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Native FKBP12 Engineering by Ligand-Directed Tosyl Chemistry: Labeling Properties and Application to Photo-Cross-Linking of Protein Complexes in Vitro and in Living Cells

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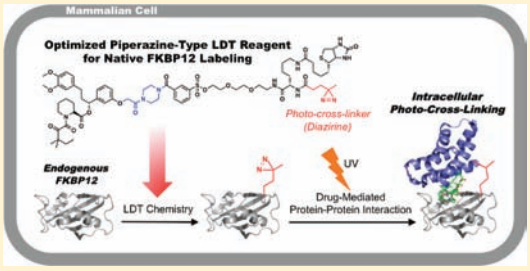
Supporting Information

ABSTRACT: The ability to modify target "native" (endogenous) proteins selectively in living cells with synthetic molecules should provide powerful tools for chemical biology. To this end, we recently developed a novel protein labeling technique termed ligand-directed tosyl (LDT) chemistry. This method uses labeling reagents in which a protein ligand and a synthetic probe are connected by a tosylate ester group. We previously demonstrated its applicability to the selective chemical labeling of several native proteins in living cells and mice. However, many fundamental features of this chemistry remain to be studied. In this work, we investigated the relationship between the LDT reagent structure and labeling properties by using native FK506-binding protein 12 (FKBP12) as a target protein. In vitro experiments revealed that the length and rigidity of the spacer structure linking the protein ligand and the tosylate group have significant effects on the overall labeling yield and labeling site. In addition to histidine, which we reported previously, tyrosine and glutamate residues were identified as amino acids that are modified by LDT-mediated labeling. Through the screening of various spacer structures, piperazine was found to be optimal for FKBP12 labeling in terms of labeling efficiency and site specificity. Using a piperazine-based LDT reagent containing a photoreactive probe, we successfully demonstrated the labeling and UV-induced covalent cross-linking of FKBP12 and its interacting proteins in vitro and in living cells. This study not only furthers our understanding of the basic reaction properties of LDT chemistry but also extends the applicability of this method to the investigation of biological processes in mammalian cells.



INTRODUCTION

The chemical modification of proteins is a key component in chemical biology. This technique allows diverse synthetic molecules, such as fluorescent dyes, affinity labels, NMR/MRI probes, or cross-linking reagents, to be attached to proteins. The resulting derivatized proteins serve as powerful tools for investigating biological processes. During the past few decades, various protein modification (labeling) methods have been reported.[1] However, most of them are applicable only to purified proteins in test tubes, i.e., the field of chemical protein modification has been developed mainly for in vitro research. Since the focus of biology has recently shifted from protein biochemistry in vitro to understanding the behavior and function of proteins in their native environments, the development of new methods for the chemical labeling of target proteins in the context of living cells is now strongly demanded.[2] The most popular strategy relies on the use of peptide or enzyme tags.[3,4] In this approach, target proteins are expressed as a fusion with a tag sequence in cells, and then labeled using a chemical or enzymatic reaction with a designed probe. Although undoubtedly valuable, this tag-based approach is restricted to recombinant proteins. The ability to label nontagged, "native" (endogenous) proteins of interest in living systems should greatly facilitate the functional analysis of proteins in their physiological context. Yet, the development of such methods is still in its infancy.[5] Classical bioconjugation reactions are capable of modifying native protein surfaces (most commonly lysine and cysteine side chains),[6] but lack target specificity among the various cellular components. Activity-based protein profiling is a new technique by which specific native enzymes can be labeled with small-molecule probes in cells and animals.[7] However, in spite of its power in proteomic research, this labeling procedure leads to the inactivation of the labeled enzymes. We recently reported affinity-guided 4-dimethylaminopyridine (DMAP) catalysts that facilitate the

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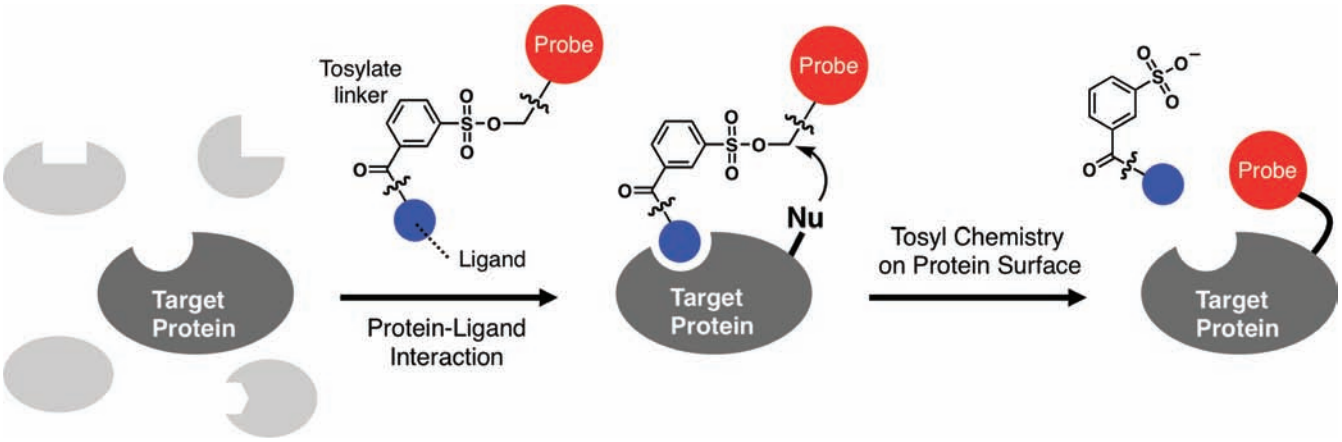

Figure 1. Schematic illustration of the basic principle of the LDT chemistry.

specific chemical acylation of native proteins on the surface of living cells;[8] however, this approach does not currently work well inside cells. Clearly, several breakthroughs are required to develop a tool for the selective, noninvasive chemical labeling of native proteins in living biological systems.

To address this challenge, we recently developed a novel protein labeling methodology termed ligand-directed tosyl (LDT) chemistry (Figure 1),[9] which is based on the principle of affinity labeling.[10] LDT chemistry involves the use of labeling reagents in which a protein ligand and a synthetic probe of choice are connected by an electrophilic phenylsulfonate (tosylate) ester group. As shown in Figure 1, by exploiting the specific protein–ligand interaction, the LDT reagent selectively binds to the target protein, covalently transferring the synthetic probe to a nucleophilic amino acid residue near the ligand-binding pocket (active site). Concomitant with this process, the ligand moiety is cleaved off. Thus, this method provides a powerful way to attach diverse synthetic probes to target proteins in a traceless manner with high site specificity and target selectivity.[11] In addition, since the ligand moiety occupies the active site during the labeling reaction process, functionally essential amino acid residues can be protected from labeling. Therefore, the function of the labeled protein is preserved in this method. The appeal of this chemistry is its applicability to native proteins in living systems. Indeed, we carried out the selective chemical modification of several endogenous proteins in living cells and even in mice.[9] However, many fundamental properties of LDT chemistry remain unclear. In particular, it is essential to understand the following two features for its general use as a native protein engineering tool: (i) how the design of LDT reagents affects the labeling outcome (labeling efficiency, site, and specificity); and (ii) what amino acid residues can be targeted with LDT chemistry.

Here we report a thorough study of the labeling characteristics of LDT chemistry and its application to the detection of protein–protein interactions by photo-cross-linking using native FK506-binding protein 12 (FKBP12) as a target protein. We first examined the effects of the spacer structure connecting the protein ligand and the tosylate group on labeling properties. In vitro experiments revealed that the length and rigidity of the spacer moiety significantly affected the overall labeling yield and labeling site. In addition to histidine, which we reported previously,[9] tyrosine and glutamate residues were identified as amino acids targetable by LDT chemistry. Through the testing of various spacer structures, piperazine was found to be optimal in terms of labeling efficiency and specificity. Using a piperazine-based LDT reagent, we introduced a photoreactive probe to native FKBP12 and demonstrated the UV-induced covalent cross-linking of protein–protein complexes in vitro as well as in cultured mammalian cells.

RESULTS AND DISCUSSION

Design of Various LDT Reagents for FKBP12 Labeling. We previously designed LDT reagent **8** for the labeling of native FKBP12 (Figure 2).[9a] This compound contains the synthetic ligand of FKBP12 (SLF) as a specific and high-affinity ligand.[12] The SLF ligand was linked to the phenyl ring of the tosylate group via an ethylenediamine spacer, and biotin was introduced as a detection probe. We previously demonstrated the labeling of FKBP12 in vitro as well as in Jurkat cells using reagent **8**.[9a] However, as described below, the overall labeling yield was moderate. This motivated us to improve the labeling efficiency and, more importantly, to investigate the detailed relationship between LDT reagent design and labeling properties. In general, the reaction efficiency in affinity-based protein surface labeling strongly depends on the spacer structure that connects a protein ligand to a reactive group because the spacer region mainly determines the spatial position and proximity of the reactive group relative to a nucleophilic amino acid on the surface of the target proteins.[13] Thus, we decided to synthesize a range of new LDT reagents with different spacer lengths and rigidity (Figure 2). Several types of synthetic probes, including fluorescent dye, biotin, and photo-cross-linker, were also evaluated. Synthesis was carried out as shown in Figure 3 and in the Supporting Information (SI). For instance, reagents **2**–**4** were synthesized as follows. A mono-Boc-protected form of the diamine spacer was amidated with 3-(chlorosulfonyl)benzoyl chloride, which was followed by sulfonate esterification with the alcohol-derivatized coumarin probe **15**. After deprotection of the terminal Boc group, the carboxymethylated SLF ligand (**31**) was condensed to generate the LDT reagents for FKBP12 labeling. All of the final compounds were fully characterized by NMR and high-resolution mass spectrometry.

Dependence of Labeling Yield on the Spacer Structure. At first, we evaluated the labeling efficiency of LDT reagents **1**, **2**, **3**, and **4** with different spacer lengths (Figure 2). These contain 7-diethylaminocoumarin (Dc) as the fluorescent probe. Native FKBP12 labeling was performed in vitro by incubating each LDT reagent with purified recombinant (wild-type) FKBP12

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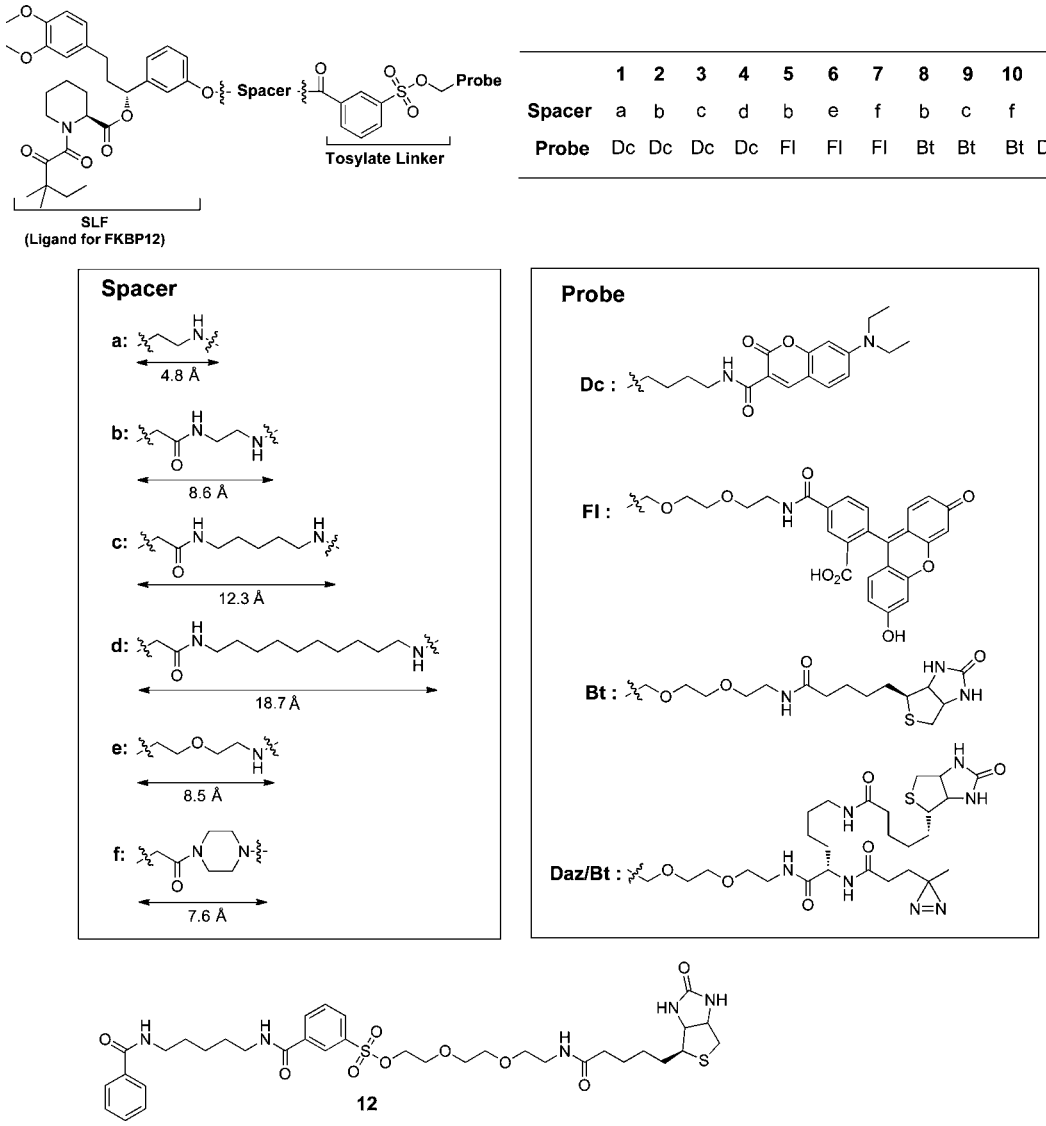

	1	2	3	4	5	6	7	8	9	10	11
Spacer	a	b	c	d	b	e	f	b	c	f	f
Probe	Dc	Dc	Dc	Dc	Fl	Fl	Fl	Bt	Bt	Bt	Daz/Bt

Figure 2. Molecular structures of LDT reagents for FKBP12 (**1**−**11**) and control compound **12**. SLF, synthetic ligand of FKBP12; Dc, 7-diethylaminocoumarin; Fl, 5-carboxyfluorescein; Bt, biotin; Daz, diazirine.

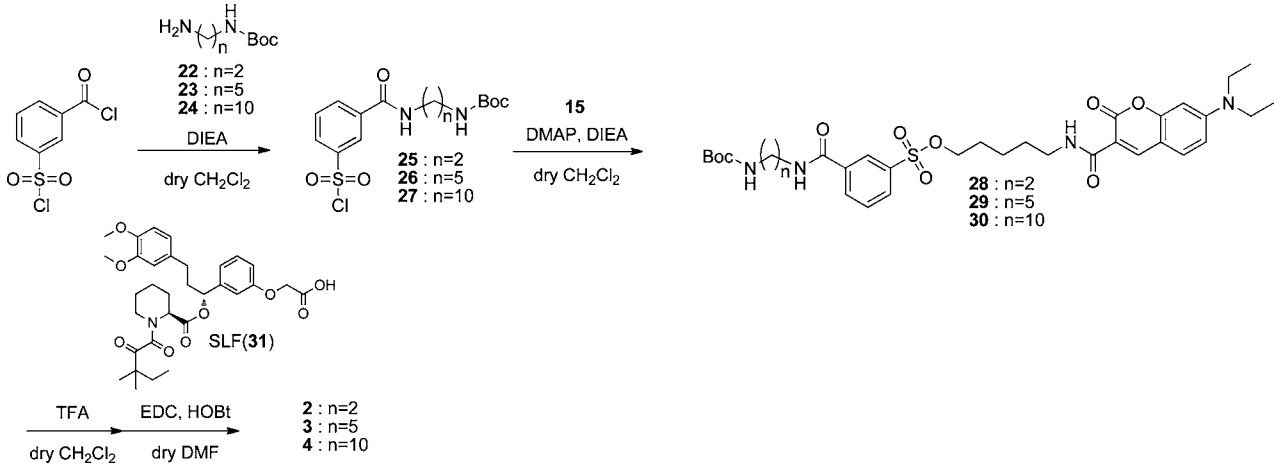

Figure 3. Synthetic scheme of LDT reagents **2**−**4**.

in a buffer solution. As shown by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence gel imaging (Figure 4a), FKBP12 was covalently modified with the Dc dye by reagents **1**, **2**, and **3** (lanes 1−3, respectively). Using a standard marker, the labeling yields after 48 h of incubation were estimated to be 3% for reagent **1**, 19% for reagent **2**, and

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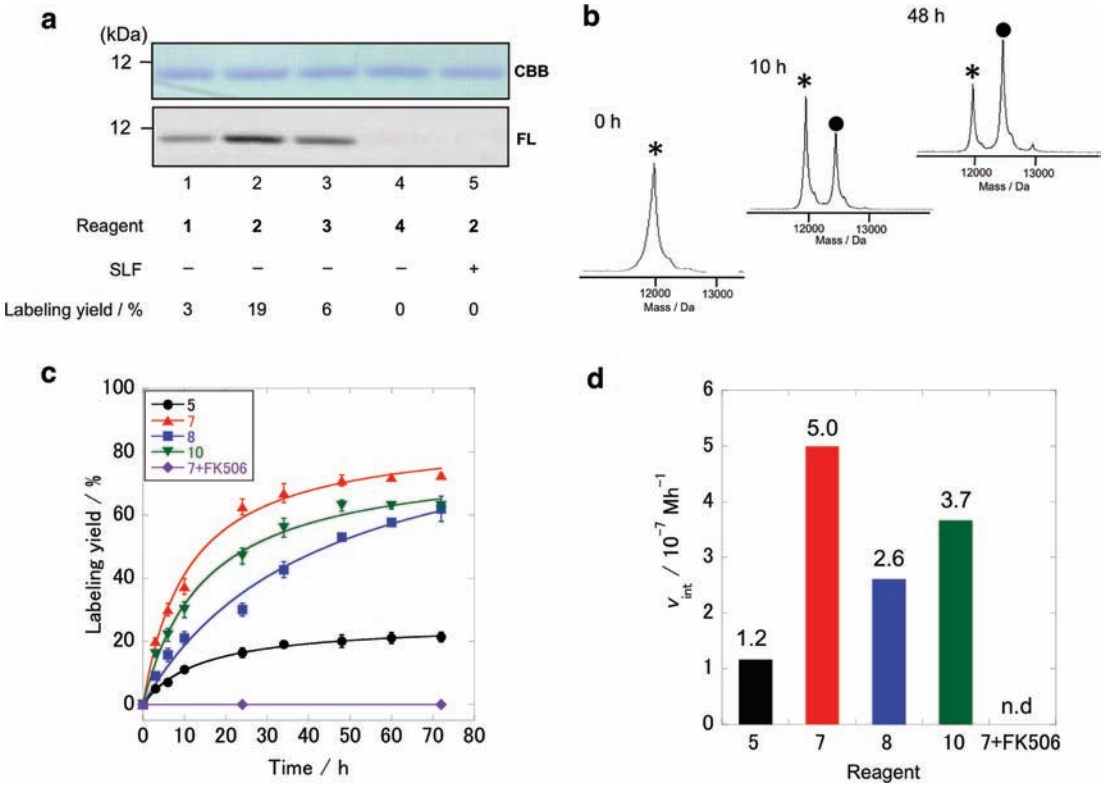

Figure 4. In vitro FKBP12 labeling. (a) SDS-PAGE analysis by *in gel* fluorescence imaging (FL) and Coomassie brilliant blue (CBB) staining. Reaction conditions: 11 μM FKBP12, 11 μM LDT reagent, 110 μM SLF (lane 5), 50 mM Tris buffer, pH 8.0, 37 °C, and 48 h. The labeling yields were determined by using an independently prepared conjugate of the Dc dye and carbonic anhydrase II as a standard marker. (b) MALDI-TOF mass analysis of FKBP12 labeling by **7**. Reaction conditions: 20 μM FKBP12, 20 μM **7**, 50 mM Tris buffer, pH 8.0, 37 °C. *, native FKBP12 (MW 11,914); ●, Fl-labeled FKBP12 (M.W. 12,404). (c) Time course of reaction yields of FKBP12 labeling by several LDT reagents. **5** (●), **7** (▲), **8** (■), **10** (▼), and **7** in the presence of FK506 (◆). (d) Initial rates ($M{\cdot}h^{-1}$) of FKBP12 labeling estimated from the data shown in (c). n.d., not detected.

6% for reagent **3** (Figure 4a and Table 1). The labeling by reagent **2** was completely abolished in the presence of excess free SLF ligand (lane 5), indicating that the labeling occurred by an affinity-driven proximity effect. On the other hand, no noticeable labeling was observed for reagent **4**, which contained the longest spacer (lane 4). These results indicate that: (i) the spacer length between the protein ligand and the reactive tosylate group is a key factor controlling labeling efficiency and (ii) there is an optimal spacer length for FKBP12 labeling.

Table 1. Yields of FKBP12 Labeling with Various LDT Reagents

			yield (%)	
cmpd	spacer	probe	12 h	48 h
1	a	Dc	0[a]	3[a]
2	b	Dc	12[a]	19[a]
3	c	Dc	3[a]	6[a]
4	d	Dc	0[a]	0[a]
5	b	Fl	11[b]	21[b]
6	e	Fl	10[b]	18[b]
7	f	Fl	45[b]	71[b]
8	b	Bt	23[b]	53[b]
9	c	Bt	5[b]	28[b]
10	f	Bt	33[b]	63[b]
11	f	Daz/Bt	35[b]	60[b]

[a]Yields determined by SDS-PAGE/*in-gel* fluorescence analysis. [b]Yields determined by MALDI-TOF-MS analysis.

Next, we attempted to optimize the structure of the LDT reagent further by changing the conformational rigidity of the spacer region. The three fluorescein (Fl)-type LDT reagents **5**, **6**, and **7** were tested (Figure 2). Their spacer lengths were kept the same or almost identical to that of reagent **2**, which contains spacer b (Figure 2). After 48 h of FKBP12 labeling, the labeling yields were evaluated by MALDI-TOF mass spectrometry (Figure 4b). The labeling yield of reagent **6** (18%), which contained the flexible ether spacer e, was slightly lower than that of reagent **5** (21%), which contained the original spacer b (Table 1). Remarkably, LDT reagent **7**, with the rigid cyclic piperazine spacer f, gave a significantly improved labeling yield (71%). The initial rate of the labeling reaction was also significantly enhanced for reagent **7** compared to that for reagent **5** (c and d of Figure 4).[14] Reagent **7**-mediated labeling failed to proceed in the presence of FK506 because the binding of reagent **7** to FKBP12 was competitively inhibited. A similar tendency for the spacer effect was also observed for the biotin (Bt)-type LDT reagents **8** and **10** (Table 1 and c and d of Figure 4).[15] These results clearly indicate that the length and rigidity of the spacer moiety significantly affect the labeling efficiency of LDT chemistry. The rigid piperazine spacer may orient the tosylate ester group more closely toward a nucleophilic amino acid residue to enhance the proximity effect on the surface of FKBP12.

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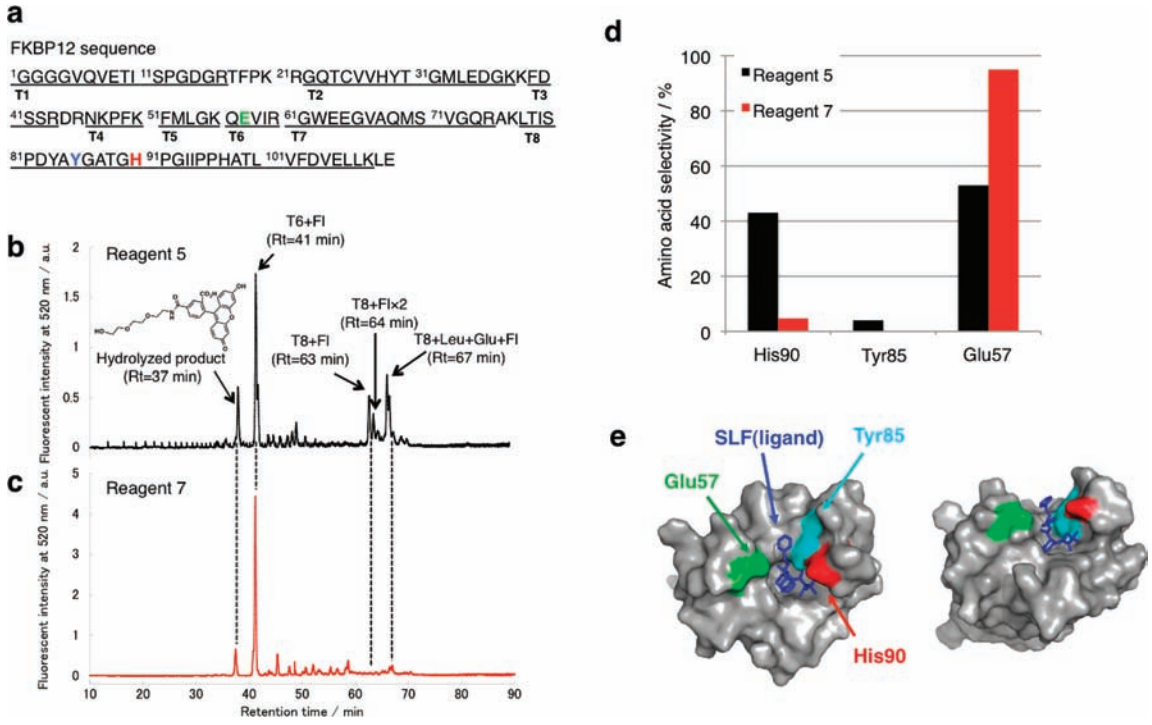

Figure 5. Evaluation of labeling sites and site-selectivity in FKBP12 labeling. (a) The primary sequence of FKBP12 and the assignment of each fragment generated by trypsin digestion. (b and c) RP-HPLC traces of trypsin-digested Fl-labeled FKBP12 generated using **5** (b) and **7** (c). The peaks were monitored by fluorescence detection (λ_{ex} = 490 nm, λ_{em} = 520 nm). (d) Relative amino acid (reaction site) selectivities of **5** and **7**. (e) The crystal structure of the FKBP12-SLF complex (PDB: 1FKG).

Control of the Labeling Site by the Spacer Structure of the LDT Reagents. Characterization of the labeling sites may help us to understand the aforementioned spacer effect more clearly. Thus, we identified the modified amino acids using LDT reagents **5** and **7**. As shown in Figure 4b and Figure S2 in the SI, FKBP12 was predominantly modified at a ratio of one fluorescein group per protein (single labeling). The Fl-labeled FKBP12 was purified and digested by trypsin. The resulting peptide fragments were analyzed by HPLC and MALDI-TOF mass spectrometry. In the case of ethylenediamine-type LDT reagent **5**, three major single-labeled fragments (T6+Fl, T8+Fl, and T8+Leu+Glu+Fl) and a small fraction of a double-labeled fragment (T8+Fl × 2) were obtained (a and b of Figure 5). Tandem mass analysis identified the modified amino acids (labeling sites) as Glu57 in T6+Fl, His90 in T8+Fl and T8+Leu+Glu+Fl, and His90 and Tyr85 in T8+Fl × 2 (Figure S3 in the SI). Quantification of the fluorescent peak areas of these fragments by HPLC analysis led to the following relative labeling selectivity among these three amino acids: 53% for Glu57, 4% for Tyr85, and 43% for His90 (Figure 5d). On the other hand, for piperazine-type LDT reagent **7**, a dominant single-labeled fragment (T6+Fl) and a tiny fraction of another single-labeled fragment (T8+Fl) were obtained (Figure 5c). The labeled amino acids were confirmed by tandem mass spectrometry as Glu57 and His90 in T6+Fl and T8+Fl, respectively, which are consistent with the results obtained for reagent **5**. However, to our surprise, the relative labeling selectivity for these two amino acids was greatly different from that of reagent **5**, that is, 96% for Glu57 and 4% for His90 (Figure 5d). Intriguingly, in the crystal structure of the SLF/FKBP12 complex,[16] these three amino acids, i.e., Glu57, Tyr85, and His90, are located at almost the same distance (6−8 Å), but in different directions from the phenoxy ring of the SLF ligand (Figure 5e and Figure S4 (SI)). This distance corresponds well with the estimated spacer lengths of LDT reagents **5** (8.6 Å) and **7** (7.6 Å). The labeling by reagent **7** was highly specific to Glu57, whereas that by reagent **5** occurred at either Glu57 or His90. These results demonstrate that the optimal piperazine spacer not only increased the labeling yield but also enhanced the selectivity of the labeling site.

In order to evaluate whether piperazine is a universal optimal spacer, we next investigated the spacer effect on the labeling of another protein, human carbonic anhydrase II (CAII). The results are summarized in Figures S5−S14 and Tables S2 and S3 in the SI. Unlike the case for the aforementioned FKBP12 labeling, reagent **53** with the ethylenediamine spacer was more efficient than reagents **52** and **54** containing the cadaverine spacer and the piperazine spacer, respectively. The labeling was highly specific to His3 when reagent **52** was used.[9] On the other hand, the use of reagent **53** led to the labeling predominantly at Glu69, and to much lesser extents at His4 and His64. In the case of reagent **54**, the three residues, His3, His4 and Glu69, were approximately equally modified. These results clearly indicate that the optimal spacer structure varies depending on the target protein. This is very reasonable because the availability and spatial distribution of nucleophilic amino acids around the ligand-binding pocket differ among (native) proteins. Therefore, for each protein target, a certain degree of optimization of the spacer structure should be effective to achieve high(er) labeling efficiency. In this regard, identifying amino acid residues that can be modified by LDT chemistry is essential. Once we know reactable amino acid residues, the de novo prediction of suitable spacer structures should be facilitated (to some extent) by in silico molecular modeling based on the crystal structure of the target protein−ligand complex (when it is available).

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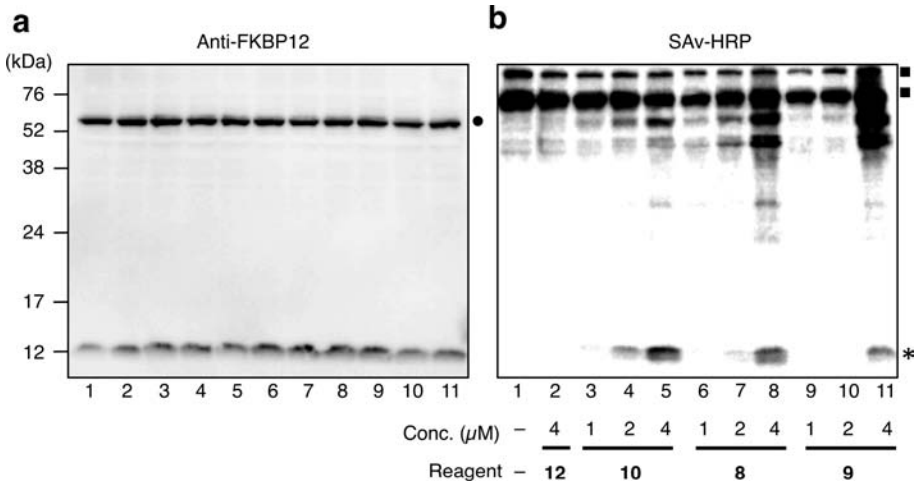

Figure 6. Chemical biotinylation of FKBP12 in Jurkat cells using **8**–**10** and **12**. Reaction conditions: Jurkat cells (4×10^6 cells), 1–4 μM LDT reagent, serum-free RPMI 1640 medium, 37 °C, and 18 h. After labeling, the cells were washed, lysed, and analyzed by Western blotting. (a) Detection by anti-FKBP12 antibody; ●, nonspecific band. (b) Detection by SAv-HRP; ■, endogenous biotinylated protein; *, Bt-labeled FKBP12.

Although it is difficult to predict the relative order of nucleophilicity of the three labeled amino acids in FKBP12 at physiological pH, it can in general be assumed as follows:[6] His > Glu > Tyr. Clearly, the amino acid selectivity observed in this study was not consistent with this expected order. As described above, the amino acid selectivity varied by changing the spacer structure. These results strongly suggest that in LDT chemistry the amino acid selectivity is not controlled by the relative nucleophilicity of amino acid residues but by other factors such as the proximity effect.

The present study demonstrates that, in addition to the previously reported His residue,[9] Glu and Tyr residues can also react with LDT reagents when the proximity effect operates. Therefore, as far as we investigated, LDT chemistry facilitates protein surface modification via: (i) the *N*-alkylation of a His residue to form a C–N(imidazole) bond, (ii) the *O*-alkylation of a Glu residue to form an ester bond, and (iii) the *O*-alkylation of a Tyr residue to form a phenol ether bond. The latter two reactions are of particular significance. The esterification reaction and the Williamson-type ether synthesis reaction have been demonstrated to proceed using LDT chemistry, which are otherwise impossible in an aqueous solution.[17,18] The ester bond is expected to be more labile and susceptible to hydrolysis than the ether bond and the C–N(imidazole) bond. Indeed, we noticed that the ester bond of the labeled Glu57 residue underwent partial hydrolysis during the protease digestion process (Figure S15 in the SI).[19]

Endogenous FKBP12 Labeling in Living Cells. It is of importance to examine if the labeling tendency observed in the aforementioned in vitro experiments is reflected in intracellular labeling. Thus, we next conducted intracellular endogenous FKBP12 labeling experiments using the three Bt-type LDT reagents **8**, **9**, and **10** (Figure 1). Jurkat cells were incubated in culture medium containing the reagent for 18 h at 37 °C. The cells were then lysed and analyzed by SDS-PAGE and Western blotting (Figure 6). Western blotting using anti-FKBP12 antibody confirmed the endogenous expression of FKBP12 (~12 kDa) in the Jurkat cell line (Figure 6a). As shown in lanes 5, 8, and 11 of Figure 6b, for all the LDT reagents, bands corresponding to biotinylated FKBP12 were clearly observed using streptavidin–horseradish peroxidase conjugate (SAv-HRP). In addition, in all cases, band intensity increased when the reagent concentration was increased from 1 to 4 μM. In particular, labeling by reagent **10** gave a clear band even at 2 μM (lane 4). When the SLF ligand in reagent **10** was replaced with a nonligand group (compound **12**), no FKBP12 labeling occurred, and only several bands, which probably corresponded to endogenous biotinylated proteins, were detected (lane 2). Thus, the observed biotinylation of FKBP12 was indeed driven by specific protein–ligand interactions. Among the three reagents, the order of labeling efficiency was **10** > **8** > **9**, which was in good agreement with the efficiency obtained in the in vitro labeling experiments described above. Reagents **8** and **9**, which contained flexible spacers, exhibited some degree of off-target biotin labeling to proteins of ~50–60 kDa, whereas the piperazine-type reagent **10** was highly specific to FKBP12. Overall, the use of the piperazine spacer improved the labeling efficiency and specificity of native FKBP12 labeling not only in vitro but also in mammalian cells. We also confirmed that the piperazine-type reagent **10** can label endogenous FKBP12 in live HeLa cells, as observed in Jurkat cells (Figure S16 in the SI).

We previously demonstrated the selective labeling of endogenous carbonic anhydrase I (CAI) in red blood cells (RBCs) using LDT chemistry.[9a,c] However, because CAI is highly abundant in RBCs (>100 μM), it was not clear whether LDT chemistry could be applied to other natural proteins that are expressed at low levels. The intracellular concentration of FKBP12 is reported to be a few μM in Jurkat cells.[20] Therefore, the present study proved the applicability of LDT chemistry to low-abundance proteins in mammalian cells.

Photo-Cross-Linking of Protein–Protein Complexes in Vitro and in Living Cells. Protein–protein interactions (PPIs) play key roles in all cellular signaling processes. Therefore, the development of techniques for detecting and characterizing PPIs in living cells is highly desirable. Photo-cross-linking is a powerful approach for this purpose.[21] In this strategy, a photoreactive cross-linking reagent is attached to a protein of interest, and any interacting protein is covalently cross-linked with the target protein by light irradiation. The resulting cross-linked protein complexes are analyzed by standard procedures including Western blotting and mass spectrometry. While other existing methods such as yeast two-hybrid technique,[22] protein complementation assay,[23] and tag-mediated

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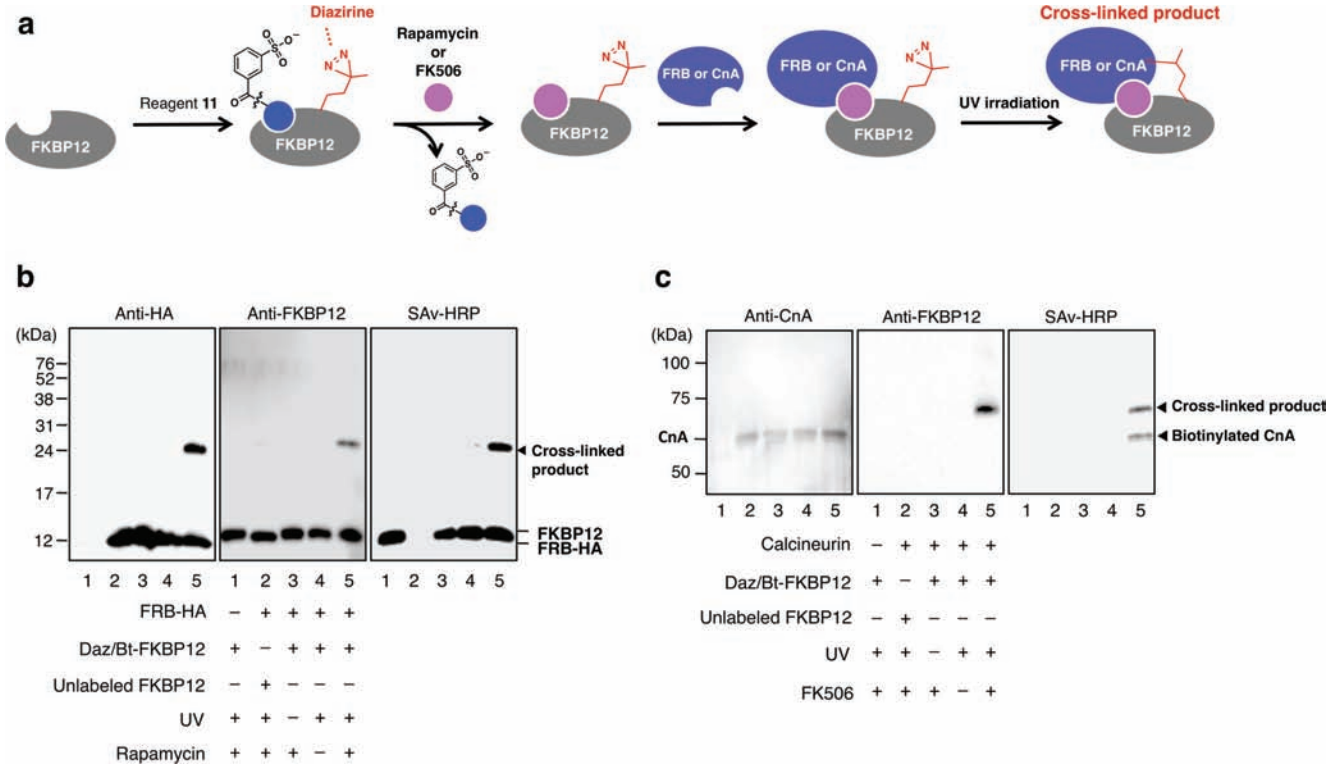

Figure 7. In vitro photo-cross-linking. (a) Schematic illustration of the strategy. (b) Western blotting analysis of photo-cross-linking of Daz/Bt-FKBP12 with FRB-HA. Reaction conditions: 1 μM Daz/Bt-FKBP12 (postlabeling solution) or unlabeled FKBP12, 3 μM FRB-HA, 5 μM rapamycin, UV light (365 nm, 20 min), 50 mM Tris buffer, pH 8.0, on ice. (c) Western blotting analysis of photo-cross-linking of Daz/Bt-FKBP12 with CnA. Reaction conditions: 0.1 μM Daz/Bt-FKBP12 (postlabeling solution) or unlabeled FKBP12, 0.3 μM Cn, 1 μM FK506, UV light (365 nm, 20 min), 50 mM Tris buffer, pH 8.0, on ice.

chemical protein cross-linking methods[24] detect PPIs of recombinant fusion proteins, photo-cross-linking can, in principle, detect the interactions of endogenous proteins. However, the photo-cross-linking approach has rarely been used in living cells because of the lack of methods to introduce photo-cross-linkers selectively into target proteins in the cellular context.[25] As demonstrated above, LDT chemistry allows us to introduce various synthetic probes of choice to native FKBP12 in a traceless manner. Taking advantage of this feature, we sought to demonstrate the tethering of a photo-cross-linker to FKBP12 and the subsequent photo-cross-linking of the engineered FKBP12 to other interaction partners in vitro and in mammalian cells (Figure 7a). FKBP12 is known to form a heteromeric protein complex with FKBP12–rapamycin binding domain of mTOR (FRB) or with calcineurin only in the presence of the immunosuppressive drug rapamycin or FK506, respectively.[26]

The piperazine-based LDT reagent **11** (Figure 2) was designed to introduce diazirine (Daz),[27] a photo-cross-linker, and a biotin tag to FKBP12. Prior to the cell-based experiments, we conducted in vitro labeling and photo-cross-linking experiments using purified protein components. First, it was shown that reagent **11** can label FKBP12 with a yield of 60% to generate Daz/Bt-labeled FKBP12 (Daz/Bt-FKBP12) (Table 1).[28] After labeling, the Daz/Bt-FKBP12-containing solution was mixed (without purification) with different concentrations of recombinant FRB fused to an HA epitope tag (FRB-HA) in the presence of rapamycin. The samples were UV irradiated at 365 nm for 20 min and then analyzed by Western blotting. A new band was observed, whose apparent molecular weight (~24 kDa) was consistent with the sum of the molecular weights of FKBP12 and FRB-HA (Figure 7b, lane 5). Importantly, this band reacted with not only anti-FKBP12 antibody but also anti-HA antibody and SAv-HRP, confirming that it was the expected cross-linked product. This band was present neither in the nonirradiated sample (lane 3) nor in the irradiated sample without rapamycin (lane 4). We also examined the photo-cross-linking of FKBP12 with another interacting protein, calcineurin (Cn). A solution of Daz/Bt-FKBP12 and recombinant Cn (a complex of CnA and CnB) was UV irradiated in the presence of FK506. As shown in Figure 7c, Western blotting using SAv-HRP showed the appearance of two new bands (lane 5). These bands did not appear without UV irradiation (lane 3) or without FK506 (lane 4). The upper band was reasonably assignable to the cross-linked complex of FKBP12 and CnA, because its apparent molecular weight (~72 kDa) matched the sum of the molecular weights of the two proteins. On the other hand, the lower band corresponded to biotinylated CnA in a noncross-linked form. This species was likely formed by the detachment of the probe moiety from Daz/Bt-FKBP12 after the formation of the photo-cross-linked complex. It is conceivable that the ester bond between the probe and Glu57 of FKBP12 was hydrolyzed during the Western blotting experiment.[19] These two successful examples clearly indicate that the SLF ligand was easily expelled from the ligand-binding pocket of labeled FKBP12 by rapamycin or FK506 to form a ternary complex. This highlights a unique advantage of LDT chemistry that enables a target protein to be labeled with the restoration of its natural binding ability.

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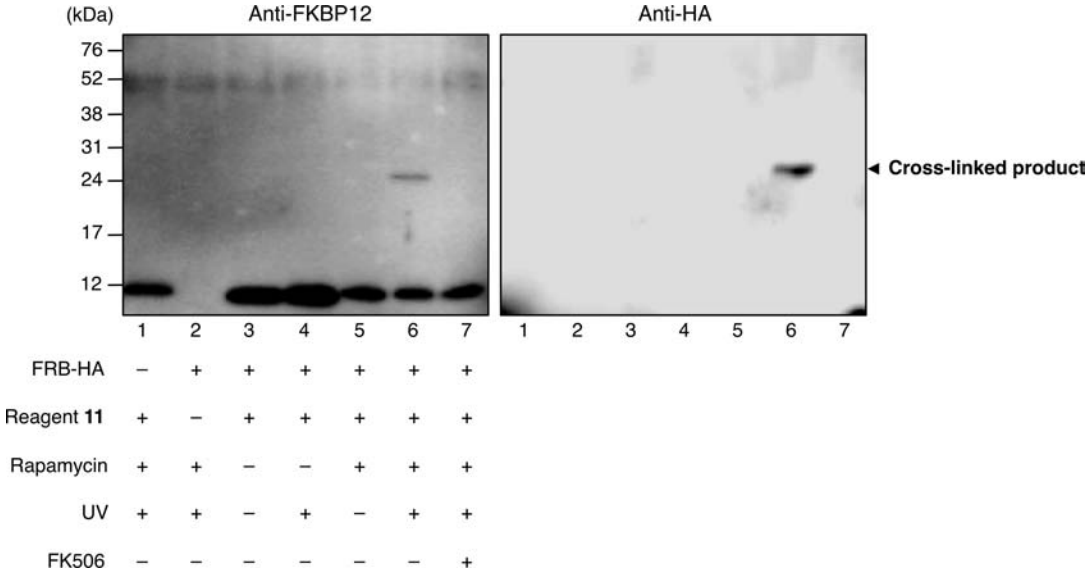

Figure 8. Photo-cross-linking of endogenous FKBP12 with its interacting protein (FRB-HA) in live cells. After HeLa cells transiently expressing FRB-HA were incubated with **11** (5 μM) for 18 h, the cells were UV irradiated (20 min) in the absence or presence of rapamycin (5 μM) and lysed. All biotinylated proteins were captured and purified with NeutrAvidin beads and subjected to Western blotting analysis.

Encouraged by the above results, we moved to cell-based photo-cross-linking experiments. At first, we confirmed the successful attachment of the Daz/Bt probe to endogenous FKBP12 in HeLa cells using reagent **11** (Figure 8). Initially, we attempted to photo-cross-link the complex formed between endogenous Daz/Bt-FKBP12 and endogenous mTOR or CnA in the presence of rapamycin or FK506, respectively. Unfortunately, we were unable to detect any cross-linked species by Western blotting. This may be mainly due to the intrinsically quite low expression level of the endogenous interaction partners in mammalian cells. Thus, we decided to employ HeLa cells that were transiently transfected with FRB-HA (HeLa/FRB). HeLa/FRB cells were incubated in culture medium containing reagent **11** for 18 h. After washing, the cells were incubated with rapamycin for 20 min and subsequently UV irradiated for 20 min. The cells were lysed, and all biotinylated proteins were isolated using avidin beads. In-cell photo-cross-linking was then analyzed by Western blotting. As shown in Figure 8, we detected a new higher-molecular weight band (lane 6). As observed in the in vitro photo-cross-linking experiments described above, the apparent molecular weight of the new species (~24 kDa) was consistent with the sum of the molecular weights of FKBP12 and FRB-HA. This new band cross-reacted with anti-FKBP12 and anti-HA antibodies, verifying that it was the cross-linked product of endogenous FKBP12 and FRB-HA. All negative control experiments yielded no cross-linked species (Figure 8). Although less clear, the photo-cross-linked product could also be detected without the isolation step using avidin beads (Figure S18 in the SI).

Overall, using LDT chemistry, endogenous FKBP12 was converted to a semisynthetic, photo-cross-linkable protein in living mammalian cells. Its ability to trap (cross-link) interacting proteins covalently by UV light irradiation was also demonstrated. Even though we needed to express the interacting partner protein by transfection, this work represents the first example in which an endogenous protein was selectively labeled and used for PPI detection by photo-cross-linking in a cellular context. We believe that the photo-cross-linking of endogenous protein pairs would become feasible in the future by further improvement of intracellular labeling yield and photo-cross-linking efficiency.

■ CONCLUSION

Using FKBP12 as a target protein, we clarified the basic properties of LDT chemistry in detail. In the design of LDT reagents, the length and rigidity of the spacer moiety between the protein ligand and the tosylate group was found to be a key determinant of overall labeling yield and labeling site. In addition to histidine, which we identified previously,[9] tyrosine and glutamate residues were identified as amino acids that are targetable with LDT chemistry.[18] Together with the data of CAII labeling, the present results highlight the importance of the spacer structure in controlling the proximity effect between the reactive group and a nucleophilic amino acid on the protein surface, which in turn determines all of the labeling properties. The rigid cyclic piperazine spacer was clearly superior to other flexible linear spacers in terms of labeling efficiency, site specificity, and target selectivity (in the FKBP12 labeling in vitro and in living cells). Furthermore, using the piperazine-based LDT reagent **11**, which contained a photoreactive diazirine, endogenous FKBP12 was converted to a semi-synthetic, photo-cross-linkable protein in an in situ manner in HeLa cells. The engineered endogenous FKBP12 was capable of covalently capturing (cross-linking) an interacting protein by UV light irradiation.

So far, LDT-mediated protein labeling is slow, requiring hours to days to proceed to completion. This limitation is attributed to the intrinsic moderate reactivity of the tosyl ester electrophile currently used. The moderate reactivity, on the other hand, is a key factor of LDT chemistry, which contributes to suppress both nonspecific (off-target) labeling and hydrolytic decomposition of the LDT reagents. Nevertheless, to expand the utility of this technique further, it is strongly desired to enhance the labeling rate. However, simply increasing the reactivity (electrophilicity) of the tosylate ester group is expected to result in increased hydrolysis (and nonspecific reactions with nontarget molecules). This dilemma, which should be common to all affinity labeling methods based on

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electrophilic reagents, might be overcome by introducing a design concept utilizing supramolecular chemistry. Indeed, we have recently demonstrated that some LDT reagents having self-assembling properties show improved resistance to hydrolysis (in the absence of the target protein) due to protection of the reactive tosylate moiety from water by the rather hydrophobic interior of aggregates.[9c] We also found that the self-assembled aggregates are disrupted by complexation of the reagent with the target protein, which is followed by labeling reaction. Although our research is now in progress, such efforts should improve the LDT-mediated protein labeling technique.

In closing, the results of this study not only demonstrate the potential of LDT chemistry as a tool for bioengineering and functional analysis of natural proteins in their native environments but also provide important guidelines for the design of new affinity labeling reagents for selective chemical (native) protein modification.

EXPERIMENTAL SECTION

Synthesis. The synthesis of reagents **8**, **12**, and **52** has been reported previously.[9a] All other synthetic procedures and compound characterizations are described in the Supporting Information.

General Materials and Methods for the Biochemical/Biological Experiments. Unless otherwise noted, all proteins/enzymes and reagents were obtained from commercial suppliers and used without further purification. UV−visible spectra were recorded on a Shimadzu UV-2550 spectrophotometer. SDS-PAGE and Western blotting were carried out using a Bio-Rad Mini-Protean III electrophoresis apparatus. Chemiluminescent signals were detected with an LAS 4000 imaging system (Fuji Film). Reversed-phase HPLC (RP-HPLC) was carried out on a Hitachi LaChrom L-7100 system equipped with LaChrom L-7400 UV and L-7485 fluorescence detectors, and a YMC-Pack ODS-A column (5 μm, 250 mm × 4.6 mm) at a flow rate of 1.0 mL/min. UV detection was at 220 nm and fluorescence detection was at 520 nm with excitation at 480 nm. All runs used linear gradients of acetonitrile containing 0.1% TFA (solvent A) and 0.1% aqueous TFA (solvent B). MALDI-TOF MS spectra were recorded on an Autoflex III instrument (Bruker Daltonics) using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. Matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry/mass spectrometry (MALDI-QIT-TOF MS/MS) analysis was performed by Dr. Masaki Yamada (Shimadzu Corporation) with a Shimadzu Biotech AXIMA QIT mass spectrometer.

Preparation of Recombinant FKBP12 and FRB-HA. Recombinant FKBP12 was obtained by bacterial expression as a fusion protein with glutathione *S*-transferase (GST) and a factor Xa cleavage site at its N-terminus. The gene encoding FKBP12 was amplified by PCR from the pC_4EN-F1 vector (ARIAD Pharmaceuticals) with the 5′-primer (5′-GGA ATT CAT CGA AGG TCG TGG AGG CGG AGG AGT GCA GGT GGA AAC-3′) and the 3′-primer (5′- CGC GGC CGC TTA TTC CAG TTT TAG AAG CTC CAC-3′) and subcloned into the *Eco*RI and *Not*I sites of the pET-41a vector (Novagen) to yield pGST-FXa-FKBP12. Recombinant FRB was obtained as a fusion protein with GST and a factor Xa cleavage site at its N-terminus and an HA epitope tag at its C-terminus. The gene encoding FRB-HA was amplified by PCR from the pC_4R_H-E vector (ARIAD Pharmaceuticals) with the 5′-primer (5′- GGA ATT CAT CGA AGG TCG TGG AGG CGG AAT CCT CTG GCA TGA GATGTG G-3′) and the 3′-primer (5′- CGC GGC CGC TTA TGC GTA GTC TGG TAC GTC GTA-3′) and subcloned into the *Eco*RI and *Not*I sites of the pET-41a vector (Novagen) to yield pGST-FXa-FRB-HA. The PCR amplified sequences were verified by DNA sequencing.

The expression vectors were transformed into *Escherichia coli* BL21 Star (DE3) (Invitrogen). The cells were grown in LB media containing kanamycin at 37 °C to an optical density (660 nm) of 0.6, at which time the expression of the fusion protein was induced by the addition of 0.5 mM IPTG. After growth for an additional 5−7 h at 37 °C, the cells were harvested by centrifugation. The cell pellets were resuspended in 50 mM HEPES, 100 mM NaCl, 10% glycerol, pH 7.2, and lysed by sonication. The proteins were purified from the soluble fraction of the lysate using a glutathione-Sepharose column chromatography (GE Healthcare) and dialyzed against 50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, pH 8.0. The GST tag in the fusion protein was cleaved with factor Xa at 20 °C for 16 h. The resulting (tag-free) FKBP12 was purified by passing it through a benzamidine-Sepharose column (GE Healthcare), followed by a glutathione-Sepharose column. The concentrations of FKBP12 and FRB-HA were determined by measuring the absorbance at 280 nm using molar absorption coefficients of 9,860 and 23,150 M^{-1} cm^{-1}, respectively.

In Vitro Labeling of FKBP12 (and CAII). FKBP12 (11 μM) was incubated with LDT reagent (11 μM) in the absence or presence of SLF (110 μM) in Tris buffer (50 mM, pH 8.0) at 37 °C for 48 h. For compounds **1**−**4**, aliquots were taken and mixed with an equal volume of 2 × SDS-PAGE loading buffer. The samples were resolved by 15% SDS-PAGE, and the coumarin-labeled FKBP12 was detected by in-gel fluorescence imaging. The 1:1 conjugate of diethylaminocoumarin and carbonic anhydrase II was used as a standard marker to determine the labeling yields on the basis of the relative fluorescence intensity.[9a] After fluorescence imaging, the gel was stained with CBB. For compounds **5**−**14**, aliquots were taken at different time points, and the labeling yields were determined by MALDI-TOF MS.

CAII labeling was performed under the following condition: 20 μM CAII, 20 μM LDT reagent, 50 mM HEPES buffer, pH 7.2, 37 °C. Aliquots were taken at different time points, and the labeling yields were determined by MALDI-TOF MS.

Peptide Mapping of Fluorescein-Labeled FKBP12. FKBP12 (20 μM) was incubated with **5** or **7** (20 μM) in Tris buffer (50 mM, pH 8.0) at 37 °C After 48 h, the labeled FKBP12 was purified by size-exclusion chromatography using a TOYOPEARL HW-40F column (Tosoh Corporation) and dialyzed against HEPES buffer (50 mM, pH 8.0) with a Spectra/Por dialysis membrane (MWCO: 3,500) (Spectrum Laboratories). The resulting solution was concentrated using an Amicon Ultra 3K centrifugal filter (Millipore). Urea (at a final concentration of 2.0 M) and trypsin (trypsin/substrate ratio = 1/10 (w/w)) or lysyl endopeptidase (LEP) (LEP/substrate ratio = 1/50 (w/w)) were added to this solution. After incubation at 37 °C for 18 h, the digested samples were applied to RP-HPLC. The trypsin-digested labeled fragments were analyzed by MALDI-TOF MS. The LEP-digested labeled fragments were further characterized by MALDI-QIT-TOF MS/MS.

Chemical Biotinylation of Endogenous FKBP12 in Jurkat Cells. Jurkat cells were incubated at 37 °C in serum-free RPMI 1640 medium containing **8**, **9**, or **10** (1, 2, or 4 μM). After 18 h, the cells were washed twice with phosphate-buffered saline (PBS), lysed using NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2.5 mM EDTA, 1% Nonidet P-40), and mixed with 2 × SDS-PAGE loading buffer. The samples were resolved by 15% SDS-PAGE and electrotransferred onto an Immun-Blot PVDF membrane (Bio-Rad). The biotinylated products were detected with SAv-HRP (Invitrogen) using Chemi-Lumi One (Nacalai Tesque). The immunodetection of FKBP12 was carried out with an anti-human FKBP12 antibody (Abcam) and anti-rabbit IgG antibody-HRP conjugate (GE Healthcare).

In Vitro Photo-Cross-Linking Experiments. FKBP12 (11 μM) was incubated with **11** (11 μM) in Tris buffer (50 mM, pH 8.0) at 37 °C. After 48 h, the labeling yield was determined by MALDI-TOF MS using CHCA as the matrix. For photo-cross-linking with FRB-HA, the postlabeling solution containing 60% Daz/Bt-FKBP12 was mixed with FRB-HA in the presence or absence of rapamycin in a 96-well plate (final concentration of FKBP12 = 1 μM, FRB-HA = 3.0 μM, rapamycin = 5 μM). For photo-cross-linking with Cn, the postlabeling solution was mixed with recombinant Cn (a complex of CnA and CnB) (Enzo Life Sciences) in the presence or absence of FK506 as described above (final concentration of FKBP12 = 0.1 μM, Cn = 0.3 μM, FK506 = 1 μM). The plate was placed on ice and exposed to 365 nm light by using a UV lamp (8 W) (UVP) at 4 cm distance from the top of the plate (without the lid). After UV irradiation for 20 min, aliquots were taken and mixed with an equal volume of 2 × SDS-PAGE

loading buffer. The samples were analyzed by Western blotting as described above. For photo-cross-linking with FRB-HA, (a) anti-human FKBP12 antibody and anti-rabbit IgG antibody-HRP conjugate, (b) anti-HA tag antibody (abcam) and anti-rabbit IgG antibody-HRP conjugate, and (c) SAv-HRP were used. For photo-cross-linking with CnA, (a) anti-carcineurin A antibody (abcam) and anti-rabbit IgG antibody-HRP conjugate and (b) SAv-HRP were used.

Intracellular Photo-Cross-Linking Experiments. HeLa cells were transiently transfected with pC_4-R_HE plasmid (ARIAD Pharmaceuticals), which encodes FRB-HA, using Lipofectamine LTX (Invitrogen). After 5 h of transfection, endogenous FKBP12 labeling was performed by incubating the cells in serum-free DMEM containing **11** (5 μM) at 37 °C for 18 h. Subsequently, the medium was replaced with serum-free DMEM containing or noncontaining rapamycin (5 μM). As a negative control, the cells were incubated with FK506 (30 μM) and rapamycin (5 μM). The cells were then exposed to 365 nm light by using a UV lamp (8 W) at 4 cm distance from the top of the cell culture dish (without the lid) on ice for 20 min. The cells were washed twice with PBS and lysed using NP-40 lysis buffer. After centrifugation, the lysate was dialyzed against PBS with a Spectra/Por dialysis membrane (MWCO: 3,500). Biotinylated proteins were isolated by incubating the lysates with immobilized NeutrAvidin protein beads (Pierce) at 4 °C for 1 h. The beads were washed 10 times with PBS containing 0.05% Tween and boiled in SDS-PAGE loading buffer containing 5 mM biotin. The samples were analyzed by Western blotting using (a) anti-human FKBP12 antibody and anti-rabbit IgG antibody-HRP conjugate, (b) anti-HA tag antibody (abcam) and anti-rabbit IgG antibody-HRP conjugate, and (c) SAv-HRP.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1−S18, Tables S1−S3, the experimental details of the synthesis, and complete ref 3j. This material is available free of charge via the Internet at http://pubs.acs.org.

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