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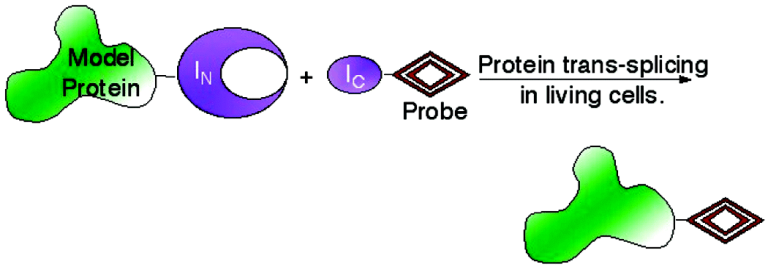

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Protein Semi-Synthesis in Living Cells

Izabela Giriat and Tom W. Muir*

Laboratory of Synthetic Protein Chemistry, The Rockefeller University, New York, New York 10021

Received February 18, 2003; E-mail: muirt@rockefeller.edu

Characterization of proteins in the context of a living cell is of crucial importance for a complete understanding of their function. Although classical genetics provides powerful tools for studying the function of proteins in cells, genetic approaches (e.g., mutagenesis, gene knockouts, and overexpression) have limitations both in terms of the range of modifications that can be made and in terms of the temporal control that is possible.[1] One way to address the former limitation is to develop chemistry-based techniques that allow the range of functional groups in targeted cellular proteins to be broadened. Approaches to this have included the in vivo chemical modification of acceptor domains fused to the protein of interest[2] and the expansion of the genetic code by nonsense suppression methodology.[3] Herein we describe a novel approach in which a synthetic molecule is ligated to a protein (i.e., semi-synthesis) inside living cells. This strategy is expected to increase the diversity of chemical modifications available for probing cellular proteins.

In principle, protein trans-splicing could be adapted to the target-specific semi-synthesis of proteins in vivo, thereby allowing for a modular approach to the incorporation of chemical probes. Protein splicing is an autocatalytic process in which an intervening sequence, termed an intein, is removed from a host protein. In protein trans-splicing the intein is split into two pieces, and activity is regained upon reconstitution of these fragments.[4] The process has found a variety of applications in vitro, including protein semi-synthesis[5] and segmental isotopic labeling.[6] In addition, split inteins have been used to cyclize proteins in vivo[7] and to study protein−protein interactions in living cells.[8] Protein trans-splicing has many of the attributes necessary for the semi-synthesis of proteins inside cells, namely inteins are promiscuous with respect to their flanking sequences,[4] split inteins associate exclusively with their corresponding partner, and specifically for the *Synechocystis* sp. (*Ssp* DnaE) system,[9] the components have high affinity for each other.[10]

We envisioned the general methodology summarized in Figure 1 to produce semi-synthetic proteins in living cells. The protein of interest is expressed in cultured cells with the first half of the naturally occurring *Ssp* DnaE split intein (I_N) fused to its C terminus. Then, a semi-synthetic polypeptide, comprising the second half of the intein (I_C) covalently linked to a synthetic probe and a protein transduction domain (PTD) peptide, is added to the cell media. The PTD peptide delivers the I_C probe into the cells, whereupon it can associate with its complementary half, I_N, triggering protein splicing. This results in the removal of the intein and ligation of the probe to the selected protein through a normal peptide bond.

To test this methodology we designed a model system in which the target protein is the green fluorescent protein (GFP) and the probe is a short synthetic peptide, based on the FLAG epitope. Preliminary in vitro studies confirmed that protein trans-splicing occurred between purified recombinant GFP-I_N and synthetic I_C-FLAG to give GFP−FLAG (see Supporting Information). A key feature of our methodology is the ability to shuttle the I_C probe (in this case I_C-FLAG) into cells using PTD peptides. These peptides

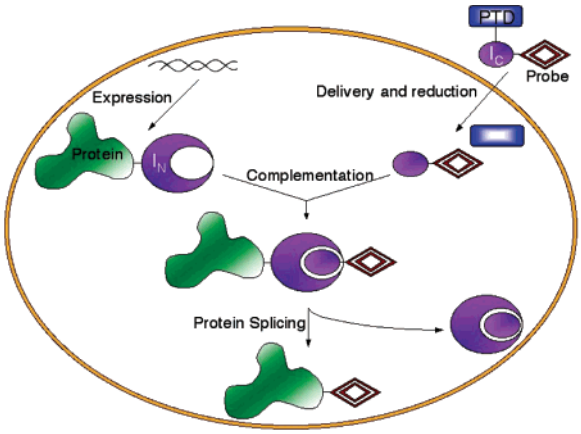

Figure 1. Principle of semi-synthetic protein trans-splicing in living cells. I_N is the first half of the naturally occurring *Ssp* DnaE intein, I_C is its second half, and PTD is protein transduction domain.

have the ability to translocate through cellular membranes and transport cargo molecules into the cytosol of cells.[11] In the present study, we chose to use a PTD corresponding to residues 43−58 of the *Drosophila* homeotic transcription factor, ANTP.[12] An efficient procedure was developed for linking ANTP to the I_C-FLAG cargo through a disulfide bond.[13] Cell culture studies demonstrated that ANTP could internalize the disulfide-linked I_C-FLAG and that, once inside the reductive environment of the cytoplasm, the cargo was released from the PTD (see Supporting Information).

We then investigated whether semi-synthetic trans-splicing could occur inside mammalian cells transiently transfected with a plasmid encoding GFP-I_N driven by a constitutively active promoter. Specifically, CHO cells expressing GFP-I_N were treated with ANTP-S-S-I_C-FLAG and incubated for 3 h. The cells were then washed several times with phosphate-buffered saline (PBS), harvested, and lysed under alkylating conditions to prevent splicing upon lysis.[14] The soluble fraction was immunoprecipitated (IP) with anti-GFP or anti-FLAG beads, and the captured proteins were loaded onto an SDS-PAGE gel and blotted against GFP or FLAG. This procedure allowed us to detect the presence of proteins containing either GFP or FLAG alone (i.e., the reactants or potential side-products) or both GFP and FLAG (i.e., the desired product). The results of this experiment are shown in Figure 2A. A single band of the expected molecular weight for the semi-synthetic product (∼29 kDa) appeared when transfected cells were treated with the ANTP-S-S-I_C-FLAG peptide (lanes 4, 8, 12, and 16). Importantly, this new band cross-reacted with both the anti-GFP and anti-FLAG antibodies, confirming it was the desired product, GFP−FLAG. In contrast, this new band was not present in nontransfected/untreated cells (lanes 1, 5, 9, and 13), nontransfected cells treated with the ANTP-S-S-I_C-FLAG peptide (lanes 2, 6, 10, and 14), and transfected cells with no peptide added (lanes 3, 7, 11, and 15).

Interestingly, GFP-I_N co-immunoprecipitated with I_C-FLAG, indicating a noncovalent complex between the two components

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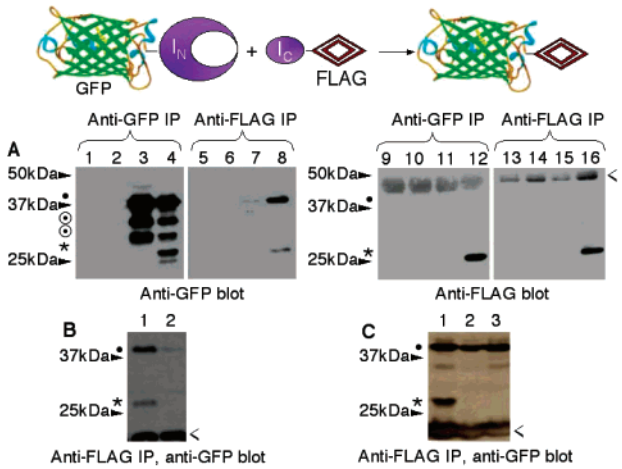

Figure 2. Semi-synthetic trans-splicing between GFP-I_N and I_C-FLAG in CHO cells [schematically shown in top panel]. Cells treated as indicated below were lysed, and the soluble fraction was immunoprecipitated and blotted as indicated in the panels. (A) Nontransfected/untreated cells (lanes 1, 5, 9, and 13), nontransfected cells treated with 100 μM ANTP-S-S-I_C-FLAG (lanes 2, 6, 10, and 14), transfected cells expressing GFP-I_N with no peptide added (lanes 3, 7, 11, and 15), and cells expressing GFP-I_N treated with 100 μM ANTP-S-S-I_C-FLAG (lanes 4, 8, 12, and 16). (B) Cells expressing GFP-I_N treated with 100 μM ANTP-S-S-I_C-FLAG (lane 1) and cells expressing GFP-I_N mixed at the time of lysis with nontransfected cells treated with 100 μM ANTP-S-S-I_C-FLAG (lane 2). (C) Cells expressing GFP-I_N treated with 100 μM ANTP-S-S-I_C-FLAG (lane 1), cells expressing GFP-I_N treated with 100 μM ANTP-S-S-I_C(SR)-FLAG (lane 2), and cells expressing GFP-I_N(Ala^{250}) treated with 100 μM ANTP-S-S-I_C-FLAG (lane 3). The ligation product GFP-FLAG is denoted by *, GFP-I_N is denoted by •, proteolysis fragments thereof by ⊙, and background bands are denoted by <.

(Figure 2A, lane 8). This suggests that protein splicing, and not split-intein association, is the rate-determining step in the process, which is in accordance with previous studies on *Ssp* DnaE-mediated protein trans-splicing.[10,15] Proteolytic fragments of GFP-I_N were evident; however, these did not associate or react with I_C-FLAG (Figure 2A, lanes 3, 4), indicating that degradation was within the I_N component.

Further control experiments were performed to completely rule out the possibility that splicing was taking place in vitro (i.e., after lysis) and to attribute product formation to protein trans-splicing. The former issue was addressed by mixing, at the time of lysis, transfected cells expressing GFP-I_N, but not treated with the peptide, with nontransfected cells treated with the peptide. Thus, both components of the trans-splicing system were present throughout the in vitro manipulations, thereby allowing us to determine if the splicing reaction could take place after lysis. As shown in Figure 2B, no splicing or split-intein association was detected (compare lanes 1 and 2). To confirm that product formation was due to protein splicing, an experiment was performed using inactive forms of I_N or I_C.[16] Although the inactive I_N and I_C analogues were able to associate with their complementary component, in neither case was trans-splicing observed (Figure 2C, lanes 2 and 3). Thus, product formation occurs via protein trans-splicing in the living cells. The trans-splicing reaction appears to be specific since only one protein was tagged with FLAG (Figure 2A, lane 16). Finally, the generation of the semi-synthetic product provides unequivocal proof that I_C-FLAG was released from ANTP since this process (i.e., disulfide reduction) unmasks the reactive cysteine within I_C required for splicing.

This is the first report of protein semi-synthesis in a cellular context. Using protein trans-splicing and PTD peptides, a synthetic molecule—a polypeptide (as in this study) or, minimally, a cysteine derivative bearing any chemical moiety—can be ligated to a protein of interest in vivo. In the present study incorporation of the probe takes place at the C terminus of the protein;[17] however, by interchanging the split intein components, it should be possible to introduce the probe at the protein N terminus as well. Furthermore, since the reaction is triggered by the addition of one of the components onto the media of cells already expressing the other component, temporal control is inherent to the system. Semi-synthetic protein trans-splicing could become a valuable tool for the analysis of proteins within their natural context. In principle, probes such as fluorophores, cross-linkers, unnatural amino acids, and other sensors can now be added onto cellular proteins to study function.

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Supporting Information Available: Full experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) Synthetic ANTP was conjugated to synthetic I_C-FLAG using a nucleophilic disulfide exchange reaction. For future applications we have developed a methodology based on expressed protein ligation (EPL) to create the I_C-probe component of the system. See Supporting Information.
(14) Protein splicing requires two reactive cysteines to take place. Lysis under alkylating conditions (PBS containing 1% (w/v) docecylmaltoside (DM), 10 mM iodoacetamide, 2 mM *N*-ethyl-maleamide, and protease inhibitors) blocks all cysteine sulhydryl groups, therefore preventing splicing.
(15) As determined on the basis of phosphoimaging analysis ~35% of the associated intein halves had spliced at the 3 h time point. The overall efficiency of the semi-synthetic protein trans-splicing reaction could not be easily determined, because GFP-I_N was being continually expressed (and so therefore not depleted) and because the ANTP-S-S-I_C-FLAG peptide was added in large excess.
(16) The reactive cysteine in I_N was mutated to an alanine (GFP-I_N(Ala^{250}). The reactive cysteine in I_C was alkylated, and an extra cysteine was added onto the N-terminus of the peptide for disulfide linkage to ANTP (I_C-(*S*R)-FLAG).
(17) The current system has been applied successfully to the semi-synthesis of four other proteins in vivo. See Supporting Information.

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