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Effective delivery of antisense peptide nucleic acid oligomers into cells by anthrax protective antigen

Daniel G. Wright *, Ying Zhang, John R. Murphy

Molecular Medicine Section, Department of Medicine, Boston University School of Medicine, Boston, MA, USA

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ABSTRACT

Peptide nucleic acid (*PNA*) is highly stable and binds to complementary RNA and DNA with high affinity, but it resists cellular uptake, thereby limiting its bioavailability. We investigated whether *protective antigen* (*PA*, a non-toxic component of anthrax toxin) could transport antisense *PNA* oligomers into reporter cells that contain luciferase transgenes with mutant β-globin *IVS2* intronic inserts, which permit aberrant pre-mRNA splicing and impair luciferase expression. *PNA* oligomers antisense to mutant splice sites in these *IVS2* inserts induced luciferase expression when effectively delivered into the cells. *PNA* 18-mers with C-terminal poly-lysine tails [*PNA*$(Lys)_8$] demonstrated modest sequence-specific antisense activity by themselves at micromolar concentrations in *luc-IVS2* reporter cell cultures. However, this activity was greatly amplified by *PA*. Antisense *PNA*$(Lys)_8$ with but not without *PA* also corrected the *IVS2-654* β-globin splice defect in cultured erythroid precursor cells from a patient with β-thalassemia [genotype, *IVS2-654*(β^0/β^E)], providing further evidence that anthrax *PA* can effectively transport antisense *PNA* oligomers into cells.

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Advances in molecular genetics have led to a detailed understanding of the genetic basis of many congenital and acquired diseases, encouraging the development of reagents that can control gene expression selectively, both as experimental tools and candidate therapeutics. Antisense oligonucleotides have been studied extensively in this regard [1–5], and a number of nucleic acid analogs and mimics have been developed as antisense reagents [6–11], including peptide nucleic acid (*PNA*) [9,12].

PNA, first described in 1991 by Nielsen et al. [13], is a DNA mimic in which the phosphate deoxyribose backbone of DNA is replaced by a pseudo-peptide polymer to which nucleobases are attached *via* methylene carbonyl linkers. *PNA* oligomers bind to complementary RNA with very high affinity and specificity, and binding is minimally affected by the secondary structure of RNA transcripts. *PNA* oligomers also resist nuclease and protease digestion and are biologically stable [1,9,13]. Moreover, because *PNA* oligomers lack a repetitively charged backbone, they do not interact with polyanion-binding proteins, which may complicate the actions of nucleotide analogs when used as gene-targeting agents [14].

While antisense *PNA* oligomers do not trigger degradation of mRNA transcripts at sites of binding, they can effectively block mRNA translation [9,12], and they can also control splicing of pre-mRNA transcripts when alternative splice sites are targeted [10,11]. This latter effect is of particular interest, since many human gene transcripts undergo alternative splicing to generate splice variants with different functions [11].

Despite these positive gene-targeting attributes, unmodified *PNA* resists cellular uptake and therefore has limited bioavailability [15]. This limitation has impeded its development as a candidate therapeutic, although effective cellular delivery of *PNA in vitro* has been achieved to some extent by the addition of lysine residues [10,16] or charged membrane penetrating peptide sequences [15,17]) or with cationic liposomes as carriers of *PNA* conjugates [18].

In this study, we examined the ability of anthrax *protective antigen* (*PA*) to deliver antisense *PNA* oligomers into cells. Microbial toxin proteins, such as diphtheria and anthrax toxin, have evolved functional domains that allow them to enter cells *via* receptor-mediated endocytosis and then access the cytoplasm *via* trans-endosomal membrane pores [19–23]. Unlike diphtheria toxin (a single protein), anthrax toxin consists of multiple proteins: a non-toxic 83 kDa polypeptide [*protective antigen* (*PA*)] that mediates cell binding, endocytosis, and trans-membrane pore formation [21,22], and two separate 90 kDa proteins [*lethal factor* (*LF*) and *edema factor* (*EF*)] that mediate cell toxicity. When *PA* binds a cell through its "receptor binding" domain, a 63 kDa fragment (*PA-63*) is generated by endoproteolysis that forms heptamers, which bind *LF* and *EF* and transport them into cells *via* endocytosis and into the cytoplasm *via* trans-endosomal pores [22].

We considered *PA* to be an attractive candidate for study as a vehicle for cellular delivery of *PNA* because of its bioavailability,

* Corresponding author. Address: Molecular Medicine Branch, NIDDK, NIH, 10 Center Drive, Building 10, Room 9N314 (MS1822), Bethesda, MD 20892, USA. Fax: +1 301 402 0101.
E-mail address: dw341u@nih.gov (D.G. Wright).

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lack of toxicity, and potential for modifications that might allow for tissue specificity (e.g., replacement of the cell binding domain with cell-specific ligands [19,24]).

Materials and methods

Reagents. Recombinant anthrax *PA-63* and *PA-83* were obtained from List Laboratories (Campbell, CA). Sense and antisense 18-mer 2′-*O*-methyl (2′-*O*-Me) phosphorothioate oligonucleotides were synthesized by TRI-Link, Inc. (San Diego, CA). *PNA* 18-mers with and without C-terminal poly-lysines were synthesized by BioSynthesis, Inc. (Lewisville, TX). Oligonucleotide primers for RT-PCR were obtained from Invitrogen (Carlsbad, CA). Cell culture reagents, DMEM/F12 (Dulbecco's modified Eagle's medium/nutrient mixture F12 Ham's—liquid medium with 2 mM L-glutamine) and fetal bovine serum, were obtained from HyClone (Logan, UT).

Reporter cell lines. Chinese Hamster Ovary cells (*CHO-K1* cells), as used in studies of anthrax toxin biology (21), were obtained from ATCC (American Type Culture Collection, Manassas, VA). A *Luc-IVS2-654* reporter transgene was generated by PCR amplification of the human β-globin intron-2 sequence (*IVS2)* with primers CGCTGCTGGGTGAGTCTATGGGACCCTT and AGGGTTGGCACTGTGG GAGGAAGATAAGAG. The fruit fly luciferase gene was amplified separately from a pGL3-control vector (Promega, San Luis Obispo, CA) into 5′-*Luc* and 3′-*Luc* segments using primers TACGATTTGTGC CAGAGTCCTTC and CCATAGACTCACCCAGCAGCGCACTTGAAT, and CCTCCCACAGTGCCAACCCTATTCTCCTTC and GCCCCGACTCTAGAAT TACAC, respectively. The PCR product from 5′-*Luc* was then joined with that of *IVS2* by PCR, using flanking primer pairs. The resulting PCR product was then joined with 3′-*Luc* by PCR using outer flanking primer pairs. The final PCR product, called *Luc-IVS2*, was digested by BclI and XbaI and ligated to the BclI and XbaI digested pGL3-control vector. The *Luc-IVS2* gene insert was then released from pGL3-*Luc-IVS2* by HindIII and XbaI and inserted into a HindIII and XbaI digested pcDNA3 vector (Invitrogen). The resulting *Luc-IVS2* plasmid was used as a positive control vector for correct splicing of luciferase. It was also mutated (C to T) at position 654 to reproduce the β-*IVS2-654* β-thalassemia mutation by PCR using primers: TCTGGGTTAAGGTAATAGCAATA and TATTGCTATTACCTT AACCCAGA and the PCR product then digested with DpnI, re-annealed, and transformed into *Escherichia coli* DH5a competent cells (Invitrogen Inc.). DNA sequencing was used to confirm that the resulting plasmid (*Luc-IVS2-654*) contained the luciferase gene interrupted by the mutant *IVS2-654* intron. This plasmid and the control *Luc-IVS2* plasmid were transfected into *CHO-K1* cells separately using Effectene™ (Qiagen Inc., Valencia, CA). Cells were maintained in selective F12K medium containing G418 (InVivoGen Inc., San Diego, CA) at 400 μg/mL and 10% fetal bovine serum, 2 mM L-glutamate, and 50 U of penicillin/streptomycin. Surviving cell colonies were picked from cultures after 10 days of G418 selection, and evaluated for luciferase expression (Promega Inc.). A separate *HeLa* reporter cell line, stably transfected with a separate luciferase transgene, containing a different mutant β-globin intron-2 insert (*Luc-IVS2-705*), originally reported by Kang et al. [25], was obtained commercially from GeneTools (Philomath, OR).

Luc-IVS2-654 CHO-K1 cells or *Luc-IVS2-705 HeLa* cells, from aliquoted frozen stocks, were cultured in DMEM/F12 medium with 10% FBS in 12- and 24-well culture plates (Costar, Fisher Scientific, Pittsburg, PA) for up to 72 h. Cells grew as adherent monolayers in culture wells, and seeding concentrations ($1–5 \times 10^4$/mL) were adjusted such that the cells did not become confluent during experimental culture periods. Culture media with reagents, depending upon experimental variables, were replaced every 24 h. For certain studies, cells were incubated with 2′-*O*-methyl (2′-*O*-Me) phosphorothioate (*PS*) oligonucleotides or *PNA* oligomers, either premixed with Oligofectamine (Invitrogen) or with cell-scraping transfection [26]. For other studies, cells were incubated without physical manipulation in media alone, or in media containing *PNA* oligomers with or without recombinant anthrax *PA-63* or *PA-83*. Cultured cell protein or RNA was extracted for luciferase assays (Promega, Inc. [27]) or for RT-PCR.

RNA extraction and analysis. Total RNA from cultured cells was extracted with Trizol (Invitrogen). *Luc-IVS2* and *Luc-IVS2-654* mRNA was amplified by reverse transcription (RT)-PCR using r*Tth* polymerase and primers (TTGATATGTGGATTTCGAGTCGTC and TGTCAATCAGAGTGCTTTTGGCG) hybridized to sequences flanking *Luc-IVS2* introns, as described previously [10,24]. PCR products were separated on 7.5% non-denaturing polyacrylamide gels and visualized by ethidium bromide staining [28].

β-Thalassemia erythroid cells. Citrate-anticoagulated blood samples from a non-transfused β-thalassemia *intermedia* patient [*IVS2-654*(β^o)/β^E genotype] were generously provided to us by Dr. Edmond Ma at the Queen Mary Hospital of the University of Hong Kong. This sample was obtained from the patient following informed consent and shipped on ice *via* FedEx, arriving within 48 h. Light density mononuclear cells were separated from the blood sample by density gradient centrifugation (lymphocyte separation medium, MB Biologicals, Solon, OH) and were cultured with recombinant erythropoietin (EPO) and c-Kit ligand (SCF), as described by Lacerra et al. [29]. Fresh medium containing cytokines ± antisense *PNA*$(Lys)_8$ (300 nM), ±*PA-63* (300 ng/mL) was added on days 8 and 12 of culture, and cells were harvested on day 15. RNA was isolated from the cells, and radiolabelling PCR was performed [29]. Total cellular RNA was isolated with Trizol (Invitrogen) and analyzed by RT-PCR using rTth DNA polymerase (Perkin-Elmer Life Sciences, Norwalk, CT) and 0.2 μCi of [α-^{32}P]dATP per sample. Aberrant and correct splicing of human *IVS2-654* β-globin pre-mRNA in the *IVS2-654*(β^o)/β^E compound heterozygote were detected using a forward allele-specific β^A primer, spanning positions 50–79 of human β-globin exon 1, and a reverse primer, spanning positions 6–28 of exon 3 of the human β-globin gene, as described [29].

Results and discussion

To evaluate the ability of *PA* to deliver antisense *PNA* oligomers into cells, we developed an antisense reporter system using *CHO-K1* cells. These cells express abundant anthrax *PA* receptors and have been used extensively to study anthrax toxin biology and *PA* receptors [21]. Following a strategy reported previously [10,11,16], we generated a *CHO-K1* cell line (*Luc-IVS2-654 CHO*) that expressed a modified luciferase gene containing a human thalassemia β-globin gene intronic insert (*IVS2-654*). This intron encodes an alternative splice site that allows incorrect splicing of pre-mRNA transcripts thereby preventing the translation of transcripts to full-length protein (Fig. 1A). This *IVS2-654* intronic mutation is a common cause of β-thalassemia in Asia. Binding of antisense oligonucleotides to the aberrant splice site in the *IVS2-654* intron has been shown to correct β-globin pre-mRNA splicing and to permit expression of normal full-length β-globin in thalassemic erythroid precursor cells that carry this mutation [29].

Luc-IVS2-654 CHO cells expressed both correctly spliced and incorrectly spliced luciferase mRNA (Figs. 1C and 2B), and background luciferase activity was measurable in cell extracts (Figs. 1B and 2A). Both antisense 2′-*O*-methyl (2′-*O*-Me) phosphorothioate (*PS*) 18-mer oligonucleotides (not shown) and antisense *PNA* 18-mers (with sequences complementary to an 18-nucleobase region of the mutant β-globin intron-2 that flanked position 654) were found to correct the aberrant splicing of *Luc-IVS2-654* pre-mRNA, when transfected into the cells, and also to increase

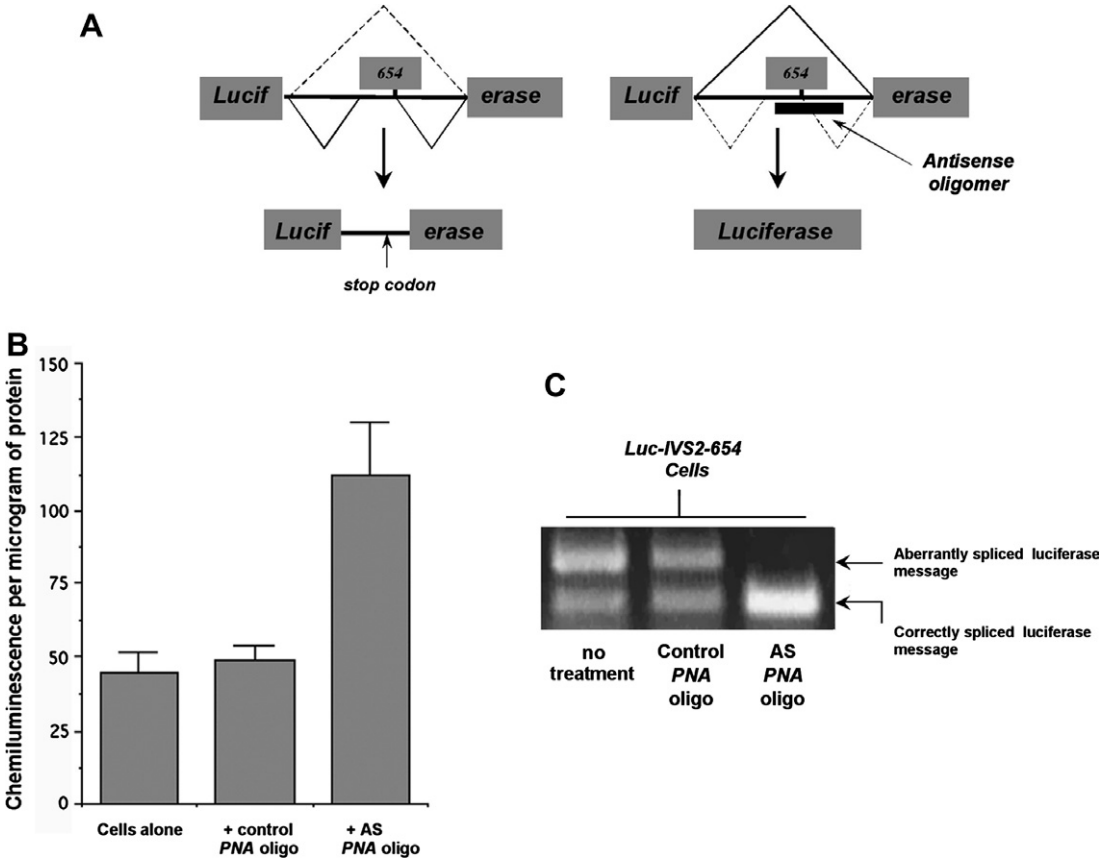

Fig. 1. (A) Diagrammatic depiction of the effect of a mutant β-globin intron-2 (*IVS2-654*) insert in the luciferase gene coding sequence on luciferase expression. Blockade of the aberrant *IVS2-654* splice site by an antisense oligomer (right-hand diagram) permits expression of a correctly spliced gene product and active enzyme. Adapted from Kang et al. [25] and Sazani et al. [10]. (B) Induction of luciferase expression and (C) correction of pre-mRNA splicing in *Luc-IVS2-654 CHO* cells following 48 h incubation with 1.0 μM antisense (AS) *PNA*$(Lys)_8$ oligomers delivered to the cells by cell-scraping transfection [16] and oligofectamine. Results in (B) represent mean values ± SEM for four replicate studies.

substantially the expression of luciferase activity in the cells (Fig. 1B and C).

Using *Luc-IVS2-654 CHO* cells to detect sequence-specific antisense activity, we next determined whether an antisense *PNA* 18-mer with a C-terminal eight-lysine oligopeptide tail [*PNA*$(Lys)_8$] could be shown to induce luciferase activity without transfection when incubated with *Luc-IVS2-654 CHO* cells along with anthrax *PA*. Antisense *PNA*$(Lys)_8$ oligomers used for these studies [GCT ATTACCTTAACCCAG-O-$(Lys)_8$ with base, A, corresponding to the mutant T at *IVS2* position 654] had the same nucleotide binding sequence as did the unmodified antisense *PS* oligonucleotides and *PNA* oligomers used to validate the *Luc-IVS2-654 CHO* reporter cell system, as shown in Fig. 1. A C-terminal poly-lysine tail was added to the *PNA* oligomers because of a finding reported by Blanke et al. [23] that C-terminal poly-lysines added to a polypeptide fragment derived from the catalytic domain of diphtheria toxin permitted this otherwise non-toxic polypeptide to use anthrax *PA* to enter and kill cells. A sense 18-mer *PNA*$(Lys)_8$ served as a control in these studies.

Both recombinant, full-length *PA-83* and "nicked" *PA-63* were evaluated, and neither form of *PA* by itself affected luciferase expression in *Luc-IVS2-654 CHO* cells. Control sense *PNA*$(Lys)_8$ oligomers also did not affect luciferase expression in these cells, with or without *PA*, under any condition of reagent dose or incubation time examined. However, antisense *PNA*$(Lys)_8$ was found to induce a modest degree of luciferase expression by itself, and this effect was substantially enhanced by both *PA-83* and *PA-63* (Fig. 2A). Enhanced luciferase expression by antisense *PNA*$(Lys)_8$ oligomers in the presence of *PA* was also associated with a shift in luciferase mRNA detected by RT-PCR to the correctly spliced form, as in control *Luc-IVS2 CHO* cells containing a luciferase transgene with a wild-type, non-mutant β-IVS2 insert (Fig. 2B).

Sazani et al. [10] reported previously that the addition of a short, C-terminal $(Lys)_4$ oligopeptide tail to *PNA* oligomers allowed detectable cellular uptake of *PNA* at micromolar concentrations. Given this report, we anticipated that antisense *PNA*$(Lys)_8$ might have some activity by itself at such concentrations, as observed. However, this activity was significantly enhanced by both *PA-83* and *PA-63* in a time and dose-dependent manner (Fig. 3A and C). Moreover, antisense *PNA*$(Lys)_8$ at concentrations as low as 30 nM was found to induce detectable increases in luciferase expression when incubated with *Luc-IVS2-654 CHO* cells together with *PA*.

The apparent ability of both *PA-63* and *PA-83* to facilitate delivery of antisense *PNA*$(Lys)_8$ into reporter cells required the C-terminal poly-lysine tail on *PNA* (Fig. 3B). Induction of luciferase expression in cell cultures by antisense *PNA* with *PA* was reduced when the number of lysine residues in the poly-lysine tail was decreased from 8 to 4 and lost completely when C-terminal lysines were reduced to two or eliminated.

PA-mediated cellular delivery of antisense *PNA*$(Lys)_8$ oligomers was also observed in human erythroid progenitor cells and in a *HeLa* cell line (*Luc-IVS2-705 HeLa*) [25], which expressed a luciferase transgene with a mutant β-globin *IVS2* intronic insert (*IVS2-705*) different from the one expressed by *Luc-IVS2-654 CHO* reporter cells. Effective antisense blockade of the aberrant splice site in *Luc-IVS2-705 HeLa* cells has been shown to induce expres-

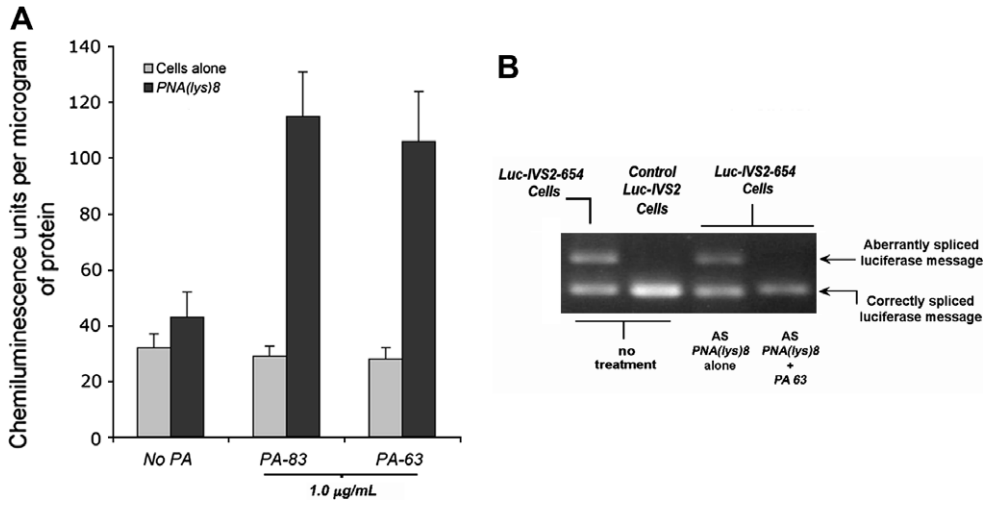

Fig. 2. (A) Induction of luciferase activity in *Luc-IVS2-654 CHO* cells by antisense *PNA*$(lys)_8$ (0.3 μM) following 72 h incubation with *PA-63* or *PA-83* (1.0 μg/mL). Results represent mean values ± SEM for four replicate studies. (B) Correction of pre-mRNA splicing in *Luc-IVS2-654 CHO* cells following incubation with 1.0 μM antisense *PNA*$(Lys)_8$ oligomers with but not without *PA-63* (1.0 μg/mL).

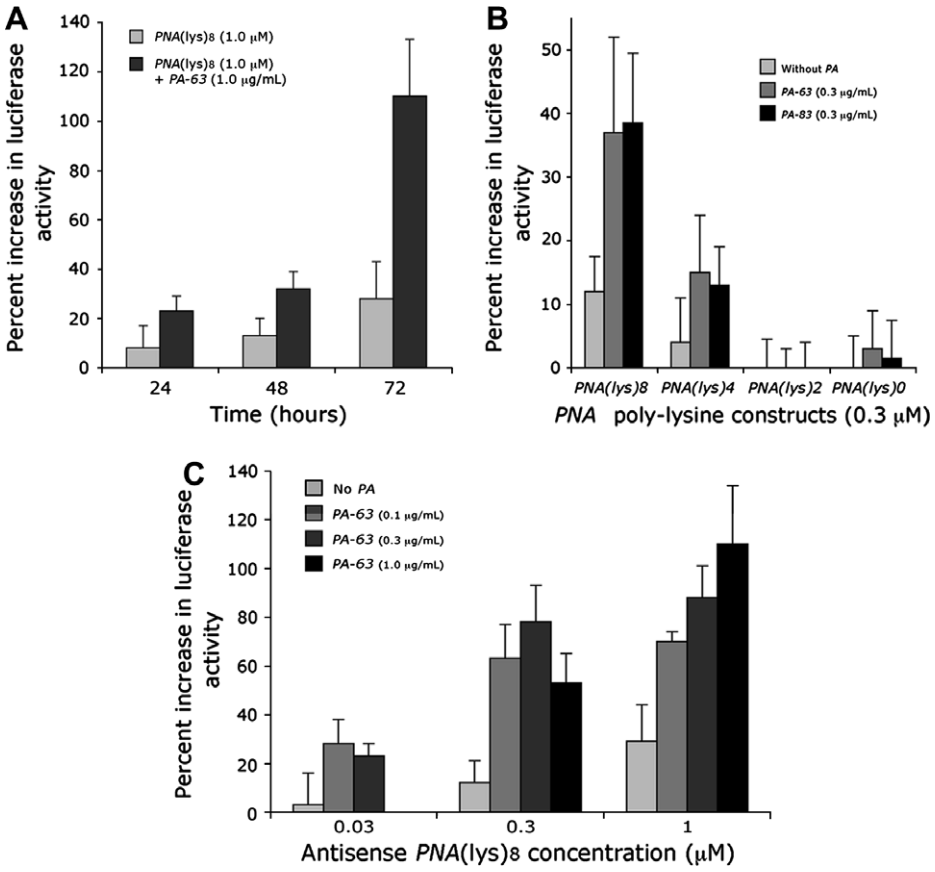

Fig. 3. (A) Induced luciferase expression in *Luc-IVS2-654 CHO* cells incubated for 24–72 h with antisense *PNA*$(Lys)_8$ oligomers at 1.0 μM with and without *PA-63*, 1.0 μg/mL. (B) Luciferase expression in *Luc-IVS2-654 CHO* cells incubated for 72 h with antisense *PNA*$(Lys)_8$ oligomers at 0.03, 0.3, and 1.0 μM both with and without *PA-63* at varying concentrations (0.1–1.0 μg/mL). (C) Induction of luciferase activity in *Luc-IVS2-654 CHO* cells by antisense *PNA* oligomers with poly-lysine tails of varying lengths [0.3 μM,PNA(Lys)8,PNA(Lys)4,PNA(Lys)2,andPNA(Lys)0] with and without *PA-63* (0.3 μg/mL). Results represent mean values ± SEM for 4–6 replicate studies.

sion of luciferase activity [25], as in *Luc-IVS2-654 CHO* cells described above. While *HeLa* cells are known to be sensitive to anthrax toxin [30], they have not been studied as extensively as *CHO* cells with regard to *PA* receptor expression. Nonetheless, *Luc-IVS2-705 HeLa* cells also demonstrated *PA*-facilitated cellular delivery of antisense *PNA* (Fig. 4A). While antisense *PNA*$(Lys)_8$ oligomers induced modest increases in luciferase expression in *Luc-IVS2-705 HeLa* cells in the absence of *PA*, induction of luciferase was substantially enhanced when *PA-63* was added to *Luc-IVS2-705 HeLa* cell cultures, as in the *CHO* reporter cells.

PA also promoted effective delivery of antisense *PNA*$(Lys)_8$ oligomers into erythroid precursor cells from a patient with

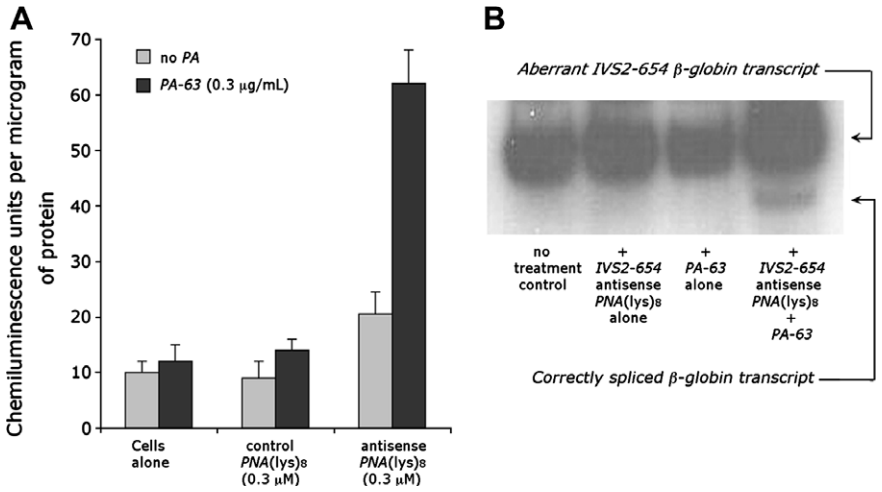

Fig. 4. (A) Induced luciferase expression in *Luc-IVS2-705 HeLa* cells by 0.3 μM antisense (*IVS2-705*) *PNA*$(Lys)_8$ with and without *PA-63* (1.0 μg/mL). Results represent mean values ± SEM for six replicate studies. (B) Correction of aberrantly spliced β^A-globin mRNA in erythroid cells of a β-thalassemia patient with the β-*IVS2-654* mutation by antisense *PNA*$(Lys)_8$ (0.3 μM) after incubation with the cells with and without *PA-63* (0.3 μg/mL).

β-thalassemia *intermedia* [*IVS2-654*$(\beta^o)/\beta^E$ genotype]. Erythroid cells from this patient expressed β-globin genes with the *IVS2-654* mutation on one allele, and incorrectly spliced β-globin gene transcripts resulting from this mutation could be detected in the cells by PCR. When antisense *PNA*$(Lys)_8$ (as used in the studies of *Luc-IVS2-654 CHO* cells described above) was added to cultures of erythroid cells from this patient by itself, no change in aberrant β-globin splicing was evident. However, correctly spliced β-globin transcripts were clearly detectable when antisense *PNA*$(Lys)_8$ was added to cultures together with *PA-63* (Fig. 4B).

These studies indicate that anthrax *PA* can serve as a vehicle for effective delivery of antisense *PNA* oligomers into various types of cells at nanomolar concentrations. While others have reported that the trans-membrane pore forming domain of diphtheria toxin can mediate delivery of oligonucleotides into cells [31], this finding has not been confirmed, and no studies of the delivery of *PNA* oligomers into cells by microbial toxin proteins have been reported previously.

Our interest in studying *PNA* and anthrax *PA* was based on several considerations. First, *PNA* oligomers are intrinsically stable and their pseudo-peptide structure is amenable to modifications whereby peptide sequences can be added either during or following synthesis [1,9,15]. Second, anthrax *PA*, available as a recombinant protein, is known to be biocompatible and non-toxic by itself. Moreover, prior studies of anthrax *PA* provided cogent leads regarding modifications of *PNA* (e.g., addition of C-terminal lysines) that might allow *PNA* to interact with *PA* and use it as a vehicle for entry into cells [23], and it is possible that alternative adducts, such as peptide sequences derived from the *LF* (anthrax "lethal factor") high-affinity *PA* binding site [20,23], might increase the efficiency of *PA*-mediated cellular delivery of *PNA* oligomers beyond that observed with *PNA(Lys)*$_8$.

An additional attraction of *PA* as a vehicle for delivering antisense *PNA* oligomers into cells is that it may be amenable to modifications that confer cell selectivity to *PNA* delivery. The fusion toxin, DAB_{389}IL-2 (or *Ontak*®), now used as an anti-cancer and immunosuppressive drug, exemplifies the potential pliability of microbial toxin proteins to achieve tissue specificity. *Ontak*®, a modified form of diphtheria toxin in which the native cell receptor-binding domain of the toxin has been replaced by the cytokine, IL-2, interacts only with cells that express high affinity IL-2 receptors [24,32,33]. Analogous modifications of the cell receptor-binding domain of anthrax *PA* might similarly allow tissue specific delivery of antisense *PNA* into cells. Replacement of the native cell receptor-binding domain of *PA* could also be advantageous to its potential for *in vivo* bioavailability by removing neutralizing antigenic epitopes [34].

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