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Recent advances in protein splicing: manipulating proteins *in vitro* and *in vivo*

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Protein splicing is an intricate self-catalyzed protein rearrangement that converts an inactive protein precursor to biologically active proteins. In the past decade, mechanistic studies and extensive engineering of the naturally occurring protein splicing elements, termed inteins, has led to the development of numerous novel technologies. These intein-based methodologies permit *in vitro* and *in vivo* protein processing in ways previously not possible using traditional biochemical and genetic approaches. Inteins have been utilized in the production of protein and peptide arrays, as molecular switches and in the reconstitution of functional proteins by split-gene techniques.

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Introduction

Protein splicing is an extraordinary post-translational processing event that involves the precise removal of an internal polypeptide segment, termed an intein, from a precursor protein with the concomitant ligation of the flanking polypeptide sequences, termed exteins [1]. Since its discovery in 1990, more than 200 inteins have been identified in all three domains of life [2]. The inteins, ranging from 128 to 1650 amino acids, share a set of highly conserved sequence motifs. The majority of known inteins appear to be bifunctional, as they also contain the characteristic motifs of a homing endonuclease that confers genetic mobility upon the intein-encoding gene. An endonuclease insertion splits the region required for splicing. A small number of inteins lack an endonuclease-coding region and are termed mini-inteins. Of special interest are the naturally occurring *trans*-splicing inteins in which a host gene is split into two separate coding regions, each fused to either the N-terminal or C-terminal portion of an intein-coding region [3]. Formation of the full-length host protein occurs when the N-terminal and C-terminal intein regions come together to reconstitute protein splicing activity.

Here, we review some of the recent advances in protein splicing research and discuss a number of intein-based applications.

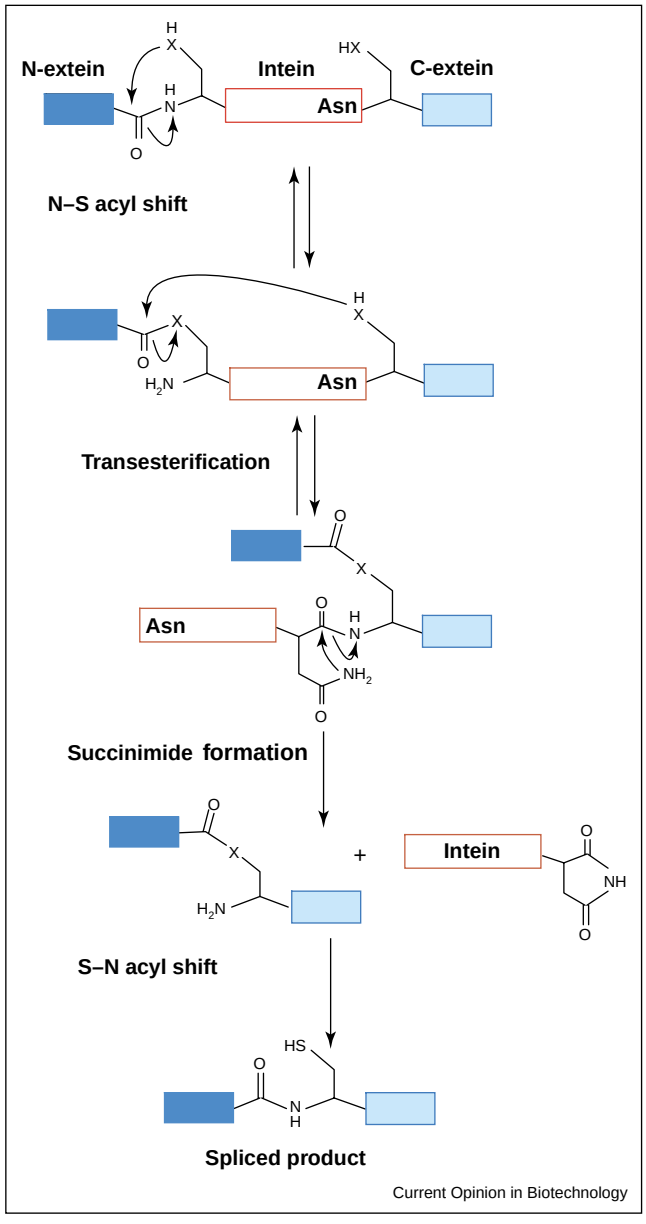
Splicing mechanism and intein family

Many inteins have been shown to self-splice *in vitro* without the requirement of external energy or protein cofactors [4]. The mechanism of protein splicing has been elucidated by the identification of key catalytic amino acid residues and intermediates (Figure 1). Most inteins start with a cysteine or serine residue that is responsible for an acyl shift at the N-terminal splice junction. The first C-extein residue following the scissile bond at the C-terminal splice site is invariably a cysteine, serine or threonine and the sulfhydryl or hydroxyl group on their sidechain nucleophilicly attacks the linkage at the N-terminal splice junction, resulting in a branched intermediate. An asparagine residue typically precedes the C-terminal splice junction and is involved in the resolution of the branched intermediate by sidechain cyclization. Interestingly, a subfamily of inteins possessing an N-terminal alanine apparently initiates splicing by a direct attack on the peptide bond at the N-terminal splice junction by the sidechain of the first C-extein residue [5]. In addition, recently identified non-canonical inteins include those with glutamate or aspartate in place of the highly conserved C-terminal asparagine and bacterial intein-like proteins, mainly possessing a C-terminal glutamate, glycine or leucine [6–9]. A study of the oceanic nitrogen-fixing cyanobacterium *Trichodesmium erythraeum* revealed a remarkable intein organization showing the presence of three inteins (including one split intein) in the *dnaE* gene encoding the catalytic domain of DNA polymerase III [10]. The study of *T. erythraeum* has also led to the first report of the coexistence of multiple inteins and introns in a single gene [11,12]. A new example of a viral intein was recently found in Mimivirus [13]. The broad range of intein properties facilitates their use in diverse protein engineering strategies.

Intein-mediated protein immobilization

The steps that underlie protein splicing consist of two acyl rearrangements, a transesterification and cyclization of an asparagine. The elucidation of the protein-splicing pathway led to the discovery that catalysis of each of the steps is often relatively independent. Formation of a thioester by an initial acyl rearrangement occurs even

Figure 1



Prototypical protein splicing mechanism. The hydroxyl- or sulfhydryl-containing sidechain of the intein N-terminal residue initiates an N–O or N–S acyl rearrangement, resulting in a (thio)ester linkage between the N-extein and C-extein. The sidechain of cysteine, serine or threonine at the C-terminal splice junction attacks the (thio)ester in a trans(thio)esterification reaction to form a branched intermediate. The intein is excised from the branch by cyclization of the intein C-terminal asparagine coupled to peptide bond breakage. A spontaneous O–N or S–N shift generates a native peptide bond. The X represents either an oxygen or sulfur atom.

when the subsequent steps are blocked by amino acid replacements (e.g. by substitution of the intein C-terminal asparagine with alanine) at the downstream splice junction [14,15]. Thioester formation is the basis for an intein-mediated purification system in which a target protein is fused to the N terminus of an intein and can be released in a thiol-induced reaction. This intein fusion system has been extended to produce recombinant proteins possessing a C-terminal thioester for ligation with synthetic peptides or recombinant proteins carrying a variety of modifications or chemical moieties [16,17]. Researchers have used this technique to incorporate various probes in a site-specific manner or to produce proteins with isotopically labeled regions for functional and structural analysis [18,19••]. Semisynthetic DNA–protein conjugates were also generated by use of a C-terminal thioester on an expressed protein and cysteinyl oligonucleotides [20,21•].

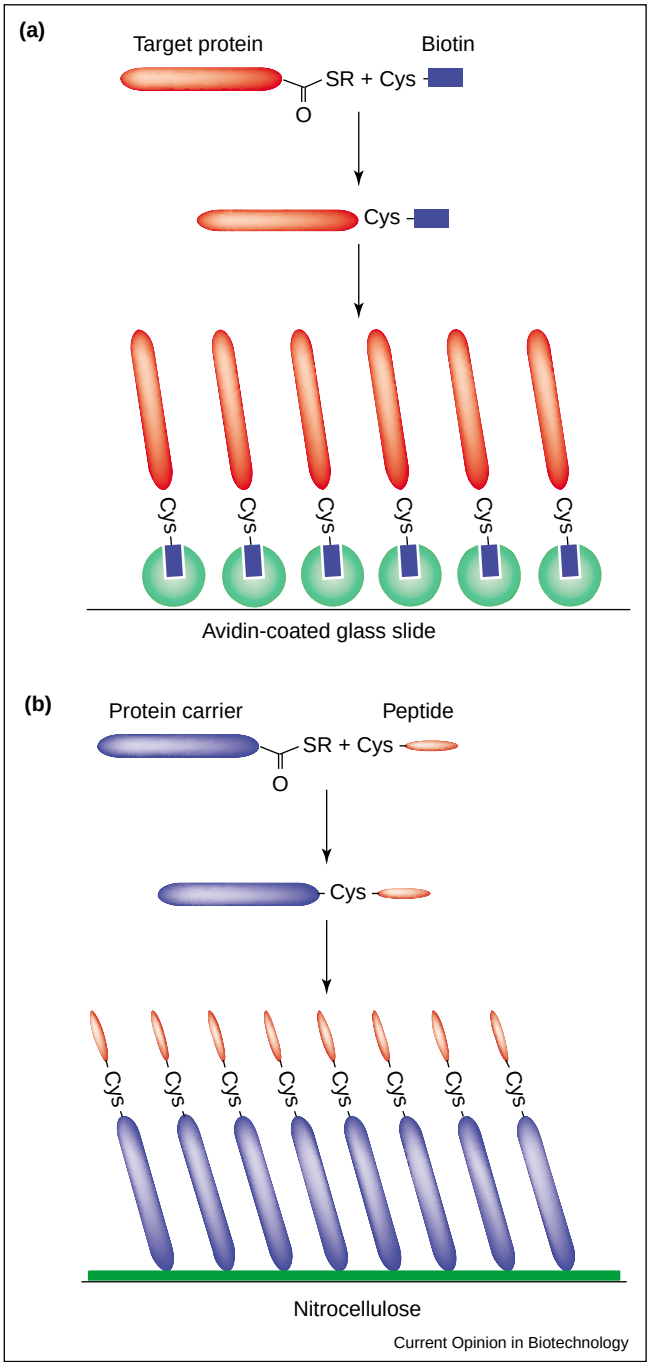
Recently, intein-mediated protein ligation has been further employed to generate protein or peptide arrays by improving binding efficiency and orientation of the target molecules. A technique was developed for the site-specific attachment of C-terminal biotinylated proteins onto avidin-coated glass slides (Figure 2a) [22•,23]. Similarly, single-chain antibodies expressed in *Escherichia coli* can be labeled for chip-based screening [24]. The immobilization of site-specifically oriented proteins might help to retain their biological activities. In addition, an extremely strong avidin–biotin linkage is beneficial to withstand various assay conditions. This scheme permits the arrays to be utilized for quantitative analysis because each target protein carries only one reactive site for the biotinylated tag, which in turn is capable of binding to avidin ligand immobilized on a glass slide.

Furthermore, a new strategy was recently demonstrated for making peptide arrays on low-cost nitrocellulose. This approach employs the intein-mediated protein ligation of synthetic peptide substrates to an intein-generated carrier protein (Figure 2b) [25•]. This method is intended to provide a simple solution to the problems associated with the variable binding of small peptide substrates to matrices. The commonly used method of synthetic peptide arrays on membrane support (SPOT synthesis) produces an excessive amount of peptide and therefore has limitations in peptide quantification and normalization. As intein-generated carrier proteins play a dominant role in binding and each carrier protein molecule has precisely one reactive site for a peptide possessing an N-terminal cysteine, the amount of peptide arrayed onto a membrane can be effectively normalized. This technique, termed intein-mediated peptide array has been applied to antibody characterization, epitope scanning and kinase assays, and resulted in an increase in sensitivity up to 10^4-fold.

Putting *trans*-splicing to work

In protein *trans*-splicing, a target gene is split into two segments and each half is fused to either the N-terminal or C-terminal portion of an intein-coding sequence; the two halves of the intein-coding sequence are not linked in

Figure 2



Immobilization of proteins for biochip production. **(a)** The site-specific attachment of C-terminal biotinylated proteins onto avidin-coated glass slides. Target proteins (red) are generated with a C-terminal thioester for ligation with a biotinylated tag (blue) for subsequent site-specific attachment to avidin (green) for biochip processing. **(b)** The intein-mediated production of peptide arrays. A thioester tagged carrier protein (blue) is ligated to target peptides (red) possessing an N-terminal cysteine. The ligated products can be efficiently arrayed onto nitrocellulose.

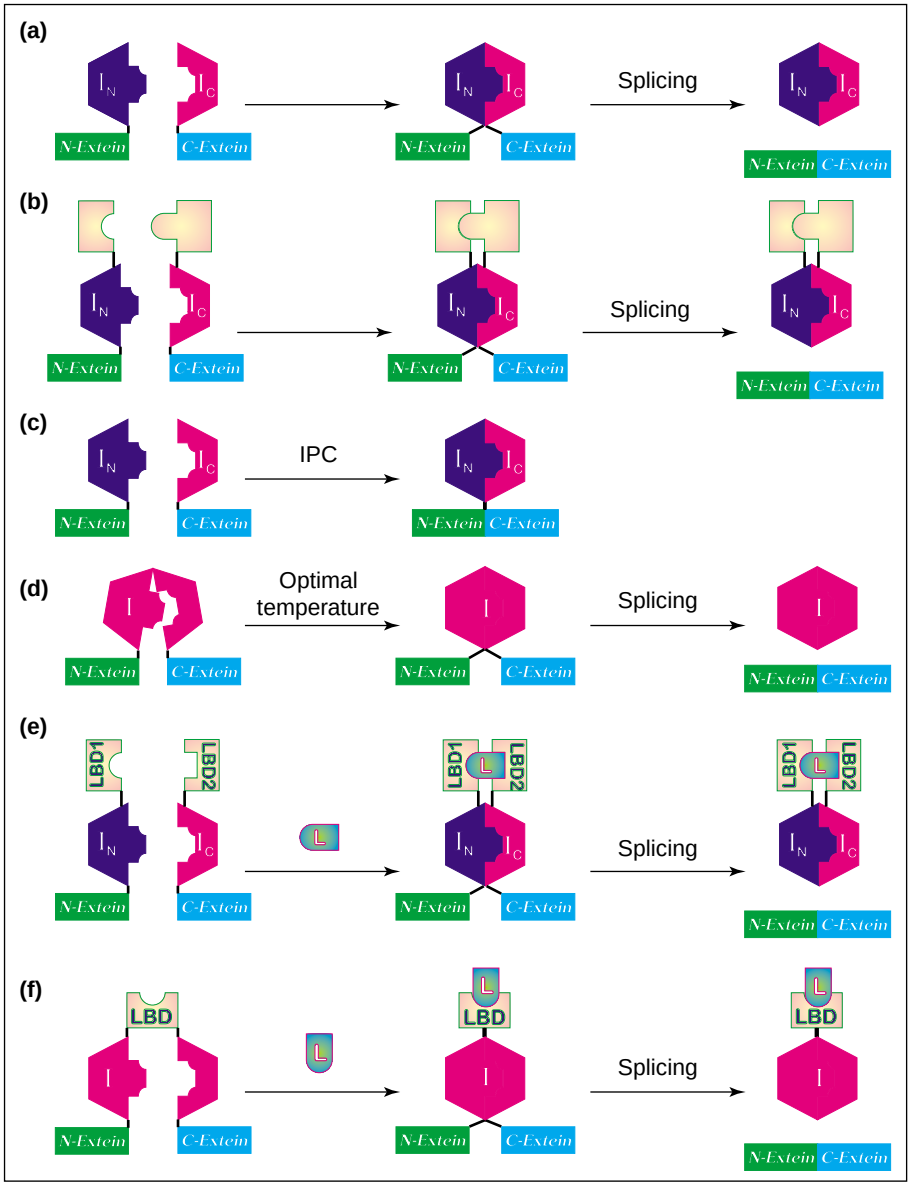
the genome. The two distant segments of the protein are then expressed as inactive truncated forms that can readily associate and regain splicing activity to generate spliced protein products (Figure 3a). In the case of *cis*-splicing, the inteins are split artificially [26,27]. The most robust tool for *trans*-splicing is the naturally occurring split intein from the catalytic subunit of a DNA polymerase III (DnaE) from the cyanobacterium *Synechocystis* sp. PCC6803. Major applications of this intein include the ligation of two expressed protein segments, *in vivo* circularization of proteins, and the generation of a cyclized peptide library [28–31]. Using the DnaE intein, it has been demonstrated that protein–protein interactions within mammalian cells can be assessed *in vivo* by reconstitution of a functional reporter from a split reporter gene by protein interactions (Figure 3b) [32]. The DnaE intein was also employed to develop a living cell imaging method to investigate nucleocytoplasmic trafficking [33••].

Exploiting the ability of inteins to associate led to the split-gene approach for preventing the spread of transgenes from genetically modified plants to the surrounding environment. Put simply, a transgene can be split into two fragments, each confined to different cellular compartments or chromosomes to reduce the chance of transgene spread. This split gene approach was tested in *Arabidopsis* cells by reconstitution of a functional β-glucuronidase (GUS) [34]. Protein *trans*-splicing occurred between two separately expressed fusion precursors: one containing the N-terminal portion of the GUS-coding sequence fused to the N-terminal DnaE intein region, and the second carrying the C-terminal portion of the GUS-coding sequence fused to the C-terminal DnaE intein region. Furthermore, a transgene containment model was systematically assessed and eventually demonstrated in *Nicotiana tabacum* by employing a herbicide resistance gene, a mutant form of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [35]. The split gene sequences were integrated into the nuclear and chloroplast genomes. The chloroplast genome is not inherited paternally in many commercially important crops and therefore plastid DNA bearing the transgene should not be spread via pollen. A plastid localization signal sequence fused to the transgene located in the nucleus can target the truncated N-terminal gene product to the chloroplast. *Trans*-splicing then occurs to yield full-length EPSPS and transgenic plants that are resistant to the herbicide glyphosate. Interestingly, these studies also led to the discovery of intein-mediated protein complementation (Figure 3c) in which functional proteins were produced when a splicing-deficient intein was used as an affinity domain to activate the truncated target protein [36].

Inteins as protein switches and drug targets

Although the role of protein splicing in the regulation of host gene expression is still unknown, the potential

Figure 3



Strategies for the production of functional proteins using inteins and conditional protein splicing. **(a)** Protein *trans*-splicing to generate active proteins. In *trans*-splicing the intein is split, with the intein N-terminal fragment (I_N) fused to the N-extein and the intein C-terminal fragment (I_C) fused to the C-extein. The two halves of the intein associate to reconstitute splicing activity. **(b)** Intein association and protein splicing induced by the dimerization of two interacting proteins fused to split intein fragments. In this case, splicing reconstitutes a reporter that can be used for cell imaging. **(c)** Intein-mediated protein complementation (IPC). IPC utilizes the heterodimerization of the intein N- and C-terminal splicing domains to bring protein fragments together and reconstitute protein activity; IPC reconstitutes protein activity without splicing. **(d)** Conditional protein splicing by a temperature-sensitive intein. **(e)** Intein association and protein *trans*-splicing induced by ligand (e.g. rapamycin) controlled heterodimerization (LBD, ligand-binding domain; L, ligand). **(f)** Conditional protein splicing controlled by a ligand-binding domain inserted into an intein.

utilization of inteins as molecular switches has been vigorously explored to implement conditional activation or inhibition of protein functions. For conditional protein splicing it is assumed that proteins are inactivated by an intein insertion and their functions can be restored upon splicing [37,38]. Furthermore, targeting inteins is of biological significance as inteins are found only in microorganisms, including bacterial and fungal pathogens, but not in human cells [39,40]. For an intein to act as a switch, it must be possible to control splicing and several approaches have been explored including the use of temperature- or pH-sensitive splicing, the mutation or splitting of inteins, inhibition or activation by small-molecule ligands or protein dimerization (Figures 3a–f).

In general, an intein-based switch system relies on rational screening for conditionally active intein variants and splicing activators or inhibitors. Temperature-sensitive inteins have been generated by various selection strategies. A temperature-sensitive splicing variant of the vacuolar ATPase subunit (VMA) intein from *Saccharomyces cerevisiae* has been used to control activation of a pair of universal transcription regulators, Gal4 and Gal80 (Figure 3d) [41••]. Gal80 is able to interact with the Gal4 enhancer to block its binding to an upstream activator sequence (UAS) inserted 5′ to a gene of interest, thereby turning the target gene on or off. It has been shown that the temperature-sensitive Gal80 intein fusion can provide temporal regulation of the Gal4/UAS system in a temperature-dependent manner in *Drosophila melanogaster*.

The temperature-sensitive intein system is limited to systems that can tolerate a temperature shift. It is therefore of considerable interest to search for small molecules that can be applied to conditional protein splicing in a broad range of organisms and cell types. The small molecule rapamycin was found to effectively trigger splicing of an engineered split VMA intein in mammalian cells [38,42••]. The split intein fragments were fused to FKBP and FRB domains, which can be dimerized by rapamycin, thereby facilitating the reconstitution of the split intein (Figure 3e). A rapid response was detected with the product being formed within 10 min of rapamycin induction. Success was also achieved using a directed evolution approach on the *Mycobacterium tuberculosis* RecA intein, with a natural ligand-binding domain inserted. Intein variants were obtained that are highly dependent on a cell-permeable small synthetic compound 4-hydroxytamoxifen (Figure 3f) [43••]. Furthermore, a switch mechanism was rationally designed by creating a chimeric intein carrying a thyroid hormone binding domain, which resulted in an allosteric intein variant capable of undergoing thyroid hormone induced splicing in *E. coli* (Figure 3f) [44•]. These successful attempts make it possible to regulate protein splicing, thereby activating arbitrary target proteins using a single small-molecule activator in a dose-dependent manner.

To target inteins present in bacterial and fungal pathogens, the ultimate goal is the discovery of small molecules or proteins that can block the protein splicing of native inteins. Although several screening systems have been developed, a compound that effectively inhibits splicing of a native intein *in vivo* has not yet been reported [40,45]. The complete inhibition of intein activity represents a major hurdle in developing an effective drug for targeting *M. tuberculosis* inteins.

Conclusions

The discovery of protein self-splicing and the investigation of the chemical mechanisms that nature employs to build and breakdown proteins has provided an enormous opportunity to develop novel strategies for protein engineering. Conditional protein splicing provides an alternative route for turning protein function on or off in a temporal and dose-dependent manner. In addition to its ability to cyclize proteins, a naturally or artificially split intein can be used to produce active proteins either by *trans*-splicing or through intein-mediated protein complementation. Furthermore, by taking advantage of a transient thioester formed by an acyl rearrangement catalyzed by an intein N-terminal cysteine residue, various forms of proteins can now be generated by combining *in vivo* expression and *in vitro* synthesis approaches. Such proteins could be used to produce protein or peptide arrays with improved binding and orientation, providing the prerequisite for high-throughput screening and quantitative analysis of protein function. These new approaches are rapidly changing our view on how proteins can be produced and targeted and therefore hold great promise to meet the needs and challenges of functional protein analysis.

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