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A photoactivatable self-localizing ligand with improved photosensitivity for chemo-optogenetic control of protein localization in living cells

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ABSTRACT

Light-mediated control of protein localization in living cells is a powerful approach for manipulating and probing complex biological systems. By incorporating a classical 6-nitroveratryloxycarbonyl (NVOC) caging group into the inner plasma membrane (PM)-localizing trimethoprim ligand, we recently developed a photoactivatable self-localizing ligand (paSL) that can rapidly recruit engineered *Escherichia coli* dihydrofolate reductase-fusion proteins from the cytoplasm to the PM upon violet (ca. 400 nm)-light illumination. However, because the photosensitivity of the NVOC-caged paSL is low to moderate, photouncaging experiments require high light intensity, which may not be ideal for many cell applications. Herein, we present a new 7-diethylaminocoumarin (DEAC)-caged paSL with improved photosensitivity. DEAC-caged paSL induced efficient protein recruitment upon violet-light irradiation, even at the low intensity under which NVOC-caged paSL does not respond. DEAC-caged paSL was insensitive to excitation light used to image green fluorescent proteins (GFPs), and it was applicable for simultaneous optical stimulation of Gαq signaling and fluorescence imaging of subsequent Ca^{2+} oscillations using a GFP-based Ca^{2+} biosensor in living cells.

Synthetic molecular systems that can rapidly manipulate specific biological processes in living cells at the post-translational level would be beneficial to interrogate cell signaling networks.[1–3] Light-induced protein translocation is one of the most promising strategies for manipulating spatiotemporal cell signaling because cells control various signaling activities at specific organelles by precisely regulating protein translocation.[4–5] As such, to control protein translocation, considerable efforts have been devoted to developing optogenetic and chemo-optogenetic methods using photoreceptor proteins[6–10] and photocaged chemical dimerizers,[11–15] respectively. However, because these techniques rely on light-induced protein heterodimerization, complicated experimental setups are required to control protein localization while observing subsequent molecular processes in living cells by fluorescence imaging. Additionally, adjusting the expression levels and stoichiometry of two-protein components (i.e., the dimerization pair) and genetically encoded fluorescent reporters is technically demanding.

To overcome this limitation, we recently developed a novel, single-protein component chemo-optogenetic approach, called paSLIPT (photoactivatable self-localizing ligand-induced protein translocation), that enables optical control of protein localization using a photoactivatable self-localizing ligand (paSL).[16] In particular, we developed a paSLIPT system targeting the plasma membrane (PM) as a tool for the spatiotemporal manipulation of signaling processes at the inner leaflet of the PM (Fig. 1a). In this system, the 6-nitroveratryloxycarbonyl (NVOC)-caged trimethoprim (TMP) ligand tethered to the myristoyl-^{D}Cys lipopeptide,[17] termed $m^{D}cTMP^{NVOC}$, is used as a PM-targeted paSL. A protein of interest is fused to the hexalysine-appended *Escherichia coli* dihydrofolate reductase (^{K6}DHFR) tag[18–20] and is expressed in the cell cytoplasm. Upon incubation with cells expressing a ^{K6}DHFR-fusion protein, paSL $m^{D}cTMP^{NVOC}$ spontaneously localizes to the inner PM with no protein binding. However, under violet (405 nm)-light illumination, the NVOC photocage is removed to produce an uncaged PM-anchored TMP ($m^{D}cTMP$), which recruits the target protein from the cytoplasm to the irradiated PM area. The $m^{D}cTMP^{NVOC}$/^{K6}DHFR-based paSLIPT system is applicable to the spatiotemporal control of signaling processes in living cells. The system can also be used in combination with fluorescent reporters of various colors. However, because the photosensitivity of the original $m^{D}cTMP^{NVOC}$ is low to moderate, the uncaging experiment requires high light intensity, which may not be ideal for many biological applications using live cells. For example,

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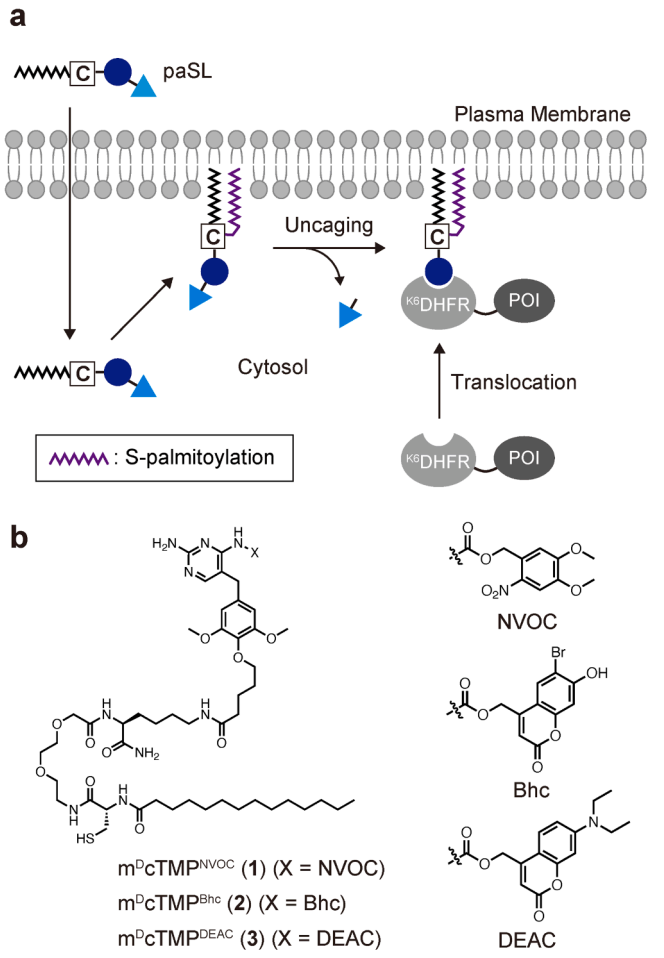

Fig. 1. The chemo-optogenetic paSLIPT system. (**a**) Schematic illustration of the paSLIPT system. (**b**) Chemical structures of paSLs used in this study.

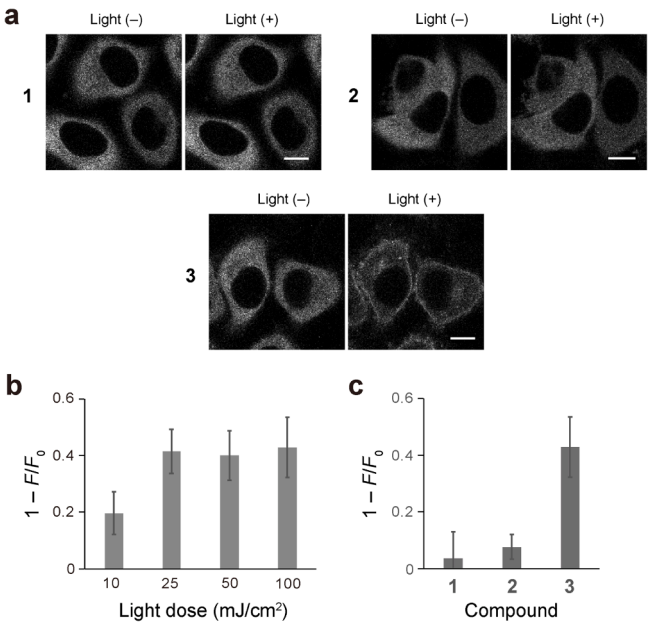

Fig. 2. Evaluation of paSLs with different caging groups. (**a**) Light-induced PM recruitment of ^{K6}DHFR-mScarlet-NES in cells. HeLa cells expressing ^{K6}DHFR-mScarlet-NES were treated with **1**–**3** (10 μM, 0.1% DMSO) for 5 min, washed, and subjected to confocal fluorescence imaging and light stimulation. Images were taken before and 5 min after light irradiation (400 nm). Scale bars, 10 μM. (**b**) Light-dose dependence of PM translocation of ^{K6}DHFR-mScarlet-NES by m^{D}cTMP^{DEAC} (**3**). (**c**) Comparison of light-induced ^{K6}DHFR-mScarlet-NES translocation efficiency using **1**–**3** under 400-nm light irradiation (25 mJ/cm^2). Protein translocation efficiency was quantified using $(1 - F/F_0)$, where F_0 refers to the cytoplasmic fluorescence intensity of ^{K6}DHFR-mScarlet-NES at 0 s (before light irradiation), and F refers to that at 300 s after light irradiation. Data are presented as the mean ± SD (n = 10 cells).

photostimulation experiments using m^{D}cTMP^{NVOC} often cause photobleaching of fluorescent reporters expressed in cells (depending on the fluorescent protein used). Herein, we present an improved paSLIPT system with high photosensitivity.

The NVOC photocage has an absorption maximum at approximately 360 nm, with very low absorption at approximately 400 nm. We therefore sought to replace the NVOC photocage of m^{D}cTMP^{NVOC} with other caging groups to improve the photosensitivity of m^{D}cTMP^{NVOC} to violet light. For this purpose, we chose 6-bromo-7-hydroxycoumarin (Bhc)[21] and 7-diethylaminocoumarin (DEAC)[22] caging groups, which have absorption spectra red-shifted from that of the NVOC group. We first synthesized Bhc- and DEAC-caged TMP derivatives and measured their absorption spectra (Fig. S1). As expected, the absorbance of the Bhc- and DEAC-caged TMP ligands at 400 nm was much higher than that of the NVOC-caged counterpart.

We next synthesized a series of paSLs, m^{D}cTMP^{NVOC} (**1**), m^{D}cTMP^{Bhc} (**2**), and m^{D}cTMP^{DEAC} (**3**), by connecting photocaged TMP to the myristoyl-^{D}Cys lipopeptide motif through a flexible 8-amino-3,6-dioxaoctanoic acid (Adox) linker (Fig. 1**b**).[23] To test and compare the utility of **1**–**3** as paSLs, we used human epithelial HeLa cells expressing ^{K6}DHFR fused to the red fluorescent protein mScarlet-I[24] and a nuclear exporting signal (NES)[25] (^{K6}DHFR-mScarlet-NES) (Fig. S2). ^{K6}DHFR-mScarlet-NES-expressing cells were treated with paSL (10 μM) for 5 min, washed, and then observed using a confocal laser-scanning microscope. For all paSLs, ^{K6}DHFR-mScarlet-NES was observed in the cytoplasm in cells before light irradiation (Fig. 2**a**). When we stimulated m^{D}cTMP^{DEAC}-treated cells under 400-nm light illumination, ^{K6}DHFR-mScarlet-NES was translocated from the cytoplasm to the PM (Fig. 2**a**). m^{D}cTMP^{DEAC}-mediated protein translocation efficiency was light dose-dependent and reached a maximum value at the light dose of 25 mJ/cm^2 (Fig. 2**b**). Under the same experimental condition, only marginal PM recruitment of ^{K6}DHFR-mScarlet-NES was observed in m^{D}cTMP^{Bhc}-treated cells, while no protein translocation occurred in m^{D}cTMP^{NVOC}-treated cells (Fig. 2**a** and Fig. 2**c**). These results indicate that m^{D}cTMP^{DEAC} serves as the paSL with the highest photosensitivity toward 400-nm light irradiation among the three compounds studied here.

Various fluorescent protein (FP)-based reporters and biosensors are currently available for investigating the dynamics of signaling molecules in living cells.[26–27] Light-based perturbation tools that are capable of photochemically stimulating specific signaling pathways while allowing subsequent visualization of molecular processes using these FP reporters would be highly beneficial. m^{D}cTMP^{DEAC} can be uncaged with high efficiency using violet (400 nm) light and is insensitive to light in the wavelength range used to excite green fluorescent proteins (GFPs) and other red-shifted FPs. We next sought to demonstrate the utility of the m^{D}cTMP^{DEAC}-based paSLIPT system as a tool to achieve simultaneous light-induced signal control and visualization of FP reporters in the same cell. For this purpose, we attempted to activate the Gαq signal and visualize subsequent calcium oscillations using a GFP-based fluorescent biosensor. The Gαq protein is one of the isoforms of heterotrimeric G proteins and is an activator of phospholipase Cβ (PLCβ).[28] Activation of Gαq triggers Ca^{2+} release from the endoplasmic reticulum to the cytoplasm and subsequent Ca^{2+} oscillations.[29] To develop a Gαq photoactivation system, a palmitoylation-deficient constitutively active form of Gαq[30] was fused at the *C*-terminus to mScarlet-tagged ^{iK6}DHFR[19] (mScarlet-^{iK6}DHFR-Gαq) (Fig. 3**a** and Fig. S2). The synthetic Gαq was co-expressed in HeLa cells with G-GECO, a genetically encoded green fluorescent Ca^{2+} indicator.[31] After the cells were treated with

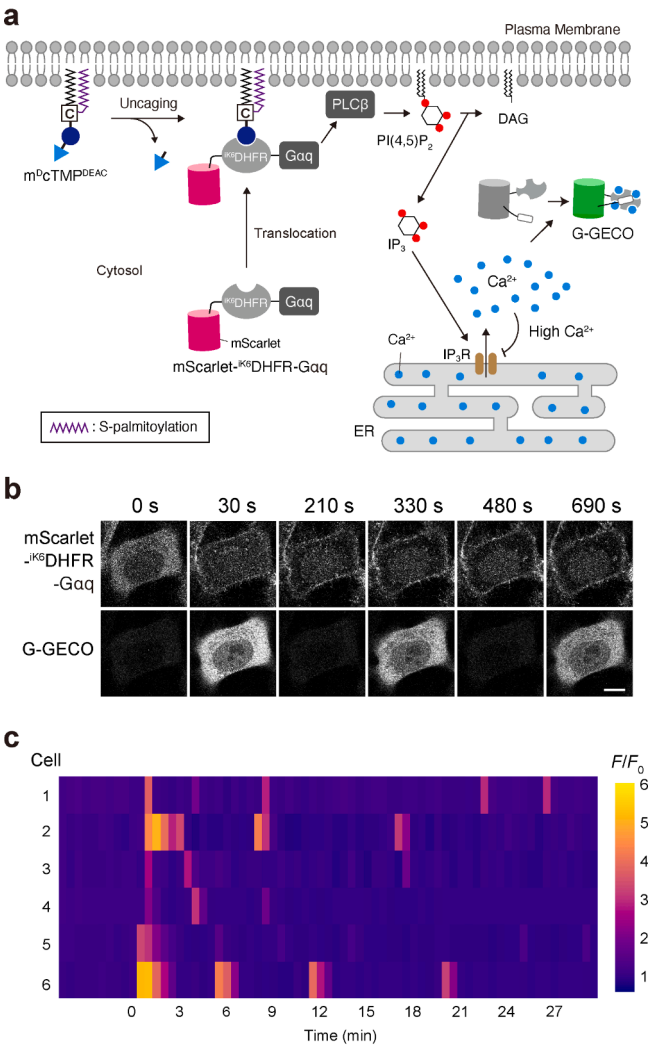

Fig. 3. Chemo-optogenetic activation of Gαq signaling and calcium oscillations. (**a**) Schematic illustration of the experimental setup. (**b**) Representative time-lapse confocal fluorescence images of a HeLa cell expressing mScarlet-^{iK6}DHFR-Gαq and G-GECO. Cells were treated with m^{D}cTMP^{DEAC} (1 μM, 0.1% DMSO) for 5 min, washed, and subjected to confocal fluorescence imaging and light stimulation. Images were taken before and after 400-nm light irradiation. Scale bar, 10 μm. (**c**) Heatmaps depicting light-induced Ca^{2+} oscillations for 6 randomly selected cells expressing mScarlet-^{iK6}DHFR-Gαq and G-GECO. Normalized fluorescence intensities (F/F_0) of G-GECO in the cytoplasm are shown.

m^{D}cTMP^{DEAC}, they were illuminated with 400-nm light (100 mJ/cm^2). Under the light-illumination condition, no bleaching of the baseline GFP fluorescence was observed. Meanwhile, upon photostimulation, mScarlet-^{iK6}DHFR-Gαq was rapidly translocated from the cytoplasm to the PM within 30 *sec*, which induced GFP fluorescence blinking, indicative of Ca^{2+} oscillations (Fig. 3**b,c**). In contrast, light-induced PM recruitment of a PLCβ-binding-deficient Gαq mutant (Fig. S2)[30] did not induce Ca^{2+} oscillations (Fig. S3**a**). Under light irradiation of mScarlet-^{iK6}DHFR-Gαq-expressing cells without m^{D}cTMP^{DEAC} treatment, protein translocation and Ca^{2+} oscillations did not occur (Fig. S3**b**). Thus, we demonstrated light-induced PM recruitment of synthetic Gαq and subsequent visualization of Ca^{2+} oscillations using the GFP-based biosensor in living cells. In addition, as described above, no decrease in GFP fluorescence was observed before and after photostimulation. Because GFP-based fluorescent biosensors tend to undergo photobleaching to some extent under the condition used for uncaging m^{D}cTMP^{NVOC} (400-nm light of >300 mJ/cm^2), the present result also demonstrated the superiority of m^{D}cTMP^{DEAC} as a tool for paSLIPT with improved photosensitivity.

In conclusion, we synthesized three myristoyl-^{D}Cys-tethered photoactivatable TMP ligands containing different photocaging groups and evaluated the ligands in terms of *in cellulo* light-induced protein recruitment efficiency. Among them, m^{D}cTMP^{DEAC} (**3**) recruited ^{K6}DHFR-fusion proteins to the PM with the highest efficiency at a low dose of 400-nm light. The present work illustrates that in paSLIPT systems, photochemical properties such as absorption wavelength and light sensitivity can be rationally and readily tuned by modularly changing the photocaging group of the paSL, which is difficult to achieve in optogenetic systems using photoreceptor proteins. By taking advantage of this modular design and using other photocaging groups, a set of paSLs that respond to various (more red-shifted) wavelengths of light may be generated.[32–34] Moreover, in this work we demonstrated that the m^{D}cTMP^{DEAC}-based paSLIPT system can be used for photochemically controlling protein recruitment and signaling activities in living cells. We also showed that m^{D}cTMP^{DEAC} is compatible with GFP-based fluorescent reporters. Given the current availability of various GFP-based biosensors used to visualize various targets, such as biomolecules, ions, second messengers, and protein activities, the combined use of the m^{D}cTMP^{DEAC}-based paSLIPT system and GFP-based biosensors (and other biosensors based on FPs including yellow to infrared red FPs) would provide a new attractive approach for the study of cell signaling networks.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors are co-inventors of a patent application related to this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2022.128865.

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