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NOTE

Intracellular protein cyclization catalyzed by exogenously transduced *Streptococcus pyogenes* sortase A

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Streptococcus pyogenes **sortase A (SpSrtA) was transduced into mammalian cells. Then SpSrtA-mediated intracellular circularization of a model protein was confirmed in a time and dose-dependent manner by Western blotting analysis. Direct transduction of SpSrtA is expected to be a strong tool for more conditional specific protein modification in mammalian cells.**

[**Key words:** Sortase A; Protein transduction; Mammalian cell; Protein modification; Cyclization]

Site-specific modification of proteins in living mammalian cells is an important yet challenging task in various research fields of bioscience and bioengineering: specifically modifying proteins of interest after translation can visualize their dynamic localization through modification (1), potentially regulate cellular events by conditionally altering the functions or localization of key proteins, and improve their stability by protein cyclization, however, currently, approaches for intracellular protein modification, which are rapid, site-specific and may not introduce perturbations into proteins of interest, are limited (2). For intracellular site-specific protein modification, chemical (3), chemoenzymatic approaches (4,5) and expressed protein ligation (EPL) (6) are now available. Among chemoenzymatic methods, sortase A (SrtA)-mediated modification was recently reported (5). SrtA is a kind of transpeptidase derived from gram-positive bacteria, recognizes a pentapeptide substrate, LPXTG, where *X* is variable, and ligates with the N-terminal amine group of another peptidyl substrates by cleaving the amide bond between T and G in the LPXTG sequence (7). In previous application studies, the target proteins bearing an LPETG sequence at their C-terminal region were specifically ligated with the N-terminal oligoglycine moiety of other protein or artificial peptidic compounds (8). The advantages of SrtA-mediated modification are as follows: compared to chemical method, the reaction specificity is so high that specific modification could be achieved even in the presence of many other proteins; pentapeptides to be attached to target proteins may not affect the folding and function of target proteins; it is relatively easy to prepare a wide variety of modifiers because the oligoglycine derivatives are easily synthesized. Therefore, intracellular SrtA-mediated modification is a promising tool in cell biology and biotechnology, although only one report about it had been published (5).

Compared with protein expression through gene transfer, protein transduction from the extracellular medium is more rapid, easily controllable in a concentration-dependent manner, and reliable because it does not need the unpredictable processes such as transformation, folding and intracellular trafficking (9). Especially, protein transduction may be more valuable for hard-to-transfect cells. On the other hand, in conventional intracellular chemoenzymatic protein modification methods, any enzymes have been expressed in living cells through gene transfer, not transduced from the extracellular medium. In this study, we first challenged to transduce SrtA into living mammalian cells through direct protein transduction. Direct transduction of SrtA was expected to enable rapid site-specific ligation in a concentration- and time-dependent manner, potentially leading to more conditional protein labeling and control.

Among a family of transpeptidases, *Staphylococcus aureus* SrtA (SaSrtA) is the most extensively employed for *in vitro* application (10), and was used for labeling membrane proteins on cell surfaces in our previous studies (11,12). However, SaSrtA is known to be Ca^{2+}-dependent (13) and difficult to be applied for intracellular modification in mammalian cells, where the calcium ion concentration is low (5,14). On the other hand, *Streptococcus pyogenes* sortase A (SpSrtA) is Ca^{2+}-independent (15), and was reported to successfully catalyze circularization of target proteins through intramolecular ligation between an LPETG sequence at their C-terminal and a GGG sequence at their N-terminal in mammalian cells (5). Accordingly, in this study, we employed SpSrtA, and SpSrtA was transduced into mammalian cells by using a commercially-available reagent for protein transduction, BioPORTER (Genlantis, San Diego, CA, USA). This reagent consisting of lipids can

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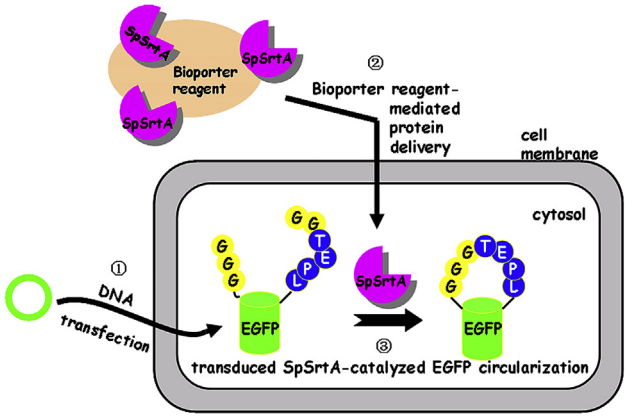

FIG. 1. Schematic illustration of protein circularization accomplished by protein transduction system. The model protein substrate is EGFP equipped with $-NH_2$-GGG and -LPETGG-COOH tags ($_{GGG}$-EGFP-$_{LPETGG}$). First, $_{GGG}$-EGFP-$_{LPETGG}$ substrate is introduced into cells by DNA transfection. Then SpSrtA is exogenously delivered into cells, using Bioporter reagent as the carrier, to catalyze the circularization of $_{GGG}$-EGFP-$_{LPETGG}$.

form non-covalent complex with cargo proteins, resulting in direct membrane translocation of the cargos (16). To visualize transduction of SpSrtA into living cells, SpSrtA was fluorescently labeled. To investigate whether SpSrtA-mediated ligation proceeds or not, circularization of enhanced green fluorescent protein (EGFP) with LPETGG and GGG sequences at the C- and N-terminal ($_{GGG}$-EGFP-$_{LPETGG}$) was evaluated by electrophoretic analysis as previously reported (5) (Fig. 1).

Cells expressing $_{GGG}$-EGFP-$_{LPETGG}$ were prepared as follows. The gene encoding $_{GGG}$-EGFP-$_{LPETGG}$ was subcloned into the *Bam*HI/*Xho*I sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) to construct an expression plasmid for $_{GGG}$-EGFP-$_{LPETGG}$ (pcDNA3.1/$_{GGG}$-EGFP-$_{LPETGG}$). This expression plasmid DNA was transfected into human embryonic kidney cells (HEK293T cells) by using lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. As a positive control, cells expressing both $_{GGG}$-EGFP-$_{LPETGG}$ and SpSrtA were prepared. The expression of proteins in HEK293T cells was confirmed by Western blotting analysis: the lysates of cells were fractionated by 12.5% SDS-PAGE; the fractionated proteins were electrotransferred onto a nitrocellulose membrane; the blots were incubated with HRP-conjugated anti-GFP antibody (Abcam, Cambridge, UK) and stained with Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan).

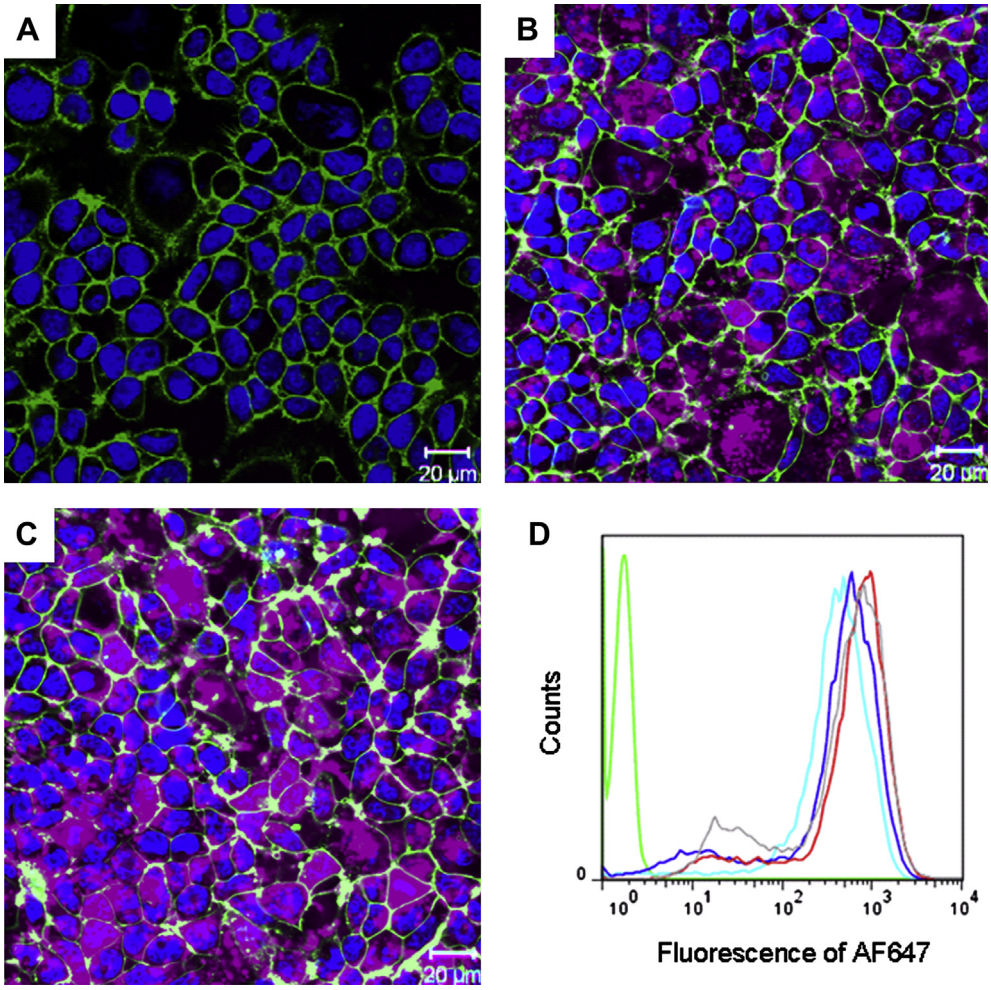

FIG. 2. AF647-SpSrtA transduction into HEK293T cells mediated by Bioporter protein transfection reagent. Fluorescent images of non-transduced cells (A) and cells transduced with the Bioporter reagent/SpSrtA complexes prepared with the different amounts of Bioporter reagent (volume: μl) and SpSrtA (concentration: μM), 10 μl/6.0 μM (B) or 15 μl/6.0 μM (C) were obtained by confocal laser scanning microscopy (CLSM). All cells were stained by 5 μg/mL Hoechst 33342 and 5 μM fluorescein-DOPE-PEG to visualize the cell nucleus and the cellular membranes. (D) The fluorescence distributions of cells treated with the Bioporter reagent/SpSrtA complexes, prepared with Bioporter reagent (μl) and SpSrtA (μM) of 6.25/2.5 (light blue line), 10/4.0 (dark blue line), 15/6.0 (red line), and 20/8.0 (gray line) were quantitatively analyzed by flow cytometry (FACS). Green line was that of non-transduced cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)