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Tracking protein–protein interaction and localization in living cells using a high-affinity molecular binder

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ABSTRACT

Probing protein–protein interactions in living cells is crucial for understanding the protein functions and developing drugs. Small-sized protein binders are considered effective and useful for such analysis. Here we describe the development and use of a repebody, which is a protein binder composed of LRR (Leucine-rich repeat) modules, for tracking protein–protein interaction and localization in real-time through live-cell imaging. A repebody with high affinity for a red fluorescent protein was selected through a phage display, fused with a green fluorescent protein, and applied for tracing a red fluorescent protein-fused target protein in mammalian cells. The potential and utility of our approach was demonstrated by tracking the rapamycin-mediated interaction between FKBP12-rapamycin binding (FRB) domain and a FK506-binding protein (FKBP) and their localization by live-cell imaging. The present approach can be widely used for the analysis of protein–protein interaction and an understanding of complex biological processes in living cells.

1. Introduction

Proteins inside cells interact with cognate biomolecules and play key roles in biological processes, including signal transduction, cell differentiation, proliferation, and programmed cell death. An understanding of such interactions and consequences in living cells is crucial to our understanding of protein functions and drug development [1]. The use of target-specific antibodies has been the primary choice as a molecular binder to track the interactions between proteins and the localization in the cells [2]. However, antibodies have difficulties penetrating and expressing within cells owing to their large size, which limits the analysis of protein–-protein interaction by live-cell imaging [3]. Antibodies are therefore mostly employed after a chemical fixation or permeabilization of the cells. In this regard, the use of small-sized protein binders as alternatives to immunoglobulin antibodies, such as fibronectin, affibody, and nanobody, have been developed [4–6]. A protein binder was fused to a reporter gene and expressed in the cells to trace the localization of a target protein [6,7]. To expand the repertoires of binders with practical applications, a new type of molecule binder with easy engineering and production will be of great significance.

We previously developed the repebody scaffold which is composed of Leucine-rich repeat (LRR) modules as an alternative to immunoglobulin antibodies [8]. The repebody scaffold was shown to have desirable properties, including easy library construction, rapid affinity maturation by modular engineering, a high expression level in bacteria, and high stability [9,10]. Herein, we present the development and use of a repebody for tracking protein–protein interaction and localization in living cells. We selected a repebody with high affinity for a red fluorescent protein through a phage display, fused to a green fluorescent protein, and used for tracing a target protein in mammalian cells by live-cell imaging. The potential and utility of the repebody was demonstrated by tracking the rapamycin-mediated interaction between FKBP12-rapamycin binding (FRB) domain and a FK506-binding protein (FKBP) in living cells.

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2. Material and methods

2.1. Cloning, expression, and purification of proteins

The genes coding for various fusion proteins as well as a repebody were cloned into the NdeI and XhoI restriction enzyme sites of a pET21a vector (Novagen, San Diego, CA). Each gene has the C-terminal hexa-histidine tag for affinity purification. The vectors were transformed into *Escherichia coli* BL21 (DE3), and induced with IPTG (0.5 mM) when the optical density reached OD_{600} between 0.5 and 0.7. Induced cells were further grown at 30 °C for 9 h and harvested by centrifugation at 4000 *g*. The cells were resuspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, at pH 8.0) and disrupted through sonication. Following centrifugation at 12,000 rpm for 1 h, supernatant was loaded onto a Ni-NTA Superflow (Qiagen, Venlo, The Netherlands). The solution was washed with 3 column volumes of 50 mM NaH_2PO_4, 300 mM NaCl, and 50 mM imidazole, at pH 8.0. Hexahistidine tagged fluorescent proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, at pH 8.0). The proteins were further purified by gel permeation chromatography (Superdex75, GE Healthcare, Little Chalfont, UK) with a phosphate-buffered saline (PBS; pH 7.4).

2.2. Selection of repebodies specific for mOrange

A repebody library was constructed by introducing random mutations into two different modules (LRRV2, LRRVe) and subjected to phage display to select a repebody specific for a red fluorescent protein (RFP) as described elsewhere [11]. A bio-panning process was performed using 5 ml immune-tubes (Greiner, Frickenhausen, Germany) coated with 1 ml of mOrange (100 μg/ml) overnight at 4 °C. The phage solution (1.0×10^{12} cfu/mL, 1 mL) was added to the tubes and incubated for 2 h at room temperature with PBST-BSA blocking solution (1% BSA in phosphate-buffered saline Tween-20). Following the removal of the phage solution, target-binding phage particles were eluted with a 0.2 M glycine-HCl solution (pH 2.2) for 15 min at room temperature. *E. coli* XL1-Blue F′ was added to the eluted phage particles, and the infected cells were cultured at 37 °C for 30 min. The cells were collected and spread on 2xYT plates containing 100 μg/mL of ampicillin, 10 μg/mL of tetracycline, and 1% glucose. The 2xYT plate was incubated overnight at 30 °C. Rounds of panning were carried out using the plated cells. Individual colonies from plated cells were seeded in a 96 deep well plate (Nunc, Roskilde, Denmark) containing 200 μl of 2xYT/ATG and grown overnight at 37 °C. Following the infection with helper phage, 100 μl of 2xYT/K was added to each well and cultured overnight at 30 °C. Repebody-displaying phage particles were purified from a culture supernatant and prepared for phage ELISA.

2.3. Construction of mammalian expression vector

For the expression of a repebopdy in mammalian cells, the gene coding for repebody-B1 was amplified by PCR and digested with XhoI and EcoRI. The fragment was then ligated into the corresponding sites in pEGFP-N1 (Clonetech Inc.), generating the expression construct, EGFP-repebody-B1.

2.4. Cell lines, transfection, and western blot analysis

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. HeLa cells were transiently transfected by LTX Lipofectamine/Plus Reagent (Invitrogen), according to the manufacturer's instructions. Cells were harvested and analyzed 30 h after transfection. Transfected cells were washed with DPBS, lysed for 10 min in lysis buffer (Tris–HCl 50 mM, pH 8, NaCl 150 mM and 1% Triton X-100). The supernatant fractions were separated using 12% SDS polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Bio-Rad, Germany) at 100 V for 2 h in ice. The membrane was incubated with a blocking buffer, phosphate-buffered saline with Tween 20 (PBST)-bovine serum albumin (BSA; PBS containing 0.1% Tween-20 and 1% BSA) and subjected to a western blot analysis using the goat anti-GFP polyclonal antibody (Abcam, Cambridge, UK) and rabbit anti-goat IgG-HRP conjugated antibody (Bio-Rad, Germany). The blots were visualized by an enhanced chemi-luminescence solution (Millipore, Darmstadt, Germany) using a LAS-3000 imaging system (Fuji-Film, Tokyo, Japan).

2.5. Live-cell imaging and data acquisition

Live-cell imaging was performed 30 h after transfection, using a Nikon A1R confocal microscope (Nikon Instruments, Japan) with CFI Plan Apo VC 60X lens. 488 nm and 561 nm lasers were used to image GFP and RFP channel, respectively. The images were analyzed with tools provided by NIS-elements (Nikon imaging software, Nikon Instruments, Japan). For analysis of cell area, we used the “ROI Statistics” method and “Time Measurement” tool in Nikon imaging software to quantitatively observe the changes of fluorescent intensities. Rapamycin was added at a final concentration of 500 nM to induce hetero-dimerization of FKBP- and FRB-fused proteins. The recruitment of a repebody and fluorescent proteins by rapamycin was observed by imaging in 30 s intervals for 5 min. Relative fluorescence units (RFU) was normalized against the initial cytosolic intensity of fluorescence prior to treatment of rapamycin, and then plotted using Microsoft ® Office Excel 2010. Ratio view and videos were also obtained using NIS.

3. Results

3.1. Selection and characterization of mOrange binding repebody by phage display

We first intended to select a repebody specific for a red fluorescent protein, mOrange. For this, we generated a repebody library by introducing random mutations into two different modules (LRRV2, LRRVe) (Fig. 1A). We chose LRRV2 and LRRVe for random mutations based on a docking simulation between the repebody and mOrange. Following five rounds of a bio-panning process against mOrange, repebody-B1 was finally selected. Repebody-B1 was shown to possess basic amino acids such as lysine and arginine as well as polar amino acids such as asparagine and glutamine at different mutation sites (Fig. 1B). We determined the binding affinities of repebody-B1 for mOrange and mCherry through ITC (Table 1 and Supplementary Fig. S1). Repebody-B1 has high binding affinities (K_D) of around 31.9 nM and 40.0 nM for mOrange and mCherry respectively. Repebody-B1 exhibited a negligible cross-reactivity against green fluorescent protein (GFP) and yellow fluorescent protein (YFP) (Supplementary Fig. S2). Although the binding affinity of repebody-B1 for mCherry was comparable to mOrange, we chose mCherry as a repebody target for further experiments by taking into account the redshift property of mCherry [12].

3.2. The applicability of repebody-B1 to mammalian cells

To test the applicability of repebody-B1 in cytosol of mammalian cells, we measured its circular dichroism (CD) spectra under reducing conditions. As a result, no significant changes in the