Phage selection of photoswitchable peptide ligands

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Supporting Information Placeholder

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 and co-workers 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 and co-workers cyclized phage-encoded peptides of the form ACX7CG 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.

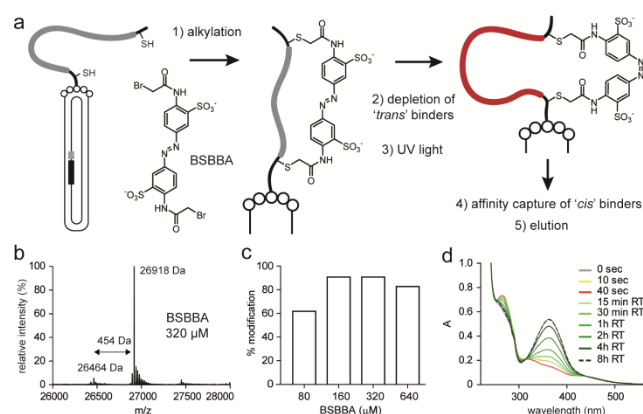

Figure 1. Phage selection of light-activated peptide ligands. (a) Schematic depiction of the selection strategy. (b) Mass spectrum of peptide genetically fused to phage coat protein pIII (domains D1 and D2) after incubation with 320 μM BSBBA for 1 hr (expected Δ mass = 454 Da). (c) Fraction of azobenzene-cyclized 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.

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.

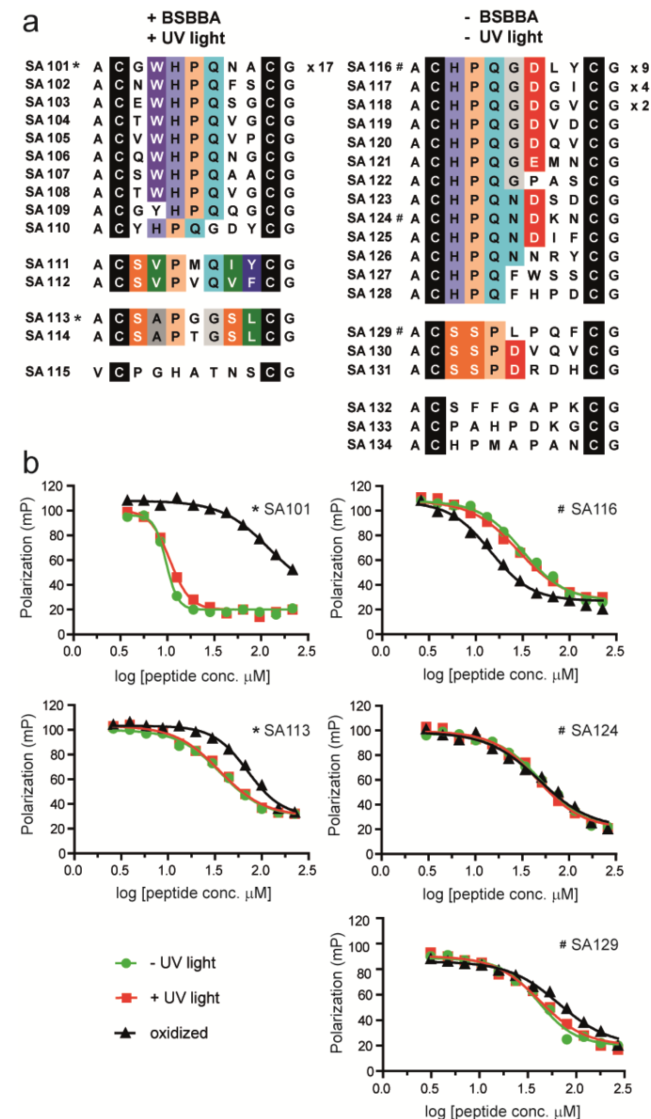

Figure 2. Phage selection of light-switchable streptavidin ligands from the CX7C peptide library. (a) Peptides isolated after cyclization of the peptide phage library with BSBBA and exposure to UV light (left column) or without alkylation and UV light exposure (right column). Sequence similarities are 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.

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 knowhow 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 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 and co-workers[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 and co-workers[18] (Figure 1a). BSBBA contains bromoacetamide that is slightly more reactive towards 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 (ACX7CG-phage; complexity: 2.3 x 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 hr) 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 and 1c).

Towards the photoswitching on phage, we found that filamentous phage exposed 20 min to a 100 watt light source (λ = 365/66; distance of 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 seconds.

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 CX7C 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 differ-

ent 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 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 peptide (Supplementary 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, Supporting 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 (Supporting 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

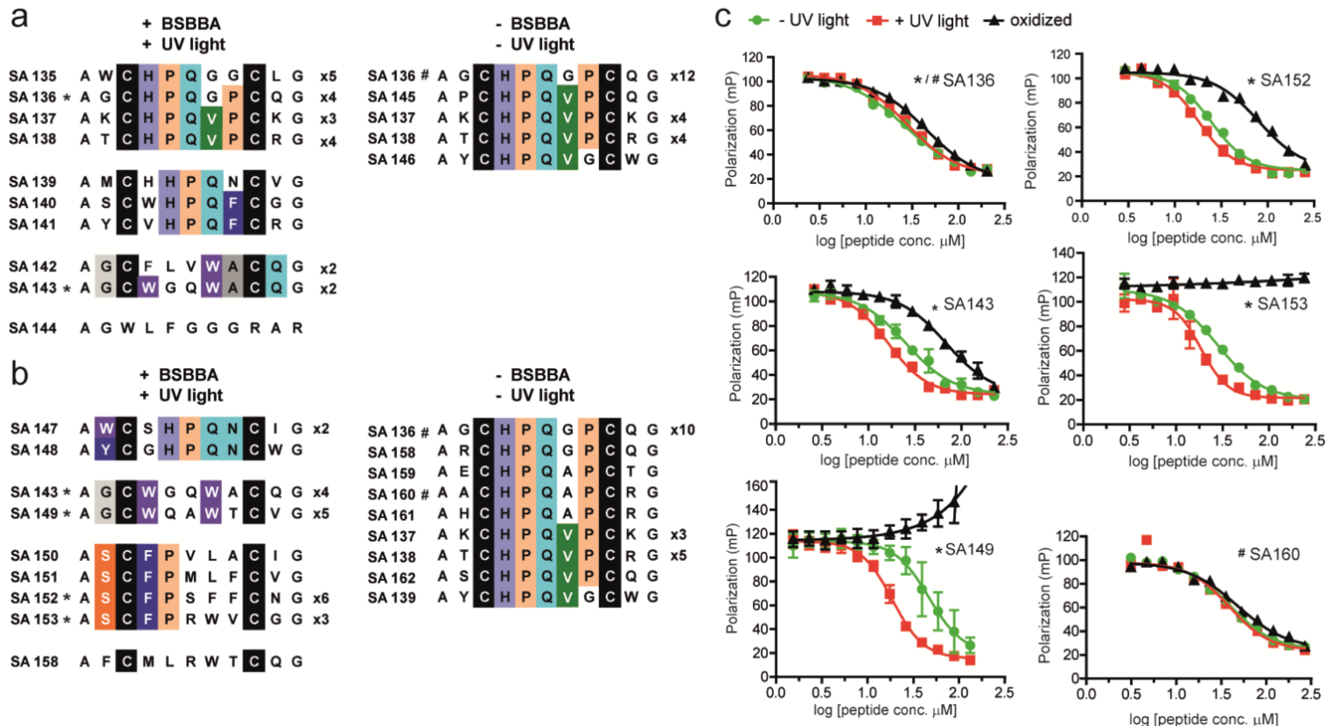

Figure 3. Phage selection of light-switchable streptavidin ligands from the CX5C library. (a and b) Peptides isolated after cyclization of the peptide phage library with BSBBA and exposure to UV light (left columns) or without alkylation and UV light exposure (right columns). The library was either depleted 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.

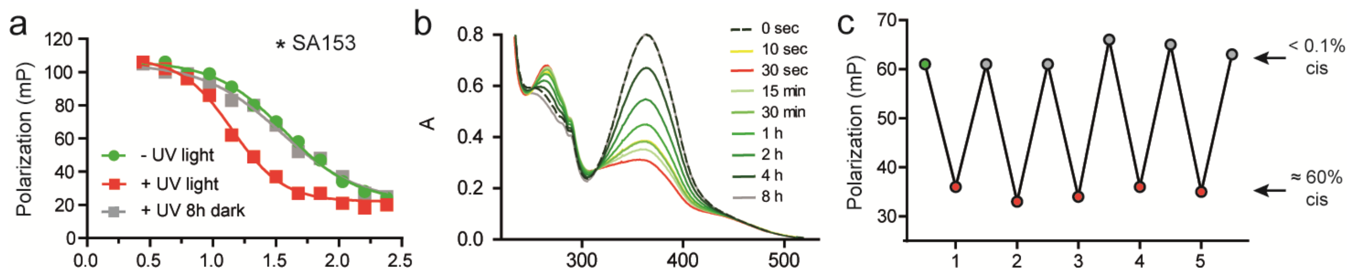

Figure 4. Switching 'on' and 'off' streptavidin-binding peptides. (a) Binding of BSBBA-cyclized peptide SA153 was measured i) before exposure to UV light, ii) immediately after UV light exposure, and iii) after UV light exposure and 8 hrs incubation in the dark. (b) Absorption spectra of SA153 after exposure to UV light for 10 or 30 seconds 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 hr (grey dots) measured in a competition fluorescence polarization assay.

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 AXCX5CXG-phage was cloned (complexity: 1.05 x 10^7 peptides) and subjected to affinity selections against streptavidin. Isolated peptides showed four different consensus sequences (Figure 3a and 3b). 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.

Table 1. Photo-responsive 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

The peptides of the XCX5CX 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 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 upon light exposure (Supplementary Table S1). The change in affinity was quantitatively reverted by incubation in dark as shown in Figure 4a and 4b. The peptides could be switched between the *cis* and *trans* conformation for several cycles without losing any activity, as shown for SA153 in Fig. 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 interests.

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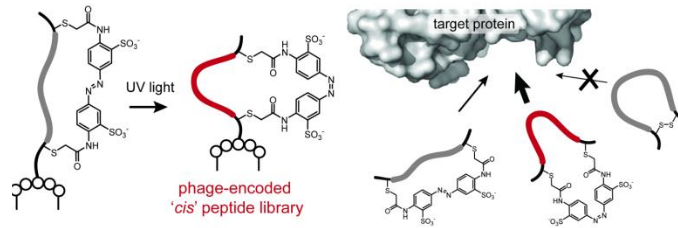
UV light
phage-encoded
'cis' peptide library
target protein

Supporting Information for:

Phage selection of photoswitchable peptide ligands

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Supplementary Materials and Methods

Synthesis of 3,3′-bis(sulfonato)-4,4′-bis(bromoacetamido)azobenzene (BSBBA)

Sodium 3,3′-bis(sulfonato)-4,4′-bis(amino)azobenzene was prepared following procedures developed by Woolley and co-workers.[1] To 291 mg (0.802 mmol, 1.00 equiv.) of this compound, bromoacetic acid (2.67 g, 19.2 mmol, 24.0 equiv.) and bromoacetic anhydride (5.00 g, 19.2 mmol, 24.0 equiv.) were added in a Pyrex Culture Tube Screw Cap, the tube put under nitrogen atmosphere and stirred at 87 °C overnight. The reaction mixture was cooled to RT, diluted to 50 mL with milliQ water and purified by reversed-phase chromatography on a Vydac C18 (218TP) column (22 x 250 mm) (Grace & Vydac, Hesperia, USA) using a solvent system of H_2O/0.1 % trifluoroacetic acid (TFA) and ACN/0.1 % TFA. After lyophilization the product was obtained in 23% yield (111 mg, 0.181 mmol).

^{1}H NMR (400 MHz, D_2O) δ 8.24 (d, *J* = 2.2 Hz, 2H), 8.14 (d, *J* = 8.8 Hz, 2H), 7.90 (dd, *J* = 8.8, 2.1 Hz, 2H), 4.20 (s, 4H) ^{13}C NMR (101 MHz, DMSO) *δ* 164.73, 146.89, 137.02, 136.47, 126.16, 120.16, 119.39, 30.56. HRMS (ESI) *m/z* calcd for $C_{16}H_{12}Br_2N_4O_8S_2^{2-}$ $[M/2]^-$ 305.9227, found 305.9204.

Peptide synthesis

Peptides were synthesized on an Advanced ChemTech 348Ω peptide synthesizer (Aapptec, Louisville, USA) by standard Fmoc (9-fluorenylmethyloxycarbonyl) solid phase chemistry using Rink Amide AM resin (0.03 mmol scale). The coupling step was carried out twice for all amino-acids using 0.12 mmol Fmoc-protected amino acid, 0.12 mmol HBTU, 0.12 mmol HOBt and 0.18 mmol DIEA in 1.3 mL DMF. Fmoc groups were removed using a 20% (v/v) solution of piperidine in DMF (2 x 2.5 mL). The peptides were deprotected and cleaved from the resin by treatment with 5 mL of cleavage solution (90% TFA, 2.5% phenol, 2.5% thioanisole, 2.5% 1,2-ethanedithiol (EDT)

and 2.5% water), for 3 hrs at RT under shaking. The resin was removed by centrifugation 1 min at 1000 rpm and filtration under vacuum. The peptides were precipitated with ice-cold diethyl ether (50 mL), incubated 30 min at - 20 °C and centrifuged 5 min at 4000 rpm. The precipitated peptides were washed and centrifuged 5 min at 4,000 rpm twice with ice-cold ether (35 and 20 mL, respectively). Samples were resuspended in water/ACN (ratio 5:2) containing 0.1% TFA and lyophilized.

Peptide cyclization with BSBBA

Crude peptide (1.00 equivalent; typically 10 mg) was reacted with BSBBA (1.1 equivalent) in 90-50% aqueous buffer (20 mM NH_4HCO_3, pH 8.0) and 10-50% ACN for 1 hr at 30 °C. The product was purified by reversed-phase chromatography on a C18 column (Vydac C18, 218TP column, 22 x 250 mm) using a solvent system of 99.9% H_2O/0.1% TFA and 99.9%ACN/0.1 % TFA. The peptide was lyophilized and the mass was confirmed by electrospray ionization (ESI) mass spectrometry.

Peptide cyclization by oxidation

Crude peptide (typically 10 mg) was dissolved in 9 mL aqueous buffer (20 mM NH_4HCO_3, pH 8.0) and 1 mL DMSO, and incubated for 1-2 days at RT. The product was purified by reversed-phase chromatography on a C18 column (Vydac C18, 218TP column, 22 x 250 mm) using a solvent system of 99.9% H_2O/0.1 % TFA and 99.9% ACN/0.1 % TFA. The peptide was lyophilized and the mass was confirmed by ESI mass spectrometry.

Buffers

Reaction buffer: 20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0

Washing buffer: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$

Modification of the peptide-D1-D2 fusion protein

Peptide-D1-D2 fusion protein (ACGSGSGSGCG-D1-D2) was expressed and purified as described by Angelini, A. *et al.*[2] 680 μg of the fusion protein in 1 mL aqueous buffer was incubated with 1 mM tris(2-carboxyethyl) phosphine (TCEP) for 1 hr at 42 °C to reduce the cysteine residues. The concentration of TCEP was reduced by size exclusion chromatography on a PD-10 column (GE Healthcare, Uppsala, Sweden) using 20 mM NH_4HCO_3 buffer, pH 8. The protein (30 μM) was incubated with increasing concentrations of BSBBA (80, 160, 320, 640 μM) in 25% acetonitrile and 75% 20 mM NH_4HCO_3 for 1h at 30 °C in a final volume of 200 μL. The protein was purified by reversed-phase chromatography on a C4 column (Vydac C4 column, 5 μm, 4.6 x 250 mm) using a solvent system of 99.9% H_2O/0.1 % TFA and 99.9%ACN/0.1 % TFA. The mass was determined by ESI mass spectrometry.

Testing photoswitching of peptide in presence of phage

A solution of filamentous phage (10^{11} phage/mL) was spiked with the peptide H_2N-AGSCHSASVCWG-$CONH_2$ cyclized with BSBBA in washing buffer at a concentration of 22 μM and placed into a 1 cm quartz cuvette. The sample was irradiated with light at the 365/66 nm peak generated by a HBO 100 mercury illuminating system (100-watt high-pressure mercury plasma lamp) at a distance of 21 cm using appropriate filters and the absorption spectra were measured at different time points.

Testing phage infectivity after BSBBA modification and UV irradiation

Phage of the CX_7C library was produced in four 0.5-L cultures as described by Rentero-Rebollo, I. *et al.*[3] Aliquots of phage (10^{11} t.u.) in 1 mL 80% 20 mM NH_4CO_3, pH 8.0, 5 mM EDTA and 20% ACN were incubated with increasing concentrations of BSBBA (final concentration: 0, 40, 80,160, 320, 640 μM) for 1 hr at 30 °C. The reactions were quenched by addition of cysteine at concentrations 20-fold higher than those of BSBBA. The number of infective phage was measured as described by Rentero-Rebollo, I. *et al.*[3] 2 mL of phage from the same preparation were transferred to a quartz cuvette and irradiated with the HBO 100 mercury illuminating system as described above. At each time point (0, 30 sec, 1, 2, 4, 8, 16 min) the solution was mixed and 20 μL were taken for phage titration.

Cloning of the phage peptide libraries

The phage peptide libraries CX_7C and XCX_5CX were cloned using a protocol described by Rentero-Rebollo, I. *et al.*[3] The following DNA primers were used:

Primer name:	Sequence (5’-3’):
Sfi1cx7cba	TATGCGGCCCAGCCGGCCATGGCAGCATGCNNKNNKNNKNNKNNKNNKNNKTGTGGCGGTTCTGGCGCTG
L3_1C5C1	TATGCGGCCCAGCCGGCCATGGCAGCANNKTGCNNKNNKNNKNNKNNKTGCNNKGGCGGTTCTGGCGCTG

Phage of these libraries display peptides with the following formats. The indicated sequence ‘AETV…’ represents the N-terminus of pIII. The complexities of the libraries are also indicated.

Library name:	Format:	Diversity:
CX_7C	ACXXXXXXXCGGSG-AETV…	2.3×10^8
XCX_5CX	AXCXXXXXCXGGSG-AETV…	1.05×10^7

Production of phage peptide libraries cyclized with BSBBA

Two 2-L flasks each containing 0.5 L 2YT media and 30 μg/mL chloramphenicol were inoculated with TG1 *E. coli* cells harbouring the CX_7C or the XCX_5CX phage library to reach an OD_{600} of 0.1. The flasks were shaken (220 rpm) for 16 hrs at 30 °C. The cells were pelleted by centrifugation at 8,000 rpm for 30 min at 4 °C. The supernatant was transferred to an ice-cold solution of 20% PEG, 2.5 M NaCl (20% of culture volume) and incubated on ice for 45 minutes. Precipitated phage were pelleted by centrifugation at 8,000 rpm for 30 min at 4 °C. The phage pellet was resuspended in 20 mL of reaction buffer and centrifuged at 4,000 rpm for 15 min at 4 °C. The supernatant was split into two fractions of 10 mL to either cyclize the peptides by BSBBA or to allow peptides to oxidize and form disulfide bridges.

Phage in the first tube were complemented with 10 mL reaction buffer and TCEP (final concentration 1 mM), and incubated at 42 °C for 1 hr to reduce the cysteines. Phage were cooled on ice and concentrated by centrifugation at 4,000 rpm in a Vivaspin-20 filter (molecular weight cut off of 100,000; GE Healthcare) to 1 mL, diluted with 9 mL ice-cold reaction buffer. This procedure was repeated to reduce the TCEP concentration to 1 μM in 1 mL. Traces of phage stuck to the filter were detached by pipetting. The solution was adjusted to 4 mL with the same buffer. 1 mL of BSBBA in milliQ water was added to the phage to reach a final concentration of 0.32 mM and the reaction was incubated at 30 °C for 1 hr. Phage were separated from non-reacted BSBBA by

precipitation with 1 mL 20% (w/v) PEG, 2.5 M NaCl on ice and centrifugation at 4,000 rpm for 30 min. The phage pellet was resuspended in 1 mL washing buffer and stored at 4°C.

Phage in the second tube were incubated with 2.5 mL ice-cold 20% PEG, 2.5 M NaCl solution for 30 min and centrifuged at 4,000 rpm for 30 min at 4 °C. The phage pellet was resuspended in 1 mL washing buffer.

Phage selection of photoswitchable streptavidin ligands

The affinity selection was performed with streptavidin Dynabeads® M-280 (Life Technologies, Carlsbad, USA). Beads were blocked by exchanging the storage solution to washing buffer containing 1% w/v BSA and 0.1% v/v Tween 20 and inverting on a rotating wheel (10 rpm) for 30 min at RT.

For the phage selection with the CX_7C library (results shown in Figure 2a), the following procedures were applied. Both, the BSBBA-modified phage (1 mL) and the oxidized phage (1 mL) were blocked in parallel by addition of 0.5 mL washing buffer containing 3% w/v BSA and 0.3% v/v Tween 20 and inversion on a rotating wheel (10 rpm) for 30 min at RT. The blocked phage were subjected to seven rounds of negative selection as follows: each phage solution was incubated with 10 μL of blocked streptavidin beads and inversed on a rotating wheel (10 rpm) for 10 min. The supernatant was collected and incubated with 10 μL of new blocked streptavidin beads. The procedure was repeated six times. Modified phage were split into two tubes (0.75 mL for each tube) and one of the two fractions was irradiated for 5 min with UV light (365/66 nm) using the HBO 100 mercury lamp as described above. Immediately after UV light exposure, each one of the three phage populations (BSBBA-modified phage irradiated, 0.75 mL; BSBBA-modified phage not

irradiated, 0.75 mL; oxidized phage with disulphide cyclized peptides, 1.5 mL) were incubated in parallel with 20 μL of blocked streptavidin beads and rotated on a wheel (10 rpm) for 30 min. The beads were washed four times with 1 mL washing buffer containing 1% BSA and 0.1% v/v Tween 20, three times with washing buffer containing 0.1% v/v Tween 20 and three times with 1 mL washing buffer. The bound phage were eluted by incubation for 5 min with 1 mL of biotin solution (30 μg/ml in milliQ water). Additionally, remaining phage were eluted by incubation of the beads in 50 μL 50 mM glycine, pH 2.2 for 5 min. The eluate was neutralized with 100 μL 1 M Tris-HCl, pH 8.0. Eluted phage was added to exponentially growing TG1 cells (OD_{600} = 0.4; 30 mL), incubated for 90 min at 37 °C, pelleted by centrifugation at 4,000 rpm for 5 min, plated on large 2YT/chloramphenicol (30 μg/mL) agar plates and incubated over night at 37 °C. From the selection with BSBBA-treated and UV light-irradiated phage and the selection with oxidized phage, new phage was produced in 0.5 mL 2YT media as described above. Two additional rounds of phage selection were performed applying identical procedures.

For the phage selection with the XCX_5CX library, two different procedures were applied (results of the first procedure are shown in Figure 3a and those of the second procedure in Figure 3b). The first procedure is exactly the same as described above for the panning with the CX_7C library. In the second procedure, the negative selection for depletion of *trans* binders was omitted.

Labeling of peptides with fluorescein

Peptides were labeled with fluorescein by incubating 1 mM peptide (typically 0.3 mg, 1.0 equiv.) with 2.5 mM 5(6)-carboxyfluorescein N-hydroxysuccinimide ester (NHS-fluorescein; 2.5 equiv. 21878 Sigma-Aldrich, St. Louis, USA) in 200 μL 99,75% v/v dry DMSO and 0.25% v/v N,N–Diisopropylethylamine (DIPEA) for 3 hrs at RT. 0.8 mL H_2O containing 0.1% TFA (v/v) was added

to the reaction mixture and the peptide purified by HPLC on an analytical C18 column (Vydac C18, 218TP column, 4.6 x 250 mm) using a solvent system of 99.9% H_2O/0.1% TFA and 99.9% ACN/0.1% TFA. The fluorescein-modified peptide was lyophilized and the mass confirmed by ESI-MS.

Measuring K_d by fluorescence polarization

Streptavidin (S4762; Sigma-Aldrich; 66 μM in washing buffer) was serially diluted in washing buffer (final concentration range 33 μM - 0.01 μM). Fluorescein-labeled peptide at a concentration of 100 nM was prepared in washing buffer. 20 μL of streptavidin and fluorescein-peptide were transferred into a well of a black 96-well half area microplate (675076 Greiner Bio-One international AG) and incubated at room temperature for at least 15 min. The fluorescence polarization of each solution was measured in a multiwell plate reader (Infinite® 200 PRO, TECAN, Maennedorf, Switzerland) using a 485 nm excitation filter and a 535 nm emission filter.

The dissociation constants (K_d) were determined by non-linear regression analyses of fluorescence polarization (F_p) versus total concentration of streptavidin $[P]_T$ using the following equation.[4]

$$F_p = F_{pmin} + (F_{pmax} - F_{pmin}) \times \left\{ \frac{\left[([L]_T + K_d + [P]_T - \sqrt{([L]_T + K_d + [P]_T)^2 - 4[L]_T[P]_T} \right]}{2[L]_T} \right\}$$

F_{pmin} and F_{pmax} are the fluorescence polarization for the free peptide and the fully bound peptide, respectively and $[L]_T$ is the total concentration of fluorescent ligand. GraphPad Prism 5 software (GraphPad software) was used for the analysis.

Competitive fluorescence polarization

20 μL of 6 μM streptavidin in washing buffer was mixed with 20 μL of 150 nM fluorescent peptide F1 (in washing buffer) and incubated for 15 minutes. 20 μL of serial dilutions of sample peptide were added. The sample peptide was either cyclized with BSBBA or a disulfide bridge. BSBBA-cyclized peptide was optionally exposed to UV light for 7 min as described above. I_{50} (total concentration of competing ligand I causing 50 % displacement of bound fluorescent peptide) was calculated with GraphPad Prism 5 software using the log(inhibitor) *vs.* response variable slope model. The K_d was determined as reported by Rossi and Taylor.[5]

Repetitive switching of light-responsive peptides

450 μL of 90 μM BSBBA-cyclized SA153 in washing buffer were subjected to four iterative cycles of i) irradiation for 2 minutes at 365/366 nm (as described above) and ii) incubation in a water bath for 1 hr at 40 °C. Before the first exposure to UV light and after each cycle, two aliquots of 30 μL were taken and their complete back-isomerization to *trans* was confirmed by measuring the absorbance at 363 nm on a NanoDrop device (NanoDrop ND-1000 UV-Vis Spectrophotometer, NanoDrop Technologies, Inc. Wilmington, DE, USA). One of the two aliquots was subjected to an additional irradiation at 365/366 nm for 2 min. 20 μL of each sample was added to 20 μL of 6 μM streptavidin in washing buffer previously incubated with 20 μL of 150 nM fluorescent peptide F1. Fluorescence polarization was measured for all samples in parallel.

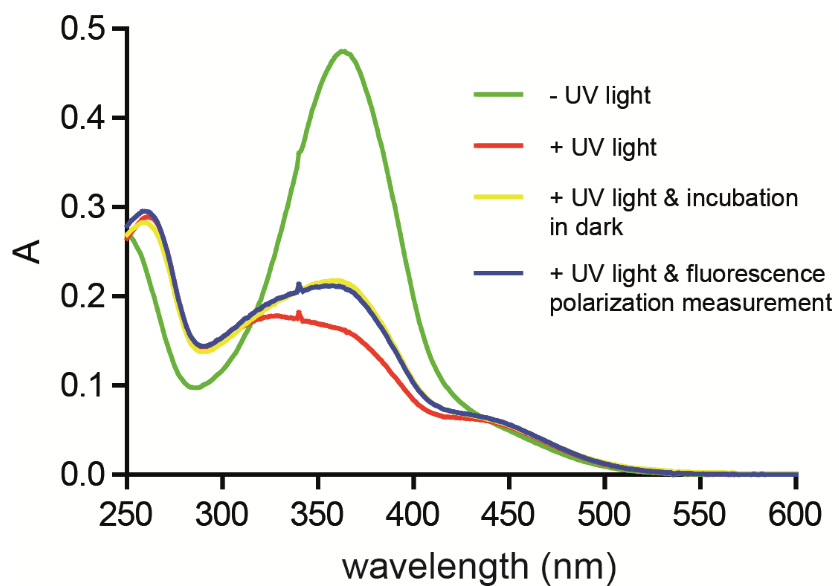
Supplementary Table

Table 1. Photo-responsive peptides. The binding affinity was measured multiple times and standard deviations are indicated.

Name	Library	Consensus sequence	Selection	K_d^{dark} [a] (μM)	K_d^{light} [a] (μM)	% *cis*	$t_{1/2}$ (min) [a]	K_d S-S [b] (μM)
SA101	CX_7C	WHPQ	+ BSBBA	< 1[c]	< 1[c]	67	66	> 20
SA113	CX_7C	SAP_GSL	+ BSBBA	4.8	4.7	71	50	9.9
SA116	CX_7C	HPQ_D	- BSBBA	3.6	4.1	73	62	1.5
SA124	CX_7C	HPQ_D	- BSBBA	6.9	6.2	72	60	6.5
SA129	CX_7C	SSP	- BSBBA	5.6	5.4	72	80	9.2
SA136	XCX_5CX	HPQ_P	+/- BSBBA	3.3	3.6	75	64	6.1
SA143	XCX_5CX	G_W__W	+ BSBBA	3.4±0.6	1.8±0.1	58	103	9.5±1
SA149	XCX_5CX	G_W__W	+ BSBBA	6.7±2	2.2±0	63	130	> 250
SA152	XCX_5CX	S_FP	+ BSBBA	3.1±0.1	2.1±0.2	74	112	11±0
SA153	XCX_5CX	S_FP	+ BSBBA	3.6±0.3	2.0±0.5	70	103	> 250
SA160	XCX_5CX	HPQ_P	- BSBBA	5.4	5.2	74	59	6.5

[a] Peptides cyclized with BSBBA, [b] Disulfide-cyclized peptides, [c] K_ds below 1 μM could not be determined accurately in the competition assay as the concentration of streptavidin monomer was 8 μM.

Supplementary Figure



Effect of fluorescence polarization measurement on peptide back-isomerization. Absorption spectra of BSBBA-modified peptide (20 μM in PBS, peptide sequence ACVWHPQVPCG) before irradiation (- UV light), at the photostationary state after irradiation at 365 nm (+ UV light), after irradiation and incubation in the darkness for 10 min (+ UV light & incubation in dark), and after irradiation and fluorescence polarization measurement in a plate reader for 10 min (+ UV light & fluorescence polarization measurement).

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