Sequence-Specific Protection of Duplex DNA against Restriction and Methylation Enzymes by Pseudocomplementary PNAs†

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ABSTRACT: A new generation of PNAs, so-called pseudocomplementary PNAs (pcPNAs), which are able to target the designated sites on duplex DNA with mixed sequence of purines and pyrimidines via double-duplex invasion mode, has recently been introduced. It has been demonstrated that appropriate pairs of decameric pcPNAs block an access of RNA polymerase to the corresponding promoter. Here, we show that this type of PNAs protects selected DNA sites containing all four nucleobases from the action of restriction enzymes and DNA methyltransferases. We have found that pcPNAs as short as octamers form stable and sequence-specific complexes with duplex DNA in a very salt-dependent manner. In accord with a strand-invasion mode of complex formation, the pcPNA binding proceeds much faster with supercoiled than with linear plasmids. The double-duplex invasion complexes selectively shield specific DNA sites from BclI restriction endonuclease and dam methylase. The pcPNA-assisted protection against enzymatic methylation is more efficient when the PNA-binding site embodies the methylase-recognition site rather than overlaps it. We conclude that pcPNAs may provide the robust tools allowing to sequence-specifically manipulate DNA duplexes in a virtually sequence-unrestricted manner.

Synthetic reagents, which are capable of sequence-specific targeting of double-stranded DNA (dsDNA),1 are of significant interest in various fields of life sciences, molecular biotechnology and medicinal chemistry as they may provide tools for highly selective, nondenaturing manipulation with designated DNA sequences. In this connection, PNA, i.e. peptide nucleic acid (1–5),2 represents one of the most promising oligonucleotide modifications exhibiting unique physicochemical and biochemical characteristics.

Until recently, three different modes of PNA binding to dsDNA (via duplex or triplex invasion and through formation of noninvasive triplex) have been described (5, 6). However, they are all have common sequence limitation: generally, one of the two dsDNA strands of the PNA-binding site must consist mostly of purines. Therefore, to expand the practical potential of PNA, an extension of the sequence repertoire for dsDNA recognition by PNAs is warranted. Some progress in relaxing the sequence requirements could be achieved by exploiting variations in the aforementioned recognition schemes (7–12). Though, the most likely way to radically widen the range of dsDNA sites that can be targeted by PNAs is to use other PNA-binding modes.

One solution is the recent development of pseudocomplementary PNAs (pcPNAs) carrying 2,6-diaminopurine (D) and 2-thiouracil (s U) instead of adenine and thymine, respectively, along with ordinary guanine and cytosine (13). This type of PNAs exhibits a distinct binding mode, double-duplex invasion (see Figure 1a), which is based on the Watson-Crick recognition principle supplemented by the notion of pseudocomplementarity. Pseudocomplementarity means that two special derivatives of initially paired normal purine and pyrimidine are structurally adjusted in such a way that they (i) do not match each other, but (ii) are capable of a stable Watson-Crick-type pairing with the natural nucleobase counterparts. The D and sU nucleobases were chosen for the design of the first pcPNAs because they satisfied both these criteria (13, 14; see Figure 1b).

Pseudocomplementary pairs of thus modified PNAs have been found to be capable of targeting the designated dsDNA sites with mixed sequence of purines and pyrimidines. Moreover, a specific pair of decameric pcPNAs has been shown to effectively block an access of T7 RNA polymerase to the corresponding promoter thus inhibiting transcription initiation (13). However, in contrast to the PNA invasion triplexes (15–18), the double-duplex invasion complexes

1 Abbreviations: PNA, peptide nucleic acid; pcPNA, pseudocomplementary PNA; D, 2,6-diaminopurine; U, 2-thiouracil; Lys, l-lysine; dsDNA, double-stranded DNA; scDNA, negatively supercoiled dsDNA; M₀, DNA methylase; R, restriction endonuclease; U, unit of enzyme quantity; EMSA, electrophoretic mobility shift assay; PFGE, pulsed-field gel electrophoresis.

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Figure 1: (a) The double-duplex invasion complex formed by a pair of pcPNAs (stippled lines) inside dsDNA site with mixed composition of all four nucleobases. (b) Modified nucleobases, D instead of A and D instead of T that are incapable of complementarily pairing due to a steric clash, prevent pcPNAs from forming the PNA–DNA duplex. Still, bulky nucleobase substitutions do not prevent pcPNA from forming a stable PNA–DNA heteroduplex with the complementary DNA strand carrying normal nucleobases.

**EXPERIMENTAL PROCEDURES**

Materials and Reagents

Synthesis and purification of pcPNAs were done as described in ref 13. Following pcPNAs have been used in this study:

- **PNA I**: H-Lys$_2$-$^8$UCDDDCD$^+$$^8$UGC-LysNH$_2$
- **PNA II**: H-GCD$^+$$^8$UG$^+$$^8$UGD-LysNH$_2$
- **PNA III**: H-Lys$_2$-$^8$UGD$^+$$^8$UCDD-LysNH$_2$

By normal peptide conventions, PNAs are written from the N terminus (H is a free amino group) to the C terminus (NH$_2$ is a terminal carboxamide). To increase the DNA binding affinity, the PNA termini were equipped with Lys amino acids. PNAs I and III have +4 charges while PNA II has +2 charges at neutral pH. PNA concentrations were determined spectrophotometrically using the following coefficients of extinction at 260 nm: 11 700 (G), 10 200 (U), 7600 (D) and 6600 (C) M$^{-1}$ cm$^{-1}$. Accordingly, 1 OD$_{260}$ corresponds to 12 µM PNA I, 10 µM PNA II, and 14 µM PNA III.

Recombinant plasmids carrying correct and singly mismatched PNA-binding sites were obtained by cloning corresponding binding sites into EcoRI (pSD1 and pSD1/m2) or BamHI (pSD2, pSD2/m2, and pSD1/2) sites of pUC19 polylinker. Plasmids used in the PNA-binding studies were cloned in the dam” strain, high-efficiency competent DH5α. Escherichia coli cells (Gibco BRL). For experiments with restriction and methylation enzymes, plasmid DNA was isolated from the dam” strain of E. coli (GM2163; New England Biolabs).

pSD2 contains the matched target for PNAs I and II, GCATGTGGTGA (the PNA-binding site is capitalized; four last letters, GAtc, correspond to the dam/DpnII recognition site). pSD2/m2 contains the mismatched target for this pair of pcPNAs, GGTATGTTTTGA (here and below, the mismatched nucleobase is underlined). pSD1 and pSD1/m2 contain matched (TTTGATCAAG) and mismatched (TTTATTCAG) targets for PNA III, respectively. pSD1/2 carries the site, TTGATCAAGCTATTTGG, which consists of matched targets for both PNA III and PNAs I/II bordering each other.

Adenovirus-2 DNA was obtained from Sigma. A 490-bp-long fragment of viral DNA containing site $^\text{CTGATCAG}$ with two terminal mismatches for PNA III was isolated by PCR amplification of the 11996–12485 bp region of adenovirus-2 genome. The PNA-binding sites in adenoviral DNA used here were verified by sequencing.

All restriction and methylation enzymes were obtained from New England Biolabs. Restriction enzymes were used in the supplemented reaction buffers. Specifically, R$^\text{Pvu}$II buffer: 100 mM NaCl, 50 mM Tris-HCl, 1 mM dithiothreitol, pH 6.0; R$^\text{Bcl}$I buffer: 100 mM NaCl, 50 mM Tris-HCl, 1 mM dithiothreitol, pH 7.9; R$^\text{Pvu}$II buffer: 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, pH 7.9. M$^\text{dam}$ was used in 25 mM sodium-citrate (pH 7.0) containing 5 mM β-mercaptoethanol and 300 µM S-adenosylmethionine.

Methods. Formation of pcPNA–dsDNA complexes and the effect of pcPNA binding on the DNA digestion with R$^\text{Bcl}$I were monitored by gel electrophoresis in TBE-containing non-denaturing 7.5% polyacrylamide gels. Typically, the DNA concentration in the reaction mixtures was less than 5 nM while the PNA concentration was always higher than 10 nM providing an excess of PNA over DNA. Unbound PNA was removed from solution by gel filtration of samples through Sephadex G-50.

DNA was visualized by ethidium bromide staining and detected by the CCD camera coupled with the IS-1000 digital imaging system/software (Alpha Innotech Corporation). All images are presented as negatives. Quantitative analysis of pcPNA binding to dsDNA was done using a gel-shift assay by measuring the normalized intensities of faster and slower migrating bands (see Figure 2), which correspond to DNA free of PNA and to PNA–DNA complexes, respectively (16, 24–26).

In experiments with M$^\text{dam}$ inhibition, the DNA samples were analyzed either by gel electrophoresis in TAE-containing 1.5% agarose gel or by pulsed-field gel electrophoresis (PFGE) in TBE-containing 1% pulsed-field certified agarose gel using the CHEF Mapper system (Bio-Rad). In case of agarose-embedded DNA, unbound PNA was removed before methylation by washing the DNA sample twice in 100 µL of methylase buffer for 30 min at 37°C. After methylation, the methylase was inactivated and the PNA–DNA complexes were dissociated at elevated temperatures in high-salt buffers thus preventing the PNA rebinding and allowing the prior PNA-protected, unmethylated sites to be digested by restriction enzyme (27). For more details of protocols in experiments on DNA protection against methylation and
restriction enzymes by pcPNAs, see the legends to the corresponding figures.

RESULTS

Double-Duplex Invasion of pcPNA Pairs. We used three pcPNAs: a pair of pseudocomplementary 10-mer PNA I/PNA II and a self-pseudocomplementary 8-mer PNA III (see Materials and Reagents for PNA sequences). First, we studied the binding of pcPNAs to matched and mismatched dsDNA targets by the electrophoretic mobility shift assay (EMSA). Figure 2 shows that PNAs I and II, when employed in combination, formed stable complexes with the correct dsDNA target but not with the singly mismatched one. As expected for double-duplex mode of PNA invasion into dsDNA, these decamer pcPNA oligomers could act only in combination: no binding to the correct DNA target was observed for individual pcPNAs. Note that melting experiments revealed no stable complexes between PNAs I and II at 37 °C (data not shown). Whether such complexes can be observed at lower temperatures, as it was demonstrated for a pair of pseudocomplementary oligonucleotides (14), requires further studies.

By using the EMSA method, we were also able to observe stable binding to dsDNA of a shorter pcPNA, octameric PNA III (see Figure 3). Here, a single pcPNA oligomer featuring a pseudo-match to itself could sequence-specifically form the double-duplex invasion complex within specific dsDNA site due to the self-complementarity of its sequence. Note that although no PNA–DNA complexes were detected when the mismatch was located inside the PNA-binding site, we observed some binding of this PNA to the dsDNA target containing the mismatch at the very end (data not shown).

To obtain additional evidence that the PNA–DNA complexes studied were formed via strand invasion, we analyzed the pcPNAs binding to negatively supercoiled DNA (scDNA) as compared to linear, nonsupercoiled dsDNA. If binding of pcPNAs to dsDNA requires an opening of the DNA double helix, one can expect the PNA–DNA complex formation to be dependent on DNA supercoiling like in case of the PNA triplex invasion (28).

Figure 3A shows that, as expected, matched binding of pcPNAs to negatively supercoiled plasmids was much more efficient as compared with binding to linearized plasmids. Note that the effect of DNA supercoiling on PNA binding was more profound in case of PNA III than in case of PNAs I/II. This can be explained in part by a high AT-content (75% A–T base pairs) and, as a result, a lower stability of the dsDNA target site for PNA III, as compared with moderate AT-content (60% A–T base pairs) of the DNA target site for PNAs I/II. Also, the observed difference in the PNA binding to two supercoiled plasmids may be due to the different native density of DNA supercoiling in both cases.

Figure 3B demonstrates that an enhanced binding of pcPNAs to scDNA allows PNA–DNA complex formation at a high, physiologically relevant salt concentration (160 mM Na+) while no complexes have been observed in case of linear dsDNA under these conditions. We expect that elevated concentrations of salt, which are known to stabilize dsDNA, should effectively inhibit the strand-invasion binding of pcPNA to linear dsDNA. Indeed, as it can be seen in Figure 4, the PNA–dsDNA complex formation is very sensitive to ionic conditions in case of linear dsDNA targets, thus additionally verifying the PNA-to-DNA strand invasion mechanism. Under physiological pH and temperature, the invasion of pcPNAs into linear dsDNA is absent at 80 mM Na+ during 0.5–1 h (and even during more prolonged incubations; data not shown).

1 Note that pcPNAs carry several positive charges to enhance, via electrostatic attraction, their binding to negatively charged DNA (22, 28–31). Hence, salt-induced inhibition of PNA-to-DNA binding can also be ascribed in part to the screening effect of Na+ and Cl– ions on the Coulomb interaction between pcPNA and dsDNA.
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![Graph](Image 57x602 to 292x744)

**Figure 4:** Dependence of the double-duplex invasion complex formation in linear dsDNA on Na⁺ concentration. 2.5 μM PNA III (squares) and 5 μM (total) PNAs I/II (circles) were incubated with pSD1 and pSD2 (both linearized by R′PvuII) at 37 °C for 35 min (PNA III) or 1 h (PNAs I/II) in 10 mM sodium-phosphate buffer (pH 6.9) containing various concentrations of NaCl (total concentration of Na⁺ is indicated in the graph). The yield of PNA–DNA complexes was determined by the EMSA in polyacrylamide gel.

Experiments on full-length plasmid targeting by PNA (Figure 3) indicate that once formed, complexes of pcPNAs with dsDNA remain very stable. Indeed, magnesium cations, which are required for restriction enzyme digestion of PNA-targeted plasmids before the EMSA analysis, strongly stabilize the dsDNA and could potentially induce dissociation of the PNA-dsDNA invasion duplexes. Our experiments show that such complexes not only quantitatively form in case of scDNA at up to 160 mM Na⁺ (and, as our preliminary data indicate, in the presence of 1 mM Mg²⁺) but also survive 1 h incubation in 10 mM Mg²⁺. In a next series of experiments, we studied the ability of pcPNA–DNA complexes to protect selected dsDNA sites from the action of restriction enzymes and DNA methyltransferases.

**Protection of dsDNA by pcPNAs against DNA Methylase.** Figure 5 shows the results of experiments on protection of dsDNA by pcPNAs against enzymatic methylation. In these “Achilles' cleavage” experiments (22, 32), we targeted pSD2 with PNAs I and II. The PNA-binding site of this plasmid overlaps (by 2 bp) only one of nearly a dozen dam/DpnII recognition sites (see Materials and Reagents for sequence). If the PNA binding shielded the corresponding site from enzymatic methylation, although quite high (about 70%), was incomplete. We explain this in terms of the limited protection of the methylation site by pcPNAs I and II. Decreased amounts of M′dam yielded more complete cleavage after methylation due to incomplete methylation of unprotected sites (Figure 5, lanes 1–3). We ascribe the limited protection against enzymatic methylation observed in this case to only partial covering of the enzyme-recognition site by the PNA-binding site. Therefore, we assumed that better protection could be achieved if the methylation site were completely covered by pcPNAs.

To check the assumption, we targeted PNA III to adenovirus-2 DNA. 35.9-kb-long adenoviral DNA contains two correct PNA-binding sites, at positions 4.0 and 29.4 kb, both of which embody the dam/DpnII recognition site, GATC. In addition, two sites partially complementary to PNA III are located at 15.3 and 32.9 kb. They have the identical sequence, GTGATCAA, which is end-mismatched (the underlined nucleobase) relative to the pcPNA binding site. Figure 6, lane 2 shows that unprotected/unmethylated adenoviral DNA is digested by R′DpnII into many short fragments. When this restriction enzyme is used after binding of 1 μM PNA III and subsequent methylation with M′dam, only a few large fragments are observed (lane 5). The lengths of the resulting DNA fragments (4, 6.5, 11, 15, and 25 kb) correspond to the PNA-assisted protection of three sites in adenoviral DNA. Thus, as expected, full protection of the two correct PNA-binding sites plus partial protection of one of the end-mismatched sites (located at 15.3 kb) was observed.

Lower concentrations of PNA III resulted in incomplete digestion of viral DNA due to incomplete binding of PNA and, as a result, lack of full protection of PNA-binding sites from enzymatic methylation (not shown). Interestingly, 3-fold increase of the PNA concentration also yielded nondigested DNA along with a pair of longer, about 29 and 32 kb, fragments again due to incomplete PNA binding (Figure 6, lane 4). Note that at a higher PNA concentration, 11 and 15 kb fragments corresponding to mismatched PNA binding were observed with much lower yield. We ascribe this effect to self-inhibition of the pcPNA invasion into dsDNA due to

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4 These are the only singly mismatched PNA-binding sites on adenoviral DNA that include the correct methylation/restriction site.

Although both end-mismatched PNA-binding sites are sequence-identical to each other, only one of them was protected from methylation by PNA III. The reason for this may be in a difference in the nucleobase sequence of flanking regions.
the polycationic character of PNA III, which by itself can stabilize the DNA double-helix at elevated PNA concentrations (see above). Prolonged incubation of viral DNA with 3 μM PNA III and replacement of the PNA solution with a fresh one did not change the digestion pattern observed in lane 4 indicating that at this PNA concentration the binding of PNA to dsDNA reached an equilibrium.

Protection of dsDNA by pcPNAs against Restriction Enzyme Cleavage. In the above experiments, the interference of pcPNAs with M'dam was demonstrated at a low salt concentration, as required for enzymatic methylation. Next, we studied the pcPNA-assisted protection of dsDNA from a restriction enzyme at a higher ionic strength that is necessary for effective digestion. In so doing, we first targeted a PvuII–PvuII fragment (~340-bp-long) of pSD1 with PNA III in a low-salt buffer, then digested the sample with R'BclI in a high-salt buffer. The binding site for PNA III includes the BclI recognition site, TGATCA, which is the only site within the plasmid for this restriction enzyme. As a control, we used the PCR-amplified 490-bp-long fragment of adenoviral DNA containing a single site, CTGATCAG, which includes the recognition site for R'BclI but contains two terminal mismatches for PNA III (underlined).6

Figure 7 shows the results of these experiments. Without PNA binding, the mixture of the two DNA fragments yields four shorter fragments upon digestion with R'BclI (see lane 4). However, if these DNA fragments were targeted with PNA III, only the adenoviral DNA fragment was completely digested by the same amount of enzyme whereas the plasmid fragment remained totally intact. These results clearly demonstrate that the formation of PNA–DNA double-duplex invasion complexes completely protects the restriction site when it is a part of the PNA-binding site.

DISCUSSION

Our data demonstrate that appropriate pairs of pcPNAs as short as octamers are able to sequence-specifically target dsDNA at sites of mixed purine-pyrimidine sequences forming stable double-duplex invasion complexes. Although the pcPNA invasion into dsDNA is inhibited at high salt concentrations, DNA negative supercoiling makes it possible to bypass this limitation. Also important is that PNA–DNA complexes formed at low ionic strength are kinetically stable at higher ionic strength, as has been already noticed before (J3). Binding of 8- and 10-mer pcPNAs to dsDNA selectively protects it from the action of restriction and methylation enzymes. Corroborating previous data on the transcription initiation inhibition (J3), the present results indicate that pcPNAs can be used for effective blocking different DNA recognizing/modifying proteins from recognition of designated sites containing all four nucleobases.

We therefore conclude that a new PNA generation, pcPNAs, which are capable of sequence-specific targeting dsDNA essentially at any chosen site, may significantly widen the prospects of various PNA applications. One of applications of pcPNAs may consist in sequence-unrestricted inhibiting the methylation for DNA rare cleavage, similar to triplex-forming PNAs (21–23, 27). Note that in experimental systems analyzed here more effective protection from enzymatic methylation was observed when the methylase-recognition site was embodied by the PNA-binding site as compared to the case of overlap. Additional experiments are required to determine whether these examples represent a general case and if optimization of PNA sequence choice, length and/or charges may further improve the technique. Binding of pcPNAs to scDNA at physiologically relevant
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salt concentrations and their interference with key enzymes, such as RNA polymerases, restriction nucleases and DNA methyltransferases, is important for possible future applications of PNA as an antigene drug.

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