Repeat Module-Based Rational Design of a Photoswitchable Protein for Light-Driven Control of Biological Processes

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Abstract: Light-driven control of biological processes using photoswitchable proteins allows high spatiotemporal interrogation or manipulation of such processes, assisting in understanding their functions. Despite considerable advances, however, the wide spread use of optical control has been hampered by a limited repertoire of photoswitchable proteins and a lack of generalized design strategy. Herein, we present a repeat module-based rational design of a photoswitchable protein composed of LRR (Leucine-rich repeat) modules using azobenzene as a photochromic ligand. Our design approach involves the rational selection of a $C_\beta$ pair between two nearby modules within a convex region and subsequent cross-linking with a photochromic ligand. We demonstrate the general utility and potential of our strategy by showing the design of three target-specific photoswitchable proteins and a light-driven modulation of the cell signaling. With an abundance of LRR proteins in nature, our approach can expand the repertoire of photoswitchable proteins for light-driven control of biological processes.

Light-driven control of biological processes using a photoswitchable protein allows spatiotemporal interrogation or manipulation of such processes, providing a fundamental insight such as demonstration of neural networks by regulating the action potential using channel rhodopsins.\textsuperscript{[1–3]} Various photosensory domains derived from naturally occurring proteins have been used for the optical control of such processes through relocalization, sequestration, fragment complementation, induced avidity, or allostery.\textsuperscript{[4–6]} Meanwhile, photochromic ligands such as azobenzene have been employed in the construction of a photoswitchable protein for the optical manipulation of protein–protein interactions.\textsuperscript{[4,5]} Photochromic ligands undergo a reversible cis–trans isomerization in response to light, inducing a conformational change in proteins when properly conjugated.\textsuperscript{[7,8]} Despite many advances, however, the use of an optical control system has been hampered by a limited repertoire of photoswitchable proteins and difficulties in the engineering of photosensory domains or photochromic ligands.\textsuperscript{[1,2,5,6]} Furthermore, the design of photoswitchable proteins has usually required painstaking optimization processes in terms of structural stability, binding affinity, and light-responsive switchability, resulting in low success rates.\textsuperscript{[1,2,5–7]}

We previously developed a protein scaffold, termed repebody, composed of LRR (Leucine-rich repeat) modules.\textsuperscript{[8]} Like other LRR proteins, the repebody comprises a concave face of a $\beta$-sheet and a convex side of the helix array, which are responsible for recognizing a cognate target and maintaining the solenoid structure, respectively, showing high physicochemical stability.\textsuperscript{[8–10]} (Scheme 1a). Herein, we present a generalized strategy to design a photoswitchable protein composed of LRR modules for light-driven control of biological processes. The repebody and an azobenzene derivative, BSBCA (3,3'-bis (sulfonate)-4,4'-bis (chloroacetimido), were used as a model protein scaffold and a photochromic ligand, respectively (Scheme 1b).\textsuperscript{[11]} BSBCA is known to reversibly undergo cis and trans conformations when exposed to near UV (370 nm) and green light (500 nm). Based on the structural and geometric analyses of the repebody and BSBCA, our approach involves the rational

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\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{scheme1.png}
\caption{Design scheme for a photoswitchable repeat protein. a) Structure of a repebody scaffold composed of LRR modules. b) Schematics for a light-responsive conformational change of a photoswitchable repebody by a photochromic ligand. c) Change in chemical structure of BSBCA during a light cycle.}
\end{figure}
selection of a Cß pair between two nearby modules within a convex region, followed by cross-linking with BSBCA to induce a conformational change in the repebody through a light cycle (Scheme 1c).

First we searched for potential Cß pairs to conjugate BSBCA between two nearby modules according to the following criteria: 1) To avoid the steric hindrance of BSBCA on the binding of a target, the candidate conjugation sites are limited to a convex region of the repebody. 2) To increase the efficiency of conjugation, the length of a Cß pair is restricted to 16 to 18 Å, the length of trans-BSBCA, and the bulky residue on the line between two Cß of a pair should be at a minimum.

To obtain insight into the selection of the Cß pair for incorporating BSBCA and consequently inducing a conformational change of a repebody, we evaluated the potential photoswitchability of the selected pairs through molecular dynamics (MD) simulations (Figure S1). Interestingly, Cß pairs for the conjugation of BSBCA diagonal to the intermodular interface of two consecutive modules exhibited a distinct resistance pattern with respect to distance compared to other pairs. As shown in Figure 1a, the Cß pairs for a diagonal conjugation revealed small resistances when compared to the resistance of pair for a perpendicular conjugation to the intermodular interface. Moreover, the pairs, which are located at equivalent diagonal positions of the pair 76/102 in other consecutive modules, showed almost the same resistance patterns as that of the pair 76/102 (Figure S2).

Based on the result, it is likely that the intermodular conjugation of BSBCA to the equivalent diagonal positions of the pair 76/102 in consecutive modules of a repebody effectively induces a light-responsive conformational change of the resulting repebody.

To assess the utility of our design strategy, we introduced cysteine mutations of several pairs into a human IgG-specific repebody (D10), which was previously developed. We selected two Cß pairs, H76C/N102C and H76C/G103C, as typical candidates for the diagonal conjugation of BSBCA. In addition, Q122C/K150C and Q146C/E174C were selected as equivalent pairs to H76C/N102C. For comparison, D77C/S123C was also selected for the perpendicular conjugation of BSBCA. Variants of five Cß pairs were constructed using D10, followed by the conjugation of BSBCA. We purified the resulting variants, and checked the conjugation efficiency of BSBCA (Figure S3). All of the variants revealed a single peak shift in their mass spectra corresponding to a 1:1 ratio between BSBCA and a repebody. This result implies a high cross-linking possibility of BSBCA with two cysteine residues in each variant. Next, we examined whether BSBCA itself conjugated to the repebody variants can undergo isomerization in response to the light. The photoswitching of conjugated BSBCA was checked by illuminating at 370 nm to induce a structural transition of BSBCA between the trans and cis forms. As a result, all of the variants revealed cis isomerization of BSBCA, leading to a reduced absorbance of their trans state (Figures 1b and S4). As predicted through the MD simulations, conjugated BSBCA of H76C/N102C variant exhibited much more switching than that of D77C/S123C variant. Based on the result, the diagonal conjugation of BSBCA can be more effective for inducing a light-responsive conformational change of a repebody scaffold compared to a perpendicular conjugation.

Next, we determined the binding affinities of the variants for human IgG in dark (trans) and UV-exposed (cis) states using a surface plasma resonance (SPR) (Tables 1 and S1). First, we checked whether the variants retained their original binding affinity even after conjugation of BSBCA in the dark with that of the wild type. H76C/N102C and its equivalent variants were shown to have comparable binding affinities for human IgG to the wild type. H76C/N102C and its equivalent binding affinity even after conjugation of BSBCA in the dark.

Table 1: Binding affinities of the photoswitchable variants.

<table>
<thead>
<tr>
<th></th>
<th>$k_a$ (M⁻¹ s⁻¹)</th>
<th>$k_d$ (s⁻¹)</th>
<th>$K_D$ (M)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10-WT</td>
<td>4.9 × 10⁸</td>
<td>1.1 × 10⁻⁵</td>
<td>2.2 × 10⁻¹</td>
<td></td>
</tr>
<tr>
<td>H76C/N102C_dark (d)</td>
<td>3.4 × 10⁸</td>
<td>5.5 × 10⁻³</td>
<td>1.6 × 10⁻⁴</td>
<td>5</td>
</tr>
<tr>
<td>H76C/N102C_UV (d)</td>
<td>6.3 × 10⁸</td>
<td>5.3 × 10⁻⁵</td>
<td>8.2 × 10⁻⁵</td>
<td></td>
</tr>
<tr>
<td>B1-WT</td>
<td>1.2 × 10⁶</td>
<td>3.6 × 10⁻⁴</td>
<td>3.1 × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>H76C/N102C_dark</td>
<td>2.7 × 10⁹</td>
<td>9.4 × 10⁻⁴</td>
<td>4.1 × 10⁻⁴</td>
<td>21</td>
</tr>
<tr>
<td>H76C/N102C_UV</td>
<td>2.9 × 10⁹</td>
<td>2.5 × 10⁻⁴</td>
<td>8.7 × 10⁻⁷</td>
<td></td>
</tr>
<tr>
<td>EgH9-WT</td>
<td>5.9 × 10⁸</td>
<td>1.5 × 10⁻⁶</td>
<td>2.5 × 10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>H76C/N102C_dark</td>
<td>1.0 × 10⁹</td>
<td>2.2 × 10⁻⁶</td>
<td>2.1 × 10⁻¹</td>
<td>1400</td>
</tr>
<tr>
<td>H76C/N102C_UV</td>
<td>4.0 × 10⁹</td>
<td>1.2 × 10⁻⁵</td>
<td>3.0 × 10⁻⁵</td>
<td></td>
</tr>
</tbody>
</table>

BBSCA. We measured the binding affinities of three equivalent variants, H76C/N102C, Q122C/K150C, and Q146C/E174C, before and after illumination (Table S1). The binding affinities of the three variants were shown to decrease by about 5- to 12-fold after illumination. We further examined the functionality of the variants as photoswitchable protein binders using an enzyme-linked immunosorbent assay (ELISA). As shown in Figures 2a and S5, all of the rebody variants showed significantly reduced signals after exposure at 370 nm, but recovered when exposed at 500 nm without a significant loss in their binding capabilities during repeated light cycles. This result demonstrates that a rebody scaffold can undergo a distinct conformational change through a light-responsive isomerization of BSBCA with reversible functionality.

To evaluate the general applicability of our design strategy, we employed other rebody units that were previously developed for mOrange (B1)\(^{[12]}\) and EGFR (EgH9)\(^{[13]}\). Using the same approach, we introduced the cysteine mutation of a C\(_p\) pair, 76/102, into B1 and EgH9, followed by the conjugation of BSBCA (B1-H76C/N102C and EgH9-H76C/N102C). We then investigated the resulting rebody variants as photoswitchable proteins. The conjugation efficiencies and photoswitchability of BSBCA were similar to that of D10-H76C/N102C (Figure S6). Both variants also retained the original binding affinities of the wild types even after the conjugation of BSBCA. Interestingly, the binding affinity of EgH9-H76C/N102C for EGFR was shown to decrease by about 1400-fold when exposed at 370 nm (Figures 3b, S11 and Table S1), leading to about a 1400-fold decrease in the binding affinity of EgH9-H76C/N102C for EGFR. Unlike D10, the nearest site from the N-terminal of EgH9 was observed to be the most effective for photoswitching. This result implies that the best site for BSBCA insertion depends on the major interactions between a rebody and a target. It is thus necessary to select the optimal site for BSBCA insertion to design a photoswitchable rebody showing the most significant change in binding affinity in response to light.

Next, we wanted to test whether a photoswitchable rebody can be used for the light-driven modulation of cell signaling. With an EgH9-H76C/N102C, we intended to turn on and off the EGF-mediated signaling process in cells in a light-driven manner. We treated EGF-expressing cells with EgH9-H76C/N102C and investigated the phosphorylation of extracellular signal-regulated kinase (pErk), a downstream component of EGF-mediated cell signaling.\(^{[14]}\)
response to a repeated light cycle (Figure 4). As a result, the pErk significantly decreased under dark or green light, which indicates that the EgH9 bound to EGFR on the cells and consequently inhibited the cell signaling by preventing the binding of EGF to its receptor. When illuminated with UV, however, the pErk was shown to recover to the half the level obtained in the presence of EGF alone. This result implies that the repebody lost its binding activity toward EGFR, and accordingly EGF-triggered cell signaling was turned on. The inhibition and recovery of pErk by the EgH9-H76C/N102C was shown to reversibly repeat in response to a light cycle. These results demonstrate that the photoswitchable repebody can be effectively used for light-driven modulation of cell signaling in living cells.

We demonstrated a generalized strategy to design a photoswitchable repebody using a photochromeic ligand. A target-specific repebody is easy to generate through a phage display and modular evolution. It is thus noteworthy that photoswitchable repebodies specific for different targets can be easily developed using the same approach. We showed the utility of a photoswitchable repebody through light-driven modulation of cell signaling in living cells. Considering abundance of proteins composed of LRR modules in nature, our approach can enable the development of diverse photoswitchable repeat proteins. The present approach is expected to effectively expand the repertoire of photoswitchable proteins for light-driven control of many biological processes.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: light-driven control · LRR proteins · photochromeic ligands · photoswitchable proteins · repebody

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