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In Vivo Imaging of a Bacterial Cell Division Protein Using a Protease-Assisted Small-Molecule Labeling Approach

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Chemical labeling strategies developed to site specifically label proteins in their native cellular milieu have been sought as attractive approaches to obviate some of the drawbacks associated with the use of genetically encoded fusions to fluorescent proteins (FPs).[1] Though elegant, many of these approaches necessitate the use of large protein domains/tags fused to target protein(s).[2] Like FPs, these protein "tags" can potentially perturb the folding and/or activity of target proteins. Small peptide-recognition sequences are more desirable, but they have lower labeling efficiencies.[3,4] Other methods such as incorporation of unnatural amino acids and metabolic installation of reporter tags are primarily governed by the ability of the enzyme(s) to tolerate the unnatural motif to be introduced.[5,6] Nevertheless, metabolic-labeling approaches have been successfully used to study glycoproteins and conjugates, which were previously inaccessible by other labeling techniques.[6] Thus, the need still exists to develop new methods that can facilitate the routine use of small-molecule probes for the in vivo study of protein dynamics.

Previously we reported an intein-mediated small-molecule approach for site-specific labeling of N-terminal cysteine-containing proteins.[7] This method, however, had some significant drawbacks, including the relatively large size of intein tag used, as well as slow and uncontrolled self-splicing of the tag, which inevitably led to a longer labeling time. In this communication, we have sought to develop a more efficient labeling strategy and have capitalized on the highly specific tobacco etch virus (TEV) NIa protease. TEV is a 3C-type cysteine protease that recognizes a seven amino acid sequence, E-X-X-Y-X-Q↓S/G (where X is any amino acid; and ↓ indicates the cleavage site). It has stringent substrate sequence requirement, with absolutely conserved residues at P_6, P_3, and P_1 positions, and as such, has been widely used for removal of affinity tags from proteins both in vitro and in vivo.[8,9] It was previously shown that mutation of the P_1' residue from S/G to cysteine does not significantly alter the efficiency of TEV cleavage.[10,11] In our current strategy (Figure 1 A), we used TEV for the rapid and controlled intracellular generation of N-terminal cysteine-containing proteins, which were subsequently labeled in a site-specific

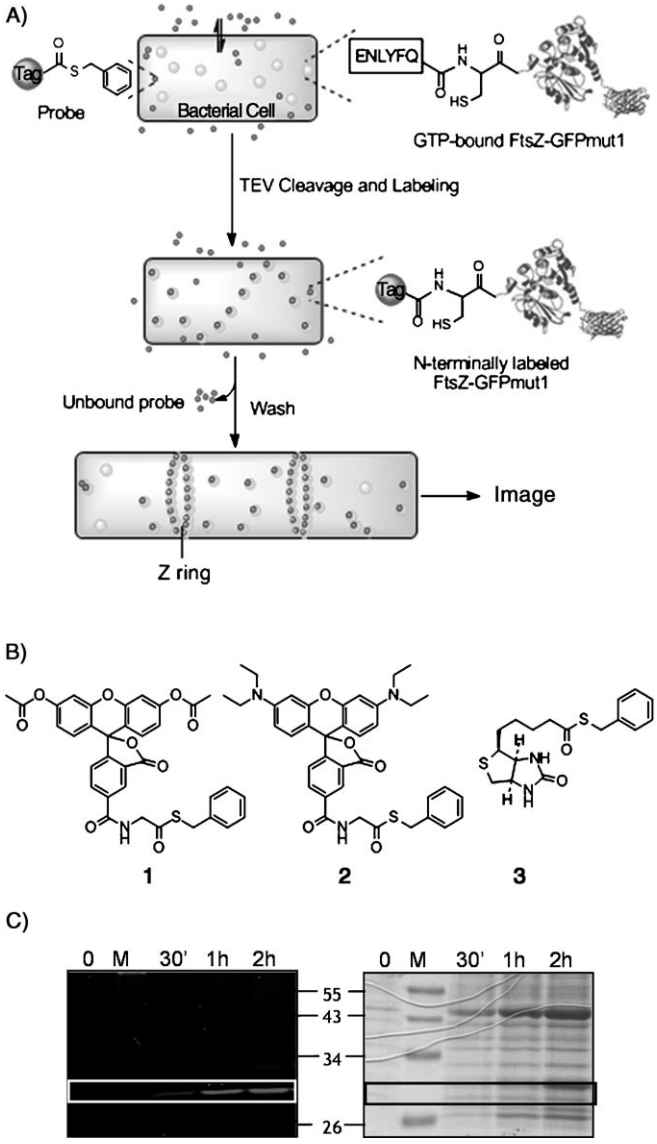

Figure 1. A) Scheme showing the TEV-assisted, small-molecule labeling approach used to study the in vivo dynamics of N-terminal cysteine-containing FtsZ–GFPmut1 (FtsZ PDB ID code: 1W5B). Following TEV cleavage and labeling, excess probe is washed away and cells are imaged. FtsZ–GFPmut1 initially occurs in spiral patterns that eventually assemble into the Z ring. B) Structures of the probes used. C) Fluorescence in-gel scanning (left) and Coomasie blue staining (right) of N-terminal cysteine-containing mouse DHFR (boxed), following in vivo cleavage and labeling. The labeled protein band was seen within 30 min of simultaneous cleavage and labeling with 25 μM of probe **2**; M: M_w marker; the sizes of the bands are given between the gels in kDa.

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and covalent manner with a set of cell-permeable, thioester-derived small-molecule probes (Figure 1B). For expression of TEV and its substrate protein in the same host cell, we made use of a pair of compatible plasmids that can be regulated independently with different chemical inducers (IPTG and arabinose). Thus far, we have demonstrated the successful application of this approach for in vivo imaging of FtsZ (an important cell-division protein) in live bacterial cells (Figure 1A).

Our first goal was to demonstrate in vitro and in vivo labeling of model proteins with an N-terminal cysteine residue by using thioester probes. The product derived from the fusion of maltose-binding protein (MBP) to mouse dihydrofolate reductase (mDHFR) was used as model protein. The P1′ cysteine residue was introduced in the TEV recognition/cleavage sequence within the linker region between the fusion partners. Fluorescein, 5′-carboxytetramethylrhodamine (TAMRA), and biotin were conjugated to benzyl thioester to give probes **1**, **2**, and **3**, respectively (Figure 1B). The conjugation did not alter the photophysical properties of the probes.[7] By monitoring the time-dependent in vitro labeling of N-terminal cysteine-containing DHFR with **1**, we observed selective labeling of DHFR within 15 min even though only $<30\%$ of the fusion was cleaved by TEV (see Figure S5 in the Supporting Information). No detectable labeling of TEV, the fusion, or MBP was observed even after 2 h (Figure S5A). In bacteria, however, TEV was found to be quite efficient, and cleaved 90–95% of the MBP–DHFR fusion at any given time (Figure S6). To label the cleaved DHFR, probe **2** (25 μM) was directly added to the culture media during cell growth. The doubling time (t_d) remained unaffected; this shows that the probe was not cytotoxic (data not shown). A labeled protein band that corresponded to DHFR was seen within 30 min of simultaneous expression and labeling (Figure 1C). Significantly, bands other than that of DHFR were not detected even after 2 h incubation; this demonstrates the feasibility of this approach for in vivo applications.

To demonstrate the utility of our TEV-assisted, small-molecule labeling approach for visualization of dynamic processes, such as cell division, we studied the localization of FtsZ—a major cytoskeletal component of the bacterial cell-division machinery.[12] FtsZ is thought to be the first protein to localize to the site of cell division, and assembles into a ring (the Z ring) that eventually contracts at septation. Functionally, it is a bacterial homologue of tubulin with a similar tertiary structure and GTPase activity. FtsZ–GFP fusions have been widely used to study FtsZ localization and Z-ring formation.[13,14] For FtsZ to be amenable for our labeling approach, we expressed the TEVseq–FtsZ–GFPmut1 fusion, which introduces the TEV recognition/cleavage sequence to the N terminus of FtsZ–GFPmut1. The green fluorescent protein (GFP) was strategically retained for further characterization of our labeling approach by colocalization and FRET-based experiments (vide infra). To ascertain that addition of the TEV tag did not interfere with FtsZ localization, individual bacterial cells were imaged (Figure 2). Analysis by using differential interference contrast (DIC) microscopy showed that the cells were filamentous (Figure 2A); this indicates that the induced level of FtsZ inhibited cell division.

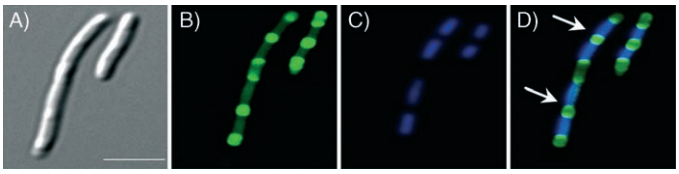

Figure 2. Localization of TEVseq–FtsZ–GFPmut1 protein to the internucleoid regions (arrows indicate the Z ring) in individual cells stained with Hoechst 33342 (5 μg mL^{-1}). A) DIC image; B) GFP channel; C) DAPI channel; D) overlay of B) and C); scale bar: 5 μm.

Under epifluorescence illumination, GFP fluorescence within the cells appeared as a series of regularly spaced bands; this is consistent with the predominant localization of TEVseq–FtsZ–GFPmut1 to internucleoid regions (Figure 2B–D). These bands might represent multiple FtsZ rings that assembled at potential division sites by maintaining their proper association with spatial markers in the cell wall, but were unable to continue septation, possibly due to interference by the GFP tag.[14]

Next, bacterial cells that simultaneously expressed TEVseq–FtsZ–GFPmut1 and TEV were labeled with **2** and imaged (Figure 3). Fluorescence signals seen in the TAMRA channel

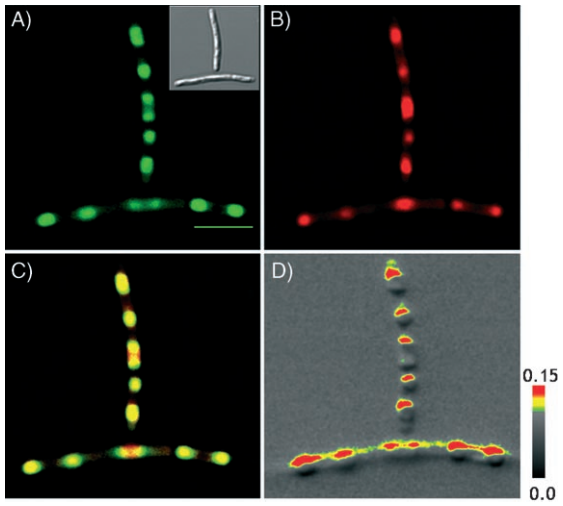

Figure 3. Fluorescence labeling of N-terminal cysteine-containing FtsZ–GFPmut1 protein in live bacteria. Fluorescence micrographs obtained from: A) GFP channel (inset: DIC image of the same field); B) TAMRA channel; C) overlay of GFP and TAMRA channels; D) ratiometric imaging of pre- and postbleach images in the donor (GFP channel) to demonstrate change in donor fluorescence following photobleaching of the acceptor. Maximal FRET changes were localized to the internucleoid regions; this is consistent with FtsZ localization; scale bar: 5 μm. The color scale to the right indicates relative FRET signals.

consistently colocalized with those of GFP, and covalent labeling was demonstrated by FRET analysis following acceptor (i.e., TAMRA) photobleaching. Ratiometric analysis of the pre- and postbleach images in the donor channel showed maximal signal enhancement in the internucleoid regions; this is consistent with FtsZ localization (Figure 3D). Though some background labeling was seen at the cell poles in control cells that expressed FtsZ–GFPmut1 (without the TEV sequence) FRET

measurements in these regions showed no changes in intensity (Supporting Information). Taken together, the data show the specific and covalent labeling of FtsZ. The ability to tag and detect FtsZ with small molecules presents an exciting paradigm for bacterial cell-division studies in whole-cell assays. This is of particular significance as Margolin and co-workers have previously reported that the key problem associated with the use of FtsZ–GFP fusion is the obligatory addition of the macromolecular GFP tag that in all likelihood interferes with FtsZ function (i.e., polymer formation).[14] Our approach, thus seeks to address this issue by replacement of the GFP tag with a small-molecule fluorophore ($M_w < 600$ Da). Work is in progress to study the dynamics of TEVseq–FtsZ (without GFP tag) following labeling with our probes to address this caveat of using GFP fusions.

In summary, we have demonstrated a method for specific in vivo labeling of target proteins that seeks to complement existing chemical-labeling approaches. Our approach is essentially "tag-free" and unlikely to perturb protein function as only a lone cysteine residue is added to the target protein following cleavage by TEV. In addition, since cleavage and labeling occur simultaneously, the oxidation of the cysteine residue is minimized; this leads to a shorter labeling time and improved labeling efficacies. A potential drawback of our approach is that TEV might become labeled in the presence of high concentrations of the protein and/or probe. This, however, does not limit the applicability of the approach as the use of two different inducers makes it possible to fine-tune the expression levels of TEV so as to minimize unspecific labeling (see theSupporting Information for further discussion). Furthermore, the high catalytic efficiency of TEV-mediated cleavage makes the requirement for high-expression levels unnecessary. Since TEV protease can be readily expressed in mammalian cells,[15] we are currently extending the use of this labeling strategy to study both cell surface and intracellular proteins in mammalian cells.

Experimental Section

Bacterial culture and labeling experiments: To monitor TEV-mediated cleavage of MBP fusions in vivo, we used MBP–mDHFR as the model protein. TEV was cloned into pACYCDuet-1 (Novagen) while mouse DHFR (mDHFR) was cloned into the compatible expression plasmid, pMAL-c2X plasmid (NEB). The modified TEV recognition and cleavage sequence (with cysteine as the P1′ residue) was appended in the linker region between MBP and mDHFR (see the Supporting Information for details). Bacterial cells transformed with pMal-C2X–MBP–mDHFR and pACYC–TEV, were grown in Luria–Bertani medium supplemented with ampicillin (100 $\mu g\,mL^{-1}$) and chloramphenicol (34 $\mu g\,mL^{-1}$). At OD_{600} ~0.4, expression of the fusion protein and TEV was induced simultaneously by the addition of IPTG (0.4 mM). For simultaneous cleavage and labeling, probe **2** (25 μM) was also added to the culture during induction. Aliquots of samples were collected at the indicated time point, centrifuged, washed with 1xPBS to remove unbound probe, and analyzed by gel (12%) electrophoresis after cell lysis in SDS sample buffer. Gels were analyzed with in-gel fluorescence scanning by using a Typhoon 9200 scanner (Amersham Biosciences; excitation at 532 nm; emission filter 580BP30, 600 PMT).

Bacterial cell imaging: FtsZ–GFPmut1 fusion was cloned into the arabinose inducible pBAD/Myc-HisB vector (Invitrogen). Overnight cultures that harbored pACYC-Duet-1–TEV and pBAD/Myc-His–FtsZ–GFpmut1 were diluted in M9 media (with 0.4% glycerol), and at OD_{600} ~0.4 protein expression was induced by addition of arabinose (0.2%, *w/v*; for FtsZ) and IPTG (10 μM; for TEV) together with TAMRA-thioester probe **2** (10 μM). Protein expression and labeling were allowed to proceed for 5 h, which corresponds to only four cell-division cycles (t_d ~80 min). At the end of this period, cells from the culture were centrifuged, washed three times with 1xPBS, and mounted on poly-L-lysine coated glass slides. Images were captured by using Olympus IX71 inverted microscope, equipped with 100X oil objective (NA 1.4, WD 0.13 mm) and CoolSNAP HQ CCD camera (Roper Scientific, Tucson, AZ, USA). The filter sets used for the different fluorophores were as follows: GFPmut1: Ex 460–480HQ, dichroic DM485, Em 495–540HQ; TAMRA: Ex BP535–555HQ, dichroic DM565, Em 570–625HQ. All images were processed by using the MetaMorph software (Version 7.1.2; Molecular Devices, PA, USA). Validation of site-specific labeling was performed with FRET measurements by using the acceptor photobleaching method.

For nucleoid staining, Hoechst 33342 dye (Molecular Probes, Oregon, USA) was added directly to the culture at a final concentration of 5 $\mu g\,mL^{-1}$, and incubated for 30 min. At the end of this period, cells were harvested, washed, and imaged by using a standard DAPI filter set (Ex BP360–370, dichroic DM400, Em BA420).

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