**Light-Switchable Proteins**

# Photocontrol of Coiled-Coil Proteins in Living Cells**

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An ability to manipulate the activity of a specific protein inside living cells offers exciting prospects for the study of protein function in vivo. Photocontrol, in particular, offers the possibility of controlling protein function with very high temporal and spatial resolution that is not easily achieved by other methods.[1] We aimed to develop an approach to the photocontrol of the coiled-coil protein structural motif inside living cells. Coiled coils in the form of basic leucine zipper (bZIP) domains are common in transcription factors that control gene expression in diverse settings.[2,3] More than 50 members of this family of DNA-binding proteins are known in humans.[4] bZIP proteins exhibit complex spatiotemporal patterns of activity. For example, timed expression of AP-1/ Jun is required for *Xenopus* development,[5] and the activation of the bZIP protein CREB in specific subsets of neurons in the amygdala is correlated with fear-memory formation.[6]

Given the importance of this protein family for transcriptional regulation, we sought to develop a general approach to
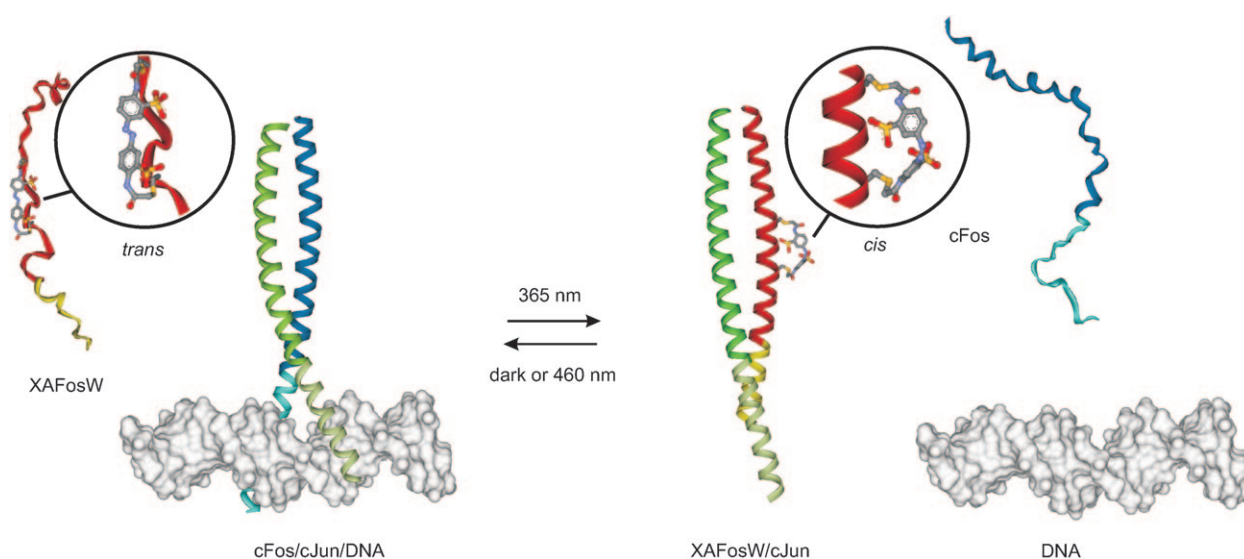


***Figure 1.*** Irradiation at 365 nm of an azobenzene-cross-linked AP-1 dominant negative peptide, XAFosW, causes *trans*-to-*cis* isomerization, which enhances helicity, favors a coiled-coil formation, and thereby leads to the inhibition of DNA binding.

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its photocontrol. Conventional optical control of protein activity through the use of caging groups is difficult to apply to coiled coils, because these structures rely on interactions between hydrophobic side chains (leucine residues), which cannot readily be caged. Instead we used an azobenzene-based cross-linker to alter the structure of dominant negative coiled-coil-forming peptides. By switching the structure of the cross-linked dominant negative peptide, we could reversibly control the activity of targeted bZIP proteins (Figure 1). We applied this approach to the photocontrol of AP-1 activity. The AP-1 transcription factor proteins Fos and Jun are perhaps the best-studied members of the bZIP family.[7] These proteins form either Fos/Jun heterodimers or less stable Jun/ Jun homodimers and bind to specific AP-1 DNA-recognition sequences.

Coiled-coil interactions have been studied extensively, and dominant negative peptides that target coiled coils have been both designed theoretically and evolved through directed experimental approaches.[2,4,8,9] By using micro-

wave-enhanced solid-phase peptide synthesis, we prepared peptide sequences based on our previously evolved dominant negative FosW peptide (see Figure S1 in the Supporting Information).[10] The bZIP domains of human wild-type cellular Fos and Jun (cFos and cJun) were recombinantly expressed, and inhibition of their DNA-binding activity by the dominant negative peptides was quantified by using electrophoretic mobility shift assays (EMSA; Figure 2).
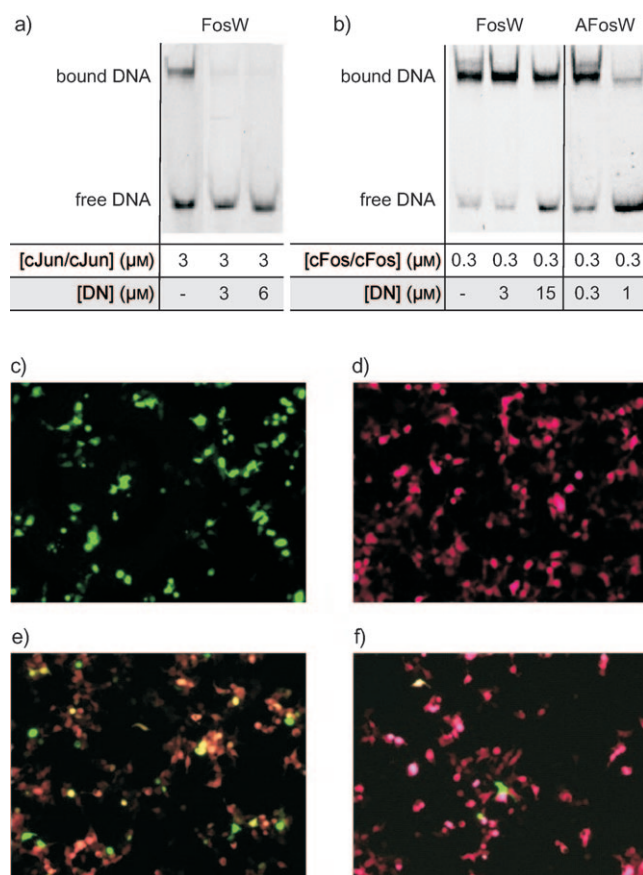


*Figure 2.* a,b) EMSA of FosW inhibition of the DNA binding of homodimeric cJun/cJun (a), AFosW inhibition of the DNA binding of cFos/cJun (b). c–f) Fluorescence images of HEK293 cells transfected with the AP-1-GFP reporter plasmid (c), the control CMV-RFP plasmid (d), both AP-1-GFP and CMV-RFP (e), AP-1-GFP, CMV-RFP, and AFosW (f). (DN = dominant negative peptide).

Whereas FosW acted as an effective dominant negative for the homodimeric cJun/cJun–DNA complex (Figure 2a), AFosW, an analogue of FosW that was created by the addition of two acidic heptad repeat motifs[11] to the N terminus of FosW, showed effective inhibition of heterodimer cFos/cJun binding to DNA (Figure 2b).

To examine the potential for the inhibition of AP-1 activity in living cells, we transfected human HEK293T cells with AFosW together with reporter plasmids by using Lipofectamine 2000. Successful transfection of the dominant negative peptide was confirmed by using a fluorescently labeled AFosW peptide (see the Supporting Information).

Cells were transfected with a plasmid containing the green fluorescent protein (GFP) reporter under the control of an AP-1 dependent promoter (AP-1-GFP; Figure 2c) and a red fluorescent protein (RFP) reporter under the control of a (cytomegalovirus) CMV promoter (CMV-RFP) as an internal control (Figure 2d). In the absence of AFosW, most cells exhibited both red and green fluorescence (Figure 2e). However, in the presence of the AFosW peptide, GFP fluorescence was significantly decreased with little effect on the RFP signal (Figure 2f). This result confirmed the specificity of AP-1 inhibition.

Next, we introduced an azobenzene-based cross-linker, 3,3′-bis(sulfonato)-4,4′-bis(chloroacetamido)azobenzene (BSBCA),[12] into the dominant negative peptides (Figure 1; see also Figure S1 in the Supporting Information). In the dark, BSBCA exists entirely in the *trans* conformation (99.9%). Photoirradiation at 365 nm isomerizes BSBCA to its *cis* form in approximately 87% yield under our experimental conditions. The *cis* form can be reisomerized to the *trans* form either by incubation in the dark or by exposure to light at 460 nm.[12] Such amide-substituted azobenzene derivatives appear relatively stable to reduction by intracellular thiols,[13] and the chloroacetyl groups of BSBCA enable the intramolecular cross-linking of proteins through covalent attachment to cysteine side chains. In the case of helical coiled-coil motifs, residues at consecutive f positions are ideal for cross-linking, because these side chains are distant from the dimerization interface, and because consecutive f positions are spaced seven residues apart, a distance compatible with the *cis* but not the *trans* form of the cross-linker.[14] Two consecutive f positions in the middle of the leucine zipper region of the dominant negative peptides were chosen for mutation to cysteine residues (see Figure S1 in the Supporting Information).

The *cis*-state lifetimes of the peptides were measured by UV/Vis spectroscopy. The observed half-lives were similar for different dominant negative peptides: approximately 20 min at 37 °C, 2 h at 20 °C, and 8 h at 4 °C (see Table S1 in the Supporting Information). Peptide structural changes upon photoisomerization were examined by circular dichroism (CD). Irradiation at 365 nm enhanced the negative ellipticity at 222 nm, an indication of increased α-helix content. When the *cis* forms of the peptides were then exposed to light at 460 nm, the dark-adapted CD spectra were recovered (Figure 3).

The effect of photoisomerization on AP-1 DNA-binding activity was studied by EMSA. When azobenzene-cross-linked FosW (XFosW) was irradiated, the DNA binding of the cJun/cJun homodimer was diminished as expected (see Figure S3 in the Supporting Information). Samples irradiated at 460 nm recovered cJun/cJun-homodimer DNA-binding activity to levels comparable to the activity of the unexposed sample (see Figure S3 in the Supporting Information). For the photocontrol of DNA binding of the cFos/cJun heterodimer, we employed a cross-linked version of the more tightly binding inhibitor AFosW (XAFosW). A similar enhancement of inhibition (ca. 10-fold) upon irradiation at 365 nm was observed (Figure 3; see also Figure S3 in the Supporting Information), whereby the irradiated XAFosW peptide
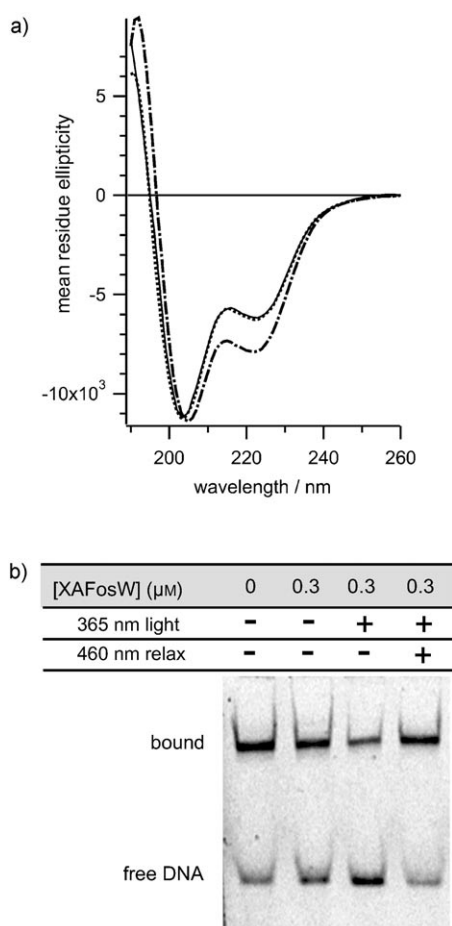
**Figure 4.** Effect of photoirradiation on cellular AP-1 activity in XAFosW-transfected 293T cells. AP-1 luciferase reporter activity relative to β-galactosidase activity was used as an indication of specific inhibition of AP-1 activity. The concentrations of AFosW or XAFosW used in transfection media are shown.

**Figure 3.** a) CD spectra of XAFosW in 10 mM sodium phosphate buffer (pH 7.0, 4°C): dark-adapted (——), irradiated at 365 nm (–•–•), then irradiated at 460 nm (•••••). b) EMSA of photocontrolled XAFosW inhibition of cFos/cJun–DNA binding.

showed comparable inhibitory potency to that of non-cross-linked AFosW (Figures 2b and 3b; see also Figure S3 in the Supporting Information).

We then tested the effect of photoirradiation on AP-1 activity in 293T cells treated with photoswitchable dominant negative XAFosW. To permit a quantitative analysis, we used a luciferase reporter (AP-1-Luc) under the control of an AP-1 promoter together with a β-galactosidase reporter under the control of a constitutive rous sarcoma virus (RSV) promoter (pRSV-Gal). Ratios of luciferase activity to β-galactosidase activity were used as an indication of specific inhibition of AP-1 activity. To isomerize the *trans* BSBCA linker to the *cis* form while limiting possible photodamage to cells, we irradiated the cells with a 365 nm light-emitting diode in short bursts, so that at the end of the incubation period, at least 50% of the linker moieties were expected to be present in the *cis* form in the irradiated cells (see the Supporting Information). When non-cross-linked AFosW was used, AP-1 activity was inhibited in a concentration-dependent but light-independent manner (Figure 4), which is consistent with our results with the GFP reporter (Figure 2). When XAFosW was used, concentration-dependent inhibition of cellular AP-1 activity was again observed, but this activity was light-
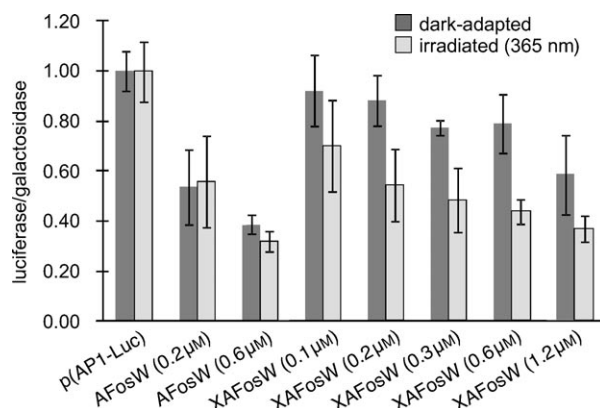
dependent, with a decrease in AP-1 activity upon photo-irradiation by as much as about 40% (Figure 4). To confirm the effectiveness of XAFosW in the photocontrol of AP-1 activity in living cells, we also used the AP-1 GFP reporter (Figure 2) in combination with flow cytometry measurements (see the Supporting Information). In agreement with the luciferase results, photoirradiation led to a selective decrease in the GFP signal. This result confirmed that the *cis* form of XAFosW is a more potent AP-1 inhibitor than the *trans* form. Photorelaxation in vivo with 460 nm blue light was not explored owing to the fast thermal relaxation at 37°C.

Importantly, the observed effects of light on the activity of AP-1 in living cells are not due to UV toxicity or a generalized inhibition of protein expression as a result of UV exposure, since there was no difference in 1) the fraction of live/dead cells, as judged by flow cytometry of propinium-iodide-stained cells, or 2) the level of β-galactosidase activity in irradiated cells versus cells incubated in the dark (see the Supporting Information); nor are these effects an endogenous response of cells to UV exposure, since no difference in AP-1 activity was observed in either assay between dark-adapted and irradiated samples to which either no peptide was added or nonswitchable AFosW was added (Figure 4; see also the Supporting Information). The activation of cJun and cFos by broad-spectrum UVA light (300–400 nm, peaking at 350 nm, doses of 250 kJ m$^{-12}$) was observed previously as part of a UV-induced stress response.[15] If such activation did occur in our case, it would be expected to counter the photocontrolled inhibition of AP-1 activity observed; however, the narrow-range UVA wavelengths ($365 \pm 5$ nm) and intensities used in this study, and the light-delivery pattern and duration, do not appear to be sufficient to trigger an endogenous UV response.

Taken together, our results indicate that cellular AP-1 activity can be photocontrolled through the introduction of the XAFosW peptide. Since this approach to photocontrol is structure-based (based on the conformational consequences of the photoisomerization of a designed chromophore and the effect of this isomerization on a well-defined protein structure), it can be extended readily to other related systems. The

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recent development of amino-substituted BSBCA analogues provides opportunities to also isomerize peptides/proteins with light in the blue and green regions of the spectrum.[16] Such derivatives may prove useful for studies involving cells that are more sensitive to UV irradiation.

The development of selection methods and computational design methods for the identification of peptide sequences that can act as dominant negatives against coiled coils is sufficiently advanced that the photocontrol of a large range of coiled-coil targets can be envisaged.[3,4,9,10] Indeed, coiled coils are found in a diverse range of proteins, in which they are involved not only in transcriptional control but also in muscle contraction, viral infection, cell signaling, mitochondrial import, and fertilization. Dominant negative peptide sequences are generally short enough to permit straightforward chemical synthesis by solid-phase methods; they can be unfolded and refolded easily, and the introduction of the photoswitch through reaction with Cys residues at f positions in the coiled-coil heptad repeat is a robust and quantitative reaction. The cellular uptake of chemically modified peptides was accomplished in a straightforward manner by using Lipofectamine during plasmid transfection. A variety of other methods for the efficient cellular uptake of modified peptides have been described, including the attachment of cell-penetrating sequences.[17] Furthermore, the cell-type-selective uptake of peptides may be possible through the introduction of targeting elements (see, for example, Ref. [18]). Such an approach would further extend the generality of this strategy for the effective spatiotemporal control of protein activity in living systems.

## Experimental Section

Peptides were prepared by microwave-based solid-phase peptide synthesis. They were then cross-linked and/or fluorescently labeled, and purified as described in the Supporting Information. bZIP proteins were produced in bacteria. DNA binding and competitive inhibition of binding was assessed by EMSA, as described in the Supporting Information. HEK293T cells were transiently transfected with plasmids and/or peptides (see Figures S1 and S4 in the Supporting Information) and characterized by fluorescence live-cell imaging, by flow cytometry, and/or in luciferase/galactosidase reporter assays, as described in the Supporting Information.

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