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"Splicing up" drug discovery. Cell-based expression and screening of genetically-encoded libraries of backbone-cyclized polypeptides☆

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ABSTRACT

The present paper reviews the use of protein splicing for the biosynthesis of backbone cyclic polypeptides. This general method allows the *in vivo* and *in vitro* biosynthesis of cyclic polypeptides using recombinant DNA expression techniques. Biosynthetic access to backbone cyclic peptides opens the possibility to generate cell-based combinatorial libraries that can be screened inside living cells for their ability to attenuate or inhibit cellular processes thus providing a new way for finding therapeutic agents.

Contents

1. Introduction

A significant number of natural products with a wide range of pharmacological activities are derived from cyclic polypeptides. In fact, peptide cyclization is widely used in medicinal chemistry to improve the biochemical and biophysical properties of peptide-based drug candidates [1,2]. Cyclization rigidifies the polypeptide backbone structure, thereby minimizing the entropic cost of receptor binding and also improving the stability of the topologically constrained polypeptide. Among the different approaches used to cyclize polypeptides, backbone or head-to-tail cyclization remains one of the most extensively used to introduce structural constraints into biologically active peptides.

Despite the fact that the chemical synthesis of cyclic peptides has been well explored and a number of different approaches involving solid-phase or liquid-phase exist [3–7], recent developments in the

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fields of molecular biology and protein engineering have now made possible the biosynthesis of cyclic peptides (Scheme 1). This progress has been made mainly in two areas, non-ribosomal peptide synthesis [8–10] and Expressed Protein Ligation/protein trans-splicing [11–16]. The former strategy involves the use of genetically engineered non-ribosomal peptide synthetases and is reminiscent of more established technologies that yield novel polyketides. The later strategy relies on the heterologous expression of recombinant proteins fused to modified intein protein splicing/trans-splicing units [17].

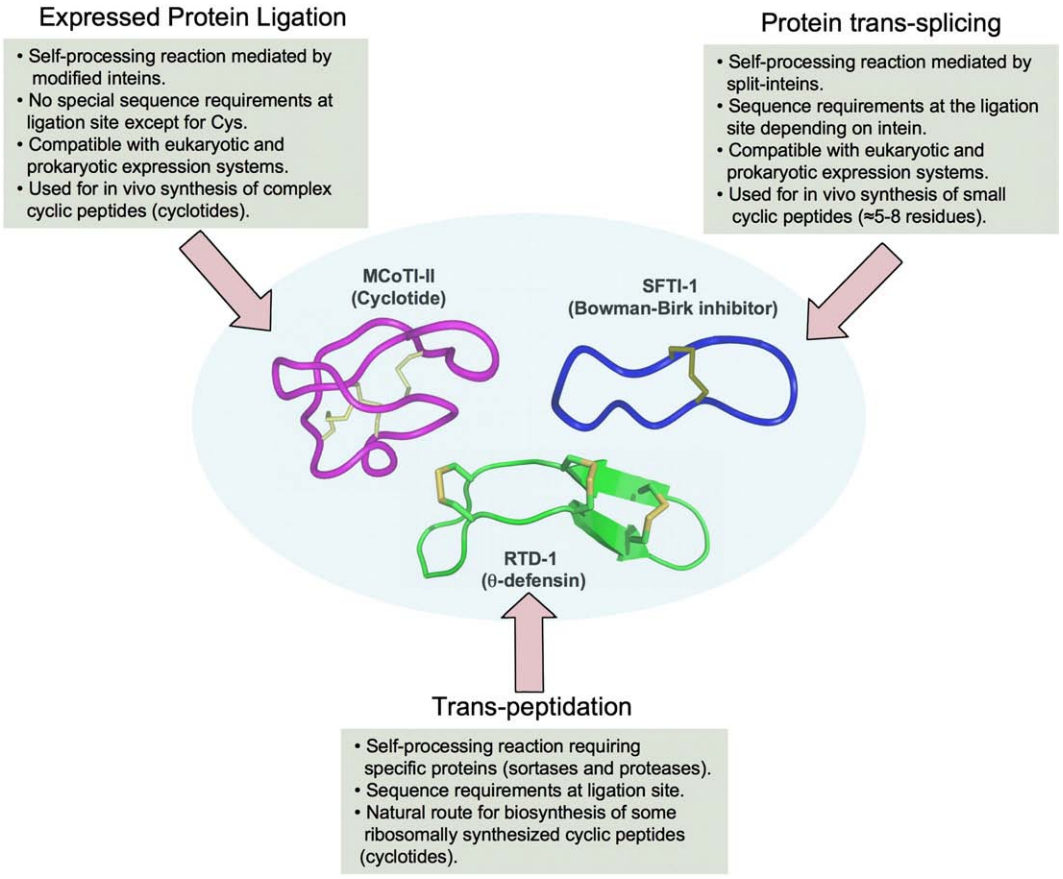
The biosynthesis of cyclic polypeptides offers many advantages over purely synthetic methods. Using the tools of molecular biology, large combinatorial libraries of cyclic peptides, may be generated and screened *in vivo*. A typical chemical synthesis may generate 10^4 different molecules. It is not uncommon for a recombinant library to contain as many as 10^9 members. The molecular diversity generated by this approach is analogous to phage-display technology. Moreover, this approach takes advantage of the enhanced pharmacological properties of backbone-cyclized peptides as opposed to linear peptides or disulfide-stabilized polypeptides. Also, the approach differs from phage display in that the backbone-cyclized polypeptides are not fused to or displayed by any viral particle or protein, but remain on the inside of the living cell where they can be further screened for biological activity in an analogous way as the yeast two-hybrid technology works [18]. The complex cellular cytoplasm provides the appropriate environment to address the physiological relevance of potential leads.

Protein trans-splicing had been successfully used by Benkovic et al. to generate backbone cyclized or polypeptides *in vivo* [12]. In this approach, the peptide to be cyclized was nested between the two split intein fragments of the naturally occurring *Ssp* DnaE split intein [19] (usually referred as *N*- and *C*-inteins) in such a way that the N-terminus of the peptide template is fused to *C*-intein fragment and *vice versa*. Protein splicing of this chimeric protein lead to the formation of the desired cyclic peptide inside *E. coli* cells. A potential limitation of this approach, however, was the requirement for specific *N*- and *C*-extein residues at the intein junction sites [20]. These amino acids were necessary for efficient protein splicing to occur, which restricts the sequence diversity within the sequence of the cyclic peptide.

An attractive alternative approach to the biosynthesis of circular polypeptides was the use of an intramolecular version of the Native Chemical Ligation reaction [21–23]. The present paper reviews the use of these processes for the biosynthesis of circular polypeptides (i.e. peptides and proteins) and it will discuss also the potential of these methods for the biosynthesis of cyclic polypeptide libraries inside living cells as a complementary source for the rapid discovery of new therapeutics.

2. Native Chemical Ligation

Native Chemical Ligation (NCL) is an exquisitely specific ligation reaction that has been extensively used for the total synthesis, semi-synthesis and engineering of different proteins [22,24–26]. In this reaction, two fully unprotected polypeptides, one containing a C-terminal α-thioester group and the other a N-terminal Cys residue, react chemoselectively under neutral aqueous conditions with the formation of a native peptide bond (Fig. 1A). The initial step in this ligation involves the formation of a thioester-linked intermediate, which is generated by a trans-thioesterification reaction involving the



Scheme 1. Summary of the technologies used for the biosynthesis of backbone-cyclized peptides. All these methods rely on the ribosomal synthesis of protein precursors that undergo protein splicing mediated by inteins, proteases or sortases. Three examples of naturally occurring backbone-cyclized peptides with potential therapeutic value are also shown in the middle of the scheme. MCoTI-II is a naturally occurring cyclotide with trypsin inhibitory activity found in the seeds of tropical squash (*Momordica conchichinensis*) [74,75] (PDB entry: 1IB9). SFTI-1 is a Bowman–Birk protease inhibitor found in the seeds of sunflower (*Helianthus annuus*) [76,77] (PDB entry: 1JBN). Both peptides have been biosynthesized using EPL [49]. RTD-1 is a primate defensin with strong antibacterial and antiviral activity [78,79] (PDB entry: 1HVZ).