A Toolkit for Manipulating Cellular Behavior: Peptide with Tryptophan-Selective Ru-TAP Complex Regioselectively Photolabeling Specific Protein in Live Cell

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Abstract

Using a chemical approach to crosslink functionally versatile bioeffectors (such as peptides) to native proteins of interest (POI) directly inside a living cell is a useful toolbox for chemical biologists. However, this goal has not been reached due to unsatisfactory chemoselectivity, regioselectivity, and protein-selectivity in *in-cellulo* protein labeling. Herein we report a highly selective photoaffinity labeling (PAL) method using a tryptophan-specific Ru-TAP complex as photocrosslinker (Trp-tag). Aside from the high selectivity, the PAL is blue light driven by a photoinduced electron transfer (PeT) and allows the bioeffector to bear an additional UV-responsive unit. The two different photosensitivities are demonstrated by blue light photocrosslinking a UV-sensitive peptide to POI. The remote-control functionality of the peptide allows POI inhibition after blue light irradiation, and reactivation upon UV photolysis. Cytoskeletal dynamics regulation is demonstrated via the unprecedented *in-cellulo* POI photomanipulation, which opens a new avenue to endogenous protein modification for novel functions.

Introduction

The functional modification of proteins of interest (POI) *in cellulo* is a powerful technique in monitoring their expressions and localizations, as well as manipulating their cellular responses. Genetic code expansion of a POI through incorporating unnatural amino acids is an effective way of expanding protein functionality¹⁻⁴. The approach however is a laborious process, in which orthogonal aminoacyl-tRNA synthetase/tRNA pairs need to be generated prior to the biosynthesis of novel functional proteins with unnatural amino acids at specific sites.

Direct chemical modification on native protein is potentially more straightforward, and may provide more functional and structural versatility than what can be achieved with genetic approach⁵⁻⁷. However, achieving selectivity towards a defined natural amino acid residue on a specific protein within an intracellular environment constitutes a great challenge. Thus, chemical modifications of POIs are commonly performed *in vitro*, to avoid interference of non-targeted proteins. For reducing those off-target reactions with plethora of bionucleophiles such as amines and thiols within cytoplasm, reagents bearing chemoselectivity toward less abundant/reactive amino acids have been intensively developed. This is the first and essential step towards chemical modifications of POI with high-specificity. Although, histidine-⁸⁻¹⁰, tyrosine-¹¹⁻¹⁵, methionine-¹⁶⁻¹⁸ and tryptophan-reactive¹⁹⁻²¹ reagents have been developed for *in vitro* labeling of native proteins, the experimental conditions are not cyto-compatible and have poor protein selectivity; therefore chemical modifications of POI cannot be achieved *in cellulo*.

Photoaffinity labeling (PAL) is a technique that involves using a simple tag-labeled affinity ligand to drive UV photocrosslinkers to label POI for ligand-protein interaction research, particularly for enriching and identifying specific proteins.²²⁻³¹ If a better chemoselective and cytocompatible photocrosslinker could be developed, PAL could be a method to directly conjugate functionally diverse bioeffectors to POI in living cells. The existing PAL has a number of flaws that make it unsuitable for chemically modifying a specific protein in living cells. First, photoinduced conjugation of the affinity ligand to POI is irreversible,

which dysfunctionalizes the POI. Second, the present photocrosslinkers utilized in PAL produce non-chemoselective reactive radical intermediates, which result in unintended heterogeneously modified proteins with reduced or lost activity. Thirdly, the current PAL methods are incompatible with other photochemical functional groups such as fluorophores or photolabile linkers since they only respond to UV light.

Herein, we provide a new PAL method using a polyazaaromatic Ru (II) complex ($\text{Ru}[(\text{TAP})_2\text{phen}]^{2+}$, TAP = 1,4,5,8-tetraazaphenanthrene, phen = 1,10-phenanthroline; Fig. 1) ³²⁻³³ in replacement of conventional UV-initiated photocrosslinker. The use of such a photo-reagent offers several important advantages. 1) It is photo-chemoselective toward the least abundant amino acid tryptophan (therefore called Trp-tag). The photoreaction originates from a thermodynamically favorable PeT process from the POI Trp indole group (electron donor, $E^+/E_{\text{Trp}} = +0.78 \text{ V vs SCE}$) ²⁷ toward the affinity ligand excited Trp-tag (Ru-TAP electron acceptor, $E^{2+*}/E^+_{\text{Ru}} = +1.15 \text{ V vs SCE}$) ³⁴, which is in vicinity of the tryptophan donor. After recombination between the so-produced ionic species and loss of two hydrogen atoms, an adduct of the Trp residue to the Ru-TAP complex is formed, leading to crosslinking of the affinity peptide to the POI (Fig. 1). The conditions for such PeT and crosslinking processes had been examined in details with different systems ²⁷⁻³⁵. 2) The residue specific PeT minimizes off-target labeling and preserves protein integrity compared to classic radical reactions. 3) The PAL needs visible (blue) excitation at 450 nm, compared to current UV excitation. This results in better cytocompatibility and in addition allows the affinity ligand to carry UV-sensitive functional groups.

In the present study, our newly developed PAL method was utilized to conjugate a multifunctional peptide to the Trp sidechain of cAMP-dependent protein kinase catalytic subunit (PKA) ³⁶⁻³⁷. Traditionally the biochemical modifications are performed *in vitro* ^{15, 38-40} but in this work we successfully performed the protein manipulation *in cellulo*. The specific targeting of POI is first demonstrated with cellular lysates and afterward *in cellulo*. Thus, even *in cellulo* conditions, our blue light PAL probe was capable of being photocrosslinked to the targeted tryptophan of PKA under 450 nm illumination, encaging the PKA whose activity could be restored under UV irradiation.

Results

Design and synthesis of PAL bioeffector probes

The designed PAL probe, Probe-1, possesses several functions. It comprises a Trp-tag derived from blue light responsive $\text{Ru}[(\text{TAP})_2\text{phen}]^{2+}$ for photocrosslinking and an affinity ligand (PKI (14-24)) for locating the Trp-tag in close proximity to a PKA Trp which also resulting in activity inhibition after 450 nm illumination (Fig. 1). Additionally, Probe-1 also contains a UV-cleavable linker, which allows for the triggered release of the inhibitor moiety to restore enzyme activity. Probe-2 is the negative control that has PKI (14-24) region scrambled. N-terminal FLAG (DYKDDDDK) tagged Probe-1 and Probe-2 were also

prepared for Western blot analysis. The synthesis steps of Trp-tag and Probes are described in Supplementary information.

Characterization of the PAL Photoadduct

According to the PeT processes²⁷⁻³⁰ with adducts productions described in the literature with the $\text{Ru}[(\text{TAP})_2\text{phen}]^{2+}$ complex⁴¹⁻⁴², the mass of the adducts always corresponds to the sum of the two species minus two hydrogen atoms, which is thus the case with the result obtained by using tryptophan^{33, 43}. The same type of results was thus expected in the Probe-1/PKA system under 450 nm light, with a conjugation site exclusively at W196 near the active site of PKA (**Fig. 2a**).

To examine the occurrence and effect of photocrosslinking, recombinant PKA (rPKA) and probe-1 were illuminated under short blue light irradiation. The photo-induced modifications were analyzed by HPLC. The chromatogram shows drops in both free Probe-1 (peak 1, $t_R = 13.6$ min) and rPKA (peak 2, $t_R = 23.5$ min), while an emerging peak (peak 2*) at $t_R = 23.1$ min increases as the irradiation time increases (**Fig. 2b**). Emerging peak 2* features a distinctive absorbance signal at 440 nm characteristic of the Trp-tag, indicating the formation of a Probe-1/PKA photoadduct. Electrospray Mass Spectrometry (ESI-MS) analysis of peak 1 is consistent with the ionic mass of Probe-1 (2,631 Da, *Supplementary Fig. 3a*). Peak 2 corresponds to protonated PKA (41,060 Da). Peak 2* shows a mass increase to 43,688 Da (*Supplementary Fig. 4*) indicating thus adduct formation. As expected, this mass increase corresponds to that by one probe-1 (2,631 Da) of two hydrogen atoms (leading to 43,688 Da). Thus, exactly what is expected for a photo-adduct formation. This result unambiguously demonstrates that a single photoadduct was successfully formed by blue light-driven PAL.

We then further verified whether PAL occurred at the intended W196 residue close to the PKA active site among the six tryptophan residues. The other tryptophan residues are either remote or buried inside the hydrophobic protein core and thus would remain unreacted. The trypsin-digested Probe-1/PKA solutions were analyzed by HPLC before and after 20 min of blue light-induced PAL (**Fig. 2c**). Isolated fractions were then analyzed by Matrix-Assisted Laser Desorption ionization Mass Spectrometry (MALDI MS). The peaks cluster **a** in the HPLC chromatogram was identified as mixture of fully and partially digested Probe-1 (*Supplementary Fig. 5 – 6*). Before irradiation, four peptides containing W30, W296/302, W196, and W221/222, are noted as HPLC peaks 1 – 4, respectively (*Supplementary Fig. 7 – 10*). The W196-containing fragment (peak 3; $t_R = 42.7$ min), where PAL was expected to occur, was further characterized by tandem MS before irradiation (*Supplementary Fig. 11*). The intensity of peak 3 notably decreases under blue light irradiation, and a new peak appears within the cluster (peak 3*; $t_R = 38.7$ min), while peaks 1, 2, and 4 remain throughout the irradiation process. This result clearly indicates that PAL occurred on the W196 fragment. Further tandem MS results of peak 3* confirm that labeling took place on the W196 residue (*Supplementary Fig. 12*).

Examining Protein-Selectivity of PAL in Cell Lysate

To examine whether the blue light PAL is protein-selective in the complex cellular micro-environment, the method was applied to a rPKA spiked in cell lysate. The two FLAG-tagged probes, FLAG-Probe-1 and FLAG-Probe-2, were subjected, after their use in PAL, to SDS-PAGE analysis and anti-PKA/anti-FLAG Western blotting (WB). The FLAG epitope on FLAG-Probe-1 did not affect the binding affinity for PKA, as evidenced by the kinase activity assay (*Supplementary Fig. 14*). The molecular weight of the PAL photoadducts generated with pure rPKA was increased from 44 kDa to 47 kDa which corresponds to one peptide photoadduct (lane 2, **Fig. 3a**), and after UV photolytic removal of the PKI moiety (lane 3, **Fig. 3a**) the molecular weight of 44 kDa was recovered. The negative control with FLAG-Probe-2 resulted in no PAL event (lane 5, **Fig. 3a**).

When analyzing the PAL reaction using rPKA spiked in cell lysate, the only extra band clearly visualized corresponded to the photoadduct as shown by the anti-PKA and anti-FLAG WB (lane 2 in **Fig. 3b**). Furthermore, the UV-triggered release of the PKI moiety was confirmed by FLAG-tag signal loss in lane 3 in **Fig. 3b**. Accordingly, the results demonstrate that the PAL event is highly PKA-selective regardless the presence of other cellular proteins.

The protein selectivity was further demonstrated in a more complex system, i.e. a cell lysate only containing endogenous PKA catalytic subunit (ePKA), the concentration of which is much less than spiked rPKA. ePKA is associated with regulatory subunits (R) as a heterotetramer (2C-2R), and its kinase activity is sequestered until secondary messenger cyclic-AMP (cAMP) triggers the release of ePKA from the heterotetramer⁴⁴. Therefore, a cell-permeable cAMP analogue, CPT-cAMP, was first incubated with MCF7 cells to liberate ePKA before harvesting the cell lysate. Subsequently, FLAG-Probe-1 was added for the PAL experiment. Anti-PKA WB analysis clearly showed a band appearing at the ~44 kDa position characteristic of the photoadduct (lane 6, **Fig. 3c**). Such band was not observed in any other lanes and disappeared upon UV irradiation (lane 7, **Fig. 3c**). Without CPT-cAMP treatment, PAL did not occur since the probe binding site of ePKA was occupied by the R subunit and remained unavailable for PAL (lane 3, **Fig. 3c**). As expected, the negative control FLAG-Probe-2 showed no PAL effect (lanes 8–10, **Fig. 3c**). In conclusion, the anti-FLAG WB also supported the fact that FLAG-Probe-1 could specifically bind to ePKA *in the complex biological mixture of cell lysate*.

The Caging and Uncaging of PKA Activity Through PAL and Photolytic Ligand Release

For investigating probe-1 concentration effect, 5 μ M PKA were incubated with Probe-1 at different concentrations to study the PKA activity in the dark, under blue light illumination (10 min, chosen from the results of **Fig 4c**), or subsequent UV (2 min) irradiation after blue light (**Fig. 4a**). Without irradiation (dark), increased concentration of Probe-1 from 0 to 20 μ M only caused a slight decrease in PKA activity due to the weak inhibitory effect of Probe-1. PKA activity was effectively lowered (caged) to 20% when

exposed to blue light at 10 and 20 μ M of Probe-1. After subsequent UV irradiation, the kinase activity returned (uncaged) to over 60%. For investigating the illumination time influence, 10 μ M PKA was incubated with 20 μ M Probe-1 under different blue light illumination times and subsequent UV irradiation (**Fig. 4c**). From 10 min illumination and beyond, a saturated caging effect was obtained with an activity recovery around 70% after UV illumination. The same experiments with Probe-2 at different concentrations and illumination times (**Fig 4b,d**) only showed a slight overall decrease in activity with blue light irradiation without indication of activity restoration upon UV irradiation. This suggests that proximity-driven labeling by probe-1 is essential for successful regioselective PKA modification.

In Cellulo PAL and Uncaging of PKA Using Two Wavelengths Light in Manipulation of Cytoskeleton Dynamics

Knowing that Probe-1 could chemically modify PKA via the established PAL method in a cytocompatible and chemo-, regio-, and protein-selective fashion, the next step was to demonstrate whether the same photo-treatments could affect activity of endogenous PKA (ePKA) *in cellulo*. Because phosphorylation of vasodilator-stimulated phosphoprotein (VASP) is critical in cytoskeletal dynamics and is regulated by the PKA pathway, the phosphorylation process was examined after PAL of ePKA with Probe-1 ⁴⁵⁻⁴⁷.

We first examined the effects of PAL on exogenous rPKA, which we could micro-inject more of to see a more substantial effect in living cells. Microinjected rPKA in the presence of Probe-1 in the dark should still possess significant activity because of the weak inhibitory effect of noncovalently attached Probe-1. As expected, 100% of the microinjected cells (Dark, n=32) showed a strong increase in VASP phosphorylation (11 times higher) compared to control (non-microinjected) cells (Ctrl, n = 27) after 1 h of incubation (**Fig. 5a,d**). After blue light irradiation, PKA–Probe-1 caged photoadduct was generated and the VASP phosphorylation could not be induced, 70% microinjected cells (Caged, n=50) were found to have a low signal of phosphorylated VASP (pVASP) similar to that of control (non-microinjected) cells (Ctrl, n=26) after 1 h of recovery incubation (**Fig. 5b,e**). This indicates that the PKA had been successfully caged in the living cells by blue light irradiation. Moreover, blue light irradiation followed by UV exposure led to 74% of microinjected cells (Uncaged, n = 33) having elevated pVASP signals (9 times higher) compared to the control (non-microinjected) cells (Ctrl, n = 34). This indicates successful PKA activity uncaging in living cell upon subsequent UV irradiation after 1 h of recovery incubation (**Fig. 5c, f**).

In a second step, we tested the *in cellulo* modification with endogenous PKA. In that case, only Probe-1 was micro-injected into REF52 fibroblast cells ⁴⁸⁻⁵⁰. After microinjection, all REF52 cells were incubated with CPT-cAMP-containing medium for 10 min to liberate ePKA from its R subunit for the *in cellulo* PAL. In the dark, 90% microinjected cells (Dark, n=41) showed increased pVASP levels similar to those of non-microinjected control cells (Ctrl, n=40) (**Fig. 5g, j**), indicating that Probe-1 did not photoreact with ePKA in the absence of irradiation. Blue light irradiation led to 76% microinjected cells (Caged, n=41) displaying 8-times pVASP intensity decrease relative to the control cells (Ctrl, n=40) (**Fig. 5h, k**). This suggests that

ePKA was caged through the Probe-1 PAL, and thus kinase activity was suppressed. Then a 10 min CPT-cAMP-free medium incubation was applied to revert the global ePKA responses (*Supplementary Fig. 17*). Afterward, a UV irradiation led to 71% microinjected cells (Uncaged, n=25) displaying a 3-times higher pVASP level than that of the control cells (Ctrl, n=40) (**Fig. 5i, l**). This demonstrates a successful ePKA chemical modification and activity manipulation *in cellulo*.

Discussion

In this study, we successfully demonstrated blue light-induced chemoselective and regioselective PAL of a POI (PKA) by a functional peptide Probe-1. Moreover, the results highlight that protein conjugation utilizing Trp-tag could be generally applicable for proximity-driven protein chemical modification directly inside living cells. *In vitro* experiments revealed that affinity binding brings the photoreactive Trp-tag unit of Probe-1 to the close proximity of the PKA active site to conjugate to Trp196, while the other five distal tryptophan residues remain unreacted. No off-target protein adduct was observed when PKA was in the presence of other intracellular predominant proteins, as analyzed in cell lysates WB experiments. As expected, PKA after PAL by Probe-1 at 450 nm yielded a “pseudo-irreversibly inhibited” enzyme which can be reactivated at intended timing by photolytic dissociation of the inhibiting moiety under UV, as confirmed by kinase assays. These significant positive results encouraged us using this methodology to chemically modify PKA in living cells and further manipulate PKA signal transduction. Microinjection of Probe-1 along with exogenous PKA and blue light irradiation suppressed the VASP phosphorylation. A subsequent UV light exposure at an intended timing uncaged the PKA activity and lead again to the PKA induced VASP phosphorylation. In addition, we also tested *in cellulo* the PAL possibility of endogenous PKA, which is more challenging due to its low abundance. Thus, when ePKA of all the cells was globally liberated by CPT-cAMP, blue light PAL by Probe-1 successfully suppressed the VASP phosphorylation and subsequent UV irradiation resulted in ePKA activity restoration with again a higher pVASP level.

In conclusion, photoaffinity-based labeling using Trp-tag expands the chemical toolkit for protein modification. Indeed, this new PAL allows the incorporation of a peptide at a desired site of POI. This generates functionally modified and expanded proteins in the cytosol of living cells via an easy chemical approach and provides an alternative to genetic code expansion approaches. We envision our novel strategy for light-controlled intracellular chemical modification of proteins in view of altering cellular behavior.

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Figures

Figure 1

A schematic representation of intracellular protein- and its residue-specific blue light photoaffinity labeling, leading to caged protein and can be reactivated by subsequent UV light to regulate its signal transduction pathway. In the present study, PKA was used as a model protein to demonstrate the feasibility of in cellulo POI modification using an easy to make peptide / Trp-tag conjugate. The chemical structure of Probe-1, which is the PAL probe designed to target PKA, is as illustrated. The UV orthogonality of this blue light photocrosslinker Trp-tag allows the installation of a UV-responsive motif, which is a photocleavable linker, on the probe. Upon the binding of the PAL probe to PKA, 450 nm blue light irradiation induces a PeT followed by adduct formation specifically between the proximate tryptophan (W196) of PKA and the Trp-tag of the peptide. The activity of the resultant PKA–Probe-1 photoadduct is temporarily halted; activity can be resumed at the desired time by subsequent UV irradiation to phosphorylate its downstream substrates, such as vasodilator-stimulated phosphoprotein (VASP). For comparison, the PKI (14-24) was replaced by a scrambled sequence (RGG AIDTHRNR), which served as the negative control (Probe 2).

Figure 2

Identification of PKA–Probe-1 photoadduct and the modified residue. a, Schematic presentation of the process where PKA (left) forms photoadduct upon blue light irradiation (middle) with Probe-1. The photoreaction products then underwent trypsin digestion (right) for identification of PKA residue labeled by Probe-1. b, A solution mixture composed of 10 μ M PKA and 10 μ M Probe-1 was irradiated with blue light for 0, 2, 5, or 10 min, followed by HPLC analysis. Chromatograms were recorded at 212 nm for amide bond detection (blue), and 440 nm for Ru-TAP complex detection (red). Three major peaks in the chromatograms correspond to Probe-1 (peak 1, $t_R = 13.6$ min), PKA (peak 2, $t_R = 23.5$ min), and the photoadduct (peak 2*, $t_R = 23.1$ min), respectively. c, HPLC analysis of the trypsin-digested sample solution (50 μ L) composed of 10 μ M PKA alone (top) or in the presence of 20 μ M Probe-1 before (middle) or after 20 min of blue light irradiation (bottom). The chromatograms were recorded at 280 nm absorbance, and the peak-of-interest is indicated by a dashed line.

Figure 3

Western blotting analysis of recombinant and endogenous PKA after PAL with FLAG-Probes. To demonstrate the highly selective feature of the developed PAL method for a specific POI, (a) a pure solution of recombinant PKA (rPKA, 2.5 μ M) was exposed to 5 μ M of either FLAG-Probe-1 or FLAG-Probe-2, in dark, irradiated with blue light, or irradiated with blue light followed by UV light. The changes were then detected with anti-PKA and anti-FLAG antibodies, and the total proteins in each lane were visualized by Coomassie Brilliant Blue (CBB) staining. (b) The same process was also applied to cell lysate spiked with rPKA. (c) For successful PAL of endogenous PKA (ePKA), the proteins were first released from their heterotetramer form by treating MCF-7 cells with 200 μ M of CPT-cAMP. The subsequent cell lysate (2 μ g/ μ L) was then mixed with either 2 μ M of FLAG-Probe-1 or FLAG-Probe-2, followed by exposing the mixtures to the same light exposure pattern, before subjected to WB analysis and CBB staining.

Figure 4

Kinase assay of probe-modified PKA under different conditions. Various concentrations of a, Probe-1 or b, Probe-2 were mixed with 5 μM PKA and kept in the dark or exposed to 450 nm light for 10 min (blue light, caging), or further irradiated under 360 nm light for additional 2 min (blue+ UV light, uncaging). Afterwards, 10 μM PKA and 20 μM of either c, Probe-1 or d, Probe-2 were irradiated with 450 nm light at different time intervals (blue dot) and further exposed to 360 nm light for 2 min (purple dot). A control sample with 10 μM PKA alone was used to define 100% activity. The activity difference (Δ activity) between caged and uncaged PKA at different 450 nm irradiation times were calculated and plotted as function of the irradiation time (red line).

Figure 5

PAL probe-mediated FMPIC of PKA by two-color light irradiation and visualized by VASP phosphorylation. REF52 fibroblasts were microinjected with a solution containing 50 μ M rPKA and 50 μ M Probe-1, along with 10 μ M TAMRA-dextran as an indicator for cells being injected. Cells were then either a, kept in the dark, or b, immediately irradiated 10 min under 450 nm blue light for intracellular PKA caging, and c, successively exposed to 360 nm UV light 2 min for PKA uncaging. The cells were then incubated for 1 h and fixed with 4% paraformaldehyde. pVASP, actin fiber, and nucleus of the fixed cells were then stained using pVASP antibody, Alexa488-phalloidin, and DAPI, respectively. Arrows indicate microinjected cells based on TAMRA-Dextran fluorescent indicator. Quantification of pVASP fluorescence intensities of microinjected cells (arrow heads) in comparison with non-microinjected control cells (Ctrl) are presented in d, Dark (n = 32), e, Cage (n = 35), and f, Uncage (n = 33). * $p < 0.005$. REF52 cells were microinjected with a solution containing 100 μ M Probe-1 and 10 μ M TAMRA-dextran. Cells were then treated with medium containing 250 μ M CPT-cAMP followed by 10 min incubation g, in the absence of light (Dark), or h, under blue light irradiation for intracellular caging. i, The blue light irradiated cells were followed by 10 min incubation in cAMP-free medium and then exposed to UV light for 2 min (Uncaging). Cell fixation and staining, and j – l, quantification of pVASP fluorescence intensities are as described in a.

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