Inhibition of a DNA-helicase by peptide nucleic acids

Lionel Bastide\(^1,4\), Paul E. Boehmer\(^2\), Giuseppe Villani\(^3\) and Bernard Lebleu\(^4,\)*

\(^1\)Genset, 1 rue R. et S. Delaunay, 75011 Paris, France, \(^2\)Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, PO Box 016129, Miami, FL 33101-6129, USA, \(^3\)Institut de Pharmacologie et de Biologie Structurale, CNRS, 205 route de Narbonne, 31077 Toulouse, France and \(^4\)Institut de Génétique Moléculaire de Montpellier, CNRS, 1919 Route de Mende, 34293 Montpellier, France

Received September 16, 1998; Revised and Accepted November 10, 1998

ABSTRACT

Bis-peptide nucleic acid (bis-PNA) binding results in D-loop formation by strand displacement at complementary homopurine stretches in DNA duplexes. Transcription and replication in intact cells is mediated by multienzymatic complexes involving several proteins other than polymerases. The behaviour of the highly stable clamp structure formed by bis-PNAs has thus far been studied with respect to their capacity to arrest RNA polymerases. Little attention has been given to their recognition and processing by DNA helicases. In this report we have investigated the inhibitory effect of a bis-PNA on the DNA-helicase activity of the well characterized herpes simplex type I UL9 protein. Unwinding by UL9 of a synthetic substrate is significantly inhibited by a bis-PNA and the addition of the IC8 protein, which increases UL9 processivity, does not relieve this inhibition.

INTRODUCTION

Molecules that bind DNA in a sequence-specific manner are attractive candidates as tools for the modulation of gene expression and have the potential, at a later stage, to be exploited as gene-targeted pharmaceuticals. Strong candidates for such purposes are oligonucleotides and their analogues. Triple helix forming oligonucleotides bind to DNA in the major groove via Hoogsteen or reverse Hoogsteen bonds (1,2). They have been shown to inhibit transcription in vitro and to induce site-specific mutagenesis in mammalian cells (3–5).

Peptide nucleic acids (PNAs) are oligonucleotide analogues in which the deoxyribose phosphate backbone has been replaced by an uncharged N-(2-aminoethyl)glycine polymer (6,7). PNAs bind to complementary DNA or RNA stretches with affinities significantly higher than those of the corresponding DNA oligonucleotides (8). It has been shown that homopyrimidine PNAs bind to complementary sequences in duplex DNA by strand invasion and displacement of the non-targeted strand, thus resulting in the formation of a D-loop (9). Bis-PNAs in which the two PNA strands are connected by a flexible linker can form a (PNA)\(_2\)/DNA triple helix with a very high melting temperature (10). In these structures, one PNA strand is hybridized to the targeted DNA strand through Watson–Crick base pairs, while the other strand forms Hoogsteen base pairs with the PNA/DNA duplex in a parallel orientation with respect to the DNA strand in the duplex (10). Furthermore, PNAs are metabolically stable in serum as well as in cell extracts (11). These properties make PNAs good candidates for anti-gene strategies. PNAs have been shown to inhibit transcription elongation in cell extracts (9) and to prevent the binding of sequence-specific proteins, such as restriction enzymes, to their target (12). In addition, PNA strand displacement can also create artificial promoters in cell-free experiments (13). Few studies, however, have reported well-controlled intracellular effects. To our knowledge, PNA triple helix formation inside cells has been demonstrated only by experiments from the laboratory of Glazer. Using the supF mutation reporter gene as a target, the authors were able to register PNA-targeted mutations, but the efficiency of triple helix formation cannot be estimated from this study (14).

An extrapolation of the data obtained from cell-free assays to intact cells is often difficult since cellular metabolic processes such as transcription, replication or recombination are mediated by very large ‘protein machines’ (15) comprising many interacting polypeptides. These complexes deal more efficiently with secondary structures and other obstacles than isolated polymerases. As an example, all transcription and replication complexes include helicases, which utilize the energy of nucleoside triphosphate hydrolysis to unwind the DNA double helix (16). Helicases are thought to precede polymerases and their accessory proteins. It is therefore anticipated that helicases will most likely be the first proteins in these enzymatic complexes to encounter a triple helical structure. Several studies have shown that triple helices made with phosphodiester oligonucleotides are efficiently unwound by DNA helicases (17,18).

It is therefore of interest to determine whether these enzymes act on bis-PNA. In this report we investigate the inhibitory effect of a bis-PNA on the helicase activity of herpes simplex virus type I, UL9 protein (rewiewed in 19). Indeed, UL9 has been shown to be essential for DNA replication and its activity has been well characterized, thus representing a particularly suitable model for such investigations.

*To whom correspondence should be addressed. Tel: +33 4 67 61 36 62; Fax: +33 4 67 04 02 31; Email: lebleu@jones.igm.cnrs-mop.fr
Genset and are shown in Figure 1. The template strand (60mer) oligonucleotides used in this study were obtained from Oligonucleotides and PNA. Serum albumin was purchased from Boehringer Mannheim. Concentrations are expressed in moles of monomeric protein. 89 220/M/cm at 280 nm, respectively. ICP8 and UL9 protein concentrations were determined using extinction coefficients of 82 720 and 98 100/M/cm at 280 nm, respectively. ICP8 and UL9 recombinant proteins were purified as previously described (20). The concentrations of ICP8 and UL9 proteins were determined using extinction coefficients of 82 720 and 98 100/M/cm at 280 nm, respectively. ICP8 and UL9 protein concentrations are expressed in moles of monomeric protein.

Materials

Materials and Methods

Materials

ATP (disodium salt) was purchased from Boehringer Mannheim. [γ-32P]ATP (3000 Ci/mmol) was obtained from Amersham Corp. [32P]-labeled 18mer oligonucleotide. Reactions contained a 10-fold molar excess of unlabelled 18mer oligonucleotide to prevent reannealing of the unwound [5'-32P]18mer oligonucleotide.

Proteins

UL9 and ICP8 recombinant proteins were purified as previously described (20). The concentrations of ICP8 and UL9 proteins were determined using extinction coefficients of 82 720 and 89 220/M/cm at 280 nm, respectively. ICP8 and UL9 protein concentrations are expressed in moles of monomeric protein.

T4 polynucleotide kinase was obtained from Gibco BRL. Bovine serum albumin was purchased from Boehringer Mannheim.

Oligonucleotides and PNA

The oligonucleotides used in this study were obtained from Genset and are shown in Figure 1. The template strand (60mer) provides the helicase loading site and is the strand along which the UL9 protein translocates in the 3′→5′ direction. The primer strand (18mer) is displaced during helicase action. PNA589, H(Lys)2-TTJJTJTJTT-(eg1)3-TTCTCCTCTT-LysNH2; PNA684, H(Lys)2-TTJJTJTJTT-(eg1)3-TTCTCCTCTT-LysNH2. As with peptides, PNA sequences are written from the N- to the C-terminus. H and NH2 indicate a free amino group and a terminal carboxamide, respectively. eg1 denotes the 8-amino-3,6-dioxaoctanoic acid group, which serves as a linker connecting two PNA oligonucleotidemers in bis-PNA. The J base indicates pseudocytosine.

Preparation of helicase substrates

The DNA helicase substrate was constructed by annealing the 60mer oligonucleotide to the complementary 5′-32P-labelled 18mer oligonucleotide with or without PNA (either 589 or 684) in 10 mM Tris–HCl pH 8, 10 mM MgCl2, 150 mM NaCl. The solution was heated at 65°C for 10 min and slowly cooled to room temperature.

Enzyme assays

Reactions (10 µl) were performed at 37°C and contained 25 mM EPPS–NaOH, pH 8.6, 4 mM MgCl2, 5 mM ATP, 1 mM dithiothreitol, 50 mM NaCl, 10% glycerol, 0.1 mg/ml bovine serum albumin and the indicated concentrations of UL9, ICP8 and DNA or DNA/PNA substrates. Reactions were terminated at indicated times by the addition of 3 µl 90 mM EDTA, 6% SDS, 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol. Reaction products were analysed by electrophoresis through 20% non-denaturing polyacrylamide–TBE gels and DNA unwinding was quantified by phosphorimager analysis. Each experiment was performed two or three times but one gel and its quantification is presented in each figure.

RESULTS

Design of the helicase substrates and triple helix characterization

The substrates shown in Figure 1A have been designed in order to examine the efficiency of helicase-mediated unwinding of a bis-PNA/DNA triple helix. The UL9 helicase requires a single-stranded DNA loading site in order to initiate DNA unwinding in the 3′→5′ direction. The bis-PNA binding site is not located in the duplex part of the substrate but in the single-stranded portion.

This structure is related to the D-loop resulting from the strand invasion of duplex DNA by bis-PNAs as schematized in Figure 1B. Moreover, it allows an easy comparison with a substrate which is not complexed with bis-PNA, because the length of the duplex is not affected by binding of the bis-PNA. The melting temperature of this DNA–DNA duplex is therefore not influenced by bis-PNA binding, which is crucial, since the helicase activity is monitored by its ability to displace the double-stranded DNA.

The specific binding of bis-PNA589 to the 60–18mer has been assessed by DNase I footprinting analysis (data not shown). In keeping with data reported in the literature, we found that bis-PNA589 hybridizes to a complementary single-stranded oligonucleotide with a very high melting temperature of 86°C (data not shown). The PNA:DNA ratio has been optimized. To lead to >90% hybridization a 3-fold molar excess of bis-PNA589 was used.

Effect of bis PNA on the helicase activity of UL9 helicase

An excess of unlabelled primer strand was present to prevent reannealing of the labelled oligonucleotide, in order to obtain a detectable displacement of the 18mer primer strand. A 10-fold excess was necessary to achieve optimal displacement (data not shown). As shown in Figure 2 for substrate A, the UL9 DNA helicase efficiently unwound the oligonucleotide substrate. Unwinding is easily monitored by the disappearance of the duplex and by the concomitant accumulation of the labelled primer strand. As expected, unwinding required ATP. As seen in
Figure 2. Effect of bis-PNA on the helicase activity of UL9. Substrate A (40 nM), complexed or not with PNA589, was incubated with 200 nM UL9 protein and a 10-fold molar excess of unlabelled 18mer as described in Materials and Methods. At the times indicated, aliquots were removed to measure helicase activity. (A) Autoradiogram of the reaction products. Lane 1, heat-denatured substrate A; lane 2, 60 min without ATP. (B) Quantitation of the data shown in (A). ○, unmodified substrate; ●, substrate complexed with PNA589.

Figure 3. Unwinding of substrate A by ICP8. Substrate A (40 nM), complexed or not with PNA589, was incubated for 30 min with the indicated concentrations of ICP8 protein and a 10-fold molar excess of unlabelled 18mer. Lanes 1–4, substrate A with 100, 200, 400 and 800 nM ICP8, respectively; lanes 5–9, PNA589-complexed substrate A with 100, 200, 400 and 800 nM ICP8, respectively.

Figures 2 and 3, the binding of bis-PNA589 slightly lowered the electrophoretic mobility of DNA substrate A.

The binding of bis-PNA589 on the 60mer template strand significantly inhibited the helicase activity of the UL9 protein. A 2-fold inhibition was observed during the course of the reaction, at 200 nM UL9 protein (Fig. 2).

Effect of ICP8 on the UL9 helicase activity

ICP8 is a 128 kDa single-strand DNA binding protein. It forms a specific and essential complex with UL9 and greatly stimulates the helicase activity of UL9 by increasing its processivity (22). UL9 and ICP8 proteins were used at 200 nM as it has been shown that ICP8 caused maximal stimulation of DNA unwinding activity at equimolar UL9 protein concentrations (22). As indicated in Figure 3, no strand displacement activity by ICP8 alone was detected in these conditions.

The comparison of Figures 2 and 4 establishes that the addition of ICP8 increases the unwinding activity of UL9, as expected. However, the addition of ICP8 stimulated the DNA unwinding activity of UL9 to the same extent regardless of whether the duplex substrate was complexed or not with bis-PNA (Fig. 4). ICP8 therefore does not qualitatively decrease the inhibitory effect of bis-PNA589.

A control bis-PNA (PNA684) which does not bind to substrate A was used in order to demonstrate that the inhibition is specific. This control had no effect on the activity of UL9 as compared with PNA589 (Fig. 5). Similar conclusions were drawn from experiments involving bis-PNA589 and a control substrate lacking the bis-PNA589 binding site (data not shown). Altogether these data demonstrate that complementarity and binding between the bis-PNA and its DNA binding site is required in order to inhibit UL9 helicase activity. This was important to establish since it has been shown previously that synthetic oligonucleotides (phosphorothioates in particular) sometimes inhibit gene expression without binding to their nucleic acid target (23).

DISCUSSION

In this paper, we have examined the capacity of UL9, a replicative helicase, to unwind DNA substrates complexed with bis-PNAs. The effect of the HSV-1 single-stranded DNA binding protein, ICP8, which increases processivity of the UL9 helicase, was also...
investigated. Together these proteins form a complex that is required for the replication of a eukariotic viral genome. The data presented in this paper indicate that the DNA unwinding activity of the UL9 helicase was significantly reduced when a bis-PNA was bound to the template strand along which the helicase translocates. This inhibition strictly depends on binding of the bis-PNA to its complementary DNA, as demonstrated in experiments in which either the sequence of the bis-PNA or the sequence of the substrate itself was modified. The inhibitory effect of bis-PNA589 was not reduced by the addition of ICP8, at variance with data reported with the major cisplatin–DNA adduct (24). An explanation could be that the bis-PNA obstacle is sterically more obstructive than the lesion induced by cisplatin and therefore more difficult to bypass.

The inhibitory effect of a bis-PNA on the template strand can be explained by a model in which translocation of the UL9 protein in the 3′→5′ direction is impaired by bis-PNA. Inhibition due to less efficient binding of UL9 after bis-PNA hybridization seems unlikely because the single-stranded region between the PNA and the 3′-terminus is two times larger than required for the loading of UL9 (25; G.Villani and P.E.Boehmer, unpublished observations).

Previous studies have shown that triple helices made with phosphodiester oligonucleotides are unwound by the bacteriophage T4 Dda and SV40 large T antigen helicases (17,18). Triple helix formation with PNAs leads to the displacement of one DNA strand and to the formation of a D-loop. In the case of bis-PNA, the flexible linker allows the two PNA strands to form an extremely stable clamp that can explain the differences observed with respect to phosphodiester oligonucleotides. Moreover, the entirely different structure of the PNA backbone, with respect to phosphodiester oligonucleotides, may conceivably be a substrate that is poorly recognized and processed by DNA helicases.

It has been reported that replication arrest due to a defect in a replicative DNA helicase can lead to DNA breakage in *Escherichia coli* (26). Since DNA double-strand breaks represent lethal lesions, all organisms have developed repair pathways, involving, in particular, illegitimate recombination or homologous recombination (26). Thus it should be interesting to investigate the possibility that bis-PNA-targeted inhibition of replicative DNA helicases may induce homologous recombination at selected sites within cells.

**ACKNOWLEDGEMENTS**

The authors thank Dr P. Nielsen (Panum Institute, Copenhagen, Denmark) for the generous gift of the bis-PNAs used in this study and Dr J. P. Léonetti for helping and stimulating discussions. This work was supported by grants from Agence Nationale de Recherche contre le SIDA, Ligue Nationale Française de Lutte contre le Cancer and Association pour le développement de la Recherche sur le Cancer and by grant AI38335 from the National Institutes of Health to P.E.B.

**REFERENCES**