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Phage Selection of Photoswitchable Peptide Ligands

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Supporting Information

ABSTRACT: Photoswitchable ligands are powerful tools to control biological processes at high spatial and temporal resolution. Unfortunately, such ligands exist only for a limited number of proteins and their development by rational design is not trivial. We have developed an *in vitro* evolution strategy to generate light-activatable peptide ligands to targets of choice. In brief, random peptides were encoded by phage display, chemically cyclized with an azobenzene linker, exposed to UV light to switch the azobenzene into *cis* conformation, and panned against the model target streptavidin. Isolated peptides shared strong consensus sequences, indicating target-specific binding. Several peptides bound with high affinity when cyclized with the azobenzene linker, and their affinity could be modulated by UV light. The presented method is robust and can be applied for the *in vitro* evolution of photoswitchable ligands to virtually any target.

Photoswitchable ligands are used to control and study complex biological systems such as folding of proteins and peptides, enzymatic reactions, or neuronal signaling.[1−7] They are typically developed by conjugating photochromic compounds to known ligands so that exposure to light and a resulting conformational change of the photochrome changes the binding affinity of the ligand. Many light-responsive ligands are based on azobenzene, a molecule that undergoes a pronounced change in geometry upon photoisomerization from *trans* to *cis* in picoseconds.[8] It has been linked to a wide range of small molecule ligands or peptides such as α-helices or β-hairpin peptides.[1,6,9−12]

For many important protein targets, photoswitchable ligands do not exist, or they suffer from limitations such as low affinity or a small difference in affinity between the two conformers. The transformation of existing ligands into light-responsive ones by rational design is not trivial. Combinatorial strategies based on the generation and screening of large molecule libraries for light-dependent binding could potentially revolutionize the development of photoswitchable ligands. *In vitro* display techniques such as phage display allow the genetic encoding of billions of random peptides and the identification of ligands within such pools. In recent years, strategies were developed to chemically modify or cyclize genetically encoded polypeptide libraries[13,14] or to incorporate unnatural amino acids.[15,16] The availability of these novel techniques suggested that also photochromic compounds such as azobenzene could be incorporated into encoded peptides and libraries screened.

Two first strategies for evolving light-responsive ligands *in vitro* have recently been proposed independently by the groups of Ito and Derda. Ito et al. incorporated an ε-(lysine)-azobenzene photoswitch into peptides encoded by mRNA using ribosome display and amber suppression.[17] The best binder isolated to the model target streptavidin was estimated to bind with a micromolar binding constant. Derda et al. cyclized phage-encoded peptides of the form ACX_7CG with azobenzene by connecting the two cysteines.[18] The best light-responsive ligand, identified also against streptavidin, had an affinity of 452 μM in *trans* conformation and showed a more than 4.5-fold weaker apparent affinity in *cis* conformation.

Both of the two approaches generated peptide ligands that bind preferentially in *trans* conformation. This means that binders are inactivated by switching them into *cis* conformation by light. As only 70−80% of azobenzene can be switched to the *cis* conformation (for physical reasons), light cannot turn off these ligands completely. For example, if the photoswitchable peptide is an enzyme inhibitor, the concentration of active inhibitor is reduced maximally 5-fold, leaving most of the enzyme inhibited. Herein, we conceived a strategy to evolve peptide ligands that are activated by light rather than inactivated. Such peptide ligands bind the target when the azobenzene is switched to *cis*. A light-activatable ligand has the advantage that essentially all the peptide population (99.99%) is initially in the energetically favored *trans* conformation. In the example of the enzyme inhibitor, all peptide would initially be in the inactive *trans* conformation, leaving the enzyme fully active. Upon UV light exposure, all enzyme would be inhibited even if only a fraction of the ligand population is switched.

Directed evolution of light-activatable peptide ligands requires that peptides are captured in their *cis* conformation, increasing the complexity of the selection process compared to the screening for *trans* binders. Combining the expertise of the Heinis laboratory in phage display and the know how of the Wegner group in azobenzene chemistry and UV spectrometry, we herein established the photoswitching of peptides *in situ* on the surface of filamentous phage. We furthermore developed procedures to efficiently select light-responsive peptide ligands to targets of choice. While all *in vitro*-evolved ligands reported to date bind with K_ds in the high micromolar range, we aimed

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at developing binders with low micromolar or nanomolar K_ds. Affinities in this range are required for most applications.

We synthesized the photoswitchable compound 3,3′-bis(sulfonato)-4,4′-bis(bromoacetamido) azobenzene (BSBBA) in analogy to the prototype compound BSBCA developed by Woolley et al.[19,20] and applied it to cyclize the phage-encoded peptides via the side chains of two flanking cysteines in a similar way as Derda et al.[18] (Figure 1a). BSBBA contains

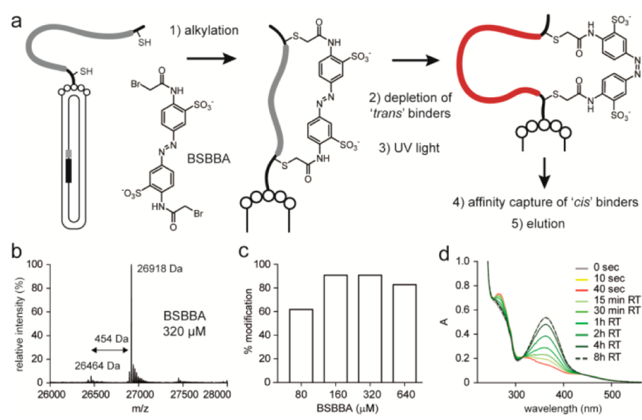

Figure 1. Phage selection of light-activated peptide ligands. (a) Schematic depiction of the selection strategy. (b) Mass spectrum of peptide genetically fused to phage coat protein pIII (domains D1 and D2) after incubation with 320 μM BSBBA for 1 h (expected Δmass = 454 Da). (c) Fraction of azobenzene-cyclized peptide-D1-D2 at different BSBBA concentrations estimated by mass spectrometry. (d) Absorption spectra of peptide cyclized with BSBBA in the presence of phage particles.

bromoacetamide that is slightly more reactive toward thiols than chloroacetamide in BSBCA.[19] We displayed peptides containing seven random amino acids flanked on each side by cysteines as fusion of pIII on filamentous phage (ACX_7CG-phage; complexity: 2.3×10^8 peptides). An engineered phage mutant deficient in three disulfide bridges in two domains of pIII was used.[21] This mutant allowed application of a broad range of reduction and alkylation conditions without affecting the functionality of the phage.[22,23] We applied harsh reaction conditions to efficiently reduce the cysteines of displayed peptides (1 mM TCEP, 42 °C, 1 h) and to alkylate the peptides quantitatively. 90% of peptide was modified at 320 μM BSBBA in 20% ACN, 80% NH_4HCO_3, pH 8, 30 °C (Figure 1b,c).

Toward the photoswitching on phage, we found that filamentous phage exposed 20 min to a 100 W light source (λ = 365/66; distance to the cuvette = 21 cm) remained fully functional. To assess if the peptides switch under these conditions, we spiked the same phage preparation with a BSBBA-cyclized model peptide (H_2N-AGSCHSASVCWG-$CONH_2$; 22 μM) and followed the switching by absorption spectrometry (Figure 1d). Maximal switching to *cis* (around 70%) was achieved in 40 s.

We performed selections against the model target streptavidin that was applied by the groups of Ito and Derda and allowed comparison of the isolated peptides.[17,18] The CX_7C phage library cyclized with BSBBA was subjected to two consecutive selection rounds against streptavidin as follows. The population was first depleted of *trans* binders by adding and discarding 7 times 10 μL magnetic streptavidin beads. The remaining phage were exposed 5 min to the UV light and incubated with 20 μL magnetic streptavidin beads to capture *cis* binders. Binders were eluted by addition of biotin and buffer with a low pH (2.2). A control experiment was performed in parallel with phage-peptides that were not cyclized with BSBBA and exposed to UV light. Sequencing of 62 clones after two rounds of selection showed strong consensus sequences. Most peptides contained a "HPQ" motif[24,25] that is characteristic for peptides binding to the biotin-binding site of streptavidin (Figure 2a, left). In the control selection without BSBBA-cyclization and UV light exposure, a similar but different consensus sequence was observed than in the actual selection, indicating that the peptides were efficiently cyclized with the azobenzene compound (Figure 2a, right). We were pleased to find consensus sequences among the isolated peptides as this is a clear indication for target-specific binders. It was the first time

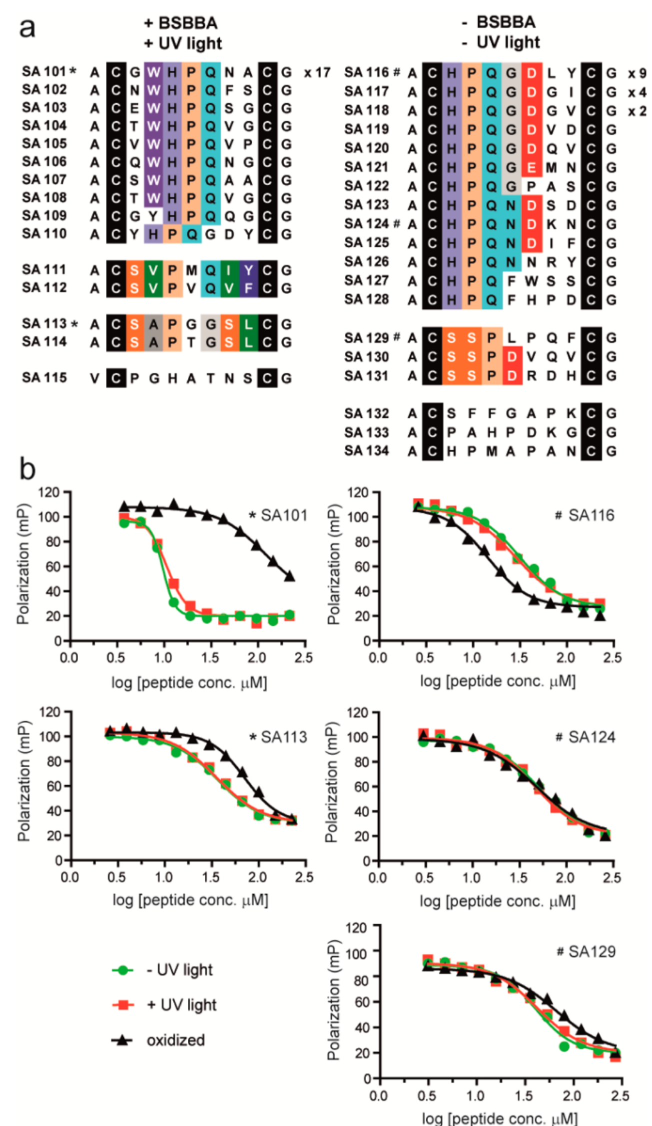

Figure 2. Phage selection of light-switchable streptavidin ligands from the CX_7C peptide library. (a) Peptides isolated after cyclization of the peptide phage library with BSBBA and exposure to UV light (left column) or without alkylation and UV light exposure (right column). Sequence similarities are highlighted in color. (b) Binding of peptides to streptavidin measured in a fluorescence polarization competition assay. Peptides are labeled with an asterisk (*) if they were selected with BSBBA and UV light and with a hash symbol (#) if they were isolated as disulfide-cyclized (oxidized) peptides.

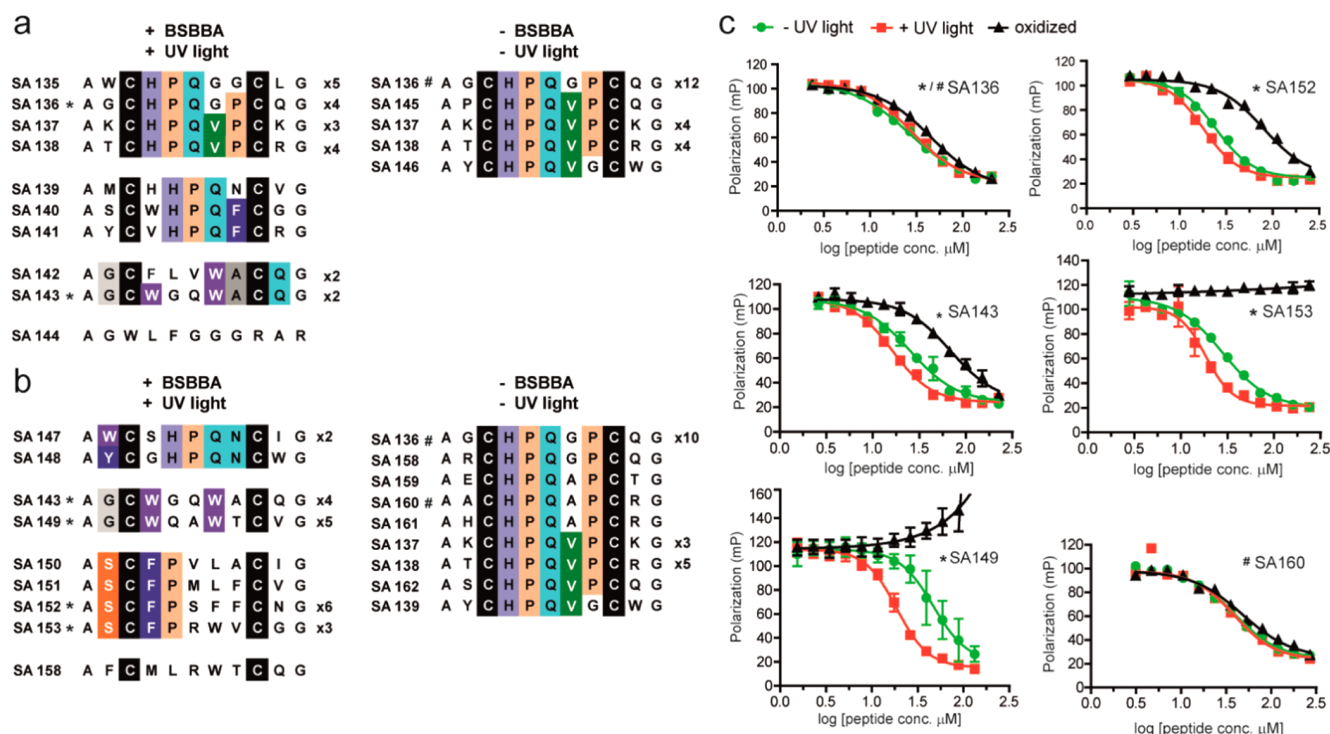

Figure 3. Phage selection of light-switchable streptavidin ligands from the CX5C library. (a, b) Peptides isolated after cyclization of the peptide phage library with BSBBA and exposure to UV light (left columns) or without alkylation and UV light exposure (right columns). The library was either depleted from *trans* binders prior to UV light exposure (a) or no negative selections were performed (b) prior to capture of *cis* binders. (c) Binding of peptides to streptavidin measured by fluorescence polarization. Peptides are labeled with an asterisk (*) if they were selected with BSBBA and UV light and with a hash symbol (#) if they were isolated as disulfide-cyclized (oxidized) peptides. The binding of light-responsive ligands was measured in independent assays, and error bars are indicated.

that a consensus sequence was found in directed evolution of azobenzene-modified peptides.

Five peptides of the selection against streptavidin were synthesized, cyclized with BSBBA, or oxidized to form disulfide-cyclized peptides, and the binding affinities measured in a fluorescence polarization competition assay. Light in the fluorescence polarization measurement did not interfere with the photoswitching of the peptides (Figure S1). All peptides competed with the disulfide-cyclized fluorescent peptide fluorescein-AECHPQGPPCIEG (F1) which binds with a K_d of 3.1 ± 0.4 μM to the biotin-binding site of streptavidin (Figure 2b, Table S1). The peptides isolated in phage selections after BSBBA-cyclization (SA101 and SA113) bound much better when modified with BSBBA than when oxidized. Conversely, peptides isolated in the control selection omitting BSBBA (SA116, SA124, SA129) bound worse or equal when BSBBA-cyclized. These results implied that the alkylation reaction on phage was successful and that peptides were isolated as BSBBA conjugates in the phage panning. All characterized BSBBA-cyclized peptides could be switched efficiently into the *cis*-conformation (50–80%), but the binding affinities of the *cis* and *trans* conformers were essentially the same (Table S1). We speculated that light-responsive ligands were not selected due to the long peptide loops: the amino acids next to the cysteines might be too flexible so that a conformational change of the linker is not transformed into a conformational change of the contact-forming amino acids. In fact, an X-ray structure of a peptide with HPQ motif in complex with streptavidin shows that the central 3–4 amino acids including HPQ are sufficient for binding.[26,27]

We subsequently turned to peptides with shorter loops. A library of the format AXCX$_5$CXG-phage was cloned (complexity: 1.05 × 10^7 peptides) and subjected to affinity selections against streptavidin. Isolated peptides showed four different consensus sequences (Figure 3a,b). Three out of the four consensus sequences were not found in the control selection in which BSBBA was omitted. This suggested that the binding of peptides of these consensus groups could potentially be modulated by light.

The peptides of the XCX$_5$CX library bound streptavidin with K_ds between 1.8 ± 0.1 and 6.7 ± 2 μM when cyclized with BSBBA (Figure 3c, Table 1). Some peptides isolated with

Table 1. Photoresponsive Peptides

name	consensus sequence	$K_d^{dark\,a}$ (μM)	$K_d^{light\,a}$ (μM)	K_d S–S^b (μM)
SA143	G_W__W	3.4 ± 0.6	1.8 ± 0.1	9.5 ± 1
SA149	G_W__W	6.7 ± 2	2.2 ± 0	>250
SA152	S_FP	3.1 ± 0.1	2.1 ± 0.2	11 ± 0
SA153	S_FP	3.6 ± 0.3	2.0 ± 0.5	>250

[a]Peptides cyclized with BSBBA. [b]Disulfide-cyclized peptides.

BSBBA did not bind streptavidin at all when cyclized by a disulfide bridge, confirming that the peptides were selected as BSBBA conjugates. Upon UV light exposure, the peptides of two consensus groups changed their binding affinity; the *cis* conformers bound up to 3-fold more tightly than the peptides in *trans* conformation (Figure 3c and Table 1). In contrast, peptides identified in the control selections without BSBBA and synthesized as BSBBA conjugates did not change their affinity

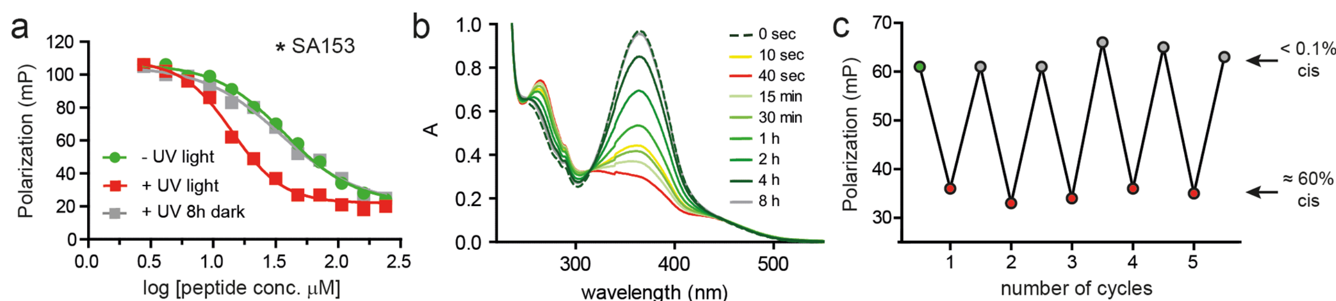

Figure 4. Switching "on" and "off" streptavidin-binding peptides. (a) Binding of BSBBA-cyclized peptide SA153 was measured (i) before exposure to UV light, (ii) immediately after UV light exposure, and (iii) after UV light exposure and 8 h incubation in the dark. (b) Absorption spectra of SA153 after exposure to UV light for 10 or 40 s and incubation in the dark for the indicated times. (c) Binding of SA153 to streptavidin after repetitive exposure to UV light (red dots) and incubation at 40 °C in dark for 1 h (gray dots) measured in a competition fluorescence polarization assay.

upon light exposure (Table S1). The change in affinity was quantitatively reverted by incubation in dark as shown in Figure 4a,b. The peptides could be switched between the *cis* and *trans* conformation for several cycles without losing any activity, as shown for SA153 in Figure 4c.

In conclusion, we were able to isolate light-responsive peptide ligands that bind with K_ds in the single-digit micromolar range and thus >300-fold better than those developed previously with other *in vitro* evolution procedures to the same model protein target. Use of a disulfide-free phage mutant and custom-made libraries allowed application of robust peptide modification procedures. An important aspect of our approach is the switching of peptides *in situ* on the phage surface to the *cis* conformation, enabling the isolation of "activatable ligands". The affinity difference between *cis* and *trans* conformation likely depends much on the target and binding site, and significantly larger changes may be obtained in selections with other proteins. The robust and general approach should be applicable for the directed evolution of light-responsive peptide ligands to virtually any target and promises to additionally fuel the fast growing field of optogenetics.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, materials, characterization of all peptides and analytical data for BSBBA. 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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