JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Fluorophore Labeling of Native FKBP12 by Ligand-Directed Tosyl Chemistry Allows Detection of Its Molecular Interactions in Vitro and in Living Cells

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Supporting Information

ABSTRACT: Introducing synthetic fluorophores into specific endogenous proteins and analyzing their function in living cells are a great challenge in chemical biology. Toward this end, we demonstrate the target-selective and site-specific fluorescent labeling of native FKBP12 (FK506-binding protein 12) in vitro and in living cells using ligand-directed tosyl (LDT) chemistry. The LDT-mediated labeling yielded a semisynthetic FKBP12 containing the Oregon green (OG) dye near the catalytic pocket. The OG-labeled FKBP12 (OG-FKBP12) acted as a fluorescent reporter that allows monitoring of its interaction with rapamycin and FRB (FKBP-rapamycin-binding domain) in vitro. We also successfully demonstrated the visualization of the rapamycin-mediated complexation of the OG-FKBP12 and FRB inside of living cells by the combined use with fluorescent protein-tag technology and Förster resonance energy-transfer imaging.

The site-specific chemical modification of proteins with fluorescent dyes is a powerful approach not only to visualize the protein itself but also to create new fluorescent reporters that allow the monitoring of specific biological events, such as protein−small molecule[1] or protein−protein interactions.[2] Traditionally, chemical protein modification has been carried out in vitro. However, with the development of chemical biology, there has been a growing need for methods that can be used to install organic fluorophores to specific target proteins in living cells. Currently, peptide or protein tag-based methods are widely used.[3] This approach permits selective protein modification in cells but is limited to label recombinant tag-fusion proteins (not natural counterparts) that are exogenously expressed. Although the tag-based method is also site specific, in most cases the tag fusion site is restricted at the N- or C-terminus of proteins. There are many situations in which a fluorophore is desired to attach at a specific site in the protein body. For example, many of semisynthetic fluorescent biosensors previously reported were prepared by installing an environment-sensitive dye into a protein scaffold at a site near the analyte-binding pocket.[1,4] The tag-mediated labeling technique may be ill-suited for this type of protein engineering approach. To overcome these limitations, our group has been developing organic chemistry-based methods for site-specific native protein labeling.[5] In particular, the recently developed ligand-directed tosyl (LDT) chemistry[6] provides a powerful means to introduce diverse synthetic probes at the vicinity of the ligand-binding pocket of target proteins with high site-specificity and target selectivity (Figure 1A). With this method, native proteins in test tubes as well as in living cells can be targeted, and the function of labeled proteins is preserved. Using this chemistry, we recently demonstrated that specific intracellular native proteins can be chemically modified and converted into a ^{19}F NMR-based biosensor[6] or photocross-linkable protein[7] in an in situ manner. However, the application of the method to generate fluorescent functional proteins has yet to be demonstrated. Given the importance of bioimaging in recent biological research, it should be invaluable to add the LDT chemistry as a new tool for fluorescent imaging of (natural) proteins in living cells. Furthermore, the combined use of the LDT chemistry with other techniques, such as the GFP technology and protein tag methods, may extend the possibility of live imaging study.

Here, we describe a successful application of the LDT chemistry for labeling native protein, FK506-binding protein 12 (FKBP12), with a fluorescent dye in vitro and in living cells. The LDT-mediated labeling yielded a semisynthetic FKBP12 containing the fluorophore near the catalytic pocket. In vitro, the labeled FKBP12 acted as a fluorescent reporter that changes its fluorescent intensity upon interaction with the drug rapamycin and FKBP-rapamycin-binding domain from mTOR (FRB). We also demonstrated the visualization of the rapamycin-induced complexation of the labeled endogenous FKBP12 and FRB in living cells by using a fluorescent protein-tagged FRB and Förster resonance energy-transfer (FRET) imaging.

On the basis of our previous study,[7] we designed and synthesized LDT reagent **1** for fluorescent labeling of FKBP12

Received: February 23, 2013
Published: April 23, 2013

dx.doi.org/10.1021/ja401956b | J. Am. Chem. Soc. 2013, 135, 6782−6785

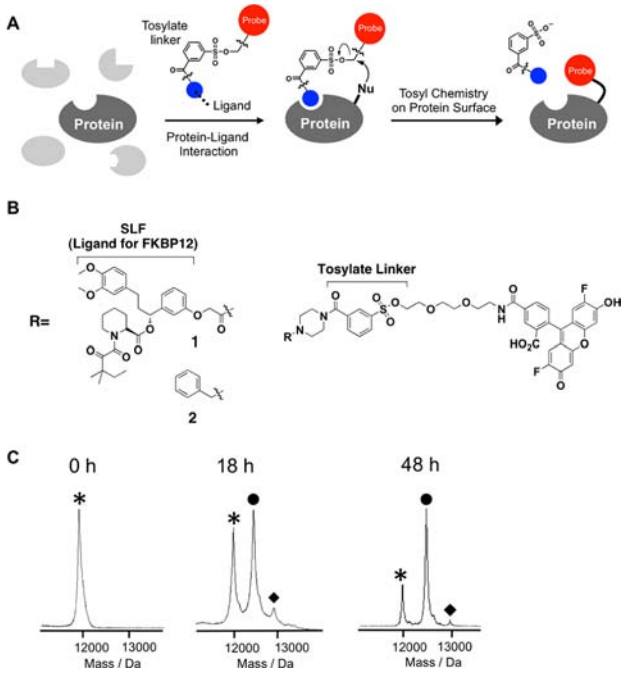

Figure 1. (A) Schematic illustration of the basic principle of the LDT chemistry. (B) Molecular structures of LDT reagents **1** and **2** used in this study. (C) MALDI-TOF mass analysis of FKBP12 labeling by **1**. *, native FKBP12 (MW, 11 914); ●, OG-labeled FKBP12 (MW, 12 439); ◆, OG × 2-labeled FKBP12 (MW, 12 964). Reaction condition: 11 μM FKBP12, 11 μM reagent **1**, 50 mM Tris buffer, pH 8.0, and 37 °C.

(Figure 1B). In the design of **1**, the synthetic ligand of FKBP12 (SLF) is connected to the Phenylsulfonate (tosyl) group via the optimized piperazine spacer, and the Oregon green (OG) dye is used as a green fluorescent molecule. OG is more photostable than fluorescein, and its fluorescence is essentially pH insensitive in the physiological pH range.[8]

We first evaluated the in vitro labeling properties of reagent **1** by incubating it with purified recombinant (wild-type) FKBP12 in a buffer solution. As shown by MALDI-TOF MS (Figure 1C and Figure S1), FKBP12 was predominantly modified with one OG group per protein (single labeling).[9] The labeling yields after 18 and 48 h incubation were estimated to be 56 and 70%, respectively.[10] The labeling by **1** was completely abolished in the presence of FK506 (a competitive ligand), demonstrating that the labeling was facilitated by an affinity-driven proximity effect (Figure S1). Conventional peptide mapping analysis revealed that Glu57 located at the proximity of the ligand binding pocket was mainly modified, which well accorded with our previous result (Figures S2 and S3).[7] It should be noted that the Glu−ester bond formed is chemically stable at neutral pH and 37 °C at least for 4 days (Figure S4).

We next examined the biosensing ability of the OG-labeled FKBP12 (OG-FKBP12). After 48 h incubation of FKBP12 with **1**, OG-FKBP12 was purified by gel filtration.[11] Upon addition of rapamycin[12] to this solution, the fluorescence of OG at 520 nm decreased up to 50% with a typical saturation behavior (Figure 2A,B). Similar fluorescence quenching occurred by the addition of AP21967 (a rapamycin analogue),[12,13] whereas only a slight decrease in the fluorescence intensity due to the dilution effect was simply observed by the addition of FK506, DMSO, or an inhibitor of carbonic anhydrase (CA) (Figure S6A). The dissociation constants (K_d) determined from the

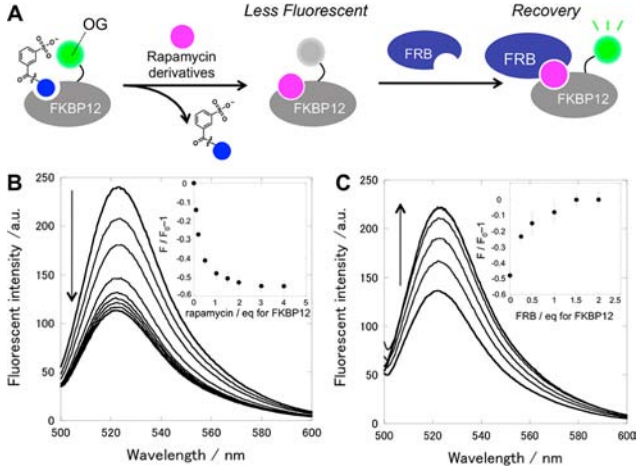

Figure 2. (A) Schematic illustration of the FKBP12-based fluorescent reporter and the detection of its interactions with rapamycin and FRB. Fluorescence spectral change of OG-FKBP12 (0.5 μM) upon (B) the first addition of rapamycin and (C) the subsequent addition of FRB. F/F_0 indicates the relative fluorescence intensity (F) in the presence of analyte against that of the initial state (F_0).

fluorescence titration curves were 0.3 and 1.0 nM for rapamycin and AP21967, respectively. These values are in the same range as the literature data (Table S1).[14] These results indicate that OG-FKBP12 works as a fluorescent biosensor selective for rapamycin derivatives. It is also demonstrated that the OG tethering by LDT chemistry never disturbs the natural ligand binding ability of FKBP12. When FRB was subsequently added to the above solution containing the OG-FKBP12/rapamycin (or AP21967) complex, the OG fluorescence was intensified and recovered up to the original intensity (Figure 2A,C). Such a fluorescence recovery did not occur by the addition of another protein CA which does not interact with FKBP12 (Figure S6B). The fluorescence titration plot showed a typical saturation curve, from which the K_d value for the interaction between the OG-FKBP12/rapamycin complex and FRB was determined to be 24 nM. This is again in good agreement with the literature value (Table S2).[15] The dual (down-and-up) fluorescence response demonstrates that OG-FKBP12 acts as a fluorescent reporter that allows the detection of not only the rapamycin−FKBP12 interaction but also the rapamycin-mediated FKBP12−FRB interaction. We previously reported a turn-on type of fluorescent biosensors prepared by the quenched LDT (Q-LDT) chemistry, which uses Q-LDT reagents containing a quencher.[16] The present example indicates that the original LDT chemistry is also applicable to convert a ligand-binding protein to a (quencher-independent) fluorescent biosensor through the attachment of a fluorophore near the binding pocket.

In order to investigate the mechanism of the fluorescence change, we evaluated fluorescence quantum yields and fluorescence lifetimes of OG-FKBP12 in different complex states (Table S3). Upon addition of rapamycin, the fluorescence quantum yield of OG-FKBP12 decreased (from 0.50 to 0.23) and the fluorescence lifetime also decreased (from 0.37 to 0.28 ns). Upon subsequent FRB addition, these parameters returned to the initial values. We also confirmed that the absorption spectrum of OG-FKBP12 does not change upon addition of rapamycin, indicating the absence of the OG dye−rapamycin interaction in the ground state (Figure S8). These results strongly suggest that the excited state of the OG

dx.doi.org/10.1021/ja401956b | J. Am. Chem. Soc. 2013, 135, 6782−6785

dye is quenched by the binding of rapamycin to FKBP12. Because there is no overlap between the fluorescence spectrum of OG (Figure 2B) and the absorption spectrum of rapamycin (Figure S9), energy transfer between them can be ruled out. Therefore, exciplex formation between rapamycin and OG or electron-transfer quenching may be involved in the fluorescence change observed in the OG-FKBP12-based biosensing system. Moreover, given that the fluorescence quenching occurred when OG-FKBP12 was mixed with rapamycin or AP21967 but not FK506, the polyene group in the rapamycin structure[12] is likely to be involved in the control of the OG fluorescence.[17]

Finally, we sought to visualize the protein−protein interaction of endogenous FKBP12 in living cells. In this experiment, FRB was fused with the red fluorescent protein DsRed2 (FRB-DsRed2).[18] The excitation spectrum of DsRed2 partially overlaps with the emission spectrum of OG, which may allow us to detect the rapamycin-mediated FKBP12−FRB interaction by intermolecular FRET (Figure 3A).[19] Prior to live cell experiment, the occurrence of intermolecular FRET was confirmed by in vitro experiments using OG-FKBP12 and purified FRB-DsRed2. Upon addition of rapamycin to a solution containing OG-FKBP12 and FRB-DsRed2, the fluorescence ratio (R) at 582 and 523 nm ($R = F_{DsRed2}/F_{OG}$) increased from 0.15 to 0.28 with the excitation of the OG dye at 480 nm (Figure S10). In contrast, no R change was observed by the addition of DMSO (Figure S10). These results clearly showed that the formation of the OG-FKBP12/rapamycin/FRB-DsRed2 complex could be monitored through intermolecular FRET.

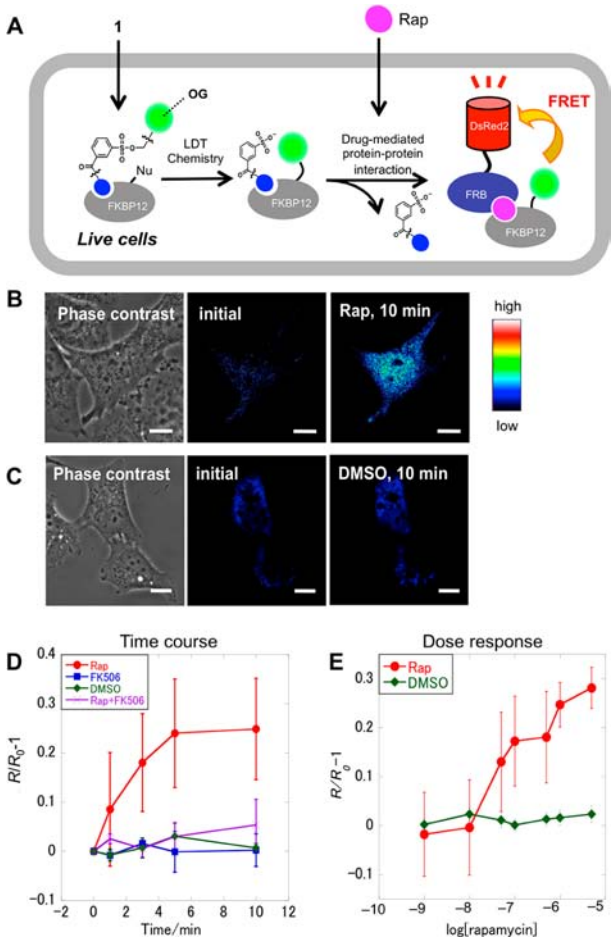

Figure 3. (A) LDT-mediated labeling and FRET imaging of endogenous FKBP12 in living cells. (B) Representative FRET images of **1**-treated A549/FRB-DsRed2 cells before (center) and 10 min after the addition of rapamycin (rap) (5 μM) (right). Phase contrast image is shown in the left panel. Scale bar: 10 μm. (C) Negative control using DMSO. (D) Time course of the R change in A549/FRB-DsRed2. (E) Dose response of the R change upon the rapamycin addition in A549/FRB-DsRed2. Rap (●), FK506 (■), DMSO (◆), Rap in the presence of excess FK506 (×). For plots in D and E, the fluorescence intensities over the entire cell(s) were quantified.

We next decided to install the OG dye into intracellular endogenous FKBP12 by the LDT chemistry. A549 cells were incubated in culture medium containing reagent **1** for 18 h at 37 °C. The cells were lysed and analyzed by SDS-PAGE and Western blotting (Figure S11). In the labeling using **1**, the band corresponding to OG-FKBP12 was clearly detected by antifluorescein antibody.[20,21] In contrast, no labeling was observed when **2** (a control reagent without the SLF ligand) was used. Quantitative time-course analysis revealed that the **1**-mediated labeling of endogenous FKBP12 reached a plateau at about 18 h, at which time ∼30% of total endogenous FKBP12 was labeled (Figure S12).[22]

Having established the in-cell labeling, we attempted to monitor the ternary complex formation of OG-FKBP12 with rapamycin and FRB in a live cell by FRET imaging (Figure 3A). A549 cells transiently expressing FRB-DsRed2 (A549/FRB-DsRed2) (Figure S14) were incubated in culture medium containing **1** for 18 h.[23] After washing, the cells were observed by confocal laser scanning microscopy. As shown in Figure 3B,D, the R value ($= F_{DsRed2}/F_{OG}$) was gradually increased for 5 min after the addition of rapamycin (5 μM),[24] whereas such a change was not observed by addition of FK506 or DMSO (Figure 3C,D). In addition, the rapamycin-induced R change was strongly suppressed in the presence of excess FK506 (Figure 3D). When we performed the same experiment using DsRed2 (no FRB)-expressing cells (A549/DsRed2), no substantial changes of the R value occurred by the rapamycin addition (Figure S17). This indicates that the FRB domain is essential for the observed R change. We confirmed that the expression level of FRB-DsRed2 does not change by the addition of rapamycin (Figure S18) and that the OG-FKBP12 degradation is negligible in the time range of FRET imaging (Figure S13).[22] Taken together, these results demonstrate that OG-labeled endogenous FKBP12 allows the detection of its complexation with rapamycin and FRB-DsRed2 by intermolecular FRET imaging. As shown in Figure 3B, the heterodimerization of OG-FKBP12 and FRB-DsRed2 was detected throughout the cell (in the cytosol and nucleus).[25] Furthermore, this system was capable of detecting the heterodimerization of the proteins in a dose-dependent manner with its detection limit of 50 nM of rapamycin (Figures 3D and S19). Because the rapamycin/FKBP12 complex and its further association with FRB (mTOR) is critical to immunosuppression,[14] the ability to monitor the molecular interaction of endogenous FKBP should be useful for the study of rapamycin-mediated immunological processes.

In summary, we have demonstrated the application of the LDT chemistry for constructing a fluorophore-modified natural protein that can be used for the detection of its molecular interactions in vitro and in living cells. Notably, in the cell experiment, all the processes from selective chemical labeling to FRET imaging were performed for an endogenous cellular

dx.doi.org/10.1021/ja401956b | J. Am. Chem. Soc. 2013, 135, 6782−6785

protein in an in situ manner using live cells. We thus anticipate that although further improvements are certainly required, the LDT chemistry should serve as a powerfully unique tool for the visualization of various endogenous proteins and their associated processes in their native habitat. In addition, this work shows that the combination of the LDT chemistry with other existing protein tagging methods is a promising strategy for live-cell fluorescence imaging.

■ ASSOCIATED CONTENT

Supporting Information

Figures and tables, synthetic procedures, compound characterization, and protein labeling methods. 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 interest.

■ ACKNOWLEDGMENTS

We thank Prof. Kenji Matsuda and Dr. Takashi Hirose (Kyoto University) for their assistance with the fluorescence lifetime measurement. T.T and T.M. acknowledge the JSPS research fellowships for Young Scientists. S.T. thanks the Inamori Foundation, the Yamada Science Foundation, and the Mochida Memorial Foundation for Medical and Pharmaceutical Research for financial support.

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(9) MS data show an additional minor peak corresponding to double-labeled FKBP12. However, its yield is very low (<3%) even after 48 h incubation (Figure S1).

(10) As we have previously reported,[6b,7] the labeling reaction does not proceed to completion due to partial hydrolysis of the LDT reagent in aqueous buffer solution.

(11) It should be noted that due to the high affinity of the SLF ligand to FKBP12 (typically K_d $<10^{-7}$ M), the cleaved SLF fragment should remain complexed with the labeled FKBP12 even after gel filtration. This was supported by a similar experiment (Figure S5).

(12) Chemical structures of rapamycin, AP21967, and FK506 are shown in Figure S7.

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(17) Based on the crystal structure of the FKBP12/rapamycin complex (Figure S3), the OG dye labeled at Glu57 is predicted to be located close enough to the polyene moiety of rapamycin for OG fluorescence quenching to occur by exciplex formation or electron transfer. It is very likely that the subsequent binding of FRB to the FKBP12/rapamycin complex separates the OG dye far from the rapamycin, eliminating the quenching.

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(20) The OG dye is detectable by antifluorescein antibody.

(21) Western blotting revealed that two other proteins were also labeled by **1** (Figure S11). The identification of these proteins is currently underway. It should be noted that the off-target labeling has little effect on the subsequent FRET imaging experiment because we evaluate the highly specific interaction between FKBP12 (OG-FKBP12) and FRB (FRB-DsRed2) induced by rapamycin.

(22) We were also able to evaluate the intracellular stability of the OG-attached endogenous FKBP12. The half-life of the labeled protein was estimated to be ∼15−20 h in living A549 cells (Figure S13). However, at the moment it remains unclear whether the intracellular degradation is due to global degradation of FKBP12 or hydrolytic/enzymatic cleavage of the labeled Glu−ester bond.

(23) In separate experiments, it was shown that the FRET sensitivity increases with incubation time for labeling and reaches a plateau at about 18 h (Figure S15). This time course is consistent with that of endogenous FKBP12 labeling in cells (Figure S12).

(24) Images and quantitative plots of the two separate emission channels are provided in Figure S16.

(25) Heterodimerization of OG-FKBP12 and FRB-DsRed2 is possible even in the nucleus because both proteins can freely enter the nucleus due to their small sizes (proteins are smaller than the nuclear pore).