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Genetic Selection of Cyclic Peptide Dam Methyltransferase Inhibitors

Todd A. Naumann,[a, b] Ali Tavassoli,[a, c] and Stephen J. Benkovic*[a]

Enzymatic methylation of specific DNA bases is fundamental to the survival and propagation of a variety of organisms, including humans. DNA methyltransferases control and regulate a variety of cellular processes, and due to the central role these processes play in the organism's lifecycle, bacterial methyltransferases are attractive targets for the development of new antibiotics. The *Escherichia coli* dam methyltransferase protein (EcoDam) is an N-6 adenine methyltransferase that methylates the GATC palindrome by transfer of a methyl group from *S*-adenosyl-L-methionine (SAM). EcoDam functions in a number of diverse and important cellular processes, the most well studied being postreplicative DNA mismatch repair[1] and control of DNA replication.[2,3] In uropathogenic *E. coli*, EcoDam activity is required for conversion to and maintenance of the virulent phenotype.[4,5]

EcoDam also effects movement of some bacterial transposons[6,7] including Tn*5* transposase (Tnp), which facilitates movement of any DNA sequence bordered by two 19 bp Tnp-binding sites termed inside end (IE), (Figure 1). EcoDam catalyzed methylation of IE inhibits transposition by Tnp. Transposition is restored by using a *dam* negative *E. coli* strain, or through the use of a mutant Tnp that is insensitive to the methylation state of IE.[8]

Here we report the development and application of a genetic-selection methodology that links in vivo EcoDam activity to that of the Tn5 transposase. The screen was combined with our split intein-mediated circular ligation of peptides and proteins (SICLOPPS)[9] technology to yield a rapid and powerful method that was used here to uncover a new class of cyclic peptide EcoDam inhibitors. SICLOPPS utilizes the *Synechocistis* sp. DnaE trans intein, degenerate oligonucleotide synthesis, and standard DNA-cloning techniques for the biosynthesis of cyclic-peptide libraries of up to $\sim 1 \times 10^8$ members (Figure 2).[10] These libraries have been used in conjunction with bacterial reverse two-hybrid systems to identify cyclic-peptide inhibitors of a variety of protein–protein interactions.[11,12]

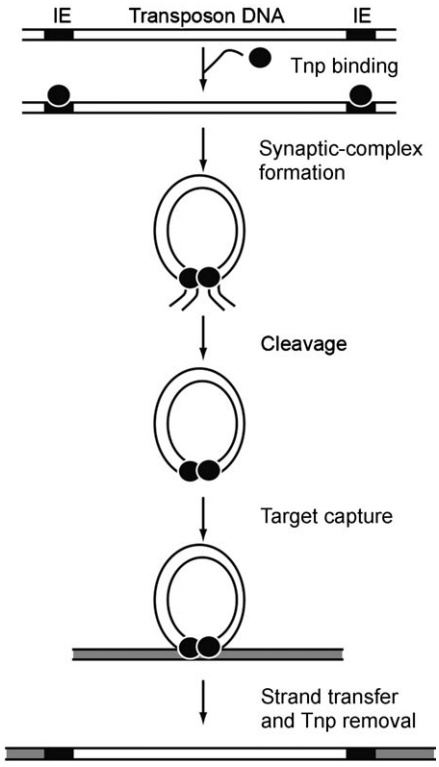

Figure 1. Tn5 functions by a "cut-and-paste" mechanism. Two monomers of Tnp bind to the 19 bp IE sequences and dimerize to form a nucleoprotein-synaptic complex. Tnp catalyzes all DNA-breaking and -joining steps, and the transposon is removed from its initial location and inserted into a new one.

In this system, SICLOPPS is expressed under conditions that are unfavorable to transposition of the methylation sensitive Tnp, due to the activity of the native EcoDam. Hence, the system will select for encoded cyclic peptides that activate transposition through inhibition of DNA methylation. SICLOPPS and chloramphenicol (Cam) resistance genes are encoded within the IE-bordered transposon, (Figure 2 A) and allow the amino acid sequence of potential cyclic-peptide inhibitors to be readily identified by the transposed element. The isolation of the transposed genes is accomplished by conjugation of F-factor DNA (which contains gentamycin (Gen) resistance gene) from the initial strain into a recipient strain that is resistant to the DNA-gyrase inhibitor nalidixic acid (Nal). Transposed SICLOPPS genes are isolated by selecting for strains that are resistant to a combination of Gen (F-factor), Cam (transposed element, including SICLOPPS), and Nal (recipient strain); this selects for cyclic-peptide inhibitors of EcoDam (Figure 3).

We tested the viability of this approach by recovering a transposed SICLOPPS gene that encoded for an EcoDam inactive cyclic peptide (CFTNVHPQFANA) from an *E. coli* strain that contained a plasmid encoding a mutant Tnp that is insensitive

[a] *Dr. T. A. Naumann,[+] Dr. A. Tavassoli,[+] Prof. S. J. Benkovic*
Department of Chemistry
The Pennsylvania State University
University Park, PA 16802 (USA)
Fax: (+1) 814-865-2973
E-mail: sjb1@psu.edu

[b] *Dr. T. A. Naumann[+]*
USDA/ARS/NCAUR
Peoria, IL 61604 (USA)

[c] *Dr. A. Tavassoli[+]*
School of Chemistry, University of Southampton
Southampton, SO16 1BJ (UK)

[+] *These authors contributed equally to this work.*

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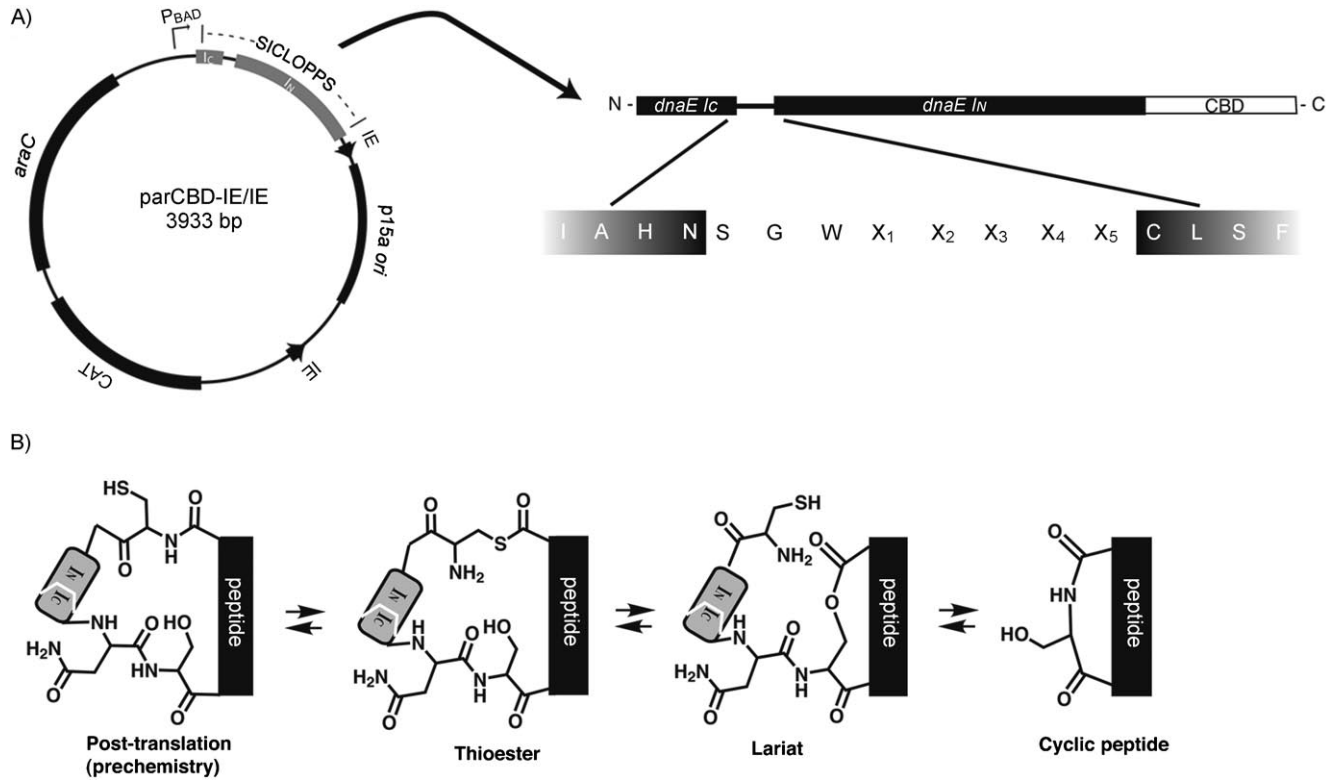

Figure 2. Intein-catalyzed cyclic-peptide production. A) Plasmid parCBD encodes the SICLOPPS gene. In this study the cyclic-peptide library contained three constant residues (SGW) followed by five random amino acids. B) After transcription and translation, the intein catalyzes a series of chemical rearrangements that result in the formation of the cyclic peptide.

to methylation of IE sequences (Tnp-I). As the control peptide does not inhibit EcoDam, we did not isolate any transposed genes from a corresponding strain with a methylation-sensitive Tnp (Tnp-S). We thus turned our attention to constructing and screening a cyclic-peptide library for inhibitors of EcoDam.

The SICLOPPS library[10,13] was constructed in the parCBD-IE/IE plasmid with oligonucleotides that encoded for cyclic peptides of type SGWXXXXX (X: any amino acid). The invariable motif of the peptide was designed to contain serine (which is a required nucleophile for intein processing), glycine (which avoids racemization during chemical synthesis), and tryptophan, which functions as a chromophore for HPLC purification. The initial library size recovered after electroporation into *E. coli* (DH5α) was estimated to be 2.4×10^7. Isolated plasmids from this library were then transformed into the selection strain (RZ212) that contained the F-factor and a plasmid that encoded Tnp-S, and recovered at an estimated size of 3.4×10^8. The library was diluted and grown under either low- (13 μM arabinose) or high-induction (130 μM arabinose) conditions on solid media. After overnight growth, cells were harvested, diluted, and plated onto new plates for four cycles. Conjugation of the F-factor into the recipient strain allowed the isolation of transposed SICLOPPS genes. Dilutions of each conjugation mixture were plated on Luria–Bertani medium (LB) with Nal + Gen, and the efficiency of F-factor transfer was estimated ($\sim1.5\times10^7$). The remaining media was plated on LB + Nal + Gen + Cam to recover recipient cells with transposon-containing F-factors coding for active cyclic-peptide inhibitors of EcoDam ($\sim$200 colonies per plate).

In order to eliminate the numbers of false positives and give a competitive advantage to the most effective inhibitors, a second round of selection was performed. A new SICLOPPS library was constructed from the first round of selectants (PCR templates) followed by cloning into parCBD-IE/IE. The ligation product was transformed directly into the selection strain at a rate of 3.4×10^5. The library was again grown on solid media under low or high induction for four days followed by conjugation of F-factors into the recipient strain. The number of colonies that grew on agar with Nal + Gen + Cam was again in the hundreds. DNA sequencing of the transposed element from randomly picked colonies indicated that many of the surviving colonies contained the same cyclic peptides. From the low-induction library eight sequenced colonies yielded the same peptide, SGWYVRNM. From the high-induction plate, one sequence contained a stop codon while the remaining seven encoded either SGWKHNGG (5 colonies) or SGWPYKWM (2 colonies). The considerable sequence homology in the genetically selected peptides should be noted, especially the conserved $[K/R]_3$–X_4–M_5 motif. This identified epitope could serve as the basis for peptidomimetic evolution of these inhibitors.[14]

The effect of the selected cyclic peptides on transposition was assayed in vivo by transforming each of the plasmid constructs that coded for the active cyclic peptides and a negative-control peptide back into the selection strain. The result-

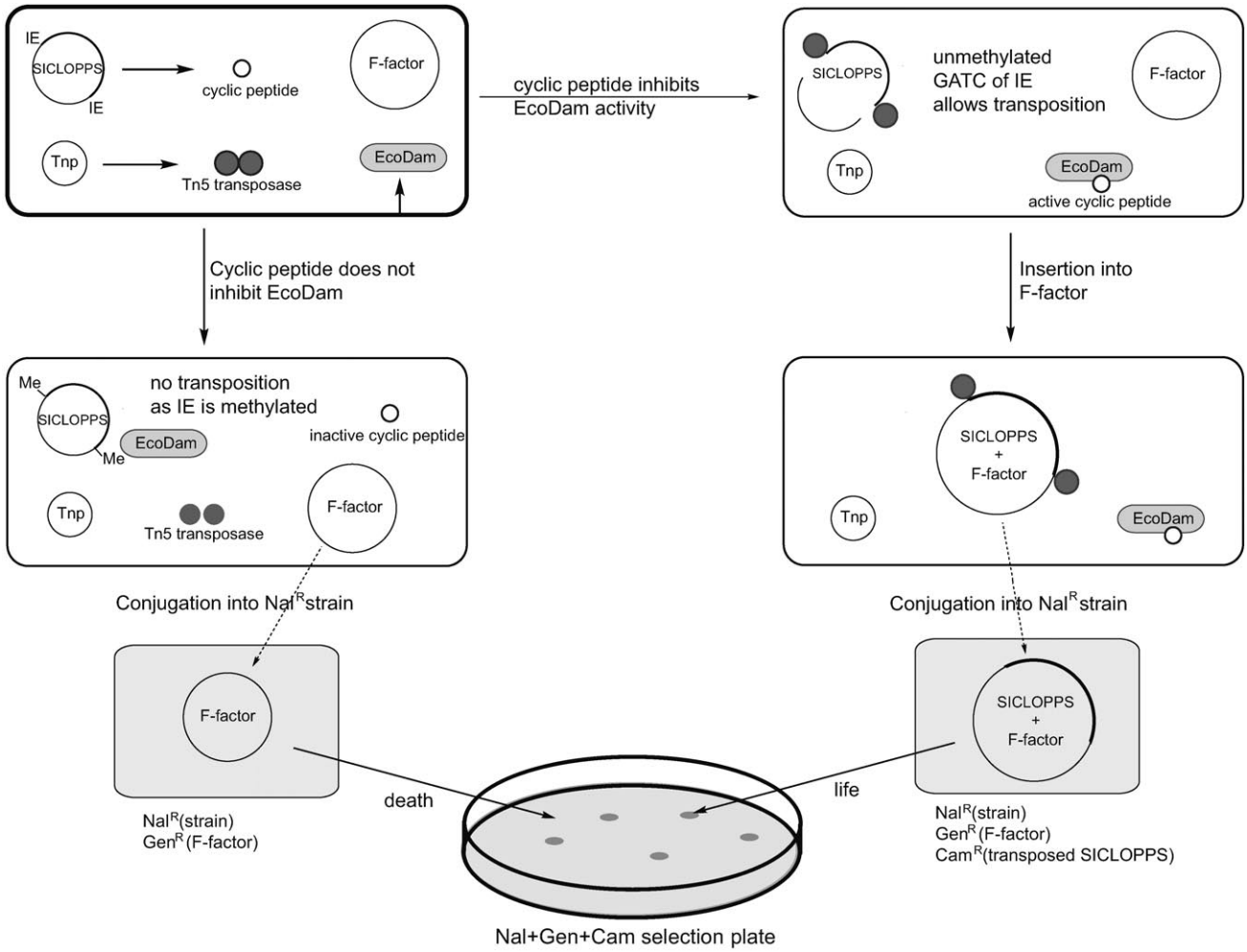

Figure 3. The selection strain is grown in the presence of arabinose to induce SICLOPPS expression. In the majority of cases the cyclic peptide is inactive against EcoDam; this allows IE methylation and no transposition. In instances in which cyclic peptides inhibit EcoDam, IE is unmethylated and transposition can occur. Transposons that insert into F-factor are isolated by conjugation into a recipient strain followed by plating on Nal + Gen + Cam. White cells represent the initial strain, RZ212, with SICLOPPS transposon plasmid parCBD-IE/IE (SICLOPPS), Tnp encoding plasmid pRZ9905 (Tnp), and the F-factor pOX-Gen (F-factor). Shaded cells represent the Nal resistant recipient 14R.

ing strains were grown, overnight, on solid media with low arabinose induction of SICLOPPS, followed by F-factor conjugation into the recipient strain. After conjugation, a small dilution was plated onto agar with Nal + Gen to determine the efficiency of F-factor transfer while the remaining volume was plated on agar with Nal + Gen + Cam to determine the frequency of transposon containing F-factors. The results (Table 1) show that the selectants increased transposition approximately tenfold relative to the negative control.

Table 1. Quantitative in vivo analysis of selected SICLOPPS genes.

Peptide	Nal + Gen [mL]	Nal + Gen + Cam [mL]	Frequency	Relative frequency
CFTNVHPQFANA	2.39×10^{7}	1	4.20×10^{-8}	1.0
SGW**KHNGG**	3.88×10^{7}	16	4.12×10^{-7}	9.8
SGW**YVRNM**	2.20×10^{7}	12	5.33×10^{-7}	12.8
SGW**PYKWM**	2.55×10^{7}	10	3.92×10^{-7}	9.3

The quantitative in vivo transposition assay confirmed increased transposition by the SICLOPPS gene product, but this only indirectly indicates EcoDam inhibition by the cyclic peptides. We wished to unequivocally demonstrate and characterize the activity of the selected cyclic peptide by using in vitro EcoDam assays. To this end we chemically synthesized the three selectants using standard peptide coupling techniques.[12] After synthesis and cyclization, the peptides were purified by reversed-phase HPLC. The chemical nature of the peptides was confirmed by comparison with biologically prepared samples by using reversed-phase HPLC and electrospray ionization mass spectrometry. The cyclic peptides and their linear counterparts were tested by EcoDam filter-binding assays,[15] at concentrations between 0 and 1 mM to determine their relative activity and IC_{50}. Concentration-dependent inhibition was observed for each cyclic peptide, while all linear peptides were inactive at 1 mM concentration. The IC_{50} for each cyclic peptide was determined by linear-regression analysis of the reciprocal of counts per minute (CPM^{-1}) per peptide con-

centration, and calculated as the concentration necessary to reduce the CPM to half of the value achieved in the absence of inhibitor (Table 2). The IC_{50} of sinefungin (a naturally occurring inhibitor of EcoDam) was also determined for comparison with the selected peptides. A cyclic-peptide inhibitor of ATIC homodimerization (SGWMFLNV)[12] was used as negative control.

Table 2. In vitro EcoDam inhibition assays.

Compound	Linear IC_{50}	Cyclic IC_{50}	*R* value
SGW**YVRNM**	>1 mM	50 μM	0.950
SGW**PYKWM**	>1 mM	121 μM	0.960
SGW**KHNGG**	>1 mM	144 μM	0.983
sinefungin	51 μM		0.993
SGW**MFLNV** (control)	>1 mM	>1 mM	

Interestingly, the relative activity of the cyclic peptides in the in vivo transposition assay closely matched their activity in the in vitro EcoDam assays. The most active peptide in both assays (SGWYVRNM) showed similar EcoDam inhibition to sinefungin, with an IC_{50} of 50 μM. The other two selected peptides were around 2–3-times less active. The linear counterparts of the selected peptides were inactive in the assay; this highlights the crucial role cyclization plays in their activity against EcoDam. The negative control was found to be inactive at concentrations of up to 1 mM.

The high concentration of SAM and DNA required, combined with their low K_d, make filter-binding assays unsuitable for determining the mechanism of inhibition. Work is currently underway with fluorescence-based in vitro assays to decipher the mechanism of action of these inhibitors.

To probe the EcoDam specificity of the cyclic-peptide inhibitors we assayed each peptide against the bacterial C-5 cytosine methyltransferase *Hha*I. All three selected cyclic peptides were ineffective against *Hha*I at concentrations of 1 mM or less. This is in contrast to the SAM analogue sinefungin, which is a broad-spectrum methyltransferase inhibitor. The cyclic peptides' apparent specificity against EcoDam is of great interest, as it could potentially allow the development of antibacterial compounds that selectively target EcoDam in the presence of the host organism's methyltransferase.

In summary, we report a new genetic selection methodology that exploits the inhibition of Tnp binding to IE by EcoDam methylation. The methodology was used to uncover a series of small-molecule inhibitors of EcoDam, the activities of which were confirmed in vivo and in vitro. These compounds represent a striking structural departure from the SAM analogues generally targeted against methyltransferases. While the genetic selection was devised for use with SICLOPPS, it could also be used in conjunction with peptide and nucleic acid aptamer libraries,[16,17] siRNA,[18] or cDNA expression libraries.[19]

Keywords: cyclic peptides · DNA methylation · EcoDam · genetic selection · peptides

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