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Strand-Invasion of Extended, Mixed-Sequence B-DNA by γ PNAs

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Abstract

In this *Communication* we show that peptide nucleic acids (PNAs), 15 to 20nt in length, when preorganized into a right-handed helix, can invade mixed-sequence double helical B-form DNA (B-DNA). Strand-invasion occurs in a highly sequence-specific manner through direct Watson-Crick base-pairing. Unlike the previously developed double-duplex invasion strategy that requires simultaneous binding of two strands of pseudocomplementary PNAs to DNA, only a single strand of γ PNA is required for invasion in this case, and no nucleobase substitution is needed.

Peptide nucleic acids (PNAs) are a promising class of nucleic acid mimics, developed in the last two decades, in which the naturally occurring sugar phosphodiester backbone has been replaced by achiral *N*-(2-aminoethyl) glycine units (Scheme 1A).¹ In addition to their ability to hybridize to complementary DNA or RNA strands, PNAs can invade double-stranded DNA.² Strand-invasion occurs predominantly in two modes, through Watson-Crick base-pairing to form a 1:1³ or in combination with Hoogsteen base-pairing to form a 2:1 (PNA:DNA) complex,⁴ depending on the target sequence. The simplicity and generality of their recognition, along with the ease and flexibility of their synthesis make PNAs an attractive class of antigene reagents. However, with the current design, not every sequence can be accessed by PNAs—in particular, those that are comprised of all four nucleobases. Mixed-sequence PNAs have been shown to be capable of invading supercoiled plasmid DNA^{5–7} and certain regions of genomic DNA,⁸ but they are not capable of invading intact, linear double helical B-form DNA (B-DNA). Recently, two approaches, “tail-clamp”^{9,10} and “double duplex invasion,”¹¹ have been developed, enabling mixed-sequence PNAs to invade B-DNA. But, they are not without limitations. The first approach still requires a stretch of homopurine target for anchoring triplex binding while the second, although more relaxed in sequence selection, requires an elaborate nucleobase substitution and the need to use two strands of PNAs to invade B-DNA—which complicates their design and greatly limits their utility.¹² In this *Communication* we show that mixed-sequence PNAs, 15 to 20 nucleotides in length, when preorganized into a right-handed helix, can invade double helical B-DNA. Strand-invasion occurs in a sequence-specific manner through direct Watson-Crick base-pairing. In this case only a single strand of γ PNA is required for invasion, and no nucleobase substitution is needed.

Recently, we showed that PNAs, which, as individual strands do not have well-defined conformations, can be preorganized into a right-handed helix by installing an (*S*)-Me stereogenic center at the γ -backbone position (Scheme 1A).¹³ These helical γ PNAs exhibit strong binding affinity for complementary DNA and RNA strands. As decamers, mixed-sequence γ PNAs are unable to invade B-DNA, but strand-invasion can be rescued by appending an acridine moiety (DNA-intercalator) to one of the termini¹³ or by replacing a cytosine nucleobase with an energetically more favorable synthetic analog.¹⁴ These results suggest that the binding free energies of the decameric γ PNAs are already near the invasion threshold and

that not much more is needed to invade B-DNA. On the basis of these findings, we speculated that alternative to the aforementioned strategies the required binding free energy may also be attained by extending the size of the γ PNA oligomers since a γ PNA-DNA is generally thermodynamically more stable than a DNA-DNA duplex. If it can be achieved, this design strategy would not only circumvent the need to attach ancillary agents to PNAs, which may compromise their recognition specificity and complicate their synthesis, but also allow γ PNAs to recognize extended DNA targets, which has been a goal of antigene design.^{2,15,16} To test this hypothesis, we synthesized a series of γ PNA oligomers with varied sizes (Scheme 1B) that are designed to bind to both the top and bottom strands of the DNA target, and assessed their invasion capability using a combination of gel-shift and enzymatic and chemical probing assays.

Gel-shift assay was performed by incubating a 291bp-PCR fragment with perfect-match (**PM**) binding site with different concentrations of γ **PNA1** through **3** in 10 mM sodium phosphate (NaPi) buffer at 37 °C for 16 hrs, followed by gel-separation and SYBR-Gold staining. Consistent with our earlier result,¹³ we did not observe binding for γ **PNA1**, a decamer, at γ PNA:DNA ratios as high as 20:1 (Figure 1A, lanes 3 & 4). However, in the case of γ **PNA2** (15mer) and γ **PNA3** (20mer), we noticed the appearance of a shifted band (lanes 5, 6, 8 & 9). The intensity of the new band, relative to that of the free DNA, gradually increased with increasing γ PNA concentrations, and the mobility decreased with increasing oligomer lengths. Formation of this complex appeared to be more efficient with γ **PNA3** than with γ **PNA2**, as reflected in the ratio of the bound-to-unbound DNA (compare lanes 8 with 5 and lanes 9 with 6). This is expected; since γ **PNA3** is longer in length it would form a thermodynamically more stable complex with DNA. Binding appeared to be unique to γ PNAs and occurred in a sequence-specific manner—neither incubation of DNA with **PM** binding site with PNAs of identical nucleobase sequence (**PNA2**, lane 7; **PNA3**, lane 10) nor incubation of DNA with single-base mismatch in the middle (**MM1**) or toward one end (**MM2**) of the binding site with γ PNAs resulted in formation of any shifted band (Figure 1B, lanes 4-9). Similar findings were also observed with γ **PNA4** (15mer) and γ **PNA5** (20mer), which were designed to bind to the top strand of the DNA target (Figure 2). Formation of the retarded band was only observed with **PM** (lanes 1-8) and none with **MM1** (lane 9) or **MM2** (lane 10). However, when an equimolar ratio of γ **PNA3** and γ **PNA5** was added, no retarded band was observed (lane 8). This was expected since the two γ PNA strands are complementary to one another; they would hybridize to each other rather than invade the DNA double helix because a γ PNA- γ PNA is thermodynamically more stable than a γ PNA-DNA duplex. A time-course study revealed that formation of this complex reached equilibrium within ~ 8 hrs of incubation and followed a pseudo-first order kinetic (Figure 3).

To confirm that these γ PNAs bind to their designated site and through a strand-invasion mechanism, we performed S1-nuclease digestion with the 3'-end of the homologous DNA strand labeled with P-32. S1-nuclease was chosen because it is known to selectively cleave single-stranded or melted regions of double-stranded DNA. Invasion of γ PNAs into DNA double helix is expected to result in a local displacement of the homologous DNA strand, which can be revealed in the form of strand cleavage following S1-nuclease digestion. Figure 4 reveals the digestion patterns of a 171bp-PCR fragment containing **PM** (lanes 1-8), **MM1** (lanes 9 & 10) and **MM2** (lanes 11 & 12) binding site following incubation with γ PNAs and PNA. Contrary to the gel-shift assay, where we did not observe any evidence of binding for γ **PNA1**, we observed cleavage on the homologous DNA strand directly across from its binding site (lanes 2 & 3). This result indicates that strand-invasion did take place because of the susceptibility of the homologous strand to S1-nuclease digestion, but the fact that no retarded band was observed in the gel-shift assay indicates that the complex was not sufficiently stable to withstand the prolonged electrophoresis. Similar cleavage patterns were observed for γ **PNA2** and γ **PNA3**, but they were more extensive in coverage—roughly 5 bases further toward

the 3'-end for γ PNA2 (lanes 4 & 5) and 5-8 bases further toward both the 3'- and 5'-end for γ PNA3 (lanes 6 & 7). This was expected based on their recognition coverage. No strand cleavage was observed with unmodified PNA3 (lane 8) or with γ PNA3 that have mismatched targets (lanes 10 & 12). This result was further corroborated by DEPC (diethyl pyrocarbonate) chemical probing assay,^{17,18} which revealed selective cleavage of the homologous DNA strand at the adenine and, to a smaller extent, guanine residues across from their binding site (Figures 1S and 2S). This was only observed with **PM** and none with **MM1** or **MM2** binding site. These results are consistent with γ PNA binding through a strand-invasion mechanism.

Further, we show that γ PNAs can be used to selectively inhibit restriction enzyme digestion. Incubation of a 321bp-DNA target containing **PM** binding site with Bgl II resulted in two DNA fragments—142 and 175bp in length (Figures 5A and B, lane 3). When the DNA target was first incubated with γ PNA3 prior to adding enzyme, the digestion was progressively inhibited with increasing γ PNA3 concentrations (lanes 4 & 5). This result indicates one of two possibilities: that either γ PNA3 bound to its target and thereby blocked the enzyme from cleaving the DNA at its restriction site, or that γ PNA3 'poisoned' the enzyme through nonspecific binding. We ruled out the latter possibility based on the mismatch result. When the DNA fragments containing **MM1** and **MM2** binding site were used, no apparent inhibition of the restriction enzyme digestion was observed (lanes 7 & 9), indicating that the presence of γ PNA3 had no effect on the activity of Bgl II. This result shows that γ PNAs can be used as molecular tools to selectively manipulate the structure of B-DNA,¹⁹ and possibly other genome-sensing applications as well.²⁰ Once formed, the invasion complex remained stable over a prolonged period, even at a relatively high ionic strength as demonstrated in this study.

In summary, we have shown that mixed-sequence PNAs, 15 to 20nt in length, when preorganized into a right-handed helix by installing an appropriate stereogenic center at the γ -backbone, can invade double helical B-DNA. We attribute the improvements in γ PNAs' ability to invade B-DNA to their preorganized structure. This structure allows them to intercept the nucleobase targets more rapidly and stabilize the invasion complex more effectively than their achiral counterparts since minimal structural rearrangement would be required prior or subsequent to invasion. The results reported herein are important because they demonstrate that the same Watson-Crick base-pairing principles that guide the recognition of single-stranded DNA and RNA can also be applied to intact double helical B-DNA. Recognition, in this case, is general to all four nucleobases, and extended target sizes that may be difficult to achieve with other classes of molecules.^{16,21-23}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Nielsen PE, Egholm M, Berg RH, Buchardt O. Science 1991;254:1497–1500. [PubMed: 1962210]
2. Nielsen PE. Acc Chem Res 1999;32:624–630.
3. Nielsen PE, Christensen L. J Am Chem Soc 1996;118:2287–2288.
4. Egholm M, Nielsen PE, Buchardt O, Berg RH. J Am Chem Soc 1992;114:9677–9678.
5. Bentin T, Nielsen PE. Biochemistry 1996;35:8863–8869. [PubMed: 8688422]
6. Smulevitch SV, Simmons CG, Norton JC, Wise TW, Corey DR. Nature Biotechnology 1996;14:1700–1704.

7. Zhang X, Ishihara T, Corey DR. *Nucleic Acids Research* 2000;28:3332–3338. [PubMed: 10954602]
8. Janowski BA, Kaihatsu K, Huffman KE, Schwartz JC, Ram R, Hardy D, Mendelson CR, corey DR. *Nat Chem Biol* 2005;1:210–215. [PubMed: 16408037]
9. Bentin T, Larsen HJ, Nielsen PE. *Biochemistry* 2003;42:13987–13995. [PubMed: 14636067]
10. Kaihatsu K, Shah RH, Zhao X, Corey DR. *Biochemistry* 2003;42:13996–14003. [PubMed: 14636068]
11. Lohse J, Dahl O, Nielsen PE. *Proc Nat Acad Sci U S A* 1999;96:11804–11808.
12. Demidov VV, Protozanova E, Izvolsky KI, Price C, Nielsen PE, Frank-Kamenetskii MD. *Proc Natl Acad Sci USA* 2002;99:5953–5958. [PubMed: 11972051]
13. Rapireddy S, He G, Roy S, Armitage BA, Ly DH. *J Am Chem Soc* 2007;129:15596–15600. [PubMed: 18027941]
14. Chenna V, Rapireddy S, Sahu B, Ausin C, Pedroso E, Ly DH. *ChemBioChem* 2008;9:2388–2391. [PubMed: 18816545]
15. Weyermann P, Dervan PB. *J Am Chem Soc* 2002;124:6872–6878. [PubMed: 12059208]
16. Jantz D, Amann BT, Gatto GJ, Berg JM. *Chem Rev* 2004;104:789–800. [PubMed: 14871141]
17. Scholten PM, Nordheim A. *Nucleic Acids Res* 1986;14:3981–3993. [PubMed: 3714469]
18. Furlong JC, Lilley DMJ. *Nucleic Acids Res* 1986;14:3995–4007. [PubMed: 3012460]
19. Komiyama M, Aiba Y, Yamamoto Y, sumaoka J. *Nat Protoc* 2008;3:655–622. [PubMed: 18388948]
20. Lin J, Lai Z, Aston C, Jing J, Anantharaman TS, Mishra B, White O, Daly MJ, Minton KW, Venter JC, Schwartz DC. *Science* 1999;285:1558–1562. [PubMed: 10477518]
21. Gowers DM, Fox KR. *Nucleic Acid Res* 1999;27:1569–1577. [PubMed: 10075986]
22. Kelly JJ, Baird EE, Dervan PB. *Proc Nat Acad Sci U S A* 1996;93:6981–6985.
23. Kielkopf CL, Baird EE, Dervan PB, Rees DC. *Nat Struct Biol* 1998;5:104–109. [PubMed: 9461074]

Figure 1.

Gel-shift assay following incubation of 0.1 μ M 291bp-DNA fragment with (A) **PM** binding site with different concentrations of γ PNAs (lanes 3-6, 8 & 9) and PNAs (lanes 7 and 10), and (B) **PM** (lanes 1-3), **MM1** (lanes 4-6) and **MM2** (7-9) binding site with different concentrations of γ **PNA3** in 10 mM NaPi buffer at 37°C for 16 hrs, followed by non-denaturing PAGE separation and SYBR-Gold staining. PNA2 and PNA3 contained the same nucleobase sequence as γ **PNA2** and γ **PNA3**, respectively, but without γ -backbone modifications.

Figure 2.

Gel-shift assay following incubation of 0.1 μ M 291bp-PCR fragment with **PM** (lanes 1-8), **MM1** (lane 9) and **MM2** (lane 10) binding site with different concentrations of γ PNAs (γ **PNA4**, lanes 2 & 3; γ **PNA5**, lanes 5, 6, 9 & 10; γ **PNA3** + γ **PNA5**, lane 8) and PNAs (PNA4, lane 4; PNA5, lane 7) under identical condition as denoted in Figure 1. γ **PNA4** and γ **PNA5** were designed to bind to the top strand of the **PM**, **MM1** and **MM2** target. γ **PNA3** and γ **PNA5** are complementary to one another.

Figure 3.

Time-dependent strand-invasion of DNA by γ PNA2 and γ PNA3. 0.1 μ M of a 291bp-PCR fragment with **PM** binding site was incubated with 1.0 μ M of each γ PNA oligomer in 10 mM NaPi at 37 °C for the indicated periods. The mixtures were separated on non-denaturing PAGE and stained with SYBR-Gold. The amounts of the bound and unbound DNA were quantified by ImageJ software.

Figure 4.

S1-nuclease digestion of a 171bp-linear B-DNA with **PM** (lanes 1-8), **MM1** (lanes 9 & 10) and **MM2** (lanes 11 & 12) binding site following incubation with γPNAs and PNA at the indicated ratios in 10 mM NaPi buffer at 37 °C for 16 hrs. The mixtures were reconstituted with 1× S1-nuclease buffer (30 mM NaOAc and 1 mM Zn(OAc)₂) before treating with S1-nuclease (10 units for 20 min at room temperature), followed by separation on denaturing gel and imaged by autoradiography. The 3'-end of the homologous DNA strand was labeled with P-32 using Klenow fragment.

Figure 5. Restriction enzyme digestion of a 321bp-PCR fragment containing **PM**, **MM1** and **MM2** binding site with γ PNA3. **(A)** A schematic diagram showing the *Bgl* II restriction and γ PNA3 binding site along with the size of the predicted DNA fragments following *Bgl* II digestion. **(B)** Restriction digestion patterns of the DNA targets containing **PM** (lanes 2-5), **MM1** (lanes 6 & 7) and **MM2** (lanes 8 & 9) binding site without (lanes 3, 6 & 8) and with (lanes 4, 5, 7 & 9) prior incubation with γ PNA3. Enzyme digestion was progressively inhibited in the presence of a perfect-match **PM** (lanes 4 & 5) and none with the single-base mismatch **MM1** (lane 7) or **MM2** (lane 9).

(B)

Oligomers

γ PNA3: H-Lys-**TATGAGACCACAGATCTAAG**-Lys-NH₂ } designed to bind to the bottom strand
 γ PNA2: H-Lys-**GACCACAGATCTAAG**-Lys-NH₂
 γ PNA1: H-Lys-**GACCACAGAT**-Lys-NH₂

DNA targets (291bp linear B-DNA)

5'.....GATATC**TATGYGACWCAGATCTAAG**CTAGCA.....3'
 3'.....CTATAG**ATACZCTGGXGTCTAGATTTC**GATCGT.....5'

154bp 5 10 5 117bp

Perfect Match (PM): W=A, X=T, Y=A, Z=T

Mismatch 1 (MM1): W=T, X=A, Y=A, Z=T

Mismatch 2 (MM2): W=A, X=T, Y=T, Z=A

γ PNA4: H₂N-Lys-**CTGGTGTCTAGATTTC**-Lys-H } designed to bind to the top strand
 γ PNA5: H₂N-Lys-**ATAGTCTGGTGTCTAGATTTC**-Lys-H

Scheme 1.

(A) Chemical structure of PNA and γ PNA, and (B) sequence of γ PNAs and a selected region of the DNA target.