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Ligand-Directed Acyl Imidazole Chemistry for Labeling of Membrane-Bound Proteins on Live Cells

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Supporting Information

ABSTRACT: Chemistry-based protein labeling in living cells is undoubtedly useful for understanding natural protein functions and for biological/pharmaceutical applications. Here, we report a novel approach for endogenous membrane-bound protein labeling for both *in vitro* and live cell conditions. A moderately reactive alkyloxyacyl imidazole (AI) assisted by ligand-binding affinity (ligand-directed AI (LDAI)) chemistry allowed us to selectively modify natural proteins, such as dihydrofolate reductase (DHFR) and folate receptor (FR), neither of which could be efficiently labeled using the recently developed ligand-directed tosylate approach. It was clear that LDAI selectively labeled a single Lys(K32) in DHFR, proximal to the ligand-binding pocket. We also demonstrate that the fluorescein-labeled (endogenous, by LDAI) FR works as a fluorescent biosensor on the live KB cell surface, which allowed us to carry out unprecedented *in situ* kinetic analysis of ligand binding to FR.

Membrane-bound receptor proteins on the cell surface are crucial for a range of intra- and intercellular signal transduction events, making them key regulatory elements in a broad range of normal and pathological processes. Due to the biological and medical importance of receptor proteins, extensive studies have been performed in recent decades, mainly by methods that have focused on isolated proteins. For an overall and in-depth understanding of structure and function, however, it is now commonly regarded that such proteins should be analyzed under the most natural conditions possible. New methodologies are therefore necessary to enable precise analyses of endogenous (natural) receptor proteins under *in situ* conditions, such as in living cell systems, in addition to conventional *in vitro* techniques.

In recent years, chemical modification of proteins with a small-molecule probe has contributed to the elucidation of protein functions in living cells,[1−5] by developing powerful methods for selective protein labeling under crude cellular conditions.[6−15] Despite their versatility, the most promising methods require the incorporation of unique reactive moieties, such as reactive protein/peptide domains, azides, or alkynes, into target proteins by genetic engineering, prior to bioorthogonal reactions for protein labeling. This prerequisite means that these approaches cannot analyze naturally expressed proteins. Chemistry-based methods potentially enable direct labeling of natural proteins without genetic manipulation. However, traditional bioconjugation reactions tend to be useful only in purely *in vitro* experiments and are not applicable to cell systems due to their insufficient labeling selectivity.[16−26]

With the aim of developing chemistry-based methods that accomplish selective modification of endogenous proteins under complicated live cell systems, we recently reported ligand-directed tosylate (LDT) chemistry for selective labeling of natural proteins.[24−26] Despite its high target- and site-selectivity, the LDT method is limited by its slow rate and low labeling efficiency. Indeed, LDT chemistry has failed to label membrane-bound receptor proteins in live cell systems to date. Here, we describe a ligand-directed acyl imidazole (LDAI) approach that can selectively label and engineer a membrane-bound folate receptor (FR) on a live cell surface (Figure 1a).

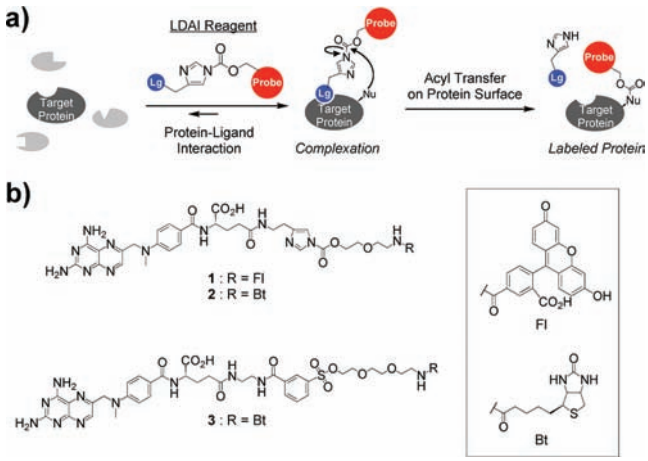

Figure 1. New ligand-directed acyl imidazole (LDAI) chemistry for selective protein modification. (a) Schematic illustration of affinity-based chemical labeling of proteins. Lg = ligand; Nu = nucleophilic amino acid. (b) LDAI (**1**, **2**) and LDT (**3**) reagents used in the present study.

Owing to the controlled reactivity of acyl imidazole and the selective binding driven by the ligand, acyl transfer from the LDAI reagent to a natural amino acid on the target protein surface was efficiently accelerated by the proximity effect to afford labeled FRs, which was not readily achieved by LDT

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chemistry. We also demonstrated the utility of LDAI chemistry by converting endogenous FR to a fluorescent biosensor on the live cell surface, which allowed us to evaluate *in situ* the binding kinetics and affinity for various FR ligands.

To label FR under live cell conditions using LDAI chemistry, two alkyloxyacyl imidazole (AI) derivatives (**1**, **2**) were designed and synthesized (Figure 1b). These reagents contain methotrexate (MTX),[27] an affinity ligand for dihydrofolate reductase (DHFR) and FR, and a probe such as fluorescein (Fl) or biotin, both of which are connected to AI as a reactive linker. It was anticipated that the nucleophilic acyl substitution reaction facilitated by the proximity effect would induce concurrent release of the ligand moiety, producing labeled active protein with a vacant binding pocket (Figure 1a). We also prepared the LDT-type labeling reagent **3** tethered to MTX for comparison.

Prior to labeling FR on live cells, we initially examined the reaction profiles of the LDAI reagent using water-soluble DHFR as a target protein. The reaction of LDAI reagent **1** with purified DHFR (m/z = 18 587) in aqueous buffer solution was traced by MALDI-TOF MS analysis, which showed that the labeling reaction proceeded in a time-dependent manner to afford DHFR singly modified with the Fl moiety of **1** (m/z = 19 086) (Figure 2a). The yield of Fl-modified DHFR was estimated to be 84%[28] after 48 h based on the peak intensity (Figure S1). On the other hand, the reaction did not occur in the presence of MTX as a competitive binding inhibitor, indicating that the labeling reaction was driven by ligand–protein recognition. The reaction was also successfully monitored by in-gel fluorescence analysis, in which the band became gradually fluorescent by Fl modification, whereas labeling was completely suppressed in the presence of MTX (Figure 2b).

The site at which LDAI reagent **1** labeled DHFR was determined by conventional proteolytic digestion and tandem mass spectrometry. Peptide mapping analysis revealed that Lys32 was uniquely modified by **1** as a single labeling site,[29] located at the entrance of the ligand-binding pocket of DHFR (Figure S3), which was consistent with the proposed mechanism based on ligand-directed labeling chemistry (Figure 1a). It was plausible that the AI moiety of LDAI reacted with the nucleophilic ε-amino group of Lys to form a carbamate bond on the DHFR surface. On the other hand, the labeling efficiency of DHFR was substantially lower (9%) in the reaction with LDT reagent **3**. Labeling selectivity of LDAI chemistry was subsequently examined using a protein mixture containing DHFR, human carbonic anhydrase I (CAI), FK506 binding protein 12 (FKBP12), bovine serum albumin (BSA), ovalbumin (OVA), and glutathione *S*-transferase (GST). Among these proteins, reagent **1** selectively labeled DHFR with Fl (Figure 2c), confirming the high protein selectivity of LDAI chemistry.

LDAI chemistry was next applied to labeling of the endogenous membrane-bound FR in live KB cells.[30] The biotin blotting analysis after live-cell labeling with LDAI reagent **2** clearly confirmed that FR was effectively modified with a biotin unit, whereas the labeling was largely diminished by addition of folic acid (FA), a strong agonist for FR (Figure 2d). In contrast to the strong band of the FR biotinylated by LDAI, a faint band of FR labeled by the LDT reagent **3** was detected. Using these band intensities, it was determined that LDAI labeling is 12-fold more efficient than LDT labeling for FR in live cells. Owing to the efficient chemical labeling of

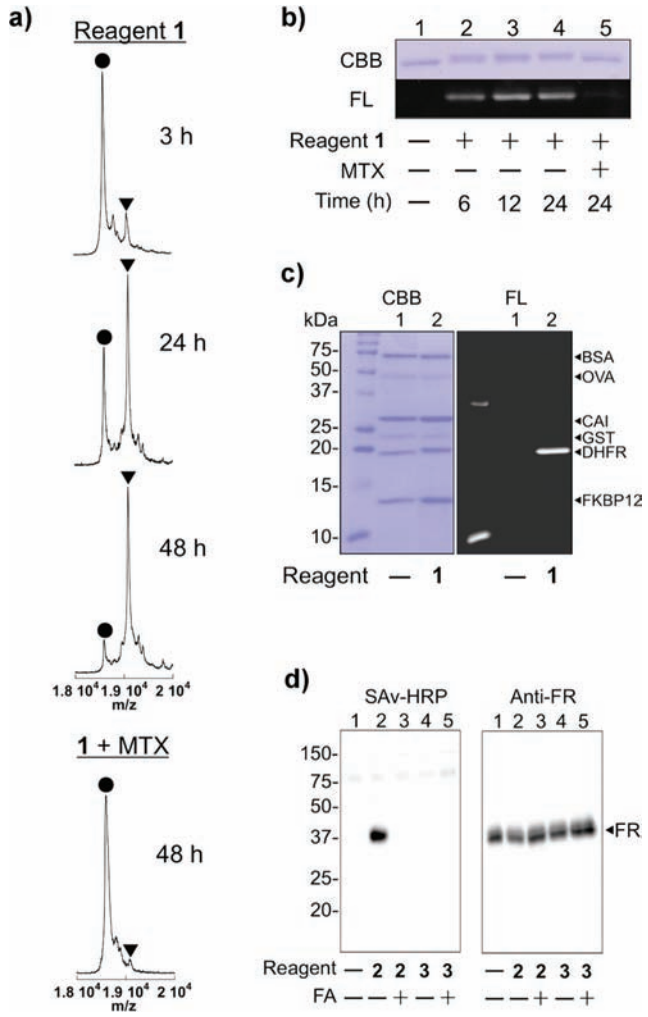

Figure 2. Analysis of the labeling reactivity and selectivity of LDAI chemistry. (a) MALDI-TOF MS analysis of the labeling of DHFR (25 μM) with LDAI reagent **1** (50 μM), or **1** in the presence of MTX (500 μM) in 100 mM phosphate (pH 6.0) at 37 °C. The peaks of unlabeled DHFR and singly labeled DHFR are marked with ● and ▼, respectively. (b) In-gel fluorescence analysis of the labeling reaction of DHFR with **1** under the same conditions as in (a). (c) Evaluation of labeling selectivity in a protein mixture containing six different proteins: DHFR (5 μM), OVA (3 μM), GST (6 μM), BSA (2 μM), CAI (5 μM), and FKBP12 (10 μM). The reaction was carried out with **1** (5 μM) in HEPES buffer (50 mM, pH 7.2) at 37 °C for 24 h. (d) Endogenous FR labeling on live cells. KB cells were treated with **2** or **3** (1 μM) in RPMI 1640 (folate-free, 10% FBS) at 37 °C for 24 h with or without FA (5 μM). Left, biotin-blotting analysis using streptavidin–horseradish peroxidase conjugate (SAv-HRP); right, Western blotting analysis using a mouse anti-FR antibody and anti-mouse IgG-HRP conjugate.

endogenous FR on the live cell membrane by LDAI, we were able to observe the fluorescently labeled FR by confocal laser scanning microscopy (CLSM). After labeling with **2** and repetitive washing, covalently biotinylated FR was visualized by addition of HiLyte647-modified fluorescent streptavidin (SAv647).[31] As shown in Figure 3a, fluorescence of HiLyte647 was clearly detectable on the outer membrane of live KB cells, but such images were not obtained upon addition of FA during the labeling reaction. The labeling efficiency was found to be 17% for 2 h on the basis of quantitative analysis of these imaging data (Figure S5), which was comparable to that of

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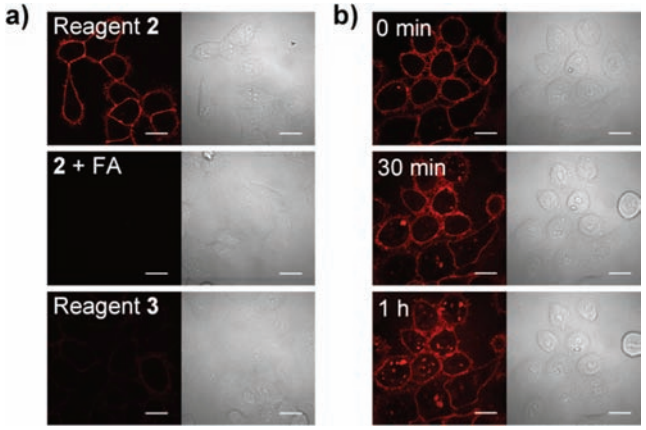

Figure 3. Fluorescence visualization of FR on live cell surface. (a) Fluorescence images of KB cells upon labeling with LDAI reagent **2** alone (top) and **2** in the presence of FA (middle) or LDT reagent **3** (bottom). The labeling reaction was conducted with 1 μM **2** or **3** in RPMI 1640 (folate-free, 10% FBS) at 37 °C for 6 h. Images were obtained after treatment with SAv647. Scale bars, 20 μm. (b) Time-lapse fluorescence image of FA-induced internalization of FR labeled with **2**. Cells were stimulated with 5 μM FA, and the biotin-appended FR was immediately labeled with SAv647 for visualization. Scale bars, 20 μm.

DHFR labeling *in vitro*. In contrast, negligible fluorescence was observed in the case of FR labeling in KB cells by **3**. More interestingly, fluorescence of SAv647-modified FR was initially observed on the cell membrane, after which it gradually moved into the cell interior over 1 h upon stimulation of FR with agonist (FA) at 37 °C (Figure 3b). Such localization changes were largely suppressed at 4 °C (data not shown). These results suggest that FR maintained its natural ligand-binding ability even after chemical modification, and the time-lapse changes in FR internalization were successfully monitored.

Surprisingly, we noted that the fluorescence of Fl tethered to FR using **1** was intensified on live KB cells by the addition of FA (Figure 4a,b), which suggested the possibility of a fluorescent biosensor comprising LDAI on live cells. We thus investigated the ligand-binding kinetics of Fl-labeled FR using this fluorescence change. The Fl fluorescence monitored by CLSM clearly increased in intensity after addition of 600 nM FA for 200 s (Figure 4c). Analysis of the intensity change gave an apparent association rate constant, which is linearly dependent on the initial concentration of FA, of k_{on} = 3.0 × 10^4 M^{-1} s^{-1} (Figures S6, S7). The k_{on} values for other ligands such as dihydrofolic acid (H2FA), tetrahydrofolic acid (H4FA), pterine (PT), MTX, and trimethoprim (TMP) were also determined and are summarized in Table 1. All ligands possess common structural features such as the substituted pterine ring or the analogous *N*-containing heterocycle (Figure S6). In addition, the fluorescence changes induced by these ligands increase with almost the same changing ratios (I_{max}/I_0 = 1.2–1.7, Table 1). These points allow us to assume that the fluorescence changes can be ascribed to the binding of these ligands. To our knowledge, this is the first study of the binding kinetics of FR ligands to be conducted in live cells.

We also carried out fluorescence titration experiments by changing the ligand concentrations under live cell conditions (Figure 4d). Analysis of the titration data by Hill's method gave the association constants (K_a) for the ligands (Table 1). The order of the ligands classified by binding affinity was consistent

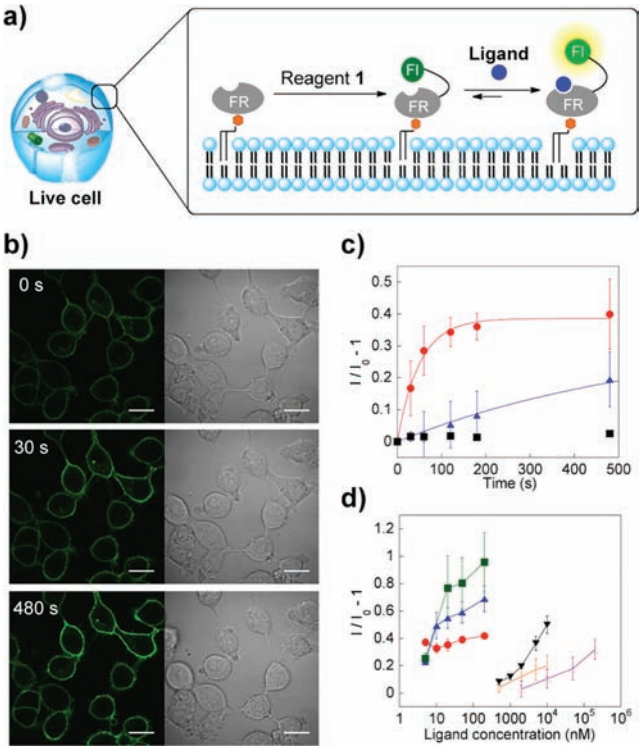

Figure 4. Functionalization of FR on live cells by LDAI chemistry. (a) Construction and fluorescence sensing of FR-based biosensor with cell-impermeable LDAI reagent **1** on live cells. (b) Time-lapse fluorescence images (0, 30, or 480 s) of Fl-labeled FR after addition of FA (600 nM). Scale bars, 20 μm. (c) Time trace plots of fluorescence intensity change in live cells induced by FA (600 nM, red circles), MTX (5 μM, blue triangles), and vehicle medium (black squares). (d) Fluorescence titration profiles of Fl-labeled FR upon addition of various ligands: FA (red solid circles), H2FA (blue solid triangles), H4FA (green solid squares), MTX (black solid down-triangles), PT (orange open circles), and TMP (purple open triangles).

with data previously determined by other methods,[32] indicating that Fl-labeled FR indeed worked as a fluorescent biosensor on live cells. By comparison of the binding kinetics with the binding affinity shown in Table 1, it was clear that the affinity of the FA derivatives to FR was mainly controlled by the ligand dissociation rate (k_{off}), and the affinity of other ligands, such as MTX and PT, was controlled by both the ligand association (k_{on}) and dissociation rates (k_{off}). Such fluorescence changes may be attributed to micro-environmental changes of the Fl induced by ligand binding,[33] thus demonstrating the advantage of tethering a small-molecule probe to a site proximal to the ligand-binding pocket for analyzing protein functions in their natural context.

In conclusion, we successfully demonstrated that AI is a novel reactive group for selective protein labeling in crude live cellular contexts. The present study also revealed that chemical modification of FR by LDAI chemistry yielded a fluorescent biosensor capable of operating on the live cell surface, which indicates the potential power of chemistry-based approaches for engineering endogenous proteins in crude contexts. We envision further applications of LDAI chemistry for protein functional analysis and engineering and for drug-target identification and drug screening in living cell systems.

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Table 1. Binding Parameters of Ligands to Fl-Labeled FR on Live Cells Determined by CLSM Study: Binding-Induced Fluorescence Intensity Change (I_{max}/I_0), Association Constant (K_a), and Association/Dissociation Rate Constants (k_{on}, k_{off})

	FA	H2FA	H4FA	MTX	PT	TMP
I_{max}/I_0	1.4 ± 0.1	1.6 ± 0.1	1.7 ± 0.3	1.4 ± 0.1	1.2 ± 0.1	1.3 ± 0.2
K_a/M^{-1}	$>10^9$	1.3×10^8	1.1×10^8	1.9×10^5	3.8×10^5	1.9×10^4
$k_{on}/s^{-1}\ M^{-1}$	3.0×10^4	2.8×10^4	1.9×10^4	4.5×10^2	3.5×10^3	ND
k_{off}/s^{-1} [a]	$<3 \times 10^{-5}$	2.2×10^{-4}	1.7×10^{-4}	2.4×10^{-3}	9.2×10^{-3}	–

[a]Values were calculated from the equation $K_a = k_{on}/k_{off}$ using experimental k_{on} and K_a values. ND = not determined.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S9, synthetic procedures, compound characterizations, and protein labeling methods using LDAI reagents. 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.

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(28) LDAI reagent **1** was gradually hydrolyzed in aqueous solution and inactivated. The half-life of LDAI reagent **1** was determined to be 20 h at pH 6.0 by HPLC analysis (Figure S2), which may suppress the completion of DHFR labeling during the reaction time (48 h, Figure S1).

(29) Based on an X-ray crystal structure of the DHFR-methotrexate complex (Figure S3), Lys32 is proximal to the ligand-binding pocket of DHFR. The distance from the γ-carboxylic acid of MTX to the ε-amino group of Lys32 is 7.3 Å, roughly equal to the length between the ligand-end amide and the reaction site of LDAI reagent. Thus, it is conceivable that the proximity effect worked well for selective Lys32 labeling.

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(31) The fluorescence signal was clearly detected even after cells were washed with FA (25 μM) (Figure S4), indicating that FR on KB cells was covalently labeled by **2**.

(32) Dissociation constants of FR for FA and the reduced FA derivatives (H2FA and H4FA) were reported to be <1 nM and 1−10 nM, respectively (Keleman, L. E. *Int. J. Cancer* **2006**, *119*, 243). Since the binding affinities for PT, MTX, and TMP were not reported, we roughly evaluated them by the competitive labeling experiment (Figure S8).

(33) The fluorescence spectrum of Fl-labeled FR on the cell surface was almost identical with that of **1** alone in its wavelength (Figure S9).