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Photomodulation of protein trans-splicing through backbone photocaging of the DnaE split intein**

Dr. Luis Berrade¹, Dr. Youngeun Kwon^{1,2}, and Dr. Julio Camarero^{1,*}

¹ Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, CA 90033, USA

² Department of Biomedical Engineering, Dongguk University-Seoul, Pildong 3-ga, Seoul, Korea 100-713

Abstract

A novel strategy to modulate the assembly and trans-splicing activity of the *Ssp* DnaE split-intein was achieved by introducing two photolabile protecting groups onto the backbone of the C-intein polypeptide. This modification was not only able to efficiently block the trans-splicing activity, but also reduce significantly the binding affinity constant between the C- and N-intein fragments. The original activity of the wild-type split intein could be fully recovered by brief exposure to UV light.

Keywords

Biotechnology; Proteins; Peptides; Photolysis; Recognition

Intein-mediated protein splicing is a naturally occurring self-processing event in which the intervening intein sequence is removed from a precursor protein and the flanking extein segments are ligated with a native peptide bond.[1–4] Protein splicing can be found to occur in cis or in trans.[5–7] In protein trans-splicing the intein self-processing domain is split in two fragments, called N-intein (I_N) and C-intein (I_C), respectively. These two intein fragments are inactive individually. However, they can bind each other with high specificity under appropriate conditions to form a functional protein-splicing domain.

Since it was initially discovered, intein-mediated protein splicing and trans-splicing have been used in a multitude of applications ranging from protein purification,[8] protein backbone cyclization,[9–12] addition of labeled tags or other moieties,[13,14] protein immobilization,[15–18] protein semi-synthesis,[7] and segmental labeling or modification.[19–22] Of particular interest is the use of conditional protein trans-splicing,[23,24] which allows controlling the activity of a particular protein by modulating the assembly of the split intein moieties and therefore its splicing activity (Fig. 1).

Recent work by Muir and co-workers has shown that the use of a reversible O-acyl backbone modification on the *Ssp* DnaE I_C can be used to modify the splicing activity of this split-intein. [25] However, the lack of trans-splicing activity associated with the different O-acyl C-intein analogues was shown to be due to the introduction of local perturbations in the active site of

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*Fax: (323) 442 1417, jcamarero@usc.edu.

the split-intein complex rather than to the inability of the fragment to associate with N-intein and adopt the canonical intein fold. Hence, although this approach may prevent trans-splicing and therefore the formation of the functional full-length protein, in some cases the binding of the two inactive intein moieties may bring the two extein fragments in close proximity (the distance between the $^{\alpha}\text{C}$ of the first N- and C-extein residues is 9.7 Å in the *Ssp* DnaE split intein[26]) in such a way that they could fold in trans and yield a functional protein in a similar way that protein-fragment complementation assays work.[27,28]

We hypothesized that the chemical introduction of backbone protecting groups on specific locations of the C-intein polypeptide could reversibly modify the splicing activity of this split intein by modulating the binding affinity between the split-intein fragments. The *Ssp* DnaE I_C polypeptide is only 36 residues long and therefore can be easily prepared by solid-phase peptide synthesis. Close analysis of the *Ssp* DnaE intein crystal structure (Fig. 2A)[26] shows that the interactions between the I_N and I_C fragments are mainly mediated by long anti-parallel β -sheets. Since β -sheet formation involves inter-strand hydrogen bonding between the backbone carbonyl oxygen and amide proton, the replacement of the amide hydrogen by a reversible bulky protecting group (i.e. introduction of a backbone protecting group) could modulate the affinity between the intein moieties and therefore its trans-splicing activity. This approach has been successfully employed for disrupting β -sheet[29] and α -helix[30] formation thus helping to minimize aggregation and improve polypeptide solubility.

We were especially interested in using photolabile protecting groups for this task. Photolabile protecting groups have been widely used in the development of photocaged versions of bioactive molecules, including inteins.[31] Photomodulation of intein assembly and splicing could in principle allow the study of protein function *in vivo* with temporal and spatial control by making use of highly focused coherent light sources.[32]

For this purpose we used the 6-nitroveratryl (3,4-dimethoxy-6-nitro-benzyl, Nvl) group,[33] a nitrobenzyl-based photolabile group that can be easily removed by UV irradiation at 365 nm. Close inspection of the split-intein structure revealed four potential Gly residues that could be used for the introduction of the nitroveratryl group. The choice of Gly residues was made based on their location in the secondary structure elements involved in the complex formation, but also to facilitate the synthesis of the corresponding backbone photocaged polypeptides. Gly residues at positions 6, 11, 19 and 31 (Scheme 1) are all involved in intermolecular β -sheets and besides Gly⁶, the rest of the Gly residues are highly conserved in the DnaE C-inteins from different species (Fig. 2B). Gly¹⁹ and Gly³¹ are also located in close proximity to the active site of the split intein. We anticipated that the introduction of a bulky aromatic group at the backbone amide groups in these positions would severely disrupt the hydrogen bond networks involved in the formation of the intramolecular β -sheets thus preventing the assembly of the split-intein and therefore its trans-splicing activity.

Accordingly, we initially synthesized four single photocaged I_C polypeptides at the positions mentioned above using Fmoc-based solid-phase peptide synthesis (Scheme 1). The synthesis of Fmoc-Gly(Nvl)-OH was carried by reductive amination of 6-nitroveratraldehyde with H-Gly-OH followed by introduction of the Fmoc group at the α -amino group (Supporting Information). The first four residues (Cys-Phe-Asn-Lys) of the C-extein were also introduced in all the I_C peptides used in this work. These four residues are required for efficient trans-splicing.[15,34] First, we evaluated the effect of backbone photocaging on the affinity between the I_C polypeptides (Table 1) and the DnaE I_N . As a model N-intein we fused the maltose binding protein (MBP) to the N-terminus of the *Ssp* DnaE I_N (MBP- I_N). The first four residues of the N-extein were also included to facilitate protein trans-splicing.[15,34] Affinity constants were calculated based on the fluorescence anisotropy change during the titration of a given I_C labeled with fluorescein isothiocyanate (FITC) at its N-terminus with increasing amounts

of the MBP-I_N. The modification of the N-terminus of different I_C peptides has been shown not to affect the activity or assembly of the DnaE split-intein.[15] The calculated affinity constants of the different single photocaged I_C peptides and MBP-I_N were only around 10 times weaker than the affinity of the unmodified wt-I_C (Table 1). Next, we evaluated their splicing activity. The trans-splicing reactions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The trans-splicing yield for the unmodified wt-I_C was $\approx 60\%$ (based on un-reacted MBP-I_N). This yield was used as a reference to estimate the effect of backbone photocaging in trans-splicing activity. Analysis by HPLC and mass spectrometry of the trans-splicing reaction confirmed the identity of the trans-spliced product as MBP-CFNK (Figs. 3A and 3C). Remarkably, no N-cleavage by-product (i.e. MBP) was detected under conditions used in this work (Fig. 3C inset and Fig. S15, Supporting Information). All the single photocaged I_C polypeptides showed a decreased trans-splicing activity compared to that of the wt-I_C (Fig. S9, Supporting Information). Peptide 11G-I_C was the less affected by the introduction of the Nvl group within the backbone, with only $\approx 60\%$ of the splicing activity of the wt-I_C. Peptides 6G-I_C and 19G-I_C both showed similar splicing activities, with $\approx 40\%$ of the splicing activity of the wt-I_C peptide. Peptide 31G-I_C was the most affected by the introduction of the Nvl group, with only $\approx 10\%$ of the splicing activity of the wt-I_C peptide. This could be explained based on the proximity of this residue to the active site of the split-intein. Thus, despite that this peptide shows similar affinity for MBP-I_N than the other single photocaged I_C peptides, the proximity of the structural disruption to the active site may affect more severely its potential splicing activity. Hence, it was clear that the introduction of a single backbone photolabile protecting group was not enough to significantly affect the binding constant between the modified I_C and I_N fragments, and more importantly to effectively block their splicing activity.

Next, we decided to explore the effect of introducing two backbone protecting groups in the same I_C polypeptide. For this purpose, we decided to use positions 19 and 31, which corresponded to the single photocaged I_C peptides that better blocked protein splicing in the single photocaged I_C polypeptides (Fig. S9, Supporting Information). Double photocaged peptide 19G,31G-I_C showed an affinity constant for MBP-I_N ≈ 50 times weaker than the observed for wt-I_C (Table 1) thus confirming the synergistic effect of adding two backbone protecting groups in disrupting the assembly of the DnaE split-intein. The splicing activity of 19G,31G-I_C was also compared with that of the unmodified wt-I_C using MBP-I_N. Interestingly, the trans-splicing activity of the 19G,31G-I_C was completely blocked after 24 h of reaction. No band corresponding to the trans-spliced product was detected by SDS-PAGE and silver staining (Fig. 4B). In comparison, the unmodified wt-I_C produced a distinguishable band corresponding to the spliced product.

Initial studies showed that Nvl-backbone photocaged peptides could be readily deprotected using UV light at 365 nm to produce in good yield unmodified wt-I_C (Fig. S11, Supporting Information). Hence as expected, irradiation of double photocaged 19G,31G-I_C with UV light at 365 nm for 20 min was able to yield a fully active I_C peptide with a trans-splicing activity similar to that of the wt-I_C (Fig. 4C). Irradiation of unmodified wt-I_C did not have any negative impact on the splicing activity of the split-intein. The kinetic analysis of the trans-splicing reactions involving wt-I_C and UV-irradiated 19G,31G-I_C confirmed also that photodeprotection of 19G,31G-I_C gave a fully active intein with similar activity to that of the wt-I_C (Fig. 3C). The analysis by mass spectrometry confirmed again the identity of the spliced product as MBP-CFNK (Fig. 3B and 3D) thus indicating that the photolysis step does not affect the trans-splicing reaction or product. As for the wt-I_C, no N-cleavage product was detected by mass spectrometry (Fig. 3D). It is also remarkable that no trans-splicing activity was detected for 19G,31G-I_C even after 72 h of reaction (Fig. S12, Supporting Information).

In summary, the current report demonstrates a novel strategy to modulate the assembly and trans-splicing activity of the *Ssp* DnaE split-intein. This was achieved by introducing two photolabile protecting groups onto the backbone of the *Ssp* DnaE I_C polypeptide. The introduction of these groups was not only able to efficiently block the trans-splicing activity of the DnaE split-intein, but also reduce significantly the binding affinity constant between the I_C and I_N intein fragments. The double photocaged I_C had an affinity constant for the I_N fragment of $5.8 \pm 0.2 \mu\text{M}$. This value is 50 times weaker than corresponding affinity constant for the unmodified wt-I_C (Table 1). This is key for the use of conditional photomodulated protein trans-splicing to study protein function *in vivo*, [35] where the high dilution conditions typically found inside cells combined with the use of photocaged inteins with low affinity could prevent the possibility of prematurely producing a functional protein by intein-mediated complementation in trans. More importantly, our results show that the original activity of the wild-type *Ssp* DnaE split-intein can be fully recovered by brief exposure of the photocaged I_C to light at 365 nm. Hence, this remarkable result could make possible the control of protein function *in vivo* with spatial and temporal control by using highly focused and coherent light sources in combination with highly reactive DnaE split inteins, such as the *Npu* DnaE split-intein (Fig. 2, entry *Npu*). [36,37] Moreover, photomodulated protein trans-splicing could be also used for the light-activated immobilization of proteins onto solid support by protein trans-splicing for the production of polypeptide micro-arrays [15,38] using photolithographic techniques.[33]

Experimental Section

See the Supporting Information for experimental details

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Photomodulated conditional protein trans-splicing is only initiated when the photocaged groups on the C-intein are removed. The use of photolabile backbone protecting groups located on the sequence of the C-intein interferes with assembly and protein trans-splicing activity. Irradiation with UV light frees the backbone of the C-intein that regains then normal activity.

Figure 2.

A) Crystal structure of the *Ssp* DnaE intein in the pre-spliced state (PDB code: 1zde).[26] DnaE I_C and I_N are shown in red and blue respectively. The I_C Gly residues used in this work to introduce photolabile backbone protecting groups are shown in green. The structural secondary elements are also shown. B) Multiple sequence alignment of the DnaE I_C for different species indicating the Gly residues used for the introduction of the backbone protecting groups. Multiple sequence alignment was performed using T-Coffee (<http://tcoffee.vital-it.ch>) and visualized using Jalview.[39] Molecular representation of the *Ssp* DnaE intein was generated using the PyMol software package.

Figure 3.

Analytical RP-HPLC traces for the trans-splicing reactions using (A) wt- I_C and (B) UV-irradiated 19G,31G- I_C polypeptides. Trans-splicing reactions between DnaE I_C and I_N constructs were performed in splicing buffer (0.5 mM EDTA, 1 mM TCEP, 2 mM semicarbazide, 50 mM sodium phosphate, 250 mM NaCl buffer, pH 7.0) at 4°C for 36 h. Analytical HPLC was performed using a linear gradient of 0–100% solvent B over 30 minutes. ES-MS analysis of peak corresponding to the trans-spliced product (MBP-CFNK) when the trans-splicing reaction was performed with (C) wt- I_C or (D) UV-irradiated 19G,31G- I_C polypeptides. Asterisks in panels C and D indicate charge states corresponding to polypeptides with estimated masses of 18 and 15 kDa, respectively. The resulting deconvoluted mass spectra (inset) are also shown showing the observed and expected (in parenthesis) masses. Arrows indicate the expected location where the N-cleavage product (MBP, 43497.04 Da) should appear.

Figure 4.

Photomodulation of protein trans-splicing by backbone photocaging of the *Ssp* DnaE I_C. A) Scheme of the trans-splicing reaction used in this work. B) SDS-PAGE analysis of the trans-splicing reaction using double photocaged I_C before and after irradiation at 365 nm for 20 min. Unmodified wt-I_C was used as control. Trans-splicing reactions between DnaE I_C and I_N constructs at 5 μM and 0.1 μM, respectively, were performed in splicing buffer (0.5 mM EDTA, 1 mM TCEP, 2 mM semicarbazide, 50 mM sodium phosphate, 250 mM NaCl buffer, pH 7.0) at 4°C for 24 h. C) Plot of the fraction of spliced product at different times relative to the maximum value obtained with unmodified wt-I_C, which typically gives yields of 60%.^[15] Error bars are the standard deviation of three independent experiments.

wt-I_C **MVKVIGRRSLGVQRIFDIGLPQDHNFLLANGAIAANCFNK**
6G-I_C **MVKVI~~X~~RRSLGVQRIFDIGLPQDHNFLLANGAIAANCFNK**
11G-I_C **MVKVIGRRSL~~X~~VQRIFDIGLPQDHNFLLANGAIAANCFNK**
19G-I_C **MVKVIGRRSLGVQRIFDI~~X~~LPQDHNFLLANGAIAANCFNK**
31G-I_C **MVKVIGRRSLGVQRIFDIGLPQDHNFLLAN~~X~~AIAANCFNK**
19G, 31G-I_C **MVKVIGRRSLGVQRIFDI~~X~~LPQDHNFLLAN~~X~~AIAANCFNK**

X = -Gly(Nvl)-

Scheme 1.

Sequences of the DnaE I_C polypeptides used in this work. Nvl stands for 6-nitroveratryl, the photolabile-backbone protecting group used to reversibly disrupt assembly and splicing activity.

Table 1

Binding and trans-splicing activities of the DnaE I_C polypeptides used in this work after 48 h of reaction. Relative trans-splicing yields are shown in parenthesis and are normalized to wt-I_C.

Peptide	Molecular weight (Da) ^a		Affinity constant ^b (μM)	Trans-splicing yield (%)
	Expected	Found		
wt-I _C	4398.2	4398.0 ± 0.8	0.12 ± 0.01	60 ± 5 (100) ^c
6G-I _C	4593.4	4593.0 ± 0.6	1.3 ± 0.1	23 ± 2 (38) ^c
11G-I _C	4593.4	4593.0 ± 0.8	1.1 ± 0.1	37 ± 3 (61) ^c
19G-I _C	4593.4	4594.0 ± 0.9	1.1 ± 0.1	24 ± 4 (40) ^c
31G-I _C	4593.4	4594.0 ± 0.9	0.93 ± 0.01	6 ± 1 (10) ^c
19G,31G-I _C	4788.5	4789.0 ± 0.7	5.8 ± 0.2	0.2 ± 0.05 (0.5) ^c

^a Average molecular weight;

^b Affinity constant for MBP-I_N;

^c Yield normalized to that obtained with wt-I_C. Trans-splicing yield for wt-I_C is ≈ 60% of the initial MBP-I_N. [15]