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Observation of NMR Signals from Proteins Introduced into Living Mammalian Cells by Reversible Membrane Permeabilization Using a Pore-Forming Toxin, Streptolysin O

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Structural information about proteins inside living cells is of crucial importance for understanding the biological events actually occurring within the intracellular environment, which are often difficult to reconstitute in an in vitro system. To achieve this goal, several "in-cell" NMR methods have been developed to directly detect the NMR signals derived from isotopically enriched molecules introduced within the living organisms.[1] Using *Escherichia coli* cells overexpressing ^{13}C- and/or ^{15}N-labeled target proteins, investigations of protein−protein interactions,[2] protein dynamics,[3] and even the determination of the protein structure[4] were performed within the "molecular crowding" environment of the bacterial cytosol, where the total protein concentration exceeds 200 mg/mL. In-cell NMR analysis using eukaryotic cells is technically more challenging because overexpression of the isotopically labeled target proteins is impossible. Several groups have shown that an ^{15}N-labeled protein microinjected into *Xenopus laevis* oocytes yields high-resolution spectra that can be used for analyses of protein−protein interactions and post-translational modifications, such as phosphorylation.[5,6] However, this method is only applicable to exceptionally large cells (e.g., 1 mm diameter for the *X. laevis* oocyte). More recently, Inomata et al.[7] reported a method in which the target protein is delivered to the cell as a fusion with the cell-penetrating peptide (CPP) from Tat of HIV. Although this method can be used for any kind of cell, including mammalian cells, the method has the limitations that the target protein has to be fused with the Tat tag and the tag must be cleaved off after translocation to the cytoplasm for NMR observation. In the present work, we have developed a method that is applicable to any kind of cell and does not require any special equipment or artificial modification of the molecule of interest.

To introduce an isotopically labeled protein into the target cells, we utilized the bacterial toxin streptolysin O (SLO), which can form a 35 nm diameter pore on the cholesterol-containing plasma membrane that allows the entry and exit of molecules with sizes up to 150 kDa.[8] Since the pore formed by SLO can be repaired by the addition of Ca^{2+} into the cytosol, the SLO-permeablized cells can be resealed after they assimilate the molecule of interest.[9] Although the SLO permeabilization and resealing methods have previously been used for the incorporation of small amounts of proteins or nucleotides into many kinds of cells,[9−11] sample preparation on the NMR scale is unprecedented.

We chose nonadherent 293F cells as the host cells and an actin-sequestering protein, thymosin $\beta4$ (T$\beta4$), as the protein to be delivered for NMR observation. To assess the efficiency of the pore formation and resealing by flow cytometry (FCM) analysis,[10] T$\beta4$ or dextran (MW 4000) conjugated with fluorescein isothiocyanate (FITC) were included during the protein incorporation, and propidium iodide (PI), which stains the cells with damaged plasma membranes, was added after the resealing step, as shown in Figure 1a. In comparison with the cells without SLO treatment, the cells treated with SLO exhibited increased FITC-positive and PI-negative populations (lower right of the FCM density plots in Figure 1b), indicating that the pore formation and resealing occurred properly. These cells would provide the NMR signals from the encapsulated molecule. On the other hand, FITC-positive and PI-positive cells were also observed (Figure 1b), which could provide unwanted signals derived from the target molecule leaking from the unrepaired pore. The FITC-negative cells indicate that the pore formation did not occur during the SLO treatment. These cells lack NMR signals, thus resulting in the reduction of the total amount of the target molecule included in the NMR sample.

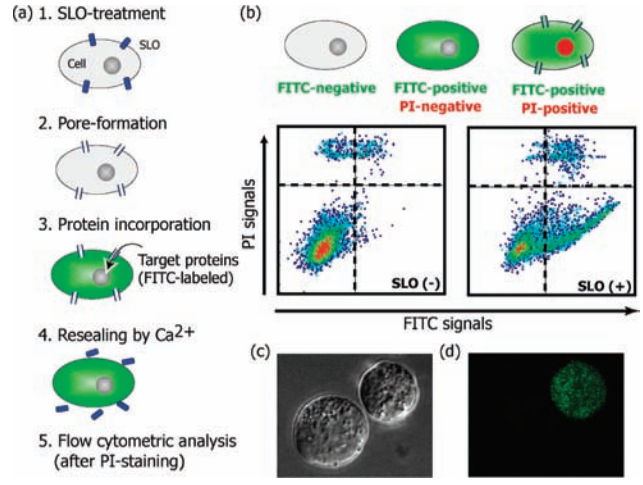

Figure 1. Incorporation of the target protein within 293F cells. (a) Schematic illustrations of SLO permeabilization and resealing of the target cells. (b) Double-staining FCM analysis of the cells (left) without and (right) with SLO treatment. (c) Differential interference contrast and (d) confocal microscope images of SLO-treated 293F cells incubated with FITC-labeled T$\beta4$.

We examined the optimal concentration of SLO for cell permeabilization. The FCM analyses revealed that more cells were FITC-positive at higher SLO concentrations, indicating that a greater number of pores were formed on the cell surface. However, the ratio of PI-positive cells also increased at the higher SLO concentrations, suggesting that some pores remained unrepaired when a large number of pores were formed on the membrane (Figure S1 in the Supporting Information). Among the concentrations we tested, a 20−40 ng/mL SLO concentration yielded optimal resealing efficiency (70−80%) with a pore-formation efficiency of 50% (Figure S1). The resealed 293F cells were placed on glass-bottom dishes for 2 h and then examined by confocal microscopy. The fluorescent images of the cell indicate

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that the Tβ4 protein was evenly distributed throughout the cytoplasm as well as the nucleus (Figure 1d). The cell without fluorescent signals (Figure 1c,d, lower left) is one without pore formation during the SLO treatment (i.e., a FITC-negative cell). The fact that the fluorescent cells adhered on the surface indicates that the cells treated with SLO are still viable (Figure 1c). SDS-PAGE of the cell lysate further confirmed that Tβ4 was present in the cytoplasm and did not undergo proteolytic degradation (Figure S2). By a comparison of the fluorescent signals of lysates from cells treated with and without SLO, the concentration of Tβ4 encapsulated within the 293F cells when the SLO-permeabilized cells were incubated with 1 mM Tβ4 was estimated to be 50 μM (Figure S3). Assuming that 2×10^7 cells (18 μm diameter) containing 50 μM Tβ4 were resuspended in 200 μL of buffer yields an estimate of 4 μM for the protein concentration of the NMR sample.

Using the optimal conditions thus established, we prepared a large amount of cells (2×10^7 cells) for NMR measurements. The resealed cells were transferred to a Shigemi tube as a suspension in CD293 medium containing 20% D_2O and supplemented with 30% RediGrad, a colloidal medium for density gravity centrifugation, to suppress the sedimentation of the cells during the NMR measurement.[12] The $^1H-^{15}N$ HSQC spectrum was recorded with 512 scans for each F1 increment (10 h measurement time) (Figure 2a). After the measurement, the suspension of the resealed cells was collected and examined by NMR spectroscopy. Almost no signals were observed from the supernatant (Figure 2b), indicating that almost all of the NMR signals originated from the Tβ4 protein within the cells. In addition, the percentages of cells stained by trypan blue were the same before and after the NMR measurement, indicating that the viability of the cells remained unchanged.

A comparison of the HSQC spectra of Tβ4 recorded under the in-cell conditions and in the buffer revealed that a number of signals underwent significant chemical shift changes (Figure 2c). However, these chemical shift changes likely did not reflect the interaction of Tβ4 with endogenous G-actin, since the line width of each signal did not change significantly in comparison with those in a previous NMR study of Tβ4 bound to G-actin performed in vitro.[13] The residues with chemical shift changes (e.g., K3 and D5) were mostly clustered by

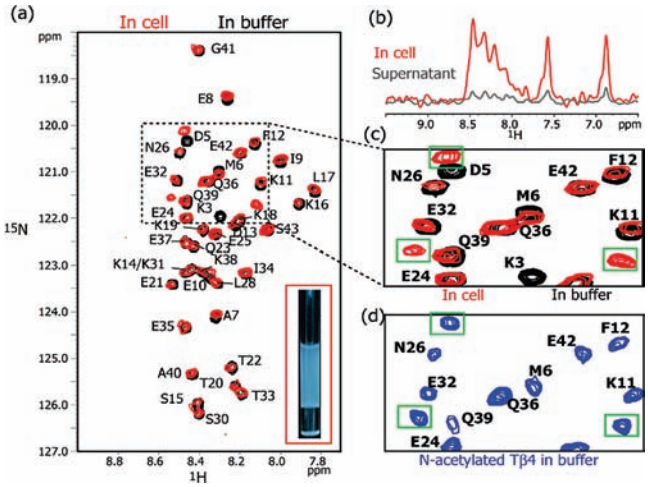

Figure 2. In-cell NMR spectra of Tβ4. (a) Overlay of $^1H-^{15}N$ HSQC spectra of Tβ4 recorded under in-cell (red) and buffer (black) conditions. The in-cell NMR sample is shown in the inset. (b) One-dimensional $^1H-^{15}N$ HSQC spectra of the resealed 293F cells (red) and the outer solution of the cell suspension (black). (c, d) Close-up views of the $^1H-^{15}N$ HSQC spectra of (c) Tβ4 under the in-cell conditions and (d) chemically synthesized Tβ4 with N-terminal acetylation. The residues with significant chemical shift changes are shown in green boxes.

the N-terminus of Tβ4. Endogenous Tβ4 is known to be enzymatically acetylated in the cytoplasm.[14] The patterns of the HSQC signals observed for in-cell Tβ4 coincided well with those of chemically synthesized Tβ4 with N-terminal acetylation (Figure 2d), indicating that the Tβ4 protein introduced within the 293F cells became acetylated in the cytoplasm as a post-translational modification.

In conclusion, we have established a new in-cell NMR method utilizing SLO permeabilization and Ca^{2+} resealing of the target cells. The advantages of this method over other existing in-cell NMR methods are that this method does not require any modification of the target protein or any specialized equipment and that it is applicable to a wide range of cells. For the further improvement of this method, cells containing a higher concentration of the target protein could be isolated using a cell sorter, thereby increasing the sensitivity of the experiment and minimizing the signal leaked from unrepaired cells. Since the cytosolic component can be replaced with any solution during the SLO permeabilization step,[15] it would be possible to use this technique to investigate protein−protein interactions by the transferred cross-saturation (TCS) method,[16] which requires a higher D_2O concentration (typically 80%). If cells are viable at high D_2O concentrations, the TCS method will identify the binding surface of the delivered protein against the endogenous intracellular components that form a complex in a fast-exchange regime.

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Supporting Information Available: Detailed experimental procedures and experimental data for sample preparations. This material is available free of charge via the Internet at http://pubs.acs.org.

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