Biochemical and Biophysical Research Communications 404 (2011) 211–216

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Fluorescent "Turn-on" system utilizing a quencher-conjugated peptide for specific protein labeling of living cells

Satoshi Arai [a], Su-In Yoon [a,b], Atsushi Murata [c], Masao Takabayashi [c], Xiaoyu Wu [d], Yixin Lu [d], Shinji Takeoka [c], Miwako Ozaki [a,b,*]

[a] Comprehensive Research Organization, Waseda University, Tokyo, Japan
[b] Waseda Bioscience Research Institute in Singapore (WABIOS), Biopolis, Singapore
[c] Department of Life Science and Medical Bioscience, Waseda University, Japan
[d] Department of Chemistry, National University of Singapore, Singapore

ARTICLE INFO

Article history:
Received 17 November 2010
Available online 24 November 2010

Keywords:
Fluorescent imaging
Small chemical probe
His-tag

ABSTRACT

A specific protein fluorescent labeling method has been used as a tool for bio-imaging in living cells. We developed a novel system of switching "fluorescent turn on" by the recognition of a fluorescent probe to a hexahistidine-tagged (His-tag) protein. The tetramethyl rhodamine bearing three nitrilotriacetic acids, which was used as a fluorescent probe to target a His-tagged protein, formed a reversible complex with the quencher, (Dabcyl)-conjugated oligohistidines, in the homogeneous solution, causing fluorescence of the fluorophore to be quenched. The complex when applied to living cells (COS-7) expressing His-tagged proteins on the cell surface caused the quencher-conjugated oligohistidines to be dissociated from the complex by specific binding of the fluorescent probe to the tagged protein, resulting in the fluorescent emission. The complex that did not participate in the binding event remained in the quenched state to maintain a low level of background fluorescence.

1. Introduction

The fluorescent probe has become the leading candidate as a tool for tracking a target protein in living cells because of its inherent sensitivity and temporal–spatial resolution [1–3]. The method using gene construction of a fused fluorescent protein such as green fluorescent protein (GFP) variants for fluorescent labeling of a target protein has become a powerful tool for biologists [4–6]. However, use of this method is restricted when the large molecular weight of a fused protein interferes with the folding and trafficking of the target protein [7]. A small fluorescent compound could therefore be expected to minimize the interference of protein dynamics and resolve this restriction [8,9]. Some studies have recently been reported to satisfy this requirement [10–12]. A general strategy has described that a short peptide tag was introduced to a protein, and a fluorophore was then designed to specifically recognize the peptide tag. In particular, a fluorescent probe for labeling a hexahistidine-tagged (His-tag) protein has attracted researchers in various fields, because the His-tag protein has been widely applied as the tag for affinity purification of a recombinant protein and for localization studies on molecules [13–16]. However, the challenge of providing a general small chemical probe remained, so that fluorescent molecules not participating in specific binding to a target protein do not need to be removed by washing with a buffer [17]. A functional fluorescent probe exhibiting enhanced fluorescence in response to His-tag recognition, *i.e.* a "fluorescent turn-on probe", is therefore required. An earlier report has described a field-sensitive system by which the Dansyl-conjugated nitrilotriacetic acid nickel complex (Dansyl-NTA-Ni^{2+}) bound to the protein tethering the hexahistidine coupled to a hydrophobic motif, resulting in a wavelength shift of the fluorescence emitted by Dansyl adjacent to the hydrophobic motif [18]. Higuchi et al. have reported a different functional probe utilizing a weakly fluorescent complex involving intramolecular coordination of the fluorophore by metals [19,20]. Although both approaches are elegant, they have never been applied for imaging living cells to our knowledge. It is assumed that the strategies just described would require sophisticated structural design utilizing a probe or a functional peptide sequence, implying that it would not be widely applicable as a general chemical probe. In addition, the type of fluorophore that can be used is restricted, although selection of the wavelength is significant in an application to living cell imaging.

We demonstrate in this paper a simple "fluorescent turn-on" system that applies a fluorescence-quenching pair with a fluorescent chemical probe to target the tagged protein and reversible quencher-conjugated peptide. The turn-on system was applied to

* Corresponding author at: Waseda Bioscience Research Institute in Singapore (WABIOS), Helios, #05-01/02, 11 Biopolis Way, Singapore 138667, Singapore. Fax: +65 6478 9416.
E-mail address: mozaki@waseda.jp (M. Ozaki).

0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.11.095

living cells and evaluated by using a fusion protein with a His-tag and trans-membrane domain. The new system showed a low artificial background and a distinct signal after binding to the His-tagged protein without needing any washing procedure. Such requirements as the recognition ability, quenching effect, and reversibility are also discussed to establish the turn-on system.

2. Materials and methods

2.1. General methods (reagents, characterization and antibodies)

FITC-NTA was synthesized according to the previous report [21]. All solvents were purchased from Kanto Chemical Industry Co. (Tokyo, Japan) and were used as received. All organic reagents were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and used without further purification. The synthesized compounds were characterized by ^{1}H NMR and electrospray ionization mass spectrometry (ESI-MS). NMR spectra were recorded in a deuterated solvent by an Oxford NMR AS400 spectrometer (400 MHz). Electrospray ionization mass spectra (ESI-MS) were recorded by an LCQ Fleet mass spectrometer (Thermo Fisher Scientific, USA). Plastic sheets coated with 0.2-mm silica gel 60 without a fluorescent indicator (Merck, Germany) were used for thin-layer chromatography (TLC). The anti-His6 monoclonal mouse antibody (His6 Ab) was purchased from Roche (11922416001, Germany) for the cell study. Anti-mouse IgG conjugated with horseradish peroxidase (HRP) was from Vector Laboratories (PI-2000, USA), and Alexa Fluor-633 goat anti-mouse IgG was from Molecular Probes (A21050, USA). The reagents for enhanced chemiluminescence labeling (ECL) and western blotting detection were from Amersham (GE Healthcare, UK). The Mini Complete™ protease inhibitor cocktail (PI) was from Roche (Mannheim, Germany), and the PVDF membranes were from Bio-Rad Laboratories (62-0177, USA). Phenyl-methyl-sulfonylurea-fluoride (PMSF) and *p*-formaldehyde were from Sigma (P7626 and P6148, USA).

2.2. Synthesis of TMR-triNTA

TMR-triNTA was synthesized according to the literature procedures [21,24]. *N,N*-Diisopropylethylamine (DIPEA, 10 μl) was added to a CH_2Cl_2 solution (1 ml) of NH_2-tri-NTA(t-Bu)$_3$ (21.0 mg, 15 μmol) and 5(6)-carboxy-tetramethylrhodamine *N*-succinimidyl ester (4.6 mg, 7.0 μmol), and the resulting mixture was stirred overnight at room temperature. The reacted solution was evaporated *in vacuo* and purified by column chromatography on silica gel ($CHCl_3$/CH_3OH = 40/1 v/v) to give TMR-triNTA(t-Bu)$_3$. This was then dissolved in TFA (2 ml) and stirred overnight. The solvent was evaporated *in vacuo* to give TMR-triNTA as a solid (5.1 mg, 42%). ^{1}H NMR (400 MHz, D_2O/CD_3CN) δ: 8.61, 8.30, 8.15, 8.10, 7.66, 7.42 (m, 3H), 7.04 (d, 2H), 6.92–6.83 (m, 6H), 3.66–3.13(m, 28H), 1.87–0.76 (m, 36H). ESI-MS *m/z*: 467.92 $[M-3H^+]^{3-}$.

2.3. Synthesis of 2′,7′-bis(2-pyridylsulfonamido)-4′,5′-dimethylfluorescein (HisZiFiT)

2′,7′-Bis(2-pyridylsulfonamido)-4′,5′-dimethylfluorescein (HisZiFiT) was synthesized according to the previous report [23]. To a solution of 2′,7′-diamino-4′,5′-dimethylfluorescein (60.8 mg, 0.156 mmol) in anhydrous pyridine (4 ml) was added 2-pyridine sulfonylchloride (200.4 mg, 0.936 mmol). The mixture was stirred at room temperature for 12 h, poured into an aqueous solution of 1 M HCl (80 ml), and the precipitate was collected. The crude compound was purified by flash column chromatography on silica gel ($CHCl_3$/CH_3OH/acetic acid = 9/1/0.05 (v/v/v)) to give HisZiFiT (10.5 mg, 10.0%) as a solid. ^{1}H NMR (CD_3OD) δ: 2.23 (6H, s), 6.34 (2H, br-s), 6.94–7.00 (1H, m), 7.42–7.48 (2H, m), 7.57 (2H, d, J = 8.0 Hz), 7.69–7.73 (2H, m), 7.79–7.85 (2H, m), 8.12–8.22 (1H, m), 8.40 (1H, d, J = 4.4 Hz) ESI-MS *m/z*: only the fragment peak was detected.

2.4. Synthesis of the quencher-conjugated peptide (Dabcyl-His6)

Quencher-conjugated His6 (Dabcyl-His6) was synthesized by Fmoc solid-phase peptide synthesis (SPPS), using Fmoc-His (Trt)-OH and HBTU/HOBt/NMM as coupling reagents (Hayashi Kasei Co.). The compound was deprotected with DMF/piperidine (20%) and TFA/EDT/Thioanisole/TIS/H_2O. The final compound was characterized by HPLC (Shimadzu Prominence) and MS (HP1100 series LC/MSD). HPLC was performed at a flow rate of 1.0 ml/min. in a C18 column (Shiseido Capcell Pak C18). MS *m/z*: 1093 $[M+H]^+$.

2.5. Fluorescence measurements

All fluorescence spectra were recorded by an RF5300PC spectrofluorophotometer (Shimadzu). The bandwidth was 5.0 nm for excitation and emission, using a 10-mm quartz cell.

2.6. Plasmid construction

His-pDisplay was constructed by replacing a DNA fragment encoding for hexahistidine residues with the sequence between the SmaI and SacII sites in pDisplay (Invitrogen, USA). In the first round of PCR, primers P1, the T7 primer (5′-TAA TAC GAC TCA CTA TAG GGA GAC-3′) and P2 (5′-ATG ATG ATG ATG ATG ATG GGG AGA TCT GGC CGG CTG-3′) were used to introduce the hexahistidine coding sequence at the 3′ end of the PCR fragment. Similarly, primers P3 (5′-CAT CAT CAT CAT CAT CAT CGG CTG CAG GTC GAC GAAC-3′) and P4, the BGH reverse primer (5′-CTA GAA GGC ACA GTC GAG GC-3′) were used to generate an overlapping region of the hexahistidine coding sequence at the 5′end. pDisplay was used as a template for the two reactions. The two PCR-amplified DNA fragments were then used as templates for the second round of PCR with primers P1 and P4. Plasmid pFLAG CMV–neuregulin containing cDNA [22] for the EGF domain of mouse neuregulin is preserved in our laboratory. His-EGF-TD was constructed by amplifying the cDNA fragments of EGF by PCR from pFLAG CMV–neuregulin, using primers P1 (5′-GCGTCGAC TCA AAC GCC ACA TCT ACA TCC-3′) and P2 (5′-GCGTCGAC CTC CTC CGC TTC CAT AAA TTC-3′) which introduced SalI restriction enzyme sites. The amplified PCR product was ligated into the TA vector, and the confirmed sequence was subcloned into the SalI site of His-pDisplay.

2.7. Cell application

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. All chemicals used were purchased from Gibco (USA). The cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere. COS-7 cells were seeded at 7×10^4 cells onto 35-mm glass-bottom dishes containing 2 ml of the fresh culture medium and transfected with the plasmid (0.5 μg) of His-EGF-TD by using the Effectene reagent (Qiagen, USA) according to the manufacturer's protocol. After 1 or 2 days, the cells were washed twice with Hanks's balanced salt solution (HBSS; Gibco, USA) and stained at room temperature by treating with either of the dyes described in the legend to Fig. 2 or His6 Ab (see Section 2) at room temperature. A His6 Ab stock solution (0.1 mg/ml) was diluted 1:20 in DMEM with 10% FBS, and the mixture added to the cells for 15 min. The cells were next washed twice with HBSS and stained for 15 min with the Alexa Fluor-633 secondary antibody (see Section 2) at a 1:300 dilution