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Light-induced protein degradation in human-derived cells

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ABSTRACT

Controlling protein degradation can be a valuable tool for posttranslational regulation of protein abundance to study complex biological systems. In the present study, we designed a light-switchable degron consisting of a light oxygen voltage (LOV) domain of *Avena sativa* phototropin 1 (AsLOV2) and a C-terminal degron. Our results showed that the light-switchable degron could be used for rapid and specific induction of protein degradation in HEK293 cells by light in a proteasome-dependent manner. Further studies showed that the light-switchable degron could also be utilized to mediate the degradation of secreted *Gaussia princeps* luciferase (GLuc), demonstrating the adaptability of the light-switchable degron in different types of protein. We suggest that the light-switchable degron offers a robust tool to control protein levels and may serves as a new and significant method for gene- and cell-based therapies.

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1. Introduction

Techniques that manipulate gene expression are typically used to study gene and protein functions in living cells. Usually, proteins may be overexpressed through the transfection of ectopic gene plasmids for gain-of-function studies. Selective gene inactivation and protein level reduction can be achieved by gene knockout through homologous recombination or by knockdown using siRNA or shRNA [1,2]. However, the drawback lies in the speed at which the biological events being studied occur, and the major determinant affecting the rate of knockdown is the half-life of the protein of interest. Cellular protein levels can be manipulated through controlled protein degradation. Controlling protein degradation can be a valuable tool for posttranslational regulation of protein abundance to study complex biological systems [3]. Several chemical-induced protein degradation systems have been developed previously. Janse reported a rapamycin-induced protein degradation in which FKBP12 and FRB interacts with each other in the presence of rapamycin and in turn directs the location of target protein into proteasome to be degraded [4]. Similar systems based on FRB and FKBP12 were developed by several other groups [2,5–7]. In addition, chemical-switchable degrons have been used to control the degradation of target protein, which can be triggered by well-defined and inert molecules, such as TMP and shield-1 [8,9]. These chemical-induced protein degradation systems allow simple and rapid control of protein stability. However, as these chemicals diffuse freely and are hard to remove, it is not possible to precisely control protein degradation at an exact location and time.

In the past few years, emerging optogenetic techniques have been increasingly utilized in life science research, combining the use of light and genetically encoded light-sensitive proteins to control the behavior of proteins in living cells and organisms [10]. Several light-switchable transgene expression systems have been developed to control gene transcription to control corresponding protein levels in mammalian cells [11–22]. However, upregulation and downregulation of protein levels are always slow by these systems, as it takes time for mRNA accumulation and degradation. To directly regulate intracellular protein levels by light, several groups have utilized the light oxygen voltage (LOV2) domain from phototropin to control protein degradation in bacteria, yeast and zebrafish [23–25]. In these systems, light-sensitive degrons were constructed by attaching a small degron at the C-terminus of AsLOV2. Upon light illumination, unfolding of the Jα helix of

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AsLOV2 would release the locking of the degrons which in turn mediate degradation of the fused protein. However, a light-switchable degron that can be used in human-derived cell lines to induce protein degradation of target protein by light has not been reported yet. In the present study, we developed a new light-sensitive degron by fusing the cODC degron to the C-terminal of *Avena sativa* phototropin 1 (AsLOV2). Our results exhibited light-induced proteasome-dependent degradation of target protein in HEK293 cells. When using the long-lived fluorescent protein mCherry as the reporter, the half-life of the fusion protein was about 3.1 h upon light illumination. We further showed that the light-switchable degron could also be used to control degradation of *Gaussia princeps* luciferase (GLuc), demonstrating that such a light-sensitive degron can be utilized to regulate the content of a secreted protein in living cells. We suggest that the light-switchable degron offers a robust tool to control protein levels and may serve as a new and significant method for gene- and cell-based therapies.

2. Materials and methods

2.1. Plasmid construction

The gene of photoreceptor AsLOV2 and degron C-terminus of human ornithine decarboxylase (cODC) was ligated by overlapping PCR and inserted into EcoRI and XhoI sites of pCDNA3.1(+) to obtain pCDNA3.1-AsLOV2-cODC. FLuc gene with Kozak sequence was inserted into pCDNA3.1-AsLOV2-cODC or pCDNA3.1(+) at NheI and EcoRI sites to obtain pCDNA3.1-FLuc-AsLOV2-cODC and pCDNA3.1-FLuc, respectively. The truncated variants of LOV2-cODC were obtained based on pCDNA3.1-FLuc-AsLOV2-cODC according the manufacturers' protocols for the TaKaRa MutanBEST kit. FLuc gene fragment in pCDNA3.1-FLuc-AsLOV2-cODC was replaced with GLuc gene by double digestion with NheI and EcoRI. Gene fragment containing mCherry-LOV2-cODC or mCherry alone was cloned into pLVX-IRES-ZsGreen retroviral expression vector by EcoRI and BamHI double digestion.

2.2. Cell culture

HEK293 and HeLa cells were cultured in high-glucose DMEM (GIBCO) supplemented with 10% FBS, 1% penicillin, and streptomycin (Invitrogen). The cells were plated in antibiotic-free high-glucose DMEM supplemented with 10% FBS for 16 h before transfection. Equal amounts (0.2 μg) of the vector with 0.6 μL of Lipofectamine 2000 were used for each well of the 48-well plate according to the manufacturer's protocol. The transfected cells were then covered with aluminum foil and kept in the dark for 24 h. For inducing protein degradation, the cells were then illuminated with 30 $\mu M\ m^{-2}\ S^{-1}$ blue light from below using an LED lamp (460 nm peak). The control cells were still maintained in darkness before measurement. The LED lamps were controlled by a relay to adjust the overall intensity of blue light.

2.3. Measuring FLuc and GLuc activity

A Synergy 2 multi-mode microplate reader (BioTek) was used to detect the chemiluminescence cells. For the Fluc activity assay, 10 μL cell lysate and 15 μL assay buffer were added into each well of a white 384 well plate (Greiner), and 15 μL 0.2 mM d-luciferin (SynChem) was added before detection. Light emission was recorded as relative light units (RLU), data were normalized based on protein concentrations measured using the DC protein assay (Bio-Rad). For the Gluc activity assay, the culture supernatants were assayed for secreted Gluc activity using a Gaussia luciferase assay kit according to the manufacturer's protocol (NEB).

2.4. Fluorescence measurement

The stable mCherry-LovD-expressing cell lines were plated on a 35 mm glass-bottom dish with phenol-red-free fresh growth medium. The sample was grown in the dark for 12 h and was either transferred to light conditions or maintained in the dark for an additional 6 h. Living cell ZsGreen and mCherry fluorescence were taken by a Nikon TE 2000-E inverted microscope equipped with a 40X water immersion Fluor lens (numerical aperture 1.3) using a FITC filter for ZsGreen and a Texas Red filter for mCherry. Images were analyzed by ImageJ software (http://rsb.info.nih.gov/ij/). The cells that stably expressed mCherry-LovD or mCherry were plated in two black 96-well glass-bottom microplates and were cultured in darkness for 24 h. The cells were then transferred to 10 $\mu M\ m^{-2}\ S^{-1}$ blue light or maintained in darkness for additional 6 h. mCherry and ZsGreen fluorescence was measured by a Synergy 2 Multi-Mode Microplate Reader, with an excitation filter of 590 BP 20 nm and an emission filter of 645 BP 40 nm for mCherry and an excitation filter of 485 BP 20 nm and an emission filter of 528 BP 20 nm for ZsGreen.

2.5. Western blot analysis

Western blot analysis was conducted using previously described procedures [26]. Briefly, equal amounts of lysates from stable cell lines were electrophoresed on 12% SDS–PAGE gel and transferred onto polyvinylidene fluoride membranes using an electroblotter. After blocking with 0.5% casein, the membranes were probed with mouse anti-mCherry (1:1000; Affinity) or mouse anti-β-actin (1:40,000; Sigma–Aldrich) antibodies. Subsequently, the cells were treated with horseradish-peroxidase-labeled secondary antibodies (1:10,000; Jackson Immuno Research). Immunoreactivity was detected using a BM Chemiluminescence Blotting kit (Roche Diagnostics) used according to the manufacturer's protocol on a Kodak In-Vivo Multispectral System FX (Carestream Health).

3. Results

3.1. Design and optimization of light-sensitive degrons

37-residue cODC is a native ubiquitin-independent degron in which a molecular mimic of a polyubiquitin chain can be recognized by proteasomes [27]. When cODC was fused to the *fruit fly* luciferase (FLuc), the FLuc activity was only 2.5% relative to the control without cODC tag in HEK293 cells (Supplementary Fig. 1). This result indicated that cODC was quite efficient in destabilizing its fusion partner and targeting it for degradation in human cells. To control the activity of cODC by light, a light-switchable degron was made by fusing cODC to the C terminus of the second LOV domain of *Avena sativa* phototropin 1 (AsLOV2) (Fig. 1A). We hypothesized that AsLOV2 is converted from a “closed” dark state into an “open” light state upon blue light illumination, resulting in the undocking and unfolding of the carboxy-terminal Jα helix, which in turn caused unlocking and release of cODC degron. The results showed that 6 h of exposure to 30 $\mu M\ m^{-2}\ S^{-1}$ blue light significantly reduced the FLuc activity of FLuc-AsLOV2-cODC-expressing HEK293 cells, the dark/light ratio of FLuc activity was 2.8 folds (Fig. 1B). However, no significant difference between light and dark conditions for FLuc-expressing cells (Fig. 1B).

To improve the efficiency of the light-switchable degron, we performed a series of truncation targeting the first 10 amino acid residues of cODC (Fig. 1C, Supplementary Table). One of the truncations, 09, in which the first 9 amino acids of cODC was deleted, exhibited enhanced stability in dark conditions, resulting in ~6-fold dark/light ratio (Fig. 1B). Truncation 10 amino acids of cODC

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