CHEMBIOCHEM

DOI: 10.1002/cbic.200700614

Site-Specific Protein Modification on Living Cells Catalyzed by Sortase

Tsutomu Tanaka,[a, b] Teruyasu Yamamoto,[a] Shinya Tsukiji,[a] and Teruyuki Nagamune*[a, c, d]

The use of enzymes is a promising approach for site-specific protein modification on living cells owing to their substrate specificity. Herein we describe a general strategy for the site-specific modification of cell surface proteins with synthetic molecules by using Sortase, a transpeptidase from Staphylococcus aureus. *The short peptide tag LPETGG is genetically introduced to the C terminus of the target protein, expressed on the cell surface. Subsequent addition of Sortase and an* N*-terminal triglycine-containing probe results in the site-specific labeling of the tagged protein. We were successful in the C-terminal-specific labeling of osteoclast differentiation factor (ODF) with a biotin- or fluorophore-containing short peptide on the living cell surface. The labeling reaction occurred efficiently in serum-containing medium, as well as serum-free medium or PBS. The labeled products were detected after incubation for 5 min. In addition, site-specific protein–protein conjugation was successfully demonstrated on a living cell surface by the Sortase-catalyzed reaction. This strategy provides a powerful tool for cell biology and cell surface engineering.*

Introduction

Proteins on the surfaces of cells are involved in many cellular processes such as growth, differentiation, immune response, cell–cell communication, and reversible adhesion to the extracellular matrix.[1] To elucidate the function of cell surface proteins, site-specific modification of proteins in living cells with synthetic molecules is a widely used technique, as it enables protein manipulation without loss of protein function. For instance, the incorporation of biophysical probes such as fluorophores and photoaffinity labels has been particularly useful for investigating protein structure, function, cellular localization, and protein–protein interaction networks. Although chemical approaches using maleimide–cysteine conjugation are suitable for protein engineering in vitro,[2] these methods cannot be applied to modify cellular proteins because they are "residue specific" rather than "site-specific". Consequently, considerable efforts have been directed toward the development of new methods that enable site-specific incorporation of synthetic probes into cellular proteins in living cells.[3–6] The previously reported tetracysteine tag[7,8] is a short peptide containing four neighboring cysteines that allows specific labeling with biarsenical derivatives in living cells; however, its specificity is relatively low, and in some cases reduction of the unreactive cysteine residues is required. Intein-based protein splicing is another promising method that was designed for site-specific and covalent modification of cellular proteins.[9,10] The shortcomings of this method are the large tag size of the intein (123 a.a. DnaE(N) intein) and a relatively slow labeling process. Thus, we believe that development and improvement of cellular protein engineering methods are still required for site-specific protein modification in living cells.

Enzymatic approaches for site-specific protein labeling in living cells have attracted much attention because the substrate specificity of enzymes enables "site-specific" protein modification.[5,6,11–18] For example, human O^6-alkylguanine transferase (hAGT) has been used for site-specific protein labeling by irreversibly transferring the alkyl group of O^6-benzylguanine derivatives to one of the cysteine residues of the target protein.[12,13]

Post-translational modification of acyl carrier protein (ACP) by phosphopantetheine transferase (PPTase) also allows site-specific modification of target proteins.[14–16] Although these methods were highly site-specific in living cells, the large tag size of hAGT (207 a.a.) and ACP (70 a.a.) may affect protein expression, function, and localization. It is desirable to use a shorter peptide tag to minimize these improper labeling effects and to monitor or control the function of cell surface proteins efficiently through neighboring labeled probes.

Walsh et al. recently reported a short peptide tag (12 a.a.) that allows site-specific labeling by PPTase.[17] In the case of biotin ligase, the tag size is also very short (15 a.a.); however,

[a] *Dr. T. Tanaka, T. Yamamoto, Dr. S. Tsukiji, Prof. T. Nagamune*
Department of Chemistry and Biotechnology
Graduate School of Engineering, The University of Tokyo
7-3-1, Hongo, Bunky-ku, Tokyo, 113-8656 (Japan)
Fax: (+81) 3-5841-7328
E-mail: nagamune@bioeng.t.u-tokyo.ac.jp

[b] *Dr. T. Tanaka*
Present address:
Organization of Advanced Science and Technology, Kobe University
1-1, Rokkodaicho, Nada-ku, Kobe, 657-8501 (Japan)

[c] *Prof. T. Nagamune*
Department of Bioengineering
Graduate School of Engineering, The University of Tokyo
7-3-1, Hongo, Bunky-ku, Tokyo, 113-8656 (Japan)

[d] *Prof. T. Nagamune*
Center for NanoBio Integration, The University of Tokyo
7-3-1, Hongo, Bunky-ku, Tokyo, 113-8656 (Japan)

this method requires a two-step reaction.[18,19] Because these labeling methods can only incorporate limited probes (that is, CoA- and biotin-derived molecules) into short peptide tags, the development of a new enzymatic labeling method, which allows the incorporation of various probe molecule types onto target proteins tagged with a short peptide, is still required.

We focused on Sortase A (SrtA), a transpeptidase from *Staphylococcus aureus*.[20] Sortase is present on the cell surface of Gram-positive bacteria and catalyzes the covalent linkage of surface proteins to cell wall peptidoglycan, a process important for bacterial infection into the host.[21,22] SrtA recognizes the LPXTG sequence, cleaves between the Thr and Gly residues, and subsequently links the carboxyl group of Thr to an amino group of N-terminal glycine oligomers by a native peptide bond. Recombinant soluble SrtA has been used for peptide–protein or protein–protein ligation in vitro[23,24] because of its greater substrate specificity.[25,26] However, just a few applications of SrtA for specific labeling of surface proteins on living mammalian cells have been reported.[27] Herein we demonstrated the Sortase-mediated site-specific modification of proteins on the surface of living cells. Recombinant osteoclast differentiation factor (ODF) appended with an extracellular C-terminal LPETGG sequence was employed as a model cell surface target protein.[28] Sortase-mediated labeling of the cell surface protein with biotin or synthetic fluorophore was performed. We also succeeded in protein–protein conjugation on the living cell surface. This methodology will provide powerful tools for cell surface engineering.

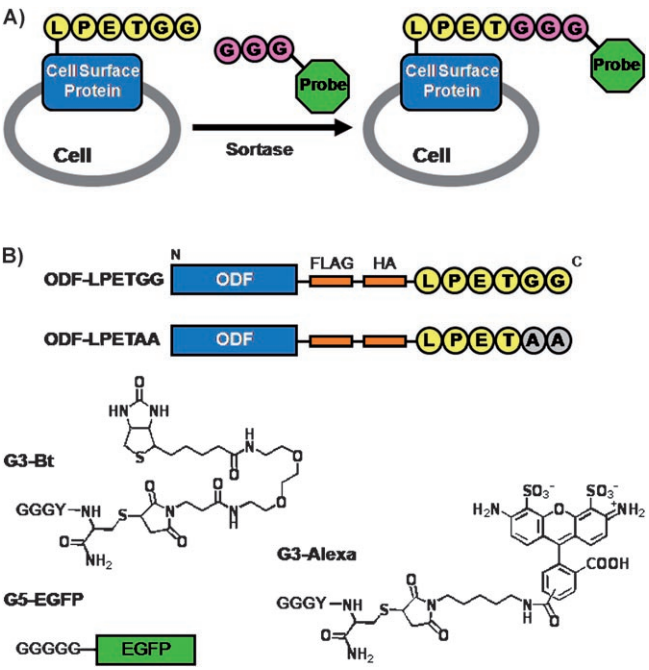

Figure 1. A) Sortase-mediated ligation of N-terminal triglycine derivatives to C-terminally LPETGG-tagged cell surface proteins. B) ODF constructs, G3-Bt, G3-Alexa, and G5-EGFP used in this study.

Results and Discussion

General strategy

The aim of this study was to establish transpeptidation-based cell surface protein engineering using SrtA. SrtA is one of the most well-studied transpeptidases, and its substrate specificity is significantly strict.[25,26] Therefore, it is suitable for site-specific labeling of proteins in cellular contexts. The principle of this method is illustrated in Figure 1 A. The tag with substrate sequence of SrtA, LPETGG, is genetically introduced to the C terminus of the target protein, which is expressed on the cell surface. Incubation of the cells with a short triglycine derivative and SrtA then allows site-specific labeling of the LPETGG-tagged cell surface protein. We designed short triglycine derivatives attached to a biotin (G3-Bt) or an Alexa Fluor 488 (G3-Alexa) as model probes (Figure 1 B). These probes were easily synthesized by standard solid-phase peptide synthesis.

Expression of LPETGG-tagged protein on the living cell surface

We selected ODF as our model cell surface protein. ODF, also known as TRANCE[29] and RANKL,[30] is a type II membrane protein that consists of an N-terminal intracellular region and a C-terminal extracellular region. We genetically fused the LPETGG sequence to the C terminus of ODF following FLAG and HA tags, used for the detection of protein expression (Figure 1 B). As a negative control, the LPETAA sequence was introduced to the C terminus of ODF. These constructs, designated as ODF-LPETGG and ODF-LPETAA, respectively, were efficiently expressed in HEK 293T cells for 24 h after transient transfection. To confirm cell surface expression of tagged ODF, we performed fluorescent immunostaining using FITC-labeled anti-HA and anti-FLAG tag antibodies, which were analyzed by confocal laser scanning microscopy (CLSM). HEK 293T cells were transiently co-transfected with plasmids encoding ODF-LPETGG (or ODF-LPETAA) and a monomeric red fluorescent protein with a nuclear localization sequence (mRFP-NLS) as a transfection marker. Figure 2 clearly shows that only transfected cells were labeled with FITC-

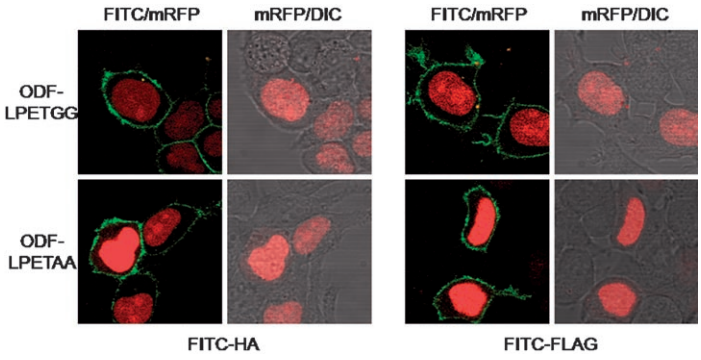

Figure 2. Immunofluorescence staining of HEK 293T cells transiently co-transfected with ODF-LPETGG (top) or ODF-LPETAA (bottom) and mRFP-NLS using FITC-HA (left) or FITC-FLAG (right).

labeled anti-tag antibodies, confirming that the additional LPETGG (or ODF-LPETAA) motif at the C terminus is exposed to the cell surface and is spatially accessible by large molecules such as anti-tag antibodies and SrtA. This accessibility is important for the subsequent SrtA-mediated labeling reaction.

Site-specific labeling of LPETGG-tagged proteins on the living cell surface

For the site-specific labeling of cell surface proteins, HEK 293T cells were transiently co-transfected with plasmids encoding ODF-LPETGG and mRFP-NLS. Cells were incubated with G3-Bt and SrtA with His-tag (His_6-SrtA) in serum-containing medium for 4 h at 37 °C, and were then washed to remove excess probe and enzyme. Cells were subsequently treated with Alexa Fluor 488-labeled streptavidin (SAv-488) and analyzed by CLSM. As shown in Figure 3 A, only cells transfected with ODF-LPETGG were efficiently labeled with the biotin tag. In contrast, no biotinylation was observed in the absence of His_6-SrtA or on cells expressing the alanine-containing negative control (ODF-LPETAA), indicating that the biotin labeling is enzyme-dependent and highly specific because of the excellent sequence specificity of SrtA. Western blotting analysis using a streptavidin–horseradish peroxidase conjugate (SAv-HRP) confirmed the covalent attachment of biotin to ODF-LPETGG (Figure 3 B). Nonspecific labeling of biotin to other cellular proteins was not detected. We also succeeded in direct labeling of the synthetic fluorophore, G3-Alexa, to ODF-LPETGG (Figure 3 C). These results suggest that our strategy may allow direct labeling of various kinds of chemical compounds to cell surface proteins with the LPETGG tag at the extracellular C terminus. In addition, this strategy was generally applicable to other cell lines including CHO and HeLa cells (data not shown). We subsequently tested the influence of the medium on the labeling reaction, and did not observe any clear effects (Figure 4). Notably, the Sortase-mediated transpeptidation reaction can be carried out in serum-containing or serum-free medium, or in PBS without any additional cofactors. This is in contrast to most other chemical labeling methods that require the reaction to be performed in buffer solutions or in the presence of appropriate cofactors.[12,16–19]

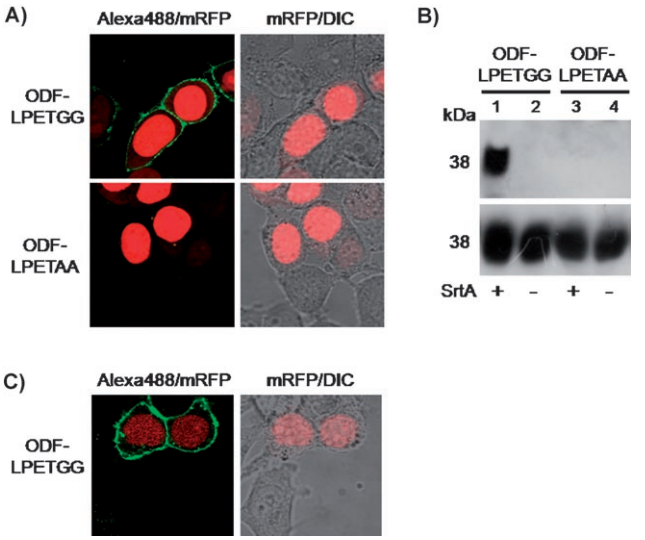

Figure 3. A) Labeling of ODF with biotin on cell surfaces. HEK 293T cells transiently co-expressing ODF-LPETGG (top) or ODF-LPETAA (negative control, bottom) and mRFP-NLS were labeled with G3-Bt at 37 °C for 4 h. Biotinylation was detected with SAv-488. Confocal images show overlays of Alexa488 and mRFP fluorescence (left) and mRFP fluorescence and DIC images (right). B) Western blotting analysis of biotinylation of ODF-LPETGG (38.3 kDa) (lane 1) using SAv-HRP (top) and anti-FLAG antibody (bottom). Negative controls are shown in the absence of His_6-SrtA (lane 2) or with ODF-LPETAA (lanes 3 and 4). C) Labeling of ODF with G3-Alexa on an ODF-LPETGG expressing cell.

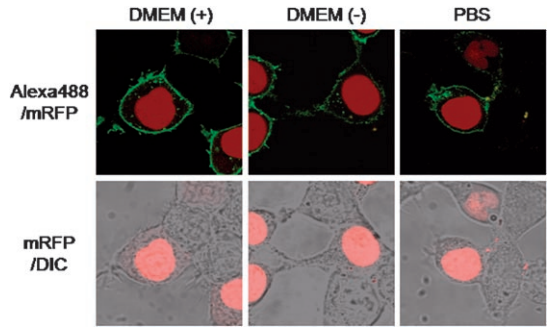

Figure 4. Influence of cell culture medium on the biotinylation reaction. The reaction was conducted in serum-containing DMEM (left), serum-free DMEM (center), or PBS (right) for 4 h.

Protein–protein conjugation by SrtA on the living cell surface

We next attempted to perform protein–protein conjugation on cell surfaces. An enhanced green fluorescent protein containing a pentaglycine motif at the N terminus[31] (G5-EGFP) was used as a model protein. HEK 293T cells co-expressing ODF-LPETGG and mRFP-NLS were incubated with G5-EGFP and His_6-SrtA for 4 h and then imaged by CLSM. Our results clearly show that EGFP was conjugated to the ODF on the cell surface (Figure 5). The formation of the covalent ODF–EGFP conjugate

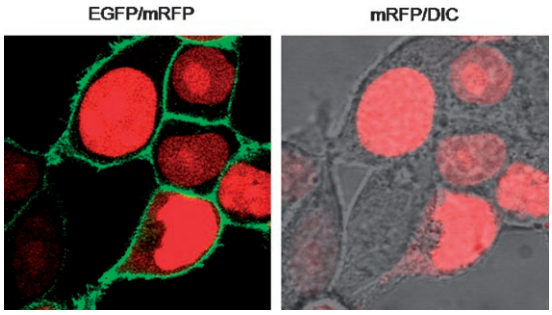

Figure 5. Protein–protein conjugation on cell surfaces. HEK 293T cells transiently co-expressing ODF-LPETGG and mRFP-NLS were incubated with 10 μM G5-EGFP and 30 μM His_6-SrtA at 37 °C for 4 h. Confocal images show overlays of EGFP and mRFP fluorescence (left) and mRFP fluorescence and DIC image (right).

was also confirmed by Western blotting (data not shown). Control experiments using ODF-LPETAA-transfected cells or in the absence of His_6-SrtA showed no EGFP conjugation (data not shown). To our knowledge, this is the first example of specific conjugation of a cell surface protein by an exogenously prepared protein.

In general, the expression of membrane proteins fused with GFP (or other proteins) is more difficult than the expression of GFP-fused cytosolic proteins. Our strategy will be applicable to the semisynthesis of membrane proteins at the surfaces of living cells.

Time course of the labeling reaction at the cell surface

Next, we evaluated the time course of the labeling reaction. HEK 293T cells were transfected as described above and incubated with G3-Bt or G5-EGFP. After incubation, cells were washed and imaged by CLSM. Figure 6A shows that the biotinylated ODF could be detected after only 5 min incubation, and thus, this technique will be useful for future applications such as pulse–chase labeling or receptor trafficking. Western blotting analysis also shows that the amount of biotinylated ODF increased with incubation time. In the case of G5-EGFP, despite the much larger size of G5-EGFP (~28 kDa) relative to G3-Bt (~1 kDa), the protein–protein conjugation was sufficiently fast and could be detected after 15 min incubation (Figure 6B). Although the hydrolysis reaction of labeled ODF by Sortase occurs competitively, the transpeptidation reaction is dominant in the presence of excess probe, because the k_{cat} value is 12-fold greater than that of hydrolysis.[26]

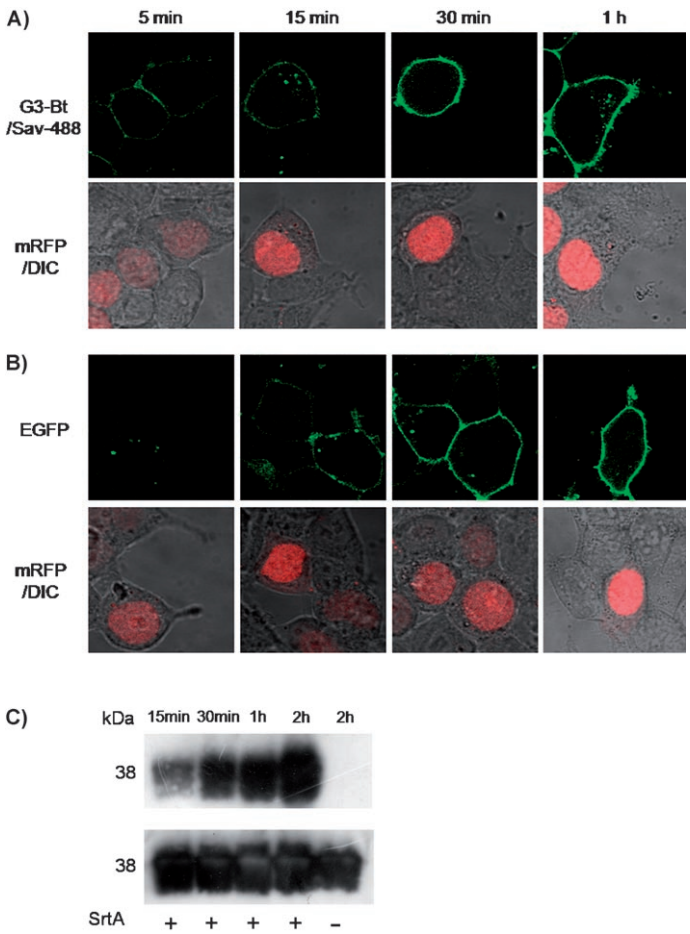

Figure 6. Time course of the transpeptidation reaction on the surface of HEK 293T cells expressing ODF-LPETGG. A) Labeling with G3-Bt. B) Conjugation with G5-EGFP. C) Western blotting analysis of biotinylation of ODF-LPETGG using SAv-HRP (top) and anti-FLAG antibody (bottom). Negative controls are shown in the absence of His_6-SrtA.

Cell viability under labeling reaction conditions

Because SrtA is originally a bacterial protein, the potential toxic effects of our procedure toward mammalian cells were assessed by MTT assay. Cells were incubated in DMEM containing SrtA and/or labeling reagents for 24 h. Figure 7 shows that no effect on cell viability was observed under all experimental conditions. These findings also support the applicability of this method for labeling specific proteins on the surfaces of living cells.

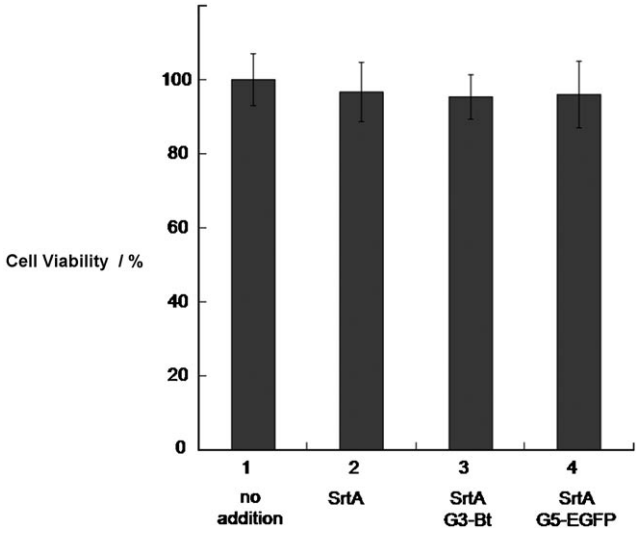

Figure 7. Cell viability evaluated by MTT assay after treatment under the following conditions: 1) no addition, 2) 30 μM His_6-SrtA, 3) 10 μM G3-Bt and 30 μM His_6-SrtA, and 4) 8 μM G5-EGFP and 30 μM His_6-SrtA.

Conclusions

We have developed a general method for site-specific labeling of cell surface proteins using Sortase. The efficient labeling of a cell surface protein with a small peptide or a large protein fragment was demonstrated. The Sortase-mediated transpeptidation reaction is covalent, highly specific, sufficiently rapid, and allows the ligation of N-terminal oligoglycine polypeptides to C-terminally LPETGG-tagged cell surface proteins. Also notable is that the tags reported herein are smaller than those previously reported.[9–18] In our work, efficient labeling under cell culture conditions and the lower Sortase and probe concentrations required (30 and 10 μM, respectively, that is, 85 and 98% lower) relative to those reported previously,[27] demonstrates

the great technical importance of Sortase-mediated labeling methods. In addition, it was shown for the first time that a whole protein can be site-specifically and very efficiently ligated to a cell surface protein, suggesting extension of this ligation method to protein semisynthesis at the cell surface, for example, in receptor reconstruction.[32,33]

Herein we employed SrtA from *Staphylococcus aureus*, the recognition sequence of which is LPXTG. Gram-positive bacteria have several kinds of Sortase with different substrate specificities.[22] It may be possible to perform multi-probe labeling of cell surface proteins by using Sortases with different recognition sequences. In addition, engineering of the substrate specificity of Sortase was recently reported.[34] This approach is also useful for multi-labeling of cell surface proteins.

Although this approach is restricted to modification of the C terminus of the target protein, it should be possible to introduce modifications at the N terminus by interchanging the reaction motifs. By combining these techniques with powerful in vitro chemical peptide and protein synthesis, a variety of peptides and proteins containing new functional groups could, in principle, be introduced to the cell surface and cell surface proteins. Further efforts will be continued to extend this strategy to labeling of intracellular proteins.

Experimental Section

Construction of expression plasmids: Pyrobest DNA polymerase (Takara) was used for PCR, and all PCR-amplified sequences were verified by DNA sequencing. The expression plasmid for His-tagged SrtA (His_6-SrtA) was constructed as follows: The gene encoding SrtA (corresponding to amino acids 60–206) was obtained by PCR from genomic DNA of *Staphylococcus aureus* (ATCC Number: 10832D) using the 5′ primer (5′-GGG GTA CCC AAG CTA AAC CTC AAA TTC CG-3′) and the 3′ primer (5′-GCG AGC TCT TAT TTG ACT TCT GTA GCT ACA AAG ATT TTA CG-3′), and was subcloned into the KpnI/SacI sites of pET-30b(+) (Novagen) to yield pET30b-SrtA. In *E. coli*, the plasmid expresses the protein construct M-[His_6]-SSG-[thrombin site(LVPRGS)]-GM-[S-tag(KETAAAKFERQHMDS)]-PDLGT-[SrtA(60–206)].

The expression plasmid for ODF containing FLAG, HA, and LPETGG sequences at its C terminus, ODF-LPETGG, was constructed as follows: The gene encoding ODF was obtained by PCR from ODF cDNA (kindly provided by Prof. Hisataka Yasuda) using the 5′ primer (5′-CCG CTC GAG GAT ATC ATG CGC CGG GCC AGC CGA GAC TAC GGC-3′) and the 3′ primer (5′-GGA CCG CGG TCA TGC GGC CGC CTT GTC GTC GTC ATC CTT GTA GTC AGC CAT GGC GTC TAT GTC CTG AAC TTT GAA AGC CCC-3′), and was subcloned into the EcoRV/SacII sites of pBluescript II SK(−) (Stratagene) to yield pBSK-ODF. The gene encoding ODF-LPETGG was then obtained by PCR from pBSK-ODF using the 5′ primer (5′-TCG ATA AGC TTG ATA TCC ACC ATG CGC CGG GCC AGC CGA GAC TAC GGC AAG TAC C-3′) and the 3′ primer (5′-GCC TCG AGT CAG CCA CCA GTT TCC GGC AGA GAG CCA CCA GCA TAA TCT GGA ACA TCA TAT GGA TAA GAT CCG CCC TTG TCG TCG TCA TCC TTG TAG TC-3′), and was subcloned into the HindIII/XhoI sites of pcDNA3.1(+) (Invitrogen) to yield pcDNA-ODF-LPETGG. In mammalian cells, the plasmid expresses the fusion protein [ODF]-AMA-[FLAG(DYKDDDDK)]-GGS-[HA(YPYDVPDYA)]-GGS-LPETGG.

The expression plasmid for ODF-LPETAA, pcDNA-ODF-LPETAA, was constructed by introducing mutations into the pcDNA-ODF-LPETGG with a QuikChange site-directed mutagenesis kit (Stratagene) using the 5′ primer (5′-GCT GGT GGC TCT CTG CCG GAA ACT **GCT GCC** TGA CTC GAG TCT AGA GGG CCC G-3′; mutated sites are in boldface) and the 3′ primer (5′-C GGG CCC TCT AGA CTC GAG TCA **GGC AGC** AGT TTC CGG CAG AGA GCC ACC AGC-3′). In mammalian cells, the plasmid expresses the fusion protein [ODF]-AMA-[FLAG(DYKDDDDK)]-GGS-[HA(YPYDVPDYA)]-GGS-LPETAA.

The expression plasmid for mRFP containing a nuclear localization sequence, mRFP-NLS, was constructed as follows: The gene encoding mRFP-NLS was obtained by PCR from pDsRed-Monomer-N1 (Clontech) using the 5′ primer (5′-GCG GTA CCA TGG ACA ACA CCG AGG ACG TC-3′) and the 3′ primer (5′-CGC GGC CGC TCT ATA CCT TTC TCT TCT TTT TTG GAT CTA CCT TTC TCT TCT TTT TTG GAT CTA CCT TTC TCT TCT TTT TTG GAT CCT GGG AGC CGG AGT G-3′), and was subcloned into the KpnI/NotI sites of pcDNA3.1(+) (Invitrogen) to yield pcDNA-mRFP-NLS. In mammalian cells, the plasmid expresses the fusion protein [mRFP]-$(DPKKKRKV)_3$.

Expression and purification of His_6-SrtA: The plasmid pET30b-SrtA was transfected into *E. coli* BL21(DE3). The cells were grown in LB medium to an OD (600 nm) value of 0.8, at which time expression of the protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM. After growth for an additional 16 h at 27 °C, the cells were harvested by centrifugation. The cell pellets were resuspended in 50 mM phosphate, 300 mM NaCl, pH 7.0 and lysed by sonication. His_6-SrtA was purified from the soluble fraction of the lysate by TALON metal affinity resins (Clontech) according to the manufacturer's protocol, and dialyzed against 50 mM Tris-HCl, 150 mM NaCl, pH 8.0. The concentration of purified His_6-SrtA was determined using a BCA protein assay kit (Pierce).

Synthesis of G3-Bt and G3-Alexa: The peptide H-Gly-Gly-Gly-Tyr-Cys-NH_2 was synthesized manually on a Rink amide resin by standard Fmoc-based solid-phase peptide synthesis protocol. Fmoc-Gly-OH, Fmoc-Tyr(*t*Bu)-OH, and Fmoc-Cys(Trt)-OH were used as building blocks. Fmoc deprotection was carried out with 20% piperidine in DMF, and coupling reactions were carried out with a mixture of Fmoc-amino acid, diisopropylcarbodiimide (DIC), and HOBt in DMF. Following chain assembly, global deprotection and cleavage from the resin was carried out with TFA containing 2.5% triisopropylsilane (TIS) and 2.5% H_2O. The crude peptide products were precipitated by Et_2O and purified by reversed-phase HPLC using a semipreparative YMC-Pack ODS-A C_{18} column with a linear gradient of 0.1% aqueous TFA and CH_3CN containing 0.1% TFA. The purified peptide was reacted with EZ-Link maleimide PEO_2-biotin (Pierce) (2 equiv) in 100 mM Tris-HCl, pH 8.0 for 3 h and purified by reversed-phase HPLC to yield G3-Bt. The peptide was identified by MALDI-TOF MS (Matrix; CHCA): calcd for $[M+H]^+ = 980.40$; obsd 980.21. G3-Alexa was synthesized similarly. The purified peptide was reacted with Alexa Fluor 488 C5-maleimide (Molecular Probes) (1.5 equiv) in 100 mM Tris-HCl, pH 8.0 for 1.5 h and purified by reversed-phase HPLC to yield G3-Alexa. The peptide was identified by MALDI-TOF MS (Matrix; CHCA): calcd for $[M+H]^+ = 1152.84$; obsd 1152.87.

Cell culture, plasmid transfection, and immunofluorescence staining: HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal bovine serum at 37 °C under 5% CO_2. Plasmid co-transfection was carried out using Lipofectamine and PLUS reagent (Invitrogen) according to the manufacturer's protocol. After expression for 24 h, the cells

were washed with PBS and incubated with 5 μg mL^{-1} FITC-labeled anti-HA antibody (FITC-HA; Bethyl Laboratories) or FITC-labeled anti-FLAG antibody (FITC-FLAG; Sigma) in PBS at room temperature for 25 min. After washing again, the cells were observed with a Leica TCS NT confocal laser microscope (Leica Microsystems). The fluorescence images were acquired using the 488-nm line of an argon laser for excitation and a 530-nm band-pass filter for emission, or the 568-nm line of a krypton laser for excitation and a 590-nm long-pass filter for emission. The specimens were viewed using a 100× oil immersion objective.

Transpeptidation reaction on cell surfaces: HEK 293T cells were co-transfected as described above and incubated for 24 h. The transpeptidation reaction was performed by incubating the cells with fresh medium containing 30 μM His_6-SrtA and 10 μM G3-Bt, G3-Alexa, or G5-EGFP for appropriate time intervals at 37 °C, and the cells were washed with PBS. In the case of G3-Bt, the cells were subsequently treated with 5 μg mL^{-1} Alexa Fluor 488-labeled streptavidin (Molecular Probes) at room temperature for 15 min in PBS and then washed again. The cells were observed with a confocal laser microscope as described above.

For Western blotting analysis, the transpeptidation reaction was carried out for 24 h. The cells were then washed and lysed in 10 mM HEPES, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, and 1 mM PMSF, pH 7.4. The protein samples were fractionated by 10% SDS-PAGE and electrotransferred onto a PVDF membrane. The blots were incubated with anti-FLAG antibody (Sigma) or streptavidin–horseradish peroxidase conjugate (Invitrogen) and stained using Chemi-Lumi One (Nacalai Tesque).

Cell viability assay: HEK 293T cells (5×10^3 cells per well) were precultured in a 96-well plate for 24 h. The medium was then replaced by DMEM containing the following; 1) no addition, 2) 30 μM His_6-SrtA, 3) 10 μM G3-Bt and 30 μM His_6-SrtA, or 4) 8 μM G5-EGFP and 30 μM His_6-SrtA, and cells were incubated for 4 h. After 24 h incubation at 37 °C, an MTT assay was performed using the Cell Counting Kit-8 (Dojindo) according to the manufacturer's protocol. In the case of condition 4), the cells were washed with DMEM before the assay to remove excess G5-EGFP.

Acknowledgements

We are grateful to Professor Hisataka Yasuda for kindly providing the plasmid encoding the cDNA of ODF. This work was supported by the Center for NanoBio Integration (Research and Development in a New Converting Field Based on Nanotechnology and Materials Science, MEXT).

Keywords: cell surface engineering · peptide tags · protein–protein conjugation · site-specific labeling · Sortase

[1] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, *Molecular Biology of The Cell*, 4th ed. Garland Science, New York, **2002**.
[2] G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, **1996**, pp. 3–23.
[3] G. Guignet, R. Hovius, H. Vogel, *Nat. Biotechnol.* **2004**, *22*, 440–444.
[4] J. A. Prescher, D. H. Dube, C. R. J. Bertozzi, *Nature* **2004**, *430*, 873–877.
[5] N. Johnsson, K. Johnsson, *ChemBioChem* **2003**, *4*, 803–810.
[6] N. Johnsson, N. George, K. Johnsson, *ChemBioChem* **2005**, *6*, 47–52.
[7] B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **1998**, *281*, 269–272.
[8] C. Hoffmann, G. Gaietta, M. Bünemann, S. R. Adams, S. Oberdorff-Maass, B. Behr, J. P. Vilardaga, R. Y. Tsien, M. H. Ellisman, M. J. Lohse, *Nat. Methods* **2005**, *2*, 171–176.
[9] I. Giriat, T. W. Muir, *J. Am. Chem. Soc.* **2003**, *125*, 7180–7181.
[10] J. P. Pellois, T. W. Muir, *Angew. Chem.* **2005**, *117*, 5859–5863; *Angew. Chem. Int. Ed.* **2005**, *44*, 5713–5717.
[11] C. W. Lin, A. Y. Ting, *J. Am. Chem. Soc.* **2006**, *128*, 4542–4543.
[12] A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, K. Johnsson, *Nat. Biotechnol.* **2003**, *21*, 86–89.
[13] A. Keppler, H. Pick, C. Arrivoli, H. Vogel, K. Johnsson, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9955–9959.
[14] L. Vivero-Pol, N. George, H. Krumm, K. Johnsson, N. Johnsson, *J. Am. Chem. Soc.* **2005**, *127*, 12770–12771.
[15] J. Yin, F. Liu, X. Li, C. T. Walsh, *J. Am. Chem. Soc.* **2004**, *126*, 7754–7755.
[16] J. Yin, A. J. Lin, P. D. Buckett, M. Wessling-Resnick, D. E. Golan, C. T. Walsh, *Chem. Biol.* **2005**, *12*, 999–1006.
[17] Z. Zhou, P. Cironi, A. J. Lin, Y. Xu, S. Hrvatin, D. E. Golan, P. A. Silver, C. T. Walsh, J. Yin J, *ACS Chem. Biol.* **2007**, *2*, 337–346.
[18] I. Chen, M. Howarth, W. Lin, A. Y. Ting, *Nat. Methods* **2005**, *2*, 99–104.
[19] M. Howarth, K. Takao, Y. Hayashi, A. Y. Ting, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7583–7588.
[20] S. K. Mazmanian, G. Liu, H. Ton-That, O. Schneewind, *Science* **1999**, *285*, 760–763.
[21] R. P. Novick, *Trends Microbiol.* **2000**, *8*, 148–151.
[22] G. K. Paterson, T. J. Mitchell, *Trends Microbiol.* **2004**, *12*, 89–95.
[23] H. Mao, S. A. Hart, A. Schink, B. A. Pollok, *J. Am. Chem. Soc.* **2004**, *126*, 2670–2671.
[24] R. Parthasarathy, S. Subramanian, E. T. Bode, *Bioconjugate Chem.* **2007**, *18*, 469–476.
[25] R. G. Kruger, B. Otvos, B. A. Frankel, M. Bentley, P. Dostal, D. G. McCafferty, *Biochemistry* **2004**, *43*, 1541–1551.
[26] X. Huang, A. Aulabaugh, W. Ding, B. Kapoor, L. Alksne, K. Tabei, G. Ellestad, *Biochemistry* **2003**, *42*, 11307–11315.
[27] M. W. Popp, J. M. Antos, G. M. Grotenberg, E. Spooner, H. L. Ploegh, *Nat. Chem. Biol.* **2007**, *3*, 707–708.
[28] H. Yasuda, N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, E. Tsuda, T. Morinaga, K. Higashipo, N. Udagawa, N. Takahashi, T. Suda, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 3597–3602.
[29] B. R. Wong, J. Rho, J. Arron, E. Robinson, J. Orlinick, M. Chao, S. Kalachikov, E. Cayani, F. S. Bartlett, W. N. Frankel, S. Y. Lee, Y. Choi, *J. Biol. Chem.* **1997**, *272*, 25190–25194.
[30] D. A. Anderson, E. Maraskovsky, W. L. Billingsley, W. C. Dougall, M. E. Tometsko, E. R. Roux, M. C. Teepe, R. F. DuBose, D. Cosman, L. Galibert, *Nature* **1997**, *390*, 175–179.
[31] T. Tanaka, N. Kamiya, T. Nagamune, *FEBS. Lett.* **2005**, *579*, 2092–2096.
[32] F. I. Valiyaveetil, R. MacKinnon, T. W. Muir, *J. Am. Chem. Soc.* **2002**, *124*, 9113–9120.
[33] F. I. Valiyaveetil, M. Sekedat, T. W. Muir, R. MacKinnon, *Angew. Chem.* **2004**, *116*, 2558–2561; *Angew. Chem. Int. Ed.* **2004**, *43*, 2504–2507.
[34] M. L. Bentley, H. Gaweska, J. M. Kielec, D. G. McCafferty, *J. Biol. Chem.* **2007**, *282*, 6571–6581.

Received: October 14, 2007
Revised: December 2, 2007
Published online on February 22, 2008

