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Communication

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Direct Conjugation of Peptides and 5-Hydroxymethylcytosine in DNA

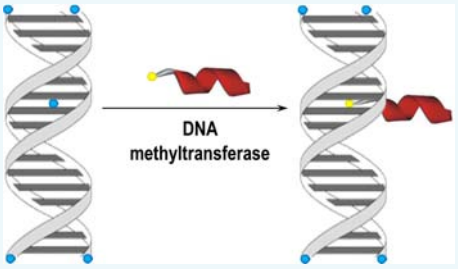
Saulius Serva*[,†,‡] and Arūnas Lagunavičius[†]

[†]Thermo Fisher Scientific Baltics, Graičiūno 8, Vilnius LT-02241, Lithuania

[‡]Department of Biochemistry and Molecular Biology, Faculty of Natural Sciences, Vilnius University, MK Čiurlionio 21/27, LT-03101 Vilnius, Lithuania

Supporting Information

ABSTRACT: Recent discovery of functional 5-hydroxymethylcytosine in vertebrate genomes prompted for elaboration of methods to localize this modification at the nucleotide resolution level. Among several covalent modification-based approaches, atypical activity of cytosine-5 DNA methyltransferases to couple small molecules to 5-hydroxymethylcytosine stands out for acceptance of broad range of ligands. We went further to explore the possibility for methyltransferase-maintained coupling of compounds possessing autonomous functions. Functionalization of DNA was achieved by direct conjugation of chemically synthesized peptides of regular structure. Sequence, residue, and position-specific coupling of DNA containing 5-hydroxymethylcytosine and different peptides has been demonstrated, with the nature of the resulting conjugates confirmed by protease treatment and mass spectrometry. Coupling products were compatible with affinity-driven separation from the unmodified DNA. This approach highlights an emerging avenue toward the enzymatic, sequence-specific DNA functionalization, enabling a single step merge of the DNA and peptide moieties into a bifunctional entity.



INTRODUCTION

Almost 150 natural modified nucleotides are found in nucleic acids, most of them in RNA.[1] In mammalian DNA, 5-methylcytosine (mC) for a long time was exclusively regarded as the "fifth base" and just recently joined by 5-hydroxymethylcytosine (hmC).[2,3] Prominent roles established for 5-methylcytosine[4,5] raised strong interest for the hmC, and indeed, quantitative and functional connection between mC and hmC was confirmed for mammalian cells[6−8] along with identified enzymes driving this process.[3,9]

Availability of the hydroxyl group present in hmC has been considered for sample enrichment and specific labeling. While antiserum-based enrichment suffers from dependency on the density of targets,[7] T4 bacteriophage β-glucosyltransferase-driven glucosylation reaction of hydroxyl moiety reportedly avoids this limitation.[10,11] HmC can be further modified with help of bacterial DNA methyltransferases HhaI and SssI using small thiol- or selenol-bearing molecules,[12] thus providing no immediate functionality to the resulting products. Moreover, the same enzymes have been found to perform in vitro hydroxymethylation of target cytosine in DNA.[13] Remarkably, removal of the hydroxymethyl group from the hmC executed by the same enzymes has also been observed.[13]

Nucleobase-residing hydroxyl group of hmC makes it unique among other DNA nucleotides. Generally, hydroxyl groups are considerably reactive, providing an attractive target for further modification. Peptides are promising candidates for conjugation with DNA. In nature, proteins or peptides are seldom conjugated to DNA and permanent linkage offers an exclusive possibility to combine properties of both into one molecule. Previously, peptides have been attached to oligonucleotides to improve cellular delivery of antisense or antigen compounds,[14] and preparation of DNA vaccines,[15] immobilization of peptides or proteins on DNA arrays,[16,17] decoration of supramolecular DNA structures with protein functions,[18,19] and even modification of enzymes with molecular springs that consist of single stranded DNA were elaborated for the control of enzyme activity.[20−22]

In general, there are two main strategies enabling the conjugation of peptides to DNA: total stepwise solid-state synthesis and solution-state or solid-state fragment coupling.[23] Different chemistries are employed for coupling; thioether formation, disulfide linkages, or "click chemistry" provides access to peptide−DNA conjugates.[24,25] Most often, 5′ or 3′ terminal hydroxyls of oligonucleotide are used for the conjugation, posing certain restrictions on the functionality of the conjugates and making it impossible to employ DNA fragments lacking them. Recently, native chemical ligation was reported for the synthesis of internally modified oligonucleotide−peptide conjugate.[26] Regardless of the strategy, oligonucleotide−peptide conjugation requires either special phosphoamidite compounds introduced during the synthesis of substrate DNA or organic chemistry approaches for coupling, making

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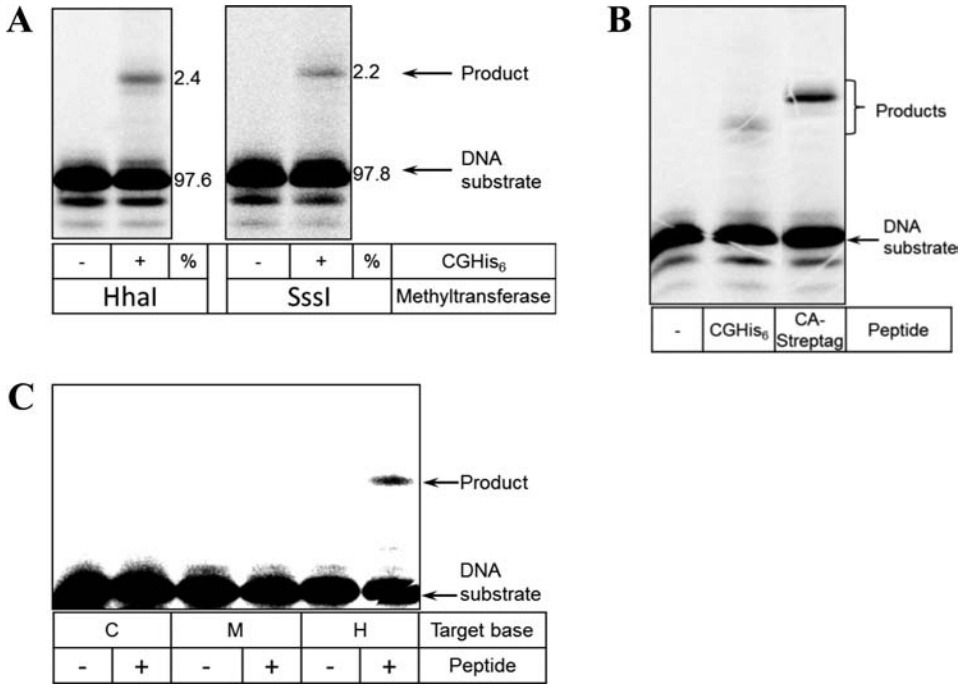

Figure 1. DNA methyltransferase-maintained coupling of DNA and peptides. (A) 5 nM DNA duplex containing hmC at the methyltransferase target position (on ^{33}P-labeled strand) was treated with 200 nM HhaI or 500 nM SssI in the absence (−) and presence (+) of 5 mM peptide N-CGHHHHHH for 1 h at room temperature. Next to coupling reactions, percentiles of substrate/product distribution are provided. (B) 5 nM DNA duplex containing hmC at the target position (on ^{33}P-labeled strand) was treated with 500 nM HhaI in the absence (−) and presence of 5 mM peptides N-CGHHHHHH (marked as CGHis$_6$) or N-CAWSHPQFEK (marked as CA-Streptag), respectively, for 1 h at room temperature. (C) 10 nM DNA duplex containing cytosine (marked as C), 5-methylcytosine (marked as M), or 5-hydroxymethylcytosine (marked as H) at the methyltransferase target position (on ^{33}P-labeled strand) was treated with 5 μM HhaI in the absence (−) and presence (+) of 5 mM peptide N-CGHHHHHH for 1 h at room temperature.

them barely compatible with standard molecular biology techniques and equipment.

In summary, there is an apt interest in 5-hydroxymethylcytosine as novel, naturally occurring nucleobase in DNA. Its chemical structure is permissive and favorable for further modification. We report exploration of innate functionality present in hmC by direct conjugation of chemically synthesized peptides. Sequence, residue, and position-specific coupling of DNA containing 5-hydroxymethylcytosine to different peptides have been demonstrated by the reaction, novel for DNA methyltransferases. The resulting products were compatible with affinity-driven separation from the unmodified DNA. This approach highlights an emerging avenue toward site-specific DNA functionalization enabling the merge of the properties of DNA and protein worlds executed by enzyme-driven single step approach.

■ RESULTS AND DISCUSSION

The ability of cytosine-5 DNA methyltransferases HhaI and SssI to perform conjugation of 5-hydroxymethylcytosine with thiol- or selenol-possessing entities has been described recently.[12] It became immediately evident that the compounds involved in conjugation are small molecules, devoid of any inherent structural regularity. More important, these compounds provide no immediate functionality onto the resulted conjugation products, so serving merely as intermediates for further modification. The major scope of our work was directed to targeted coupling of 5-hydroxymethylcytosine with peptides to render the resulting conjugate properties typical for proteinaceous compounds. The general principle of the coupling was the use of cofactor-free DNA methyltransferase for mediating the extension of hydroxymethyl moiety of target nucleobase defined by the recognition sequence of enzyme used for conjugation. In particular, cytosine−5 DNA methyltransferases HhaI and SssI were demonstrated to maintain the coupling reaction.

Fully complementary DNA duplex substrate containing modified inner cytosine in sequence 5′-G<u>C</u>GC-3′ was formed by annealing of two synthetic oligodeoxyribonucleotides. One strand contained 5-hydroxymethylcytosine and another contained 5-methylcytosine, both at the target position of corresponding methyltransferases. This substrate was treated with cofactor-free HhaI or SssI in the presence of octapeptide N-CGHHHHHH (CGHis$_6$ in Figure 1 A). The reaction yielded product, migrating in denaturing PAA gel clearly more slowly than DNA substrate (Figure 1 A, lanes "+"). Densitometric analysis of product composition revealed the outcome of the reaction accounting for 2.4% (in case of HhaI) or 2.2% (SssI) of the total amount of substrate DNA involved in this reaction. As demonstrated in Figure 1 A, control reactions performed by omitting peptide (lanes "−") yielded no product.

To address substrate specificity, reactions including HhaI and hmC-containing DNA duplex were performed including peptides N-CGHHHHHH and N-CAWSHPQFEK (CA-Streptag in Figure 1 B). As shown in the corresponding lanes of Figure 1 B, products derived from different peptides possess different mobility in a gel in concordance with the different mass of the conjugation partners (1001.05 versus 1232.37 Da) involved in the coupling reaction. It should be noted the

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variable efficiency of the coupling reaction regardless of the same concentration of peptides used, emphasizing the importance of the inherent properties of substrate for the reaction outcome.

Specificity of DNA methyltransferase HhaI-maintained coupling of hmC with peptides was addressed by treating DNA substrate, containing differently modified target base. In particular, DNA duplex substrate containing cytosine along with 5-methylcytosine and 5-hydroxymethylcytosine at the target position for enzyme was used in this experiment. Complementary strand invariantly contained 5-methylcytosine at the corresponding position, ensuring orientation of active site of DNA methyltransferase toward residue on partner strand. DNA substrates were treated with HhaI in the presence of peptide N-CGHHHHHH. As illustrated in Figure 1 C, the only substrate competent for coupling is the one containing hmC at the target position (see sample H, lane "+"). Neither cytosine nor 5-methylcytosine is competent for coupling, demonstrating the specificity of HhaI methyltransferase toward the hydroxymethylated target base.

Proteinaceous properties of DNA and peptide conjugates were addressed next. Following the HhaI-dependent coupling of appropriate DNA duplex and peptide N-CGHHHHHH or N-GSGHHHHHHHHHH (GSGHis$_{10}$ in Figure 2), respectively, samples were aliquoted and one set subjected to proteinase K treatment. As shown in Figure 2, left, reactivity of enzyme toward both peptides is of comparable level. Since their molecular masses differ almost 1.6-fold (1001 Da vs 1590 Da), so differs the mobility of conjugation products in PAA gel. The following treatment by proteinase K significantly truncates the peptide counterpart (see Products section in Figure 2). Product pattern of proteinase K treatment proves the sequence and structural differences of conjugated peptides. Of special note is the absence of cysteine at the N-terminal position in case of peptide N-GSGHHHHHHHHHH, pointing to the possibility for the thiol-independent mechanism for methyltransferase-maintained hmC and peptide conjugation. Conjugation products were also characterized by mass spectrometry. DNA duplex was subjected to HhaI-dependent conjugation involving peptide N-CAWSHPQFEK (CA-Streptag), digested by nuclease P1 with subsequent phosphatase treatment and resulting nucleosides analyzed by an integrated HPLC/ESI-MS. Nucleoside-based compound, matching the expected product of hmC conjugation to CA-Streptag, was observed (see Supporting Information, Part 2, Figure S1).

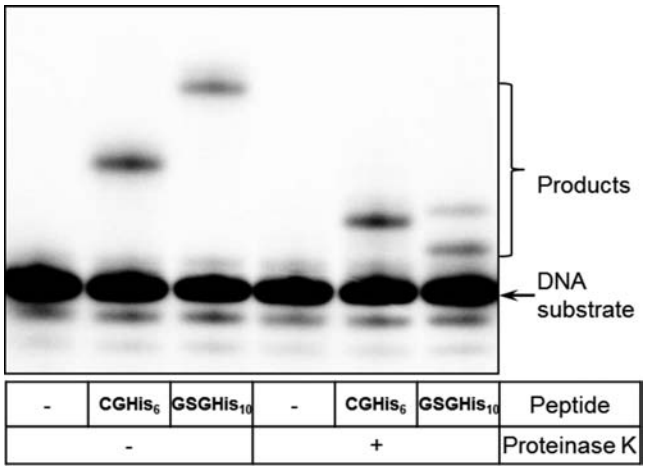

Figure 2. Proteinaceous properties of conjugation products. 10 nM DNA duplex containing hmC at the target position (on ^{33}P-labeled strand) was treated with 1 μM HhaI in the absence ((−) on top row) and presence of 5 mM peptides N-CGHHHHHH (marked as CGHis$_6$) or N-GSGHHHHHHHHHH (marked as GSGHis$_{10}$), respectively, for 2 h at room temperature. Samples subjected to 15 min proteinase K treatment at 55 °C in the presence of 0.4% SDS are indicated as (+) in bottom row.

Functionality of DNA−peptide coupling products was demonstrated by affinity purification using Ni-chelate resin. Briefly, DNA substrate bearing hmC was treated by either HhaI (Figure 3, left part) or SssI (right part) and peptide N-CGHHHHHH, reaction outcome represented by lanes marked as N/A. Then samples were applied on resin prepared in spin column format, incubated for 15 min at room temperature to bind, washed, and eluted using subsequently 50 mM (first eluate) and 500 mM (second eluate) imidazole solution. As shown in Figure 3, 50 mM imidazole was found to be unsuitable for elution, while 500 mM was effective. Purification efficiency was in range of 1600-fold (1677 times for HhaI and 1593 times for SssI), calculated as the difference in substrate to product band intensity ratio in samples before and after purification.

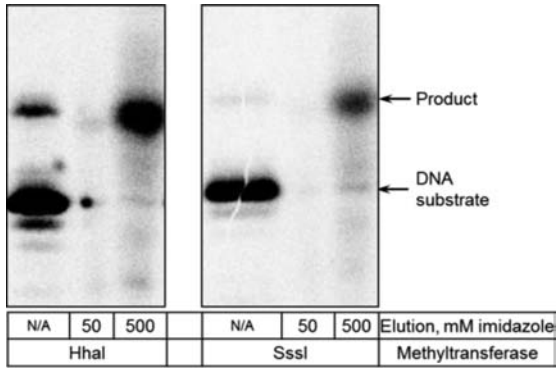

Figure 3. Purification of DNA−peptide coupling products. 10 nM DNA duplex containing hmC at the methyltransferase target position (on ^{33}P-labelled strand) was treated with 1 μM HhaI (left part) or 1.6 μM SssI (right part) and 5 mM peptide N-CGHHHHHH for 1 h at room temperature (indicated as N/A). Then samples were applied on Profinity IMAC resin, washed and eluted using either 50 mM or 500 mM imidazole solution, as indicated.

5-Hydroxymethylcytosine, present in genomic DNA, received much attention upon recent rediscovery of this moiety. Analysis methods of hmC detection were elaborated focusing on either single nucleotide-precision readout of its position or enrichment of DNR fragments possessing this residue. Approaches reported so far enable sequence independent modification of hmC. Generally, they are incapable of rendering the product with genuine properties exploitable without further chemical and/or enzymatic manipulation. We went further to perform targeted sequence-specific coupling of DNA to achieve novel functionalities and so exploit hmC as a universal anchor point for peptide conjugation. The cornerstone of this approach is broad range of sequence preferences driven by specificity of cytosine−5 DNA methyltransferases, the class of enzymes currently hosting 134 members with established specificities toward 61 unique sequences in DNA[27] (retrieved February 2015). Several of them already proved to generate hmC in sequence-specific manner,[12] so making enzymes a

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multitool for both target preparation by hmC introduction and further functionalization, described in this study. This target and tool range offers broad conjugation site repertoire, even though the presence of cytosine at the recognition sequence is required.

Occurrence of transient DNA−protein linkage is common in biological systems, while permanent conjugates proved valuable in manipulations at the molecular level. However, multistep and often harsh conditions for production of such conjugates raise a significant burden for broad acceptance. Peptides, in particular chemically synthesized ones, are beneficial partners due to the diverse structural and functional properties. Conjugation, demonstrated in this study, involved peptides without any chemical alterations, pointing to the capability of employing naturally occurring peptides, too. The targeted sequence-specific mode of DNA conjugation precludes the need for 5′- or 3′-terminus accessibility, required for other conjugation approaches, or the prior internal modification of the substrate DNA by chemical means.

Products of DNA conjugation with proteinaceous compounds were demonstrated to be employable for execution of properties conferred by peptide counterpart. Following the coupling, modified DNA is readily separated from the fraction lacking the 5-hydroxymethylcytosine by affinity chromatography. Purified fraction becomes available for a number of applications, among them sequence readout, immobilization on microchips either in DNA-directed mode[28] or relying on peptide-conferred properties. DNA-directed immobilization of peptides opens new avenues to prepare biochip surfaces, adding power of Watson−Crick base pairing as multiplexing tool.

Conjugation of DNA with peptides possessing cell-penetration properties should be useful for directing of DNA into certain compartments of the given cell. As transfection efficiency of DNA alone is rather poor, addition of positively charged peptide facilitates the process.[23] Combination of DNA methyltransferases possessing different recognition sequences should allow introduction of different peptide-based transfection-directing signals onto the same DNA molecule, so providing with possibility for combination of destinations inside a recipient cell. This approach can potentially be useful both for delivery of the DNA sequence of choice and for research of properties of the peptides of interest. Also, such products might be employed for preparation of efficient DNA vaccines, as introduction of the appropriate epitope into the DNA significantly increases immunologic response.[15]

In conclusion, the propensity of cytosine DNA methyltransferases to maintain conjugation of 5-hydroxymethylcytosine containing DNA with peptides of regular structure has been demonstrated. We confirm the functionality of DNA−peptide conjugation products. This type of DNA−peptide coupling offers numerous advantages including direct functionalization of DNA by proteinaceous compounds, virtually limitless range of peptides as coupling partners, targeted sequence-specific mode of DNA conjugation, substrate peptides requiring no alterations on the structure or composition of reaction, voiding use of special equipment and chemicals. This provides possibilities for another dimension of DNA manipulation, opening ways for merge of the properties of DNA and protein worlds executed by enzyme-driven single step approach.

■ ASSOCIATED CONTENT

Supporting Information

Methods of conjugation and MS data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00165.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: saulius.serva@gf.vu.lt.

Notes

The authors declare no competing financial interest.

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