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Article

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Tagging Transferrin Receptor with a Disulfide FRET Probe to Gauge the Redox State in Endosomal Compartments

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ABSTRACT: Although the basic process of receptor-mediated endocytosis (RME) is well established, certain specific aspects, like the endosomal redox state, remain less characterized. Previous studies used chemically labeled ligands or antibodies with a FRET (Fluorescence Resonance Energy Transfer) probe to gauge the redox activity of the endocytic pathway, with a limitation being their inability to track the apo receptor. New tools that allow direct labeling of a cell-surface receptor with synthetic probes would aid in the study of its endocytic pathway and function. Herein, we use a peptide ligase, butelase 1, to label the human transferrin receptor 1 (TfR1) in established human cell lines with a designer disulfide FRET probe. This strategy enables us to obtain real-time live cell imaging of redox states in TfR1-mediated endocytosis, attesting a reducing environment in the endosomal compartments and the dynamics of TfR1 trafficking. A better understanding of endocytosis of different cell surface receptors has implications in designing strategies that hijack this natural process for intracellular drug delivery.

INTRODUCTION

Receptor-mediated endocytosis (RME) is a process that cells take up extracellular ligands (nutritional and signaling molecules and viruses) intracellularly.¹⁻³ In RME, the initial internalized vesicles containing the receptor-ligand complexes are first fused with early endosomes, which then sort the endocytic contents for degradation or recycling.⁴⁻⁶ Traditionally, the study of RME uses fluorophore-labeled ligands.⁷⁻⁹ However, as the receptor-ligand complex may dissociate in the cell,¹⁰⁻¹² such an approach is inadequate to track the bona fide status of the ligand-free receptor. Another widely used method is genetic fusion of receptors with a large fluorescent protein, such as GFP or its variants, of which some are redox-sensitive.¹³⁻¹⁶ But this strategy can only allow the receptor to be functionalized with genetically encoded molecules, which may hinder some applications in certain experimental settings due to their large size or sensitivity to pH change. By contrast, synthetic molecules can overcome the limitations associated with genetically encoded reporters, which represent an alternative powerful toolbox to detect various intracellular molecules, including H₂S, reactive oxygen and nitrogen species (ROS/RNS) and glutathione (GSH).¹⁷⁻²¹ Despite their usefulness, being non-protein specific, most of these synthetic probes can only be applied to whole cells or a targeted organelle. There is an increasing need for methods that can monitor the change patterns of a detected molecule or event in a cell or organism that is uniquely correlated with the activity of a specific protein.²² Therefore, combining the advantages of protein-specific labeling

technology and versatile artificial synthetic probes may provide novel bioanalytical tools.

Understanding the redox biology in RME has significant implications in drug delivery research. The endocytic compartments are believed to have a reducing environment, which has led to the design of one type of antibody-drug conjugates that contains a reducible disulfide linker for intracellular drug release.^{23,24} However, a study using an anti-Her2 mAb conjugated to a fluorophore through a disulfide concluded that the Her2-mediated endocytic pathway was largely oxidising, which prevented efficient release of the fluorophore from the antibody in breast carcinoma SKBr3 cells.²⁵ Interestingly, a subsequent study using a folic acid-derived, S-S linked FRET probe led to the opposite conclusion that the recycling endosomes indeed maintained a reducing environment in the nasopharyngeal KB cells.²⁶ Because cellular redox systems are dynamically regulated,²⁷ it is possible that these conflicting results are due to different cell systems or experimental setups in these studies or to that the cargo molecules endocytosed by different receptor systems may be subjected to different redox status in their respective endocytic pathways. In these regards, a strategy that can functionalize a specific receptor directly with an artificial probe would further expand current toolkits for RME studies. Depending on the properties of installed “payload” on the protein, various targeted molecules or cellular events could be traced or detected accordingly, such as the redox activity in endosomal compartments in a specific receptor trafficking pathway.

So far there are many chemical and enzymatic strategies for site-specific protein labeling, providing unprecedented opportunities to study protein structure and function. Among these, enzymatic tagging of proteins is very attractive owing to

their substrate specificity and biocompatible working conditions. Several enzymes have been used to label cell surface proteins,

Scheme 1. General strategy for live cell imaging of redox states during TfR1 trafficking. The disulfide-based redox FRET probe was conjugated to the C terminus of TfR1 via butelase-mediated ligation (BML). An increase of fluorescence indicates the reduction of the disulfide bond in the endosomes. ①: Butelase 1 catalyzes the labelling of TfR1 (which contains a C-terminal NHV tripeptide motif) by the FRET probe which contains an N-terminal GI dipeptide; ②: Transferrins loaded with iron ions (holotransferrins) bind TfR1 to form holotransferrin-TfR1(FRET) complex; ③ The holotransferrin-TfR1(FRET) complex is internalized through clathrin-dependent pathway; ④ The redox-sensitive disulfide bond of the TfR1-attached FRET probe is cleaved to de-quench BODIPY, resulting in green fluorescence emission; ⑤ Upon releasing the irons, the complex of apotransferrin-TfR1 (with reduced FRET probe) is recycled back to cell surface which can go on for next round of trafficking.

but with some inherent limitations, such as slow kinetics, the need for a multistep labeling procedure and requirement for a large recognition tag in the protein substrates, or the difficulty to synthesize the probes.²⁸⁻³¹ Herein, we show that butelase 1, a broadly useful Asn-specific peptide ligase that cuts an Asn-Xaa bond in a peptide substrate and religates the Asn with the amino end of another,³²⁻³⁵ is an ideal enzyme that can overcome these drawbacks. In this study, we use butelase-mediated ligation (BML) to site-specifically label the human transferrin receptor 1 (TfR1) with a synthetic redox-activable FRET probe to visualize, in real time, the redox state of the endocytic pathway (**Scheme 1**). This is conceptually different from previous studies where a fluorophore-labeled ligand or antibody was used to track receptor-mediated endocytosis and the detected activities were associated with the ligand or the antibody. Our approach is also of potentially general utility to study the traffic of other cell surface receptors using similar or different biophysical probes.

EXPERIMENTAL SECTION

Methods for HPLC and Mass Spectrometry. Analytical HPLC analyses were performed using a Shimadzu HPLC system equipped with the proper column. The buffer system for all the analyses was buffer A (H₂O containing 0.045% TFA) and buffer B (90% acetonitrile in H₂O containing 0.045% TFA). For mass spectrometry, small compound and peptide mass spectra data were obtained on a Thermo ScientificTM LTQ XLTM with ESI ion source.

Plasmids construction. Plasmids pcDNA3.2/DEST/hTfR-HA-NHV, which encodes TfR1 containing butelase 1 recognition site (NHV) at its C-terminal end, was firstly constructed from pcDNA3.2/DEST/hTfR-HA (Addgene plasmid # 69610) by using Gibson assembly kit (NEB). To increase the flexibility of NHV in the TfR1-NHV, a flexible linker (GGGSGGGGS) was inserted between the HA tag and NHV sequence. The sequence integrity was confirmed by DNA sequencing. Then, the gene expression cassettes of TfR1 and TfR1-NHV (both with the HA tag) were then individually cloned into Lenti-EF1a plasmid to give Lenti-EF1a-TfR1 and Lenti-EF1a-TfR1-NHV, respectively, which were used to

transfect the cells and obtain the cells expressing TfR1 and TfR1-NHV.

Establishment of cell lines with stable expression of TfR1-NHV and TfR1. Cells were transfected with the above prepared Lenti-EF1a-TfR1-NHV or Lenti-EF1a-TfR1 plasmid along with two packaging vector PMD2.G and Pspax2 by using lipo3000 (Thermo Fisher Scientific). After 12 h of transfection, the old medium was removed from the cell culture and the fresh DMEM complete medium was added inside. The virus was harvested by collecting the supernatant at 48 h post transfection. The collected supernatant was added into the cells in 1:1 ratio with the fresh medium and cells were further incubated for 24 h. After the incubation, cells were incubated in the fresh medium. Then puromycin (3 $\mu\text{g}/\mu\text{L}$) was added into the cell culture to enrich the infected positive cells for 72 h.

FACS (fluorescence-activated cell sorting) analysis of labeled cells with Probe 1 and 2. Cells were washed with PBS (pH=7.4) for three times, then treated with trypsin for 3-5 min to detach them from the well, followed by addition of DMEM complete medium to neutralize trypsin activity. After that, cells were washed again with PBS (pH=7.4) for three times, followed by addition of 200 μL PBS (pH=6.5) containing 25 μM probe **1** or **2** (**Figure 1A**) and butelase 1 (0.125 μM) with gentle mixing. Cells were then incubated in 37 $^{\circ}\text{C}$ incubator for 30 min. After centrifugation, the supernatant was discarded. Cell pellets were washed three times with PBS (pH=7.4). Cells labeled with probe **1** were resuspended in PBS (pH=7.4) and filtered with cell strainer to remove the cell clots and transferred to flow cytometry tubes for analysis. Cells labeled with probe **2** were further incubated with Streptavidin conjugated with FITC for 30 min. Then cells were washed with PBS, filtered and subjected to FACS analysis.

Immunostaining detection of HA-tagged TfR1-NHV. After cell labeling via BML, cells were fixed with 4% PFA directly on 35 mm cell culture dish for 20 min, and then washed three times with PBS. The cells were treated with PBS containing 1% Triton X-100 for 10 min. For the staining, cells were incubated in the blocking solution (PBS with 0.1% Triton X-100 plus 10% Donkey serum) for 0.5 h at room temperature. The anti-HA antibodies (Sigma, clone HA-7 (H3663)) was diluted at 1:500 in the blocking solution, which was then added to the culture dish for incubation for 1 h at room temperature. Cells were washed four times with PBST (PBS with 0.1% Triton X-100) and then treated with anti-mouse antibody (Thermo Fisher Scientific, Rabbit anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 546) diluted at 1:300 in blocking solution for 1 h at room temperature. Then cells were washed four times with PBST and incubated with DAPI (1 $\mu\text{g}/\text{mL}$, diluted in PBS) for 10 min at room temperature for nuclear counterstain. Cells were visualized using a confocal microscope under 100 X magnification.

Peptide-based probes synthesis. Detailed synthetic procedure and characterization of final products and key intermediates could be found in the Supporting Information.

RESULTS AND DISCUSSION

Site-specific labeling of a cell surface protein using BML. Cells rely on the TfR1 endocytosis process for iron uptake.^{36,37} TfR1 is a homodimeric type II transmembrane protein with a

small N-terminal cytoplasmic domain, a single-pass transmembrane region and a large extracellular C-terminal domain. Each TfR1 dimer binds two transferrins with a two-fold symmetry and the C-terminal end of TfR1 is not involved in the binding of transferrin to TfR1.³⁸⁻⁴¹ TfR1 is one of the most characterized cell surface receptors and its trafficking pathway has been well established. Each transferrin binds two irons with extremely high affinity to form diferric transferrin (also called holotransferrin). The holotransferrin-TfR1 complex is internalized through clathrin-dependent pathway. Generally, this complex first enters early endosomes where iron is released from transferrin in the acidic environment. The resulting apotransferrin (iron-free protein) remains bound to TfR1 until the complex recycles back to the cell surface, where it dissociates from the receptor which can then undergo another cycle of trafficking.^{42, 43}

To label TfR1 via BML, a tripeptide butelase recognition motif NHV preceded by an HA tag was introduced at the C-terminus of TfR1. Although we have previously shown that butelase 1 can efficiently label live bacterial cell-surface proteins (OmpA) in phosphate buffer (pH 6.5),³⁴ it was unclear whether butelase 1 could retain its activity in nutrient media, considering the culture conditions and sensitivity of the much more complex mammalian cells. So two 293T cell lines expressing TfR1 w/o the NHV motif and TfR1-NHV, respectively, were first treated with butelase 1 and the fluorogenic probe **1** (20 μM), a good nucleophilic peptide substrate of the ligase (**Figure 1A**), in DMEM media (pH 6.5) supplemented with 10% FBS for 20 min at 37 $^{\circ}\text{C}$. As a control, cells were also treated with **1** in the absence of butelase 1. Confocal fluorescence microscopy analysis showed that only cells expressing TfR1-NHV were labeled, which had clear fluorescence on the rims. No obvious fluorescent staining was observed in 293T cells expressing TfR1 w/o NHV or in cells expressing TfR1-NHV treated in the absence of butelase 1 (**Figure 1B**), indicating that there are no endogenous butelase-reactive sequences on the cell surface. To further confirm site-specific modification of TfR1-NHV in 293T cells, a biotinylated peptide **2** (**Figure 1A**) was synthesized to test whether it could be attached to TfR1-NHV via BML. Labeled cells were collected at different time points (0, 5, 10, 20, 45 and 60 min) and subjected to Western blot analysis using HRP-streptavidin. Results show that compared with the non-labeled cells (0 min), a special band corresponding to TfR1 could be detected as early as 5 min of incubation and the intensity of band increased drastically after 20 min (**Figure 1C**). In addition, FACS (fluorescence-activated cell sorting) analysis was performed on the cells labeled with the same probes **1** and **2** (**Figure 1A**), which further showed that only cells expressing TfR1-NHV were successfully labeled by BML (Supporting information (SI), **Figure S1**). All these results together show that BML is a selective and efficient method to decorate mammalian cell surface as long as there is a membrane protein carrying an exposed NHV motif.

Characterization of peptide-based redox-sensitive probes. To achieve real-time redox state imaging during ligand-induced endocytic receptor trafficking, we prepared a disulfide-linked fluorescent probe **3** (**Figure 1A** and SI, **Figure S2-S5**), in which the fluorescence of the fluorophore is quenched by the quencher via intramolecular FRET until the disulfide bond is reductively cleaved. Probe **4** was used as the control (**Figure 1A** and SI, **Figure S6-S12**). Both **3** and **4**

were designed to have no native peptide bonds between the fluorophore and the quencher to avoid proteolytic dequenching. We first studied disulfide cleavage kinetics in a buffer. Each probe (10 μ M) was dissolved in PBS containing varying concentrations of reduced GSH (from 0.5 mM to 10 mM), set similar to its physiological range.^{44, 45} As expected,

only probe **3** was responsive to GSH to emit enhanced fluorescent signal, while **4** did not show any significant fluorescent enhancement (**Figure 1D**). This indicates that **3** could be employed as an effective reporter of the redox state in the cellular compartments where the labeled protein resides.

Figure 1. Site-specific labeling of TfR1-NHV with peptide-based probes using butelase 1 and their characterization. (A) Chemical structures of the labeling probes used in this study. In **3**, the FRET pair was linked via a disulfide bond. In **4**, the FRET pair has an acetamide linkage instead of the S-S linkage in **3**. (B) Confocal microscopy of fluorescence-labeled 293T cells. (C) Western blot analysis of biotin-labeled TfR1-NHV on 293T cell surfaces by BML from 0 to 60 min. See SI for experimental details. (D) In vitro characterization of **3** and **4** in the presence of different concentrations of reduced GSH (from 0.5 mM to 10 mM) in PBS (pH 7.4). The excitation wavelength was 488 nm. The data was collected using a plate reader. All fluorescence change lines of probe **4** stayed near the baseline level.

Figure 2. Live cell imaging of redox state during TfR1 trafficking. Video analysis of the redox states in endosomes in TfR1-NHV endocytic pathway in 140 min (See Supporting video file). Scale bar in all images = 50 μ m. Lysosomes were stained with lysotracker; Nuclear was stained with Hoechst. Excitation wavelength = 488 and 405 nm.

Live cell imaging of redox pattern change during TfR1 endocytosis. HeLa cells were used to evaluate the redox states in TfR1-mediated endocytosis for their superior adhesion

properties which facilitate live cell imaging studies. HeLa cells with stable expression of TfR1-NHV were generated and confirmed by butelase-mediated labeling assay (SI, **Figure**

S13). Then we continued to test whether TfR1-NHV could also be labeled with the redox-activable probe **3**. HeLa cells expressing TfR1-NHV were treated with **3** (25 μ M) in the presence of butelase 1 (0.125 μ M) at 37 $^{\circ}$ C for 20 min in the DMEM medium (pH 6.5) supplemented with 10% FBS. Then the unreacted probe and butelase 1 were removed from the culture dish and the labeled cells were re-incubated in the normal DMEM medium (10% FBS, pH 7.2) at 37 $^{\circ}$ C. To confirm the labeling of cell-surface TfR1, 10 mM reduced GSH was added to the cell culture to cleave the disulfide bond in **3** and de-quench the fluorophore. Labeled cells without reduced GSH treatment served as the control. After incubation for 45 min at 37 $^{\circ}$ C, confocal fluorescence microscopy analysis showed that the HeLa cells labeled with **3** under GSH treatment had clear fluorescent signals on the cell surface whereas the labeled cells without GSH treatment did not show significant fluorescent signal (**Figure S14**). This confirmed that **3** was successfully ligated with TfR1-NHV by butelase 1 and the labeled product TfR1-N-**3** on the cells was accessible by a reducing agent in the extracellular milieu.

With clear confirmation of TfR1 labeling by **3**, we went on to test whether this system could be used to detect the redox state in the endosomal compartments during TfR1 trafficking. Therefore, 0.25 μ M holotransferrin was added to the culture medium of the cells labeled with **3** to quickly occupy and stimulate internalization of the surface TfR1 proteins.^{46,47} After about 30-40 min, significant fluorescence signal was observed in the intracellular compartments (shown as green dots) and the signal was gradually increasing over a period of about 2 h. The fluorescence gradually accumulated around the juxtanuclear region, suggesting that the TfR1 was transported there for sorting (**Figure 2** and **Supporting video** online). We reasoned that the endosomal environment was reducing in TfR1 trafficking as the observed fluorescence could only be emitted from BODIPY in **3** after the quencher was removed by the reductive cleavage of the S-S bond.

Figure 3. Fluorescent imaging of redox states in TfR1 trafficking with HeLa cells labeled by the redox-sensitive probe **3** on TfR1 (Top). Cells labeled with the non-redox probe **4** were used as controls (Bottom). Nucleus was stained with DAPI. Lysosomes were stained with lysotracker. Scale bar = 10 μ m.

To rule out the possibility that the dequenching of fluorescence was caused by other cellular events, such as proteolysis of amide linkages in the probe, rather than by disulfide reduction, we performed a control assay. HeLa cells labeled with **3** and **4** were collected at different time (0, 2, 4, 6, 8 h), then fixed and subjected to confocal microscopy analysis. At 0 h, both groups of cells had only very weak fluorescent signals, indicating that the fluorophore in the FRET probe remained effectively quenched (**Figure 3**). But at 2, 4, 6, 8 h, only the cells labeled with **3** showed significant fluorescence staining at the cell surface, and there were also many bright green spots in the intracellular space (**Figure 3**). Consistent with the above results (**Figure 2**), this imaging data clearly showed that the internalized TfR1 molecules were accumulated at the juxtanuclear region, suggesting they may have entered recycling endosomes (**Figure 3**). These observations not only indicated the occurrence of reduction events in the endosomes but also the dynamic nature of TfR1 trafficking. By contrast, after a long incubation time of up to 8 h, the cells labeled with **4** did not show any significant fluorescent signals (**Figure 3**). Immunostaining TfR1 with an anti-HA tag antibody also indicate the co-localization of the probe and the HA tag in TfR1 (**Figure S15**), revealing that the observed fluorescence was not due to a probe fragment that might have been cut off from the labeled TfR1. Overall, our study provides direct imaging evidence that, in HeLa cells, the intra-endosomal environment in the TfR1 endocytic pathway is reducing enough for disulfide cleavage. Our result is consistent with the findings of Yang et al. who have shown that, in KB cells, the recycling endosomes involved in the

trafficking of the folate receptor have a reductive environment.²⁶ This is not surprising considering the colocalization of folate- and transferring-containing endosomes also observed by Yang et al.²⁶ However, in a separate study done on macrophages, Collins et al. found disulfide reducing activity in the lysosomes but not in the early, recycling endosomes involved in transferrin trafficking.⁴⁸ It is worth mentioning that the detection method used by Collins et al. was not as sensitive as the FRET methods used by us and Yang et al.²⁶ Nevertheless, given the dynamic nature of cellular redox systems,²⁷ the conclusions derived from this study may not apply to all cell systems. The significance of our study is that, using BML, we can achieve site-specific tagging of a membrane receptor, which allows us to more accurately study its trafficking, location as well as the biochemical properties (e.g., redox activity) of its intracellular environment, regardless of the status of ligand binding.

CONCLUSIONS

In summary, we have successfully demonstrated that butelase 1 is a robust enzymatic tool for the labeling of a cell-surface protein carrying a tiny recognition motif. In fact, the tripeptide motif (NHV) represents the smallest C-terminal peptide tag for site-specific enzymatic protein labeling known so far. In the current study, butelase 1 allowed rapid and efficient labeling of TfR1 with several probes, one being a redox-activable FRET probe. Our study enabled live cell imaging of the redox states during ligand-directed TfR1 endocytosis. It revealed that the disulfide bond could be cleaved in the endosomes during TfR1-mediated endocytosis in Hela cells, thus supporting the idea that receptor ligands may be employed as vehicles to deliver drugs into endosomes where the drug is released upon disulfide reduction.^{49, 50} In addition, techniques that target specific proteins for detection of redox potentials in particular cellular compartments are useful tools in redox biology research. Clearly, using this approach, other types of synthetic probes can also be tagged to a cell-surface protein for different studies. The versatility offered by our method makes it a very attractive alternative to the approach that relies on genetically encoded fluorescent proteins. Overall, we believe that this study will stimulate future developments of enzyme-mediated cell-surface remodeling strategies to monitor cellular events associated with a protein of interest.

ASSOCIATED CONTENT

Supporting Information

The detailed experimental details in this study could be found in the Supporting Information, which include TfR1-NHV DNA sequence, detailed procedure for the chemical synthesis of peptide-based probes and their ESI-MS and analytical HPLC characterization (PDF) and etc.

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Notes

The authors declare no competing financial interest.

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