasing a lipid moiety from its C terminus. The chemical diversity that can be introduced by this approach should permit the manipulation of a protein in a variety of ways and the generation of a variety of responses upon photocleavage. Such caging strategies hold potential for the characterization of complex biological processes in their native context, single cells or whole organisms. Direct applications might involve the photoregulation of proteins containing photocleavable localization signals and post-translational modifications.

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