Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 23, pp. 3001-3006, 1997
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0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)10136-6

SYNTHESIS AND MEMBRANE PERMEABILITY OF PNA–PEPTIDE CONJUGATES

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Abstract: Chimeric molecules consisting of peptide nucleic acid oligomers (PNAs) and peptides derived from the third helix of the homeodomain of Antennapedia are taken up by mammalian cells in culture. Uptake is independent of orientation and occurs with high efficiency, suggesting that peptide conjugates are a promising strategy for intracellular PNA delivery. © 1997 Elsevier Science Ltd.

Introduction

PNAs are DNA analogs in which the phosphate backbone has been replaced by (2-aminoethyl) glycine carbonyl units linked to the nucleotide bases through the glycine amino nitrogen and methylene carbonyl linkers.[1] In spite of the dramatically altered chemistry of the oligomer backbone, PNAs hybridize to complementary sequences by Watson–Crick base-pairing.[2] Moreover, because the PNA backbone is uncharged, electrostatic repulsion between complementary strands is avoided making hybridization more stable[1,2] and more rapid.[3] The altered chemistry also confers resistance to digestion by nucleases and proteases[4] and results in less nonsequence selective association to DNA binding proteins than is exhibited by phosphorothioate oligomers.[5]

These favorable properties confer significant advantages to PNAs for complementary recognition, and have been exploited to develop improved methodologies for hybridization in vitro[6] including quantification of telomere length,[7] isolation of transcriptionally active DNA,[8] screening for mutations,[9] pre gel hybridization,[10] and inhibition of human telomerase.[11] The variety and apparent success of these applications for PNAs suggests that they will prove valuable tools for improved in vitro hybridization protocols and might have important advantages in vivo as well. However, unmodified PNAs have not been reported to cross cell membranes,[12,13] preventing examination of their potential to control cellular processes and elucidation of their full potential.

The uptake of oligonucleotides by cells is a major problem confronting the use of any oligonucleotide motif, and it is especially pressing for PNAs because their neutral backbone linkages prevent delivery by standard protocols employing cationic lipids. Methods for improving PNA entry into cells include conjugation of PNAs to DNA[14] or to insulin-like growth factor 1 peptide,[15] although in the latter case uptake was dependent on expression of the cognate receptor on the cell surface. Several peptide sequences have been demonstrated to spontaneously cross through cell membranes and to transport peptides, proteins, and oligonucleotides into cells.[16–18] Once inside, the attached macromolecules have been observed to exert a biological effect. We report here an efficient synthetic method for obtaining hybrids between PNAs and one of these peptides, the third helix of the homeodomain of Antennapedia.[17] These conjugates cross through cell membranes and enter cells with high efficiency. This ability to deliver modified PNAs within cells should facilitate the use of PNAs for control of gene expression and allow PNAs to be compared with other oligonucleotide chemistries for in vivo efficacy.

Results and Discussion

Synthesis of PNA–peptide conjugates. We synthesized four different PNA–peptide conjugates (Table 1) with either PNA or peptide as C-terminal domain. Conjugates were designed to test if peptides could direct PNAs across cell membranes, whether full length homeodomain peptide was essential, and if conjugate orientation influenced cellular uptake. Conjugates were labeled with either fluorescein or rhodamine for visualization.

Conjugates were obtained by first synthesizing the C-terminal domain by solid-phase synthesis using automated protocols for making the peptides and manual protocols for making the PNA. Aliquots of these initial syntheses were deprotected, cleaved from the solid support, and evaluated by mass spectral analysis. Upon validation, the remainder of the protected material was used as the basis for synthesis of the N-terminal partner, again using automated synthesis to attach peptides and manual synthesis to attach the PNA. A portion of the resultant PNA–peptide conjugates was then deprotected, cleaved, subjected to further mass spectral analysis (Fig. 1B) and purified by HPLC (Fig. 1A). In all cases mass spectral analysis indicated that full length conjugate was the major product, and HPLC purification was effective at removing impurities due to failed syntheses. After derivatization with rhodamine or fluorescein and further HPLC purification the fluorescently labeled conjugates were used in conjuction with microscopy and fluorescence activated cell sorting (FACS) analysis to monitor cellular uptake.

Table 1. Sequences of PNAs and PNA–peptide conjugates (conjugates are shown N to C termini).

Homeodomain peptide - PNA (conjugate **1**) GlyGlyArgGlnIleLysIleTrpPheGlnAsnArgArgMetLysTrpLysLys-GGGTTAGACAA-Lys
Truncated homeodomain peptide - PNA (conjugate **2**) GlyGlyLysIleTrpPheGlnAsnArgArgMetLysTrpLysLysGluAsn-GGGTTAGACAA-Lys
PNA - homeodomain peptide (conjugate **3**) GGGTTAGACAA-GlyGlyGlyArgGlnIleLysIleTrpPheGlnAsnArgArgMetLysTrpLysLys
PNA - truncated homeodomain peptide (conjugate **4**) GGGTTAGACAA-GlyGlyGlyLysIleTrpPheGlnAsnArgArgMetLysTrpLysLysGluAsn

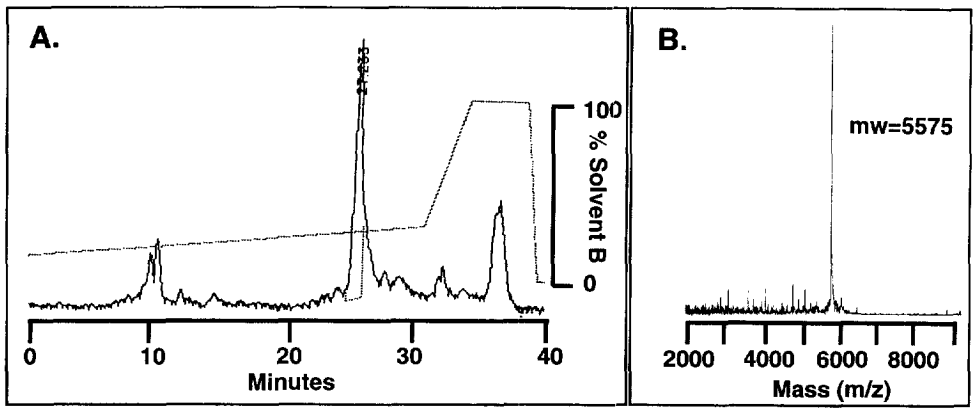

Figure 1. (A) HPLC purification of conjugate **3**. (B) Mass spectral analysis of conjugate **3** (with an N-terminal cysteine).

Uptake of PNA–peptide conjugates by cells. We incubated immortal human prostate tumor-derived DU145 cells with N-terminal rhodamine or fluorescein-labeled PNAs or the fluorescently labeled PNA–peptide conjugates for one to twelve hours, with maximal uptake reached after one hour. These oligomers were all soluble in water at concentrations of 100 μM. Cells were exhaustively washed to remove free fluorescent material that might be associated with cell surfaces.

We did not note any uptake of rhodamine or fluorescein-labeled PNAs that were not attached to the Antennapedia-derived peptide (Fig. 2C). We also did not observe any uptake of control conjugates **2** and **4** containing the truncated homeodomain sequence. By contrast, conjugates **1** and **3** containing the full sixteen amino acid peptide sequence were readily taken up by cells (Fig. 2A and D) and then localized within cells (Fig. 2B). Conjugates entered cells regardless of whether rhodamine or fluorescein was used, indicating that the identity of the reporter group was not influencing uptake. No toxicity was observed at concentrations of conjugate as high as 10 μM, and cells remained viable indefinitely after incubations with PNA–peptide chimera. Uptake was observable at concentrations as low as 500 nM. Fluorescence within cells continued to be observable after four days of additional growth in cell culture after removal of free conjugate. The strong localization of conjugate (especially apparent in Fig. 2A and B) indicates that further modification may be required for efficient delivery to the nucleus.

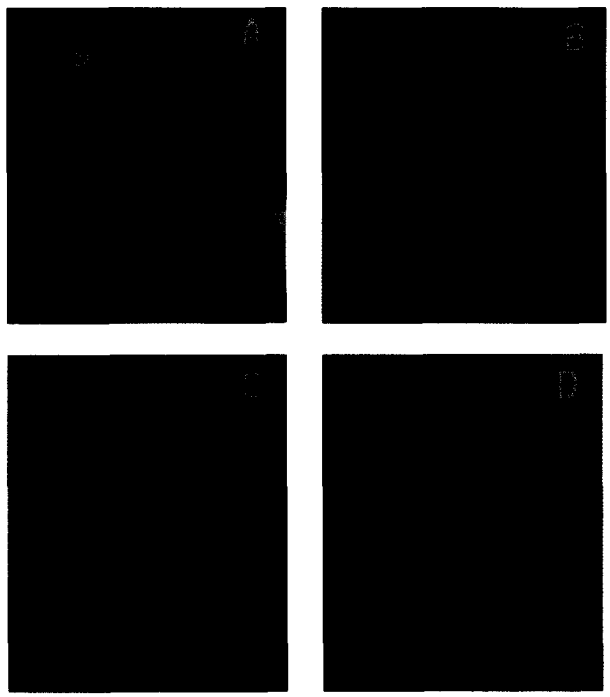

Figure 2. (A) Uptake of fluorescein-labeled PNA-homeodomain-derived peptide conjugate **1** by cells. (B) Uptake of fluorescein labeled conjugate **1** by cells visualized using nuclear staining by propidium iodide. (C) Lack of uptake of rhodamine-labeled PNA by cells. (D) Uptake of rhodamine-labeled conjugate **3** by cells. Cells are magnified 1000-fold.

Analysis of PNA uptake by flow cytometry. We used FACS analysis to quantify the percentage of cells that incorporated fluorescently-labeled PNA. We observed that 99% of cells treated with rhodamine-labeled conjugate **1** fluoresced with at least tenfold greater intensity than untreated cells or cells treated with the analogous rhodamine-labeled PNA (Fig. 3) (i.e., background fluorescence). FACS analysis of the uptake of conjugate **3** produced similar results.

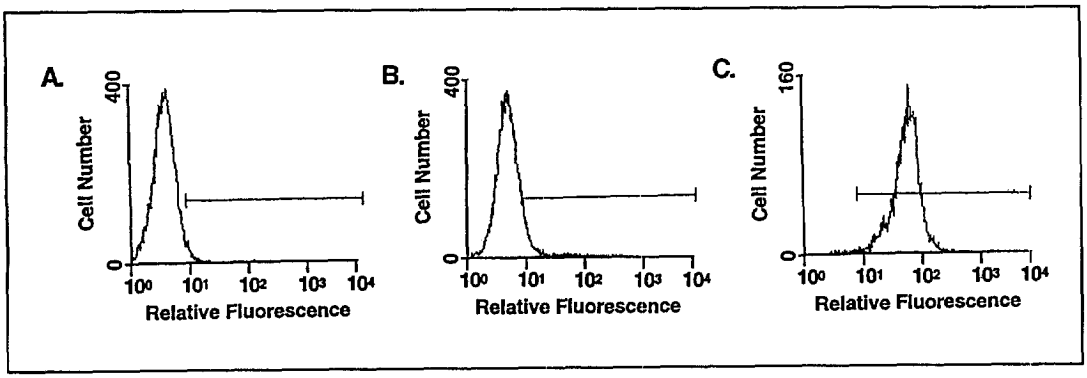

Figure 3. (A) Untreated cells. (B) Cells treated with rhodamine-labeled PNA. (C) Cells treated with PNA–peptide-rhodamine conjugate **1**. The horizontal line delineates fluorescence above background. The median relative fluorescence values were 4 (A), 5 (B), and 70 (C).

Conclusion. PNAs can significantly improve the recognition of complementary targets in vitro and if they could be delivered into cells it is reasonable to expect that PNAs would be powerful tools for sequence-specific control of biological processes and afford leads for the development of anti-gene therapeutics. We have shown that (i) PNA–peptide conjugates of up to 30 couplings can be efficiently synthesized and labeled with fluorescent groups, (ii) conjugates can be characterized by mass spectrometry and purified by HPLC, and (iii) they are efficiently taken up by cells. In vitro, PNA–peptide conjugates inhibit human telomerase as well as do unmodified PNAs, demonstrating that the attached peptide does not interfere with hybridization, but we have not observed inhibition of telomerase within cells. Further work will be needed to optimize nuclear targeting by PNAs and to confirm their ability to bind to complementary DNA and RNA sequences within cells, but these goals will be facilitated by availability of relatively inexpensive and synthetically accessible peptide import sequences.

Experimental

Reagents. PNA synthesis was accomplished by solid phase manual methods[19] using either Boc or Fmoc chemistry. Manual methods were used to afford greater control over coupling efficiency. PNA monomers were obtained from PerSeptive Biosystems (Framingham, MA). Fluorescein-5-maleimide and fluorescein isothiocyanate were obtained from Pierce (Rockford, IL). 5-(and-6)-Carboxytetramethylrhodamine succinimidyl ester (rhodamine) was obtained from Molecular Probes (Eugene, OR). All solvents and reagents were of the highest purity and dryness obtainable and were stored desiccated to maintain dryness. Small (100 mL size) bottles of 1-methyl-2 pyrrolidinone (NMP) and dimethyformamide (DMF) were purchased from Aldrich (Milwaukee, WI) and were used for dissolving the monomers and rinsing the resin just before the addition of the monomer. Smaller bottles were preferred since dryness is absolutely necessary for successful syntheses. Peptides were synthesized on a Symphony Multiplex peptide synthesizer (Rainin, Waltham MA) using Fmoc chemistry.

Mass spectral analysis. All PNAs, peptides, and PNA–peptide conjugates were analyzed by matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF) using a Voyager-DE workstation (PerSeptive Biosystems). Scan average was set at 64, the acceleration voltage was held at a constant 25000, the grid voltage was 93.9%, and the laser power varied between 1200 and 1600 (positive ion), depending upon the length and structure of the PNA–peptide to be analyzed. Higher laser power gives a higher background, but seems to be necessary for obtaining mass spectra of PNAs and PNA–peptide hybrids. The matrix which worked best for our purposes was cyano-4-hydroxycinnamic acid. A fresh solution of matrix (10 to 15 mg/mL in 50% acetonitrile, 0.1% TFA) was mixed 1:1 with each sample, then spotted on the target plate and allowed to air dry before loading into the spectrometer.

Synthesis of PNA–peptide conjugates. PNA–peptide conjugates were synthesized with either the peptide or the PNA in the C-terminal position. Depending on the orientation, either the peptide was made first by automated synthesis or the PNA was made first by manual synthesis. After completion of this initial synthesis, a small aliquot would be deprotected and cleaved, then characterized by MALDI-TOF spectrometry to ensure successful synthesis of the entire lot. Once the identity of the synthesis was confirmed, the fully protected oligomer was used as the basis for addition of the PNA by manual synthesis or a peptide by automated synthesis. We normally employed Boc protected monomers for PNA synthesis because we have found that the resulting deprotected products could be obtained in better yield and with fewer side-products. However, when the PNA was added to the N- terminus of a peptide already prepared by Fmoc synthesis, Fmoc chemistry was also used for the PNA synthesis. PNAs or PNA–peptide conjugates were purified on a Rainin HPLC system with a Dynamax detector set at 260 nm using a Delta Pak C18 300 Å column (7.8 × 300 mm) heated to 50 °C.[19]

Synthesis of fluorescein and rhodamine labeled PNAs and PNA–peptide conjugates. PNA–peptide conjugates were labeled with fluorescein or rhodamine. Fluorescein maleimide was coupled to deprotected PNA–peptide conjugates through cysteine. Rhodamine can withstand trifluormethanesulfonic acid (TFMSA) cleavage conditions as well as four hours of TFA cleavage without breaking down and was added to the N-terminus of the fully protected PNA or PNA–peptide hybrid before cleavage. After the N-terminal Boc or Fmoc protecting group was removed from the completed PNA–peptide hybrid rhodamine was coupled using diisopropylethylamine (DIPEA) to increase the pH to 9.0. Coupling was complete after thirty minutes. At least a fourfold excess of rhodamine over PNA–peptide was used, while fluorescein was used in twofold excess. After coupling the finished product was washed extensively with DMF or NMP to remove the unreacted rhodamine.

Uptake and visualization of PNAs and conjugates by cells. 50,000 DU145 cells were allowed to attach to 24-well plates in 1X Dulbecco's Modified Eagle's Media (DMEM) (Mediatech, Herndon VA) supplemented with 10% fetal bovine serum. Media was removed from cells and PNAs and conjugates were added directly for three minutes prior to addition of fresh media to bring the final concentration of oligomer to 1 μM. Cells were incubated for one to twelve hours, with maximal uptake observed after one hour. Following incubation cells were rinsed 8–12 times with phosphate buffered saline (PBS) to remove residual free fluorescent material. Cells were then treated with trypsin and transferred to Lab-TekII chamber slides (Nalge-Nunc, Rochester, NY) for visualization. After reattachment, cells were washed several times with PBS and fixed with 70% methanol. Vectashield (Vector Laboratories, Burlingame CA) mounting medium (25 μL) was added to the fixed slides. Cells were visualized using an Olympus BHS microscope with a reflected light fluorescence attachment.

Analysis by flow cytometry. Adherent populations of DU145 cells were treated with 1 μM rhodamine labeled conjugate **1**, the analogous rhodamine labeled PNA, or unlabeled PNA in media for 2 h at 37 °C. Cells were extensively washed, trypsinized, and resuspended in 0.5 mL 1 X PBS. Populations were immediately analyzed on a FACStarPlus flow cytometer using LYSYS II software (Becton Dickinson, Franklin Lakes, NJ) and a 575 nm broad band pass filter. Cell populations were gated to only measure the fluorescence in intact cells.

Acknowledgements

We would like to thank Steve Madden for peptide synthesis, Revathi Katipally for PNA synthesis, and Lauren Gollahon and Susan Hamilton for advice. This work was supported by a grant from the Robert A. Welch Foundation (I-1244). Anne Pitts was supported by NIH predoctoral training grant 5T32GM0706223. DRC is an assistant investigator with the Howard Hughes Medical Institute.

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(*Received in USA* 11 *September* 1997; *accepted* 14 *October* 1997)