Labeling of Cytoskeletal Proteins in Living Cells Using Biotin Ligase Carrying a Fluorescent Protein

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Labeling with fluorescent proteins is now widely exploited for elucidating the functions and roles of target proteins in living cells. Previously, we developed a protein labeling method by combining a fluorescent protein with a biotinylation reaction from archaeon Sulfolobus tokodaii. Biotinylation from S. tokodaii has a unique property that biotin protein ligase (BPL) forms a stable complex with its biotinylated substrate protein (BCCP). By taking advantage of this unique property, a target protein carrying BCCP in living cells can be labeled through biotinylation with BPL carrying a fluorescent protein. In the present work, to demonstrate the utility and performance of this labeling system in more detail, the cytoskeletal proteins β-actin and α-tubulin were selected as target proteins and labeled in living cells. With this approach, we succeeded in fluorescent imaging of actin filaments and microtubules in living cells, and shows the advantages of our approach over the conventional labeling methods with fluorescent proteins.

Keywords Protein labeling, fluorescent labeling, fluorescent protein, cytoskeletal proteins, biotin ligase, β-actin, α-tubulin

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Introduction

The fluorescent labeling of proteins is an indispensable approach for elucidating their functions and roles in cells. Various fluorescent labeling methods for proteins have been developed, with those based on fluorescent proteins being most widely used due to their simplicity and specificity, where target proteins are fused with fluorescent proteins by introducing genes encoding the appropriate fusion proteins into cells. Concomitant with the dissemination of fluorescent protein-based labeling methods, new labeling methods based on chemical biology have been proposed, such as tag-probe labeling technologies, in which synthetic fluorescent probes are attached to the target proteins carrying genetically encoded tags through specific interactions between the fluorescent probes and the tags. These chemical biology-based approaches allow for the labeling of proteins with synthetic fluorophores bearing various properties and controlling the timing of the labeling, thus broadening their applications in protein analysis. However, these methods require labeling procedures that are specific to each technology, which hampers their wide-spread use; also, the cytotoxicity that may accompany labeling has not yet been fully understood because of their limited use. Therefore, from the viewpoint of utility, it is important to expand the versatility of fluorescent protein-based labeling technologies in which labeling is accomplished simply by the introduction of DNA into cells.

We previously developed a protein labeling method by combining green fluorescent protein (GFP) with a biotinylation reaction from archaeon Sulfolobus tokodaii.13 In biotinylation, biotin protein ligase (BPL) mediates the attachment of biotin to the specific lysine residue of its substrate protein, biotin carboxyl carrier protein (BCCP).14 Biotinylation from S. tokodaii is unique in that BPL forms a stable complex with its product, biotinylated BCCP (Fig. 1a). By taking advantage of this unique property, we succeeded in labeling a membrane protein carrying BCCP as a tag inside living cells using a fusion protein of BPL with GFP coexpressed in the cells. In this approach, labeling is accomplished simply by DNA transfection, just as in the systems in which fluorescent proteins are directly attached to the target proteins. The cytotoxicity accompanying the labeling is expected to be limited because the fluorescent probe is comprised of proteins that do not exhibit harmful effects on cells. In addition, our method allows for attaching different types of fluorescent proteins to a target protein without reconstructing the expression system for the target protein, and also enables control of the timing of labeling in principle; these are the advantages of our system over the conventional labeling systems with fluorescent proteins.

In the current work, to demonstrate the utility and performance of our labeling system in more detail, the cytoskeletal proteins β-actin and α-tubulin were selected as target proteins and labeled in living cells. Specifically, β-actin and α-tubulin carrying BCCP on their N- or C-termini were coexpressed with BPL carrying GFP or the red fluorescent protein DsRed monomer (Fig. 1b). In the cells coexpressing cytoskeletal proteins carrying BCCP and BPL carrying the fluorescent protein, those cytoskeletal proteins are labeled by the fluorescent protein through the interaction between BPL and BCCP.
The cytoskeletal proteins assemble to form protein filaments: \(\beta\)-actin and \(\alpha\)-tubulin constitute actin filaments and microtubules, respectively. Because those protein filaments are formed by strict protein-protein interactions, subtle modifications to the cytoskeletal proteins could inhibit the formation of filaments. Thus, in this work, the potential for adverse effects of labeling was investigated by fluorescent observation of the filament structures, and the utility of our labeling system was evaluated.

### Experimental

#### Materials

The expression vectors in mammalian cells, pAcGFP1-Actin, pAcGFP1-Tubulin, and pDsRed-Monomer-C1, were obtained from Clontech. Oligonucleotides used as PCR primers in the construction of expression plasmids for fusion proteins were custom-synthesized by Gene Design (Osaka, Japan). The WST-1 cell proliferation assay system was purchased from Takara Bio (Kyoto, Japan). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA). Glass-bottom dishes used for cell culture and imaging experiments were purchased from AGC Techno Glass (Shizuoka, Japan).

#### Construction of expression plasmids for fusion proteins

The expression plasmids for the series of fusion proteins shown in Fig. 1b were used in the present work. Expression plasmids for \(\beta\)-actin and \(\alpha\)-tubulin carrying BCCP on their N- or C-termini were constructed in this study, along with the expression plasmids for \(\beta\)-actin and \(\alpha\)-tubulin carrying GFP on their C-termini. For \(\beta\)-actin and \(\alpha\)-tubulin carrying GFP on their N-termini, the commercially available expression plasmids pAcGFP1-Actin and pAcGFP1-Tubulin were used, respectively. The plasmid for a fusion protein of GFP with BPL (GFP-BPL) was constructed in our previous work, and that for a fusion protein of DsRed-monomer with BPL (DsRed-BPL) was constructed in this study. Detailed procedures for constructing the expression plasmids are described in the Supporting Information.

#### Blotting analysis

The expression of cytoskeletal proteins carrying BCCP in mammalian cells was examined by avidin-blotting analysis, as follows. HeLa cells maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) on a 35-mm culture dish were transfected with an expression plasmid of each fusion protein using Lipofectamine 2000; transfections were conducted with 2 \(\mu\)g of each plasmid and 4 \(\mu\)L of transfection reagent for approximately 5 \(\times\) 10^5 cells on a 35-mm dish according to the supplier’s instructions. Twenty four hours after transfection, the cells were lysed, and the samples were subjected to SDS-PAGE. Subsequently, the separated proteins were electroblotted on a PVDF membrane. The biotinylated fusion proteins were detected with streptavidin conjugated with alkaline phosphatase using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitroblue tetrazolium chloride (NBT) as developing reagents.

#### Fluorescence imaging of mammalian cells

The cells expressing the desired fusion proteins were imaged by confocal laser microscopy as follows. HeLa cells were seeded on a 35-mm glass-bottom dish and allowed to grow to approximately 5 \(\times\) 10^5 cells per dish for 24 h. Then, the cells were transfected with the respective expression plasmids using 4 \(\mu\)L of a transfection reagent, as described above. The amounts of plasmids used are described in the legends of figures showing imaging data; typically, 2 \(\mu\)g of a plasmid for cytoskeletal proteins carrying BCCP and 1 \(\mu\)g of a plasmid for fusion proteins of BPL with fluorescent proteins were used for labeling experiments. Twenty-four hours after transfection, the cells were imaged in fresh DMEM with 10% FBS by confocal laser microscopy on an Olympus Fluoview FV1200. The green channel was used for imaging fluorescence from GFP with excitation at 473 nm and detection over 490 – 590 nm, while the...
red channel was used for that from DsRed-monomer with excitation at 559 nm and detection over 575 - 675 nm.

Assessment of cell viability

The viability of cells expressing fusion proteins was assessed by measuring the conversion of a tetrazolium salt (WST-1) to its formazan dye by mitochondrial dehydrogenase using a WST-1 cell proliferation assay system according to the supplier’s instructions. Briefly, HeLa cells cultured on a 35-mm dish were transfected with the expression plasmids of the desired fusion proteins. Transfection was conducted under the same conditions as that used for fluorescence imaging experiments. Twenty-four hours after transfection, the cells were seeded in wells of a 96-well microplate; the number of cells seeded was adjusted to be 1.0 x 10^4 per well. After seeding, the cells were cultured for 24 h; following the addition of 10 μL of a WST-1 reagent per well, the cells were cultured for another 3 h. Finally, the absorbance at 450 nm was measured on a PerkinElmer ARVO× multilabel counter. In the same way, background absorbance was measured with culture medium containing a WST-1 reagent alone as a blank, and that value was subtracted from the absorbance of each sample.

Results and Discussion

Expression of cytoskeletal proteins carrying BCCP

The expression plasmids for a series of cytoskeletal proteins carrying BCCP (shown in Fig. 1b) were constructed in this study. The β-actin proteins carrying BCCP on their N- and C-termini were named BCCP-Actin and Actin-BCCP, respectively. In the same manner, α-tubulin proteins carrying BCCP on their N- and C-termini were named BCCP-Tubulin and Tubulin-BCCP, respectively. In these fusion proteins, appropriate linker sequences were inserted between the cytoskeletal proteins and BCCP to avoid steric hindrance between them. Following transfection with the respective plasmids, the cells were cultured for 24 h; then, the expression of each fusion protein was confirmed by avidin-blotting analysis, because BCCP from S. tokodaii has been shown to be biotinylated by endogenous BPL in mammalian cells. As shown in Fig. 2, clear bands were observed in avidin blotting around the molecular masses of the respective fusion proteins; the molecular masses calculated from amino acid sequences for BCCP-Actin, Actin-BCCP, BCCP-Tubulin, and Tubulin-BCCP are 49.7, 50.8, 57.2, and 58.6 kDa, respectively. These results indicate that the desired fusion proteins carrying BCCP were successfully expressed in mammalian cells. In addition, it is considered that the BCCP moieties on fusion proteins retain the substrate activity for biotin ligase, judging from the fact that BCCP moieties were biotinylated.

Comparing the band intensity in avidin blotting of the two types of α-tubulin proteins carrying BCCP, we observed that Tubulin-BCCP exhibited a more intense band than BCCP-Tubulin. It is deduced that there is not so a large difference in the expression level of these two proteins considering that both fusion proteins are comprised of the same protein units, BCCP and α-tubulin. Thus, difference in band intensity is considered to be mainly derived from the three-dimensional structures of microtubules constructed by the polymerization of tubulin proteins. The N- and C-termini of α-tubulin proteins are located on the inner and outer faces of microtubules, respectively; thus, Tubulin-BCCP, in which BCCP is attached to the C-terminus of α-tubulin, is more easily biotinylated by endogenous BPL than BCCP-Tubulin when it is integrated into a microtubule. This difference in the degree of biotinylation might be responsible for the difference in the band intensity in avidin-blotting analysis. Incidentally, the bands found at positions lower than that of Tubulin-BCCP were considered to have originated from the degradation products of Tubulin-BCCP.

Labeling of β-actin

First, we attempted to label the β-actin protein carrying BCCP on its N-terminus using GFP-BPL as a fluorescent probe. For this purpose, the cells were transfected with both expression plasmids for BCCP-Actin and GFP-BPL simultaneously, and 24 h after transfection, the cells were observed by confocal microscopy with a green channel. As shown in Fig. 3a, filamentary structures were clearly observed in the cells in the fluorescent image. On the other hand, such localization of fluorescence signals was not observed in cells expressing GFP-BPL alone (Fig. 3b). Thus, it is considered that BCCP-Actin was labeled by GFP-BPL through the interaction of BPL with BCCP, and the labeled BCCP-Actin molecules were successfully incorporated into actin filaments (Fig. 3c). The optimized conditions for labeling were investigated by adjusting the amount of the expression plasmid for GFP-BPL (pGFP-BPL). Thus, the cells were transfected with 0.25 - 4 μg of pGFP-BPL along with 2 μg of the expression plasmid for BCCP-Actin. Although the filamentary structures were observed under all conditions, larger amounts of pGFP-BPL caused higher background fluorescence in the cytosol (Fig. S1 in Supporting Information). Thus, in subsequent experiments, 1 μg of the expression plasmid for the fluorescent probe was employed along with 2 μg of the expression plasmid for the target protein.

As a control, the cells expressing GFP-Actin, a fusion protein in which GFP was directly attached to the N-terminus of β-actin, were imaged by confocal microscopy (Fig. 3c). As a result, cytoskeletal filaments were clearly observed just as in the cells coexpressing GFP-BPL and BCCP-Actin. This indicates that our system has the same labeling efficiency as that of the conventional approaches with fluorescent proteins under the present experimental conditions. We also conducted labeling of BCCP-Actin using DsRed-BPL as a fluorescent probe which emits fluorescence in a longer-wavelength region than GFP-BPL. Here, the cells transfected with BCCP-Actin and DsRed-BPL were observed by confocal microscopy with a red channel.
Cytoskeletal filaments were again clearly observed in the cells (Fig. 3d), while the fluorescence was observed over the whole area of the cells when DsRed-BPL was expressed alone (Fig. 3e). This demonstrates that our labeling system allows for labeling with different types of fluorescent proteins without reconstructing the expression system for a desired protein, which is regarded as a practical advantage over the conventional labeling systems with fluorescent proteins.

Incidentally, in our system, labeling in the cells should be accomplished in two ways. In the first, BCCP is biotinylated by the BPL in the fluorescent probes, resulting in formation of a tight complex of BCCP and BPL through which the labeling is completed. In the second, BCCP is firstly biotinylated by the endogenous BPL in the cells, and then the biotinylated BCCP is complexed with BPL in the fluorescent probes. Although the detailed mechanisms remain to be clarified, it can at least be concluded that biotinylation by endogenous BPL does not exert a harmful effect on the labeling, judging from the fact that the labeling reactions proceeded smoothly inside the cells.

We also attempted the labeling of β-actin carrying BCCP on its C-terminus, and observed cells coexpressing Actin-BCCP and GFP-BPL by confocal microscopy. In this case, filamentary structures were not observed in the fluorescence image, but granular spots were observed in the cytosol (Fig. S2a in Supporting Information). Judging from the results of the avidin-blotting analysis, Actin-BCCP should be expressed in the cells and retain its substrate activity for biotinylation. Thus, it was deduced that in this system Actin-BCCP was labeled by GFP-BPL but not successfully incorporated into actin filaments. As the control, cells expressing Actin-GFP, a fusion protein in which GFP was directly attached to the C-terminus of β-actin, were similarly imaged (Fig. S2b). Again, only granular spots were observed in the fluorescent image of the cells, as in the cells coexpressing Actin-BCCP and GFP-BPL. These results indicate that attaching the protein to the C-terminus of β-actin inhibits the formation of actin filaments. This behavior is considered to be reasonable, considering the fact that the C-terminus region of β-actin is located on the binding interface between the β-actin molecules in actin filaments. Incidentally, the granular spots observed might originate from the complex of Actin-BCCP with GFP-BPL/Actin-GFP entrapped into vesicles such as lysosomes.

### Labeling of α-tubulin

To further assess the utility of our labeling system, we attempted to label the cytoskeletal protein α-tubulin. First, labeling was conducted with BCCP-Tubulin, α-tubulin carrying BCCP on its N-terminus. The cells were transfected with expression plasmids for BCCP-Tubulin and GFP-BPL, and were observed by confocal microscopy 24 h after transfection.
As shown in Fig. 4a, filamentary structures were partially observed in the fluorescence image; however, they were vague as a whole. As a control, cells expressing GFP-Tubulin, the fusion protein in which GFP was directly attached to the N-terminus of α-tubulin, were also imaged (Fig. 4b). Cytoskeletal filaments were more clearly observed with this system than with that using BCCP-Tubulin and GFP-BPL. From these results, it is inferred that the complexation of BCCP-Tubulin with GFP-BPL did not proceed smoothly or that BCCP-Tubulin complexed with GFP-BPL was not effectively integrated into the microtubules. As already mentioned, the N-terminus of α-tubulin is located on the inner face of a microtubule; thus, the BCCP moiety of BCCP-Tubulin is displayed on the inner face when it is integrated into a microtubule. In this case, GFP-BPL, with larger size (54 kDa) than that of GFP (27 kDa), might not find easy access to inside of the microtubules, or integration of BCCP-Tubulin complexed with GFP-BPL into the microtubules might be impeded due to steric hindrance.

Thus, we next attempted labeling with Tubulin-BCCP, a fusion protein in which BCCP is attached to the C-terminus of α-tubulin, located on the outer surface of the microtubule. Figure 4c shows the imaging data obtained by the observation of cells coexpressing Tubulin-BCCP and GFP-BPL. In this case, fine filamentary structures were clearly observed in the cells, indicating that the complexation of Tubulin-BCCP with GFP-BPL occurs smoothly, and that the complexed Tubulin-BCCP was successfully integrated into the microtubules (Fig. 1d). As a control, the cells expressing Tubulin-GFP, a fusion protein in which GFP was directly attached to the C-terminus of α-tubulin, were also imaged (Fig. 4d). In this case, only vague fluorescence was observed, mainly in the cytosol; filamentary structures were scarcely observed. Such behavior was also observed in the previous study.20 Thus, regarding the labeling of α-tubulin on its C-terminus side, our method has a clear advantage over the conventional labeling method with fluorescent proteins. Although the origin of this advantage remains to be clarified, it is speculated that a small protein, BCCP (7 kDa), functions as a rigid spacer between GFP-BPL and α-tubulin to avoid the steric hindrance accompanied by the formation of microtubules.

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Assessment on cytotoxicity

We also investigated the cytotoxicity of our labeling system. Specifically, the metabolic activity of the cells expressing the fusion proteins was examined by monitoring the conversion of a tetrazolium salt to its formazan dye in the cells. As shown in Fig. 5, the cells expressing GFP-BPL and cytoskeletal proteins carrying BCCP exhibited no significant change in metabolic activity compared with the cells transfected with an empty vector (the expression plasmid with no target gene inserted). In these experiments, the DNA transfection and culturing were conducted under the same conditions as used for the fluorescent imaging experiments, and the activity of the cells was measured two days after transfection; fluorescent imaging experiments were conducted one day after transfection. Thus, it is considered that our labeling system scarcely affects the cell viability under the conditions used for the imaging experiments. This is regarded as one of the characteristics of our labeling system, where a protein not exhibiting harmful effects acts as a fluorescent probe.

Conclusions

In the present work, we assessed the usefulness of our labeling method with BPL carrying fluorescent proteins by selecting cytoskeletal proteins as target proteins. We clearly observed the actin filaments in the cells coexpressing BCCP-Actin and GFP-BPL (or DsRed-BPL) by fluorescent imaging. Likewise, we succeeded in observing the fine structures of microtubules in the cells coexpressing Tubulin-BCCP and GFP-BPL by fluorescent imaging. On the other hand, microtubules were not observed in the cells expressing Tubulin-GFP, demonstrating the advantage of our approach over the conventional labeling methods in which the fluorescent proteins are directly attached to the target proteins. Although our labeling system could also be constructed with a single expression plasmid using IRES (Internal Ribosome Entry Site) system, it is possible to control the timing of labeling by employing separate expression plasmids for the target protein and fluorescent probe. In addition, this approach allows for attaching different types of fluorescent proteins to a target protein without reconstructing the expression system for the target protein. Thus, our method harnesses the versatility to fluorescent proteins-based labeling technologies and could be an alternative approach to the existing fluorescent labeling methods of proteins.

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Supporting Information

The procedures for construction of expression plasmids, Fig. S1 and Fig. S2 are provided. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/

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