Fluorescent labeling of the nuclear envelope by localizing GFP on the inner nuclear membrane

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Abstract

The nuclear envelope (NE) is a double membrane that segregates nuclear components from the cytoplasm in eukaryote cells. It is well-known that the NE undergoes a breakdown and reformation during mitosis in animal cells. However, the detailed mechanisms of the NE dynamics are not yet fully understood. Here, we propose a method for the fluorescent labeling of the NE in living cells, which enables the tracing of the NE dynamics during cell division under physiological conditions. In our method, labeling of the NE is accomplished by fixing green fluorescent protein carrying the nuclear localization signal on the inner nuclear membrane based on a unique biotinylation reaction from the archaeon *Sulfolobus tokodaii*. With this method, we observed HeLa cells during mitosis by confocal laser scanning microscopy, and succeeded in clearly visualizing the difference in the timing of the formation of the NE and the nuclear lamina.

The nuclear envelope (NE) consists of the inner nuclear membrane (INM) and the outer nuclear membrane (ONM), which are joined at the site where the nuclear pore complex (NPC) resides. The ONM is continuous with the membrane network of the endoplasmic reticulum (ER), and the ONM shares fundamental properties with the ER. Prior studies showed that at the beginning of mitosis in animal cells, the NE disassembles and its membrane is mixed with the ER, and then the NE is reformed around the chromosomes where the ER serves as a source of the NE components[1–4]. The recent knowledge of the mechanisms underlying the disassembly and reformation of the NE was obtained mainly in experiments using INM proteins fused to fluorescent proteins as marker proteins[5–8]. For example, the processes of NE formation were investigated in detail using fusion proteins of integral INM proteins, i.e., Lamin B receptor[5–7] and emerin[6,8], with green fluorescent protein (GFP). However, the INM proteins undergo specific interactions with nuclear components, and thus, marker proteins based on the INM proteins exhibit their peculiar localizations, and the overexpression of these proteins could exert abnormal effects on the functions of nuclei. Therefore, to trace the native structures and dynamics of the NE, it is necessary to label the NE based on methods that do not rely on INM proteins. In this paper, we propose a method for the fluorescent labeling of the NE which does not need INM proteins.

In our method, the NE is labeled by localizing GFP on the INM based on a unique biotinylation reaction from the archaeon *Sulfolobus tokodaii* (Figure 1). 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)[9,10]. Biotinylation from *S. tokodaii* has a unique property in which BPL forms a stable complex with its product, the biotinylated BCCP[11,12]. By taking advantage of this unique property, we previously constructed fluorescent labeling systems for proteins in the living cells, in which the target proteins fused to BCCP are labeled by BPL carrying fluorophores[13,14]. In the present work, BPL was

expressed as a fusion protein with a single transmembrane domain of the human platelet-derived growth factor receptor (TM) in living cells. BPL was attached to the C-terminus of the TM (Figure 1a), and in the cells expressing the resulting fusion protein, i.e., TM-BPL, the BPL moiety should be displayed on the surface of the membrane facing cytoplasm or nucleoplasm. In addition, BCCP was attached to N-terminus of GFP carrying the nuclear localization signal (NLS) on its C-terminus (Figure 1a). The resulting fusion protein, BCCP-GFP-NLS, was coexpressed with TM-BPL in living mammalian cells. In the cells expressing both fusion proteins, BCCP-GFP-NLS is expected to be trapped mainly on the INM through the complexation between BPL and BCCP via biotinylation (Figure 1b).

Our method also utilizes the characteristics of the molecular transport in the periphery of the NPC to retain the trapped BCCP-GFP-NLS in the nuclei. It is known that the integral membrane proteins can move from the ONM to the INM through the narrow channel at the boundary between the nuclear membrane and the NPC if the size of the proteins' domains in cytoplasmic/nucleoplasmic side is <60–70 kDa[15–19]. In this case, TM-BPL synthesized on the ER or the ONM is expected to travel to the INM since the molecular size of BPL is 27 kDa. However, when TM-BPL is complexed with BCCP-GFP-NLS on the INM, its entire molecular size in the nucleoplasmic side exceeds 60 kDa; the molecular sizes of BCCP, GFP, and NLS are 7, 28, and 3 kDa, respectively. Thus, the TM-BPL complexed with BCCP-GFP-NLS is expected not to return to the ONM, resulting in the retention and concentration of the GFP moiety on the INM (Figure 1c).

To assess the feasibility of our approach, we transfected HeLa cells with the expression plasmids for TM-BPL and BCCP-GFP-NLS simultaneously, and 24 hr after transfection, the cells were observed by confocal laser scanning microscopy (Figure 2a). The fluorescence from GFP was observed mainly at the nuclear rims, as revealed from the staining of the nuclear region with Hoechst. As a control, TM-BPL was coexpressed with a fusion protein of

BCCP with GFP not carrying NLS (BCCP-GFP). In that case, the strong fluorescence signal was observed not only at the nuclear rim but also in the membrane network of the cytoplasm (Figure 2b). This pattern of distribution of the fluorescence signal is closely matched with that observed in the cells expressing TM fused to GFP on its C-terminus (TM-GFP) (Figure 2c). These results indicate that the localization of fluorescence to the nuclear rim observed in the cells expressing TM-BPL and BCCP-GFP-NLS is derived from the immobilization of BCCP-GFP-NLS on the INM, showing that our approach can be exploited as a labeling method for the NE.

Incidentally, in our system, the expression levels of BCCP-GFP-NLS and TM-BPL are crucial for the labeling of the NE. As the overexpression of BCCP-GFP-NLS can be the noise for the NE labeling, the expression of BCCP-GFP-NLS should be controlled at the relatively lower level. When the cells were transfected with the same amount of expression plasmid for both proteins (2 μg TM-BPL and 2 μg BCCP-GFP-NLS), BCCP-GFP-NLS not tapped on the INM caused the significant background, resulting in the decrease in selectivity of the NE labeling (Figure S1a). On the other hand, the number of the labeled cells was limited when the amount of the plasmid for BCCP-GFP-NLS was relatively small (2 μg TM-BPL and 0.5 μg BCCP-GFP-NLS) (Figure S1b). Thus, considering the selectivity and efficiency of the labeling, we used twice amount of the plasmid of TM-BPL to that of BCCP-GFP-NLS (2 μg TM-BPL and 1 μg BCCP-GFP-NLS) for the labeling experiments of the NE.

Next, to evaluate the structure of the NE visualized with our approach, we compared that structure with that of the nuclear lamina, which is a fibrillar network inside the nucleus. Here, for the labeling of the nuclear lamina, Lamin A fused to red fluorescent protein, mApple, on its N-terminus (mApple-Lamin A) (Figure 1a) was coexpressed in the cells. Thus, the cells were transfected with the expression plasmids for TM-BPL, BCCP-GFP-NLS, and mApple-Lamin A, simultaneously, and 24 hr after transfection, we observed the cells by

confocal microscopy on green and red channels. Cross-sectional images showed the close similarity of the distribution of the fluorescence signal from GFP to that from mApple (Figure S2a).

To compare the fluorescence signals from GFP and mApple in more detail, we reconstructed three-dimensional images of a labeled cell by stacking optical sections taken at different depths of the cell. As shown in Figure S2b, the fluorescence signals from GFP and mApple again overlapped with each other in the sectional images in the XZ and YZ planes. Incidentally, a nucleoplasmic reticulum (NR) formed by the invagination across the nucleus was observed on both channels as shown in images in the XZ plane; the NR is a structure found in a wide variety of cell types, and is regarded as a common feature of eukaryotic cells[20,21]. These results indicate that the distributions of the NE and the nuclear lamina completely coincide with each other including the NR in the cells at interphase.

Next, to trace dynamics of the NE and the nuclear lamina, we imaged the cells expressing TM-BPL, BCCP-GFP-NLS, and mApple-Lamin A during cell division by taking confocal sections every 15 min (Figure 3, Movie S1). Figure 3 shows a representative time-lapse sequence recorded from prophase through cytokinesis. The localization of fluorescent signals at the nuclear rim was completely dispersed at the beginning of metaphase (Figure 3, 30 min), showing the breakdown of the NE and the nuclear lamina at this stage. After the disappearance of the NE, fluorescence from mApple was observed uniformly in the whole area of the cytosol, demonstrating that Lamin A was completely distributed in the cell. On the other hand, we observed fluorescence from the GFP as fragments in the cell. This behavior supports the fact that the NE membrane is adsorbed into the ER network following the breakdown of NE[1–4]. After mitosis, we observed the reassembly of the NE and the accumulation of Lamin A into the NE in the daughter cells (Figure 3, 90 and 105 min, and Movie S1). We also confirmed the reproducibility of the results (Figure S3). In both examples,

the structures of the NEs observed seem to be normal before and after mitosis, showing that our labeling does not exert the significant effect on the structure of the NE.

To gain further insight into the timing of the reformation of the NE and the nuclear lamina, we took confocal images of the cells during mitosis every 2 min (Figure 4, Movie S2). Figure 4 shows a time-lapse sequence recorded from anaphase through cytokinesis. Following the formation of the NE precursors at late anaphase (Figure 4, 4 min), Lamin A began to accumulate into the newly formed NEs (Figure 4, 6 min). This result is correlated with the previous reports stating that the reformation of the nuclear lamina begins at late telophase and early cytokinesis[22,23]. The accumulation of Lamin A from the cytosol was then almost completed at the end of mitosis (Figure 4, 12 min). After mitosis, the NE continued to develop in the daughter cells along with the nuclear lamina (Movie S2).

To resolve the distribution of the NE and the nuclear lamina during mitosis quantitatively, we calculated the fluorescence intensities from GFP and mApple on some of the imaging data shown in Figure 4 with the software program ImageJ (Figure S4). At anaphase, the fluorescence signal from the GFP began to accumulate around the chromosome, showing the formation of the NE precursor (Figure S4a; Figure 4, 0 min). At late anaphase, localization of the GFP signal around the chromosome was manifested, whereas localization of the mApple signal was not still evident at this stage, showing the uniform distribution of the Lamin A in the cell (Figure S4b; Figure 4, 4 min). At telophase, the mApple signal began to overlap with the GFP signal around the chromosome, showing the formation of the nuclear lamina, and at this stage, the transport of Lamin A through the connection part of the two daughter cells was observed (Figure S4c; Figure 4, 8 min). The fluorescence signals from GFP and mApple mostly overlapped with each other at the end of mitosis (Figure S4d; Figure 4, 12 min). To the best of our knowledge, this is the first example clearly showing the difference in the timing of the formation of the NE and the nuclear lamina during cell

division by time-lapse imaging on a confocal laser microscope.

In our labeling system, relocalization of the GFP signal to the NE was observed during the reformation of the NE as shown in Figures 3, 4, and S3. This behavior might contain important knowledge on the mechanism of the NE reformation. In the previous work, it was demonstrated that the NLS cannot target the membrane protein to the NE[15]. Also, the proteins of our labeling system (BPL, BCCP, TM, and GFP) are not known to interact with the components in the nucleus. Therefore, we envisage that the relocalization of the GFP signal is derived from the property of the NE membrane.

Following the breakdown of the NE, the NE membrane is absorbed into the membrane network of the ER. Here, there is a possibility that the NE membrane does not completely mix with the ER membrane and retains its identity in part even after the breakdown of the NE. In this case, BCCP-GFP-NLS complexed with TM-BPL remains in proximity to the INM proteins via the NE membrane. Consequently, the GFP complex is concentrated around the chromosomes along with the INM proteins as the NE is reformed, resulting in the relocalization of the GFP signal on the newly formed NE. The idea that the NE does not completely lose its identity even after the NE breakdown seems to be reasonable considering the rapid reformation of the NE during mitosis. By tracing the movement of the GFP complex under the optimized conditions, we might provide the novel knowledge on the dynamics of the NE during mitosis.

METHODS

Materials.

DNA transfection reagents, Lipofectamine 2000 and X-tremeGene 9, were obtained from Invitrogen and Roche, respectively. The glass-bottom dish used for cell culture and imaging experiments were purchased from AGC Techno Glass. Oligonucleotides used as polymerase

chain reaction (PCR) primers were custom synthesized by Gene Design Inc. The plasmids, mApple-C1 and pBABE-puro-GFP-wt-lamin A, which were used for construction of the expression plasmid for mApple-Lamin A were gifts from Michael Davidson (Addgene plasmid # 54631)[24] and from Tom Misteli (Addgene plasmid # 17662)[25], respectively.

Fluorescence imaging of the cells.

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO_2. For the acquisition of fluorescence imaging data, we seeded the cells on a 35-mm glass-bottom dish coated with poly-L-lysine and allowed them to grow to approx. 5×10^5 cells per dish for 24 hr. The cells were then transfected with the respective expression plasmids using Lipofectamine 2000 or X-tremeGene 9 DNA transfection reagent according to the supplier's instructions. The amounts of expression plasmids and transfection reagents used are described in the legend of each figure. Twenty-four hours after the transfection, the culturing medium was replaced with the fresh DMEM containing 10% FBS, and we stained the cells with Hoechst 34580 by adding the stock solution of the dye to the medium at the final concentration of 1 μg/mL. The cells were then incubated for 30 min. After that, the cells were imaged in fresh DMEM with 10% FBS by confocal laser scanning microscopy on an Olympus Fluoview FV1200. The green channel was used for monitoring the fluorescence from GFP with excitation at 473 nm and detection over 490–540 nm, and the red channel was used for monitoring that from mApple with excitation at 559 nm and detection over 575–675 nm. The blue channel was also used for monitoring fluorescence from Hoechst 34580 with excitation at 405 nm and detection over 430–455 nm.

Time-lapse imaging of the cells.

HeLa cells cultured on a glass-bottom dish were transfected with the expression plasmids of TM-BPL, BCCP-GFP-NLS, and mApple-Lamin A simultaneously with X-tremeGene 9 DNA transfection reagent. Twenty-four hours after the transfection, the culturing medium was replaced with the Leibovitz's L-15 medium containing 10% FBS, and after focusing on the labeled cells, we recorded time-lapse sequences of the cells every 15 min or 2 min by confocal microscopy on green and red channels along with differential interference contrast (DIC) images. We constructed the time-lapse movies by assembling each imaging frame with the use of ImageJ software (https://imagej.nih.gov/ij/index.html).

Image processing and analysis.

We performed three-dimensional reconstruction from a series of the sectional images on ImageJ. Specifically, a series of the sectional images were taken from the bottom to the top of the labeled cell at an interval of 0.25 μm, and the three-dimensional images of the cell were reconstructed by stacking the 40 sectional images in the Z-direction. Fluorescence intensities on the sectional images were also quantified with ImageJ.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publication website.

Supplementary figures (Figures S1–S4), legends of movies, and the experimental procedures for construction of expression plasmids (PDF); Supplementary movies (Movies S1 and S2) (AVI)

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Notes

The authors declare no competing financial interest.

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FIGURE LEGENDS

Figure 1. Fluorescence labeling of the NE based on *S. tokodaii* biotinylation. (a) Domain structures of the fusion proteins used in the present work. The NLS is composed of three copies of the NLS sequence of the SV 40 large T-antigen. (b) Schematic illustration of the labeling method. TM-BPL and BCCP-GFP-NLS are coexpressed in a mammalian cell. TM-BPL is distributed in the membrane network of the cell including the INM. BCCP-GFP-NLS is localized on the INM through the complexation of BPL with BCCP. (c) Transport of the membrane protein in the periphery of the NPC. TM-BPL not complexed with BCCP-GFP-NLS can travel between the INM and the ONM. TM-BPL complexed with BCCP-GFP-NLS in the INM cannot move to the ONM because of the size constraint of the channel at the boundary between the nuclear membrane and the NPC.

Figure 2. Confocal images of the cells expressing GFP-fusion proteins. (a) Images of the cells expressing TM-BPL and BCCP-GFP-NLS. HeLa cells were transfected with 2 and 1 μg of the expression plasmids for TM-BPL and BCCP-GFP-NLS, respectively, using 4 μL of Lipofectamine 2000. Twenty-four hours after transfection, the cells were stained with Hoechst 34580 and observed by confocal microscopy on green (*left)* and blue (*second from the left*) channels for GFP and Hoechst, respectively. The *third panel from the left* represents an overlay of signals from both channels. The *right panel* is a differential interference contrast (DIC) image of the cells. (b) Images of the cells expressing TM-BPL and BCCP-GFP. HeLa cells expressing both fusion proteins were prepared and imaged in the same procedures as those in panel (a). (c) Images of the cells expressing TM-GFP. HeLa cells were transfected with 1 μg of the expression plasmid for TM-GFP using 2 μL of Lipofectamine 2000, and 24 hr after the transfection, the cells were imaged in the same procedures as those in panel (a).

Bars, 10 μm.

Figure 3. Comparison of the distribution of the NE and the nuclear lamina during cell division. HeLa cells were transfected with 2, 1, and 2 μg of the expression plasmids of TM-BPL, BCCP-GFP-NLS, and mApple-Lamin A, respectively, using 8 μL of X-tremeGene 9. Twenty-four hours after transfection, time-lapse sequences of the cells were recorded every 15 min by confocal microscopy on green and red channels for GFP and mApple, respectively. *First and second columns from the left:* Typical time-lapse sequences of the cell recorded from prophase to cytokinesis on green and red channels, respectively. *Third column from the left:* An overlay of signals from both channels. *Right column:* DIC image of the cell. Bars, 10 μm.

Figure 4. Comparison of the timing of the reformation of the NE and the nuclear lamina. HeLa cells expressing TM-BPL, BCCP-GFP-NLS, and mApple-Lamin A were prepared in the same procedures as described in the Fig. 3 legend, and time-lapse sequences of the cells were recorded every 2 min by confocal microscopy. *First and second columns from the left:* Typical time-lapse sequences of the cell recorded from anaphase to cytokinesis on green (GFP) and red (mApple) channels, respectively. *Third column from the left:* An overlay of signals from both channels. *Right column:* DIC image of the cell. Bars, 10 μm.