## Construction of a small-molecule-integrated semisynthetic split intein for *in vivo* protein ligation<sup>†</sup>

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A new split intein-based protein ligation tool that is synthetically accessible and can be used for protein semisynthesis on the cell surface and potentially inside cells has been constructed.

The ability to incorporate site-specifically a range of chemical probes such as fluorescent molecules, photocrosslinkers, unnatural amino acids, and post-translational modifications into cellular proteins will provide invaluable tools for the study of living systems. Consequently, the development of strategies that enable the specific modification of proteins in cellular contexts is an increasingly active area of chemical biology. Although several chemical labeling methods have been described to date, most of them allow only limited types of modification at the side chain of specific residue(s) within remote fusion protein-/peptide-tags.<sup>1</sup> A protein semisynthesis approach<sup>2</sup> is a powerful alternative strategy. The semisynthesis of proteins is based on the chemoselective ligation of a chemically modified peptide to a recombinant protein, thus enabling the precise incorporation of an unlimited variety of chemical probes into the target protein.<sup>2</sup> Despite such significant potential, the utility of this approach is currently limited to in vitro conditions due to the lack of protein ligation techniques which are applicable in vivo.<sup>3,4</sup> Here we report a new split intein-based protein ligation method that can be used on the cell surface and potentially inside cells.

Inteins are attractive scaffolds for the creation of *in vivo* protein ligation tools, because they can split into two halves to mediate protein splicing *in trans.*<sup>5</sup> The *trans*-splicing process allows N- and C-terminal extein polypeptides, either synthetic or recombinant in origin, to be joined together by a native peptide bond with concomitant removal of the intein complex.<sup>5</sup> In pioneering work, Giriat and Muir demonstrated the feasibility of *in vivo* protein semisynthesis by using the naturally split *Synechocystis* sp. (*Ssp*) DnaE intein.<sup>4</sup> However, the general applicability of the *Ssp* DnaE intein as well as other existing split inteins would be severely limited by their moderate-to-large sizes (*ca.* 36–180 amino acids), which make chemical synthesis of intein fragments extremely difficult or impractical. In addition, for many *trans*-splicing inteins, the intrinsic low affinity between the complementary fragments is a

limitation.<sup>5</sup> We focused on the rational design of a new split intein that overcomes these drawbacks. The Ssp DnaB intein was chosen as our starting point, because it was recently found that the DnaB intein split at residue 11, generating I<sub>N</sub> and I<sub>C</sub>, is capable of transsplicing in bacteria<sup>6</sup> and also in vitro.<sup>7</sup> The I<sub>N</sub> fragment contains only 11 amino acids, so is suitable for preparation and further modification by conventional solid-phase peptide synthesis (SPPS). However, the in vitro study reported a reactant concentration, required for trans-splicing of the pair of these I<sub>N</sub> and I<sub>C</sub> fragments, of over 10  $\mu$ M,<sup>7</sup> which will restrict its broad use for cellular applications. Consistent with this, we observed no trans-splicing of the I<sub>N</sub>-I<sub>C</sub> pair under submicromolar concentrations (vide infra). Therefore, inspired by previous two-<sup>8</sup> and three-hybrid strategies,<sup>9</sup> we aimed to confer high affinity to the complementary  $I_N-I_C$  pair by integrating an auxiliary receptor-ligand interaction as outlined in Scheme 1. Covalently fusing a small-molecule ligand and its cognate receptor protein to the C-terminus of I<sub>N</sub> and the N-terminus of I<sub>C</sub>, respectively, should generate a semisynthetic peptide-protein trans-splicing system in which the short synthetic  $I_N$  fragment can heterodimerize with the recombinant  $I_C$  efficiently to reconstitute the splicing-active complex.

To test our design strategy, a series of model  $I_N$  peptides and  $I_C$  proteins were prepared, in which the chemical probe, the N-terminal extein, is a biotin tag, and the target protein, the C-terminal extein, is the monomeric red fluorescent protein (mRFP) (Fig. 1(A)). We used *E. coli* dihydrofolate reductase (*e*DHFR) and trimethoprim (TMP) as the receptor–ligand pair, because TMP has nanomolar affinity for *e*DHFR and can be easily derivatized without substantial loss of its binding affinity.<sup>10</sup> We synthesized a 4'-carboxy-substituted TMP (Scheme S1, ESI†) and attached it to the C-terminus of  $I_N$  *via* a linker consisting a tetramer of PEG<sub>4</sub>-based amino acid.<sup>11</sup> The peptides were readily



Scheme 1 Schematic representation of the small-molecule-integrated semisynthetic split intein.

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Fig. 1 In vitro trans-splicing assays. (A) The I<sub>N</sub> peptides and I<sub>C</sub> proteins used in this study. The LRESG and SIEQD sequences in I<sub>N</sub> and I<sub>C</sub> fragments are native exteins of *Ssp* DnaB intein. TMP is shown in pink. (B) Protein products of the *trans*-splicing between I<sub>N</sub>-2 and I<sub>C</sub>-3. (C) Western blotting analysis of P-6 formation using SAv-HRP. The reactions were performed between pairs of 0.5  $\mu$ M of I<sub>N</sub> peptide and I<sub>C</sub> protein at 25 °C for 20 h.

synthesized by standard SPPS and purified by reversed-phase HPLC. Each of the protein constructs were bacterially expressed and purified from the soluble fraction by affinity chromatography.

Initial trans-splicing assays were performed by incubating each of the  $I_N$  and the  $I_C$  at an equimolar concentration of 0.5  $\mu$ M and analyzed by Western blotting using streptavidin-horseradish peroxidase conjugate (SAv-HRP). While the incubation of I<sub>N</sub>-1 and I<sub>C</sub>-3 for 20 h resulted in no splicing (Fig. 1(C), lane 4), the formation of splicing product P-6 was clearly observed in the reaction of  $I_N$ -2 and  $I_C$ -3 (Fig. 1(C), lane 3). The product band showed cross-reactivity to the anti-His-tag antibody as expected (data not shown). The formation of splicing product P-6, excised intein fragment P-7, and C-terminal cleavage product P-8 was further confirmed by MALDI-TOF-MS analysis of the reaction mixture.<sup>12</sup> In contrast, no splicing was observed between I<sub>N</sub>-2 and active site-mutated (S155A) I<sub>C</sub>-4 (Fig. 1(C), lane 6), which is consistent with product formation occurring via protein transsplicing. The total yield of P-6 was estimated to be *ca*. 50%.<sup>13</sup> This value lies in the range reported for other split inteins.<sup>7,9</sup> In addition, kinetic experiments revealed that the trans-splicing reaction follows first-order kinetics with a  $k_{\rm obs}$  of 1.1  $\times$  10<sup>-4</sup> s<sup>-1</sup> (Fig. S1, ESI<sup>†</sup>).<sup>14</sup> It should be noted that a significant amount of splicing product P-6 could be detected even within 1 h (Fig. S1, ESI†).

To further confirm that the interaction between TMP ligand and *e*DHFR plays a critical role in this peptide–protein *trans*splicing system, the reactions were carried out using  $I_N$ -2 and  $I_C$ -3 in the presence of a 20-fold molar excess of TMP, and using  $I_N$ -2 and  $I_C$ -5 lacking the *e*DHFR portion. In neither case was the product formed (Fig. S2, ESI<sup>+</sup> and Fig. 1(C), lane 5). These results provide clear evidence that the specific *e*DHFR–TMP interaction facilitates the *trans*-splicing reaction.



Fig. 2 Effects of linker length of TMP-integrated  $I_N$  peptide on *trans*splicing efficiency. The reactions were performed between pairs of 0.5  $\mu$ M of  $I_N$  peptide and  $I_C$ -3 at 25 °C for 20 h and analyzed by Western blotting using SAv-HRP.

We also investigated the effect of linker length of TMPintegrated  $I_N$  peptide on *trans*-splicing efficiency and found the tetramer of PEG<sub>4</sub>-amino acid to be optimal among peptides we tested (Fig. 2). It was clearly indicated that a linker of appropriate length is essential for the reconstitution of splicing-active intein complex in this system. Importantly, at a reaction concentration of 5 µM, the formation of P-6 could be detected using  $I_N$ -1 and  $I_C$ -3, whereas the pair of  $I_N$ -2 and  $I_C$ -3 still showed a three-fold higher *trans*-splicing efficiency by virtue of the auxiliary interaction (Fig. S3, ESI†).

To demonstrate the *in vivo* applicability of this ligation tool, we next performed the semisynthesis of a protein on the surface of mammalian cells. We chose cell membrane-bound mRFP as the model protein (Fig. 3(A)). Chinese hamster ovary (CHO) cells



Fig. 3 On-cell trans-splicing. (A) The I<sub>C</sub> constructs used in this study and the splicing product. IgS is the Ig $\kappa$  signal sequence and TMD is the transmembrane domain of the platelet-derived growth factor receptor. (B) Biotinylation of cell surface-displayed mRFP. CHO cells expressing I<sub>C</sub>-9 were treated with 0.5  $\mu$ M of either I<sub>N</sub>-1 (top) or I<sub>N</sub>-2 (bottom) at 25 °C for 8 h. The biotinylation was detected with SAv-488. Confocal images show Alexa Fluor 488 fluorescence (left) and overlays of mRFP fluorescence and DIC images (right). (C) Western blotting analysis of the biotinylation of mRFP-TMD (lane 1) using SAv-HRP. Negative controls are shown with I<sub>N</sub>-1 (lane 2) or with cells expressing I<sub>C</sub>-10 (lanes 3 and 4).

were transiently transfected with plasmid encoding I<sub>C</sub>-9, and cell surface expression of the intein-mRFP fusion was confirmed by immunofluorescence staining using FITC-labeled anti-HA and anti-Myc antibodies (data not shown).<sup>15</sup> Cells were incubated with  $I_{N}$ -2 for 8 h and washed to remove excess peptide. Cells were then stained with Alexa Fluor 488-labeled streptavidin (SAv-488) and observed by confocal laser scanning microscopy. The images showed that only surfaces of transfected cells were specifically labeled by biotin (Fig. 3(B)). Cells treated with SAv-488 alone showed no fluorescence staining (data not shown). The formation of biotinylated P-11 was unambiguously confirmed by Western blotting using SAv-HRP (Fig. 3(C), lane 1). No labeling was observed after incubation with  $I_N$ -1 or on cells expressing  $I_C$ -10 that lacks the intein domain (Fig. 3(C), lanes 2-4). These results are in accordance with the in vitro experiments described above. To our knowledge, this work represents the first demonstration of a semisynthesis of cell surface protein on living cells.

In conclusion, we have designed and constructed a new split intein tool for *in vivo* protein ligation. The small-molecule-ligandintegrating strategy allowed the creation of semisynthetic split intein in which the  $I_N$  fragment is short, hence synthetically accessible, but is capable of *trans*-splicing efficiently through the intein fragment complementation assisted by the auxiliary ligand– receptor interaction. Given the high orthogonality of *e*DHFR– TMP interaction in mammalian cells,<sup>10</sup> this system should be applicable inside living cells by combining it with intracellular peptide delivery methods.<sup>4,16</sup> Because inteins are promiscuous with respect to the extein sequences, the present protein ligation tool would find general use for semisynthesis of proteins containing a variety of chemical probes in cellular contexts.

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- 11 We designed the linker length by predicting the tertiary structure of the fusion complex of  $I_N$  and *eDHFR-I<sub>C</sub>* using the crystal structures of *Ssp* DnaB intein (PDB ID: 1MI8) and *eDHFR*-methotrexate complex (1DRE).
- 12 MALDI-TOF-MS analysis was performed in negative mode with sinapic acid as a matrix: P-6, calc. 28806, obs. 28789; P-7, calc. 35279, obs. 35286; P-8, calc. 28037, obs. 28020.
- 13 Because we could not distinguish the bands of P-6 and P-8 by SDS-PAGE/Western blotting due to the similar molecular weight, we estimated the yield of P-6 by determining the peak ratio of P-6 and P-8 by MALDI-TOF-MS analysis (*ca.* 55 : 45). However, the potential differences in ionization energy cannot be fully ruled out.
- 14 This rate constant is comparable to that for the split *Ssp* DnaE intein  $(0.7 \times 10^{-4} \text{ s}^{-1}: \text{D. D. Martin, M.-Q. Xu and T. C. Evans, Jr.,$ *Biochemistry*, 2001,**40** $, 1393–1402), the rapamaycin-inducible split VMA intein <math>(1.9 \times 10^{-4} \text{ s}^{-1})$ ,<sup>9</sup> and the split *Ssp* DnaB intein (I<sub>N</sub>–I<sub>C</sub> pair)  $(0.4 \times 10^{-4} \text{ s}^{-1})^7$ .
- 15 We confirmed that the construct I<sub>C</sub>-9 is capable of *trans*-splicing with I<sub>N</sub>-2 in a crude cell lysate (Fig. S4, ESI†). It was demonstrated that over 10  $\mu$ M of I<sub>N</sub>-1 was required to produce the splicing product P-11 in an amount comparable to that obtained using 0.5  $\mu$ M of I<sub>N</sub>-2 in the cell lysate (Fig. S4, ESI†), indicating again the advantage of the present ligation system.
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