RESEARCH PAPER

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Effects of decoy molecules targeting NFkappaB transcription factors in cystic fibrosis IB3-1 cells

Recruitment of NFkappaB to the IL-8 gene promoter and transcription of the IL-8 gene

Alessia Finotti,[1] Monica Borgatti,[1] Valentino Bezzerri,[2] Elena Nicolis,[2] Ilaria Lampronti,[1] Maria Cristina Dechecchi,[2] Irene Mancini,[1] Giulio Cabrini,[2] Michele Saviano,[3,4] Concetta Avitabile,[3,4] Alessandra Romanelli[3,4] and Roberto Gambari[2,5,*]

[1]ER-GenTech and BioPharmaNet; Department of Biochemistry and Molecular Biology; Università di Ferrara; Ferrara, Italy; [2]Laboratory of Molecular Pathology; Laboratory of Clinical Chemistry and Haematology; University-Hospital; Verona, Italy; [3]Department of Biological Sciences; Università di Napoli "Federico II"; Napoli, Italy; [4]Istituto di Biostrutture e Bioimmagini; CNR; Napoli; Italy; [5]Interdisciplinary Center for the Study of Inflammation; University of Ferrara; Ferrara, Italy

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Abbreviations: ODN, oligodeoxyribonucleitides; PNA, peptide nucleic acids; PDP, PNA-DNA-PNA chimeras; TFD, transcription factor decoy; NFkappaB, nuclear factor kappa B; IkB, IkappaB, inhibitor of NFkappaB; EMSA, electrophoretic mobility shift assay; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PAO-1, *Pseudomonas aeruginosa*, strain O1; IL-8, Interleukin 8; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; ChIP, chromatin immunoprecipitation

One of the clinical feature of cystic fibrosis (CF) is a deep inflammatory process, which is characterized by production and release of cytokines and chemokines, among which interleukin 8 (IL-8) represents one of the most important. Accordingly, there is a growing interest in developing therapies against CF to reduce the excessive inflammatory response in the airways of CF patients. Since transcription factor NFkappaB plays a critical role in IL-8 expression, the transcription factor decoy (TFD) strategy might be of interest. In order to demonstrate that TFD against NFkappaB interferes with the NFkappaB pathway we proved, by chromatin immunoprecipitation (ChIP) that treatment with TFD oligodeoxyribonucleotides of cystic fibrosis IB3-1 cells infected with *Pseudomonas aeruginosa* leads to a decrease occupancy of the Il-8 gene promoter by NFkappaB factors. In order to develop more stable therapeutic molecules, peptide nucleic acids (PNAs) based agents were considered. In this respect PNA-DNA-PNA (PDP) chimeras are molecules of great interest from several points of view: (1) they can be complexed with liposomes and microspheres; (2) they are resistant to DNases, serum and cytoplasmic extracts; (3) they are potent decoy molecules. By using electrophoretic mobility shift assay and RT-PCR analysis we have demonstrated that (1) the effects of PDP/PDP NFkappaB decoy chimera on accumulation of pro-inflammatory mRNAs in *P. aeruginosa* infected IB3-1 cells reproduce that of decoy oligonucleotides; in particular (2) the PDP/PDP chimera is a strong inhibitor of IL-8 gene expression; (3) the effect of PDP/PDP chimeras, unlike those of ODN-based decoys, are observed even in the absence of protection with lipofectamine. These informations are of great impact, in our opinion, for the development of stable molecules to be used in non-viral gene therapy of cystic fibrosis.

Introduction

Cystic fibrosis (CF) is a severe inherited disease caused by mutations of a gene encoding a chloride channel termed Cystic fibrosis transmembrane conductance regulator (CFTR).[1] Although most of the CF patients are affected by multiple organ pathologies, lung disease is the major cause of morbidity and mortality in CF. In the lung of CF patients a hyper-inflammatory condition is established, which is characterized by predominant infiltrates of polymorphonuclear neutrophils (PMNs) in bronchial lumina and increased expression of pro-inflammatory cytokines and chemokines, in particular interleukin-8 (IL-8).[2-8] Recently, it has been established that excessive IL-8 release from lung epithelial cells may also play a role in bacterial proliferation and adhesion.[9] Accordingly, IL-8 is presently considered a critical pharmacological target to reduce the excessive inflammation in CF lungs.[10] We recently described an analysis of the transcription machinery of the IL-8 gene in human bronchial epithelial cells, and found NFkappaB pathway very important.[11,12] These results are in line with several reports indicating lung inflammation in CF related to an increased NFkappaB signaling causing the induction of IL-8 gene expression.[13]

One of the possible strategies to inhibit IL-8 gene transcription is the so-called "transcription factor decoy" (TFD) approach,[14-17]

*Correspondence to: Roberto Gambari; Email: gam@unife.it
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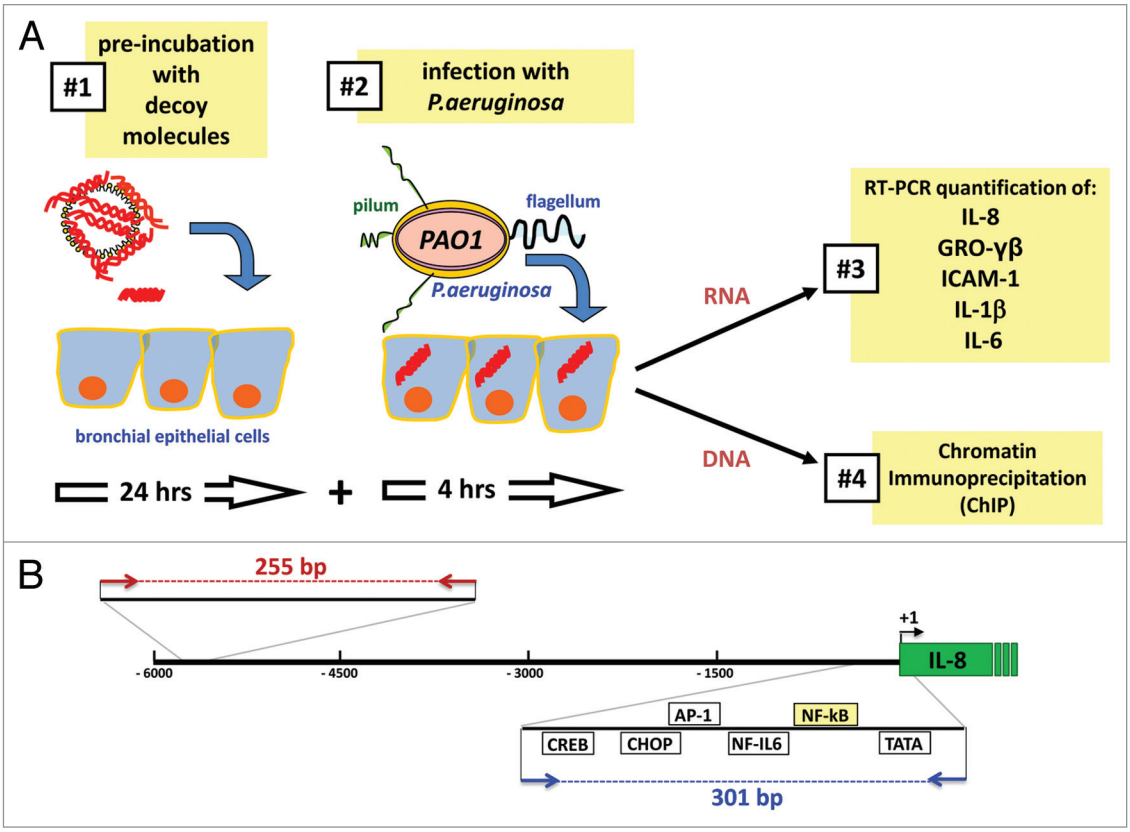

Figure 1. (A) Experimental design followed for the analysis of the effects of the transcription factor decoy (TFD) strategy employing TF decoy ODNs against NFkappaB and human bronchial epithelial cells. (#1) Human bronchial CF-derived respiratory epithelial IB3-1 cells were pre-incubated for 24 h with NFkappaB ODNs or PDP/PDP chimeras before infection with the laboratory strain of *P. aeruginosa* PAO1 (#2). After 4 h post-infection, total RNA was extracted, reverse-transcribed to cDNA and analyzed by RT-qPCR (#3). In parallel, chromatin was purified by the IB3-1 cells for chromatin immunoprecipitation assay (ChIP) (#4). In (B) representation of the genomic region located: 6 kb upstream of IL-8 gene. The location of primers used for IL-8 promoter amplification in the ChIP assay and the respective product length are indicated: PCR product obtained from IL-8 promoter amplification, containing NFkappaB binding site (301 bp, in blue); PCR product obtained using control primers flanking a genomic region: 5 kb upstream of IL-8 promoter (255 bp, in red).

based on oligodeoxynucleotides (ODNs) mimicking consensus sequences identified within the proximal promoter region of the IL-8 gene. In order to verify whether the TFD against NFkappaB interferes with the NFkappaB pathway we employed in this study chromatin immunoprecipitation (ChIP) on cystic fibrosis IB3-1 cells infected with *Pseudomonas aeruginosa* and treated with TFD ODNs against NFkappaB factors.

In consideration of the fact that an important drawback of the decoy approach designed for the modulation of gene expression is the presence of intracellular and extracellular DNases,[18] therefore, large amounts of DNA must be loaded into target cells in order to obtain biological responses leading to alteration of gene expression. On the other hand, modified oligonucleotides (either methylphosphonate or phosphorothioate) have the advantage in that they are resistant to DNase cleavage; unfortunately, these molecules are highly toxic.[18]

In this respect, in recent reports we presented the possible use of peptide nucleic acids (PNAs)[19-21] as alternative reagents in experiments aimed at the control of gene expression involving the TFD approach. In PNAs, the pseudopeptide backbone is composed of N-(2-aminoethyl)glycine units.[19] PNAs hybridize with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson-Crick double helices[21] and are resistant to both nucleases and proteases.[22] PNAs were found to be excellent candidates for antisense and antigéne therapies.[23-26] Among PNA-based molecules, we found that PNA-DNA chimeras are of great potential in gene therapy being active as decoy molecules against the NFkappaB and Sp1 transcription factors.[23,27-29]

Following these considerations, we determined the effect of PNA-DNA-PNA (PDP) chimeras mimicking the binding sites of NFkappaB on the transcription of IL-8 gene in cystic fibrosis IB3-1 cells infected by *Pseudomonas aeruginosa*. We have described this system in several reports and reviews, pointing out that after *Pseudomonas aeruginosa* exposure, IB3-1 are induced to increase accumulation of IL-8 mRNA in respect to basal levels of uninfected cells. In addition to IL-8 mRNA, other sequences induced by PAO1 are GRO-γ, GRO-α, IL-6, IL-1β, ICAM-1.[11,12] Therefore, IB3-1 cells infected by *P. aeruginosa* are an excellent system to verify (1) whether decoy molecules against NFkappaB inhibit IL-8 gene transcription and (2) whether this effect is restricted to the IL-8 gene, or affects

other genes containing NFkappaB binding sites, such as GRO-γ, IL-6, IL-1β and ICAM-1.

Results

The experimental system and preliminary assays on the specificity of the decoy molecules. **Figure 1** shows the experimental strategy followed in our study. Complexes of cationic liposomes with NFkappaB ODNs or PDP/PDP chimeras have been preincubated with cystic fibrosis IB3-1 cells 24 h before exposure to the PAO1 laboratory strain of *P. aeruginosa* (100 CFU/cell) for a further four hour time period. A scrambled ODN was used as negative control (**Table 1**). After the treatment RNA was extracted and real-time quantitative RT-PCR performed; at the same time chromatin was purified from IB3-1 cells for chromatin immunoprecipitation assay (ChIP). In the first set of experiments, described in **Figure 2**, we provided evidences that the TF decoy approach interfere(s) with the NFkappaB activity in vitro and in the IB3-1 cellular system. In order to verify the ability of this decoy ODNs to compete for the binding of NFkappaB to the sequences contained in the promoter of IL-8 gene, the NFkappaB decoy ODNs was incubated with nuclear extracts from IB3-1 cells in the presence of a radiolabeled probe 100% homologous with the NFkappaB binding sequences and performed electrophoretic mobility shift assays (EMSA). Complete inhibition of interaction of the ^{32}P-labeled probes with specific transcription factor proteins (NFkappaB/DNA complexes) has been obtained, providing the proof of principle of the competition of this NFkappaB decoy ODNs for the DNA consensus sequence contained in the promoter of the IL-8 gene. On the contrary, other ODN containing the binding sites for other transcription factors are inactive. ODN for CREB, NF-IL6, AP-1 and CHOP were selected because these transcription factors play a crucial role in the control of transcription of the IL-8 gene.[11]

Effects of decoy ODNs targeting NFkappaB on gene expression of *Pseudomonas aeruginosa* infected IB3-1 cells. The results reported in **Figure 3A** show that treatment with 0.5 μg/ml of ODN NFkappaB decoy is sufficient to obtain decreased expression of the IL-8 gene. However also the other signals from transcription factors present within the IL-8 gene promoter (**Fig. 1**) might play an important transcriptional role; this is supported by the observation that decoys for CREB, AP-1 and CHOP-1 also induce to some extent inhibition of IL-8 mRNA content in parallel experiments (data not shown). In any case, some decoy oligonucleotides are not active in inhibiting *P. aeruginosa* induced IL-8 gene increased expression, as outlined in **Figure 3B**, showing that treatment of PAO1 infected IB3-1 CF cells with NF-IL6 decoy does not lead to significant reduction of IL-8 mRNA. In order to clarify the effects of decoy molecules on the NFkappaB intracellular dynamics, we studied by chromatin immunoprecipitation (ChIP) the possible effects of the treatment with decoy ODNs on the recruitment of NFkappaB to the IL-8 gene promoters. Chromatin was isolated from uninfected IB3-1 cells, from cells infected with PAO1, and from cells infected with PAO1 and treated with 2 μg/ml of ODN NFkappaB decoy. After immunoprecipitation (**Fig. 1B**),

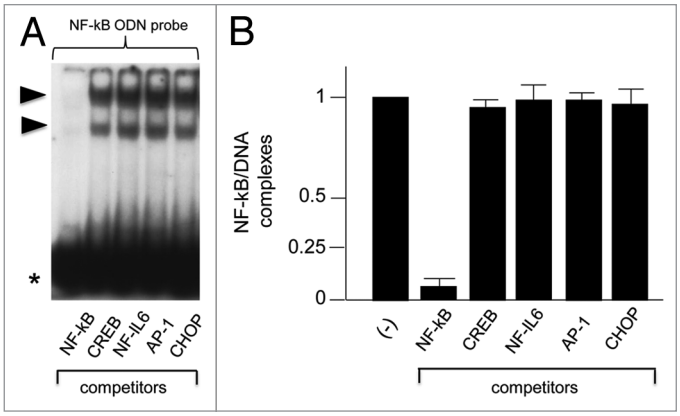

Figure 2. (A) Representative autoradiogram showing the effects of cold ODN competitors specific for NFkappaB, CREB, NF-IL6, AP-1 and CHOP transcription factors on molecular interactions between nuclear factors from IB3-1 cells and ^{32}P-labeled NFkappaB ODN (▶, NFkappaB/DNA complexes; *free probe). (B) Effects of the indicated ODN competitors on the NFkappaB/DNA complexes. Data (average ± SD of three independent EMSA experiments) represent the amount of the complexes in respect to that found in control EMSA reaction conducted in the absence of any competitors (-).

amplification of the IL-8 gene promoter segment containing the NFkappaB binding sites was performed; as a negative control a parallel PCR was also performed using primers amplifying an upstream sequence of the IL-8 gene (located at -5 Kb from the transcription start site) lacking NFkappaB consensus elements. **Figure 3B** shows one important feature of our experimental cell systems, i.e., that NFkappaB is recruited with high efficiency to the IL-8 gene promoter following infection with PAO1; second, the data clearly indicate that the NFkappaB decoy ODN interferes with this recruitment. No changes in occupancy by NFkappaB were detected when ChIP-DNA was amplified using PCR primers specific for the upstream sequence of the IL-8 gene lacking NFkappaB consensus elements.

The NFkappaB decoy PNA-DNA-PNA chimeras are strong inhibitors of IL-8 gene expression and do not require transfection reagents. While it is firmly established that the entry of oligonucleotides into eukaryotic cells can occur through a nucleic acid channel,[30] the complexation with lipofectamine (or other delivery systems) is required in vitro, since the degradation of ODNs exposed to fetal calf serum is an important drawback of these transfection approaches.[31,32] On the contrary, we have elsewhere reported that, unlike ODN-based decoys, PDP/PDP chimeras are fully resistant to serum and cytoplasmic extracts.[18,28] This information is of great impact, in our opinion, for the development of stable molecules to be used in non-viral gene therapy. Therefore, we compared the effects of ODN-based and PDP-based NFkappaB decoys on *P. aeruginosa* infected cells in the presence or in the absence of the lipofectamine transfection reagents. In the first set of experiments we confirmed that the NFkappaB decoy PNA-DNA-PNA chimera (see **Fig. 4A** for molecular structure) reproduces the activity of the NFkappaB decoy ODN on *P. aeruginosa* induced genes. In fact, the NFkappaB PDP/PDP chimera exhibits differential effects on expression of PAO1

Table 1. Sequence of synthetic oligonucleotides used in this study

Double-stranded oligonucleotides used in gel shift assays and decoy transfections[a]			
NFkappaB		5'-AGA GGA ATT TCC ACG ATT-3'	
CREB		5'-AAA ACT TTC GTC ATA CTC-3'	
NF-IL6		5'-CAT CAG TTG CAA ATC GTG G-3'	
AP-1		5'-TGT GAT GAC TCA GGT TTG-3'	
CHOP		5'-CGC TGG TGT GAT GCA CGG-3'	
SCRAMBLED		5'-CAC AAA GTG TAA CAG TCT-3'	
ChIP Q-PCR primers	**Amplified region**	**Sequences**	
IL-8 ChIP f	IL-8 promoter region	5'-TCA CCA AAT TGT GGA GCT TCA GTA T-3'	
IL-8 ChIP r		5'-GGC TCT TGT CCT AGA AGC TTG TGT-3'	
Neg ChIP f	Negative control region[b]	5'-TCC CTA AGT CAC TTT CTT CAA GTT GC-3'	
Neg ChIP r		5'-CGT GCA TTT AAT TGT GTC TTG TGG-3'	
RT Q-PCR primers	**Amplified region**	**Sequences**	**Accession number**
IL-8 f	IL-8 transcripts	5'-GAC CAC ACT GCG CCA ACA-3'	AF385628.2
IL-8 r		5'-GCT CTC TTC CAT CAG AAA GTT ACA TAA TTT-3'	
ICAM-1 f	ICAM-1 transcripts	5'-TAT GGC AAC GAC TCC TTC TCG-3'	NM_000201
ICAM-1 r		5'-CTC TGC GGT CAC ACT GAC TGA-3'	
GRO-γβ f	GRO-γβ transcripts	5'-CCG GAC CCC ACT GCG-3'	M36821
GRO-γβ r		5'-TTC CCA TTC TTG AGT GTG GCT A-3'	
IL-1-β f	IL-1-β transcripts	5'-CTC CAC CTC CAG GGA CAG GA-3'	BT007213.1
IL-1-β r		5'-GGA CAT GGA GAA CAC CAC TTG TT-3'	
IL-6 f	IL-6 transcripts	5'-CGG TAC ATC CTC GAC GGC-3'	NM_000600
IL-6 r		5'-CTT GTT ACA TGT CTC CTT TCT CAG G-3'	
GAPDH f	GAPDH mRNA	5'-GTG GAG TCC ACT GGC GTC TT-3'	NM_001404.3
GAPDH r		5'-GCA AAT GAG CCC AGC CTT C-3'	

[a]Sequence of decoy ODNs based on IL-8 promoter regulatory elements; [b]Region about 5 kb upstream of the IL-8 promoter, lacking NFkappaB binding sites.

activated genes (**Fig. 4B**), such as ICAM-1, GRO-γ, IL-1β, IL-6 and IL-8. The major inhibitory effect was found on IL-8 gene expression, confirming on one hand elsewhere reported studies on the role of NFkappaB for IL-8 gene transcription and, on the other hand, that decoy for NFkappaB might retain differential effects of genes regulated by promoters containing NFkappaB signal sequences, such as ICAM-1, GRO-γ, IL-1β and IL-6. This suggest that other transcription factors are the master regulators of these genes (such as Sp1 for IL-6 gene).[33] Despite the fact that this specific issue should be object of future investigations, the effects of the NFkappaB PNA-DNA-PNA chimeras, as shown in the insert of **Figure 4B**, reproduce those obtained using NFkappaB ODNs. The results shown in **Figure 5**, while confirming that the NFkappaB decoy ODN exhibit lipofectamine-dependent effects, provide evidence that the NFkappaB decoy PDP interferes with the NFkappaB activity without the need of lipofectamine.

Discussion

Recent studies have indicated that the transcription factor decoy (TFD) strategy targeting NFkappaB might be of interest to develop anti-inflammatory approaches for cystic fibrosis.[12,13] The first set of data of the present study demonstrate that decoy ODNs against NFkappaB interfere with the NFkappaB pathway in cystic fibrosis cells infected with *Pseudomonas aeruginosa*. This was demonstrated by chromatin immunoprecipitation (ChIP) using as a model system cystic fibrosis IB3-1 cells infected with *Pseudomonas aeruginosa*. Treatment with ODN decoys against NFkappaB led to a decreased occupancy of the IL-8 gene promoter by NFkappaB factors.

In order to develop more stable therapeutic molecules, peptide nucleic acids (PNAs) based agents were considered. PNAs are DNA mimicking molecules in which the pseudopeptide backbone is composed of N-(2-aminoethyl)glycine units.[19-21] PNAs

are resistant to both nucleases and proteases[22] and, more importantly, hybridize with high affinity to complementary sequences of single-stranded RNA and DNA, forming Watson-Crick double helices.[21] For these reasons, PNAs were found to be excellent candidates for antisense and antigene therapies.[23-26] In recent studies, PNA-DNA chimeras have been described as reagents for the transcription factor decoy approach.[27-29] PNA-DNA chimeras are PNA-DNA covalently bonded hybrids and were designed on one hand to improve the poor cellular uptake and solubility of PNAs, on the other hand to exhibit biological properties typical of DNA, such as the ability to stimulate RNaseH activity and to act as substrate for cellular enzymes (for instance DNA polymerases). The results published by Romanelli et al.[29] Borgatti et al.[27,28] and Moggio et al. firmly demonstrate that decoy molecules based on PNA-DNA chimeras are powerful decoy molecules. In respect to experiments aimed at pharmacological modification of gene expression, PNA-DNA-PNA chimeras are molecules of interest for several points of view: (1) unlike PNAs, they can be complexed with liposomes and microspheres;[28] (2) unlike ODNs, they are resistant to DNases, serum and cytoplasmic extracts;[22] (3) unlike PNA/PNA and PNA/DNA hybrids,[35] they are potent decoy molecules.[27-29]

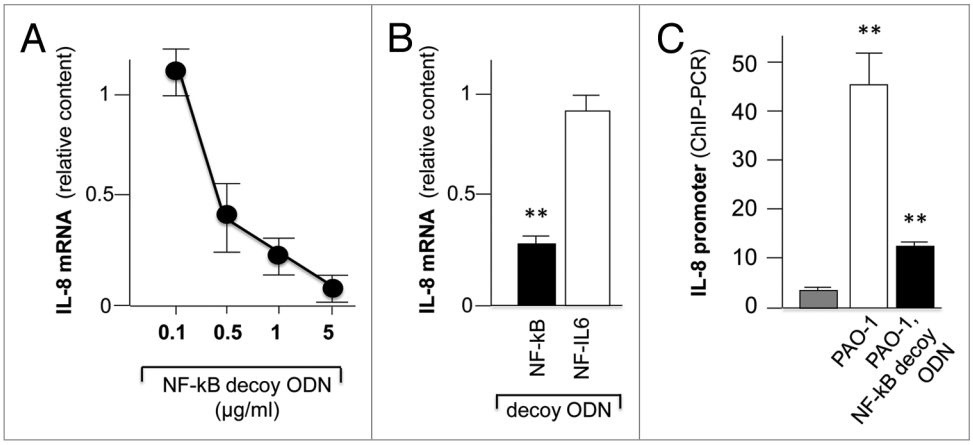

Figure 3. (A) Effect of NFkappaB decoy ODNs on *P. aeruginosa*-dependent induction of IL-8 mRNA in IB3-1 cells. Cells were pre-incubated for 24 h with the indicated concentration of NFkappaB decoy ODN, the same amounts of scrambled ODNs or medium alone before infection with *P. aeruginosa*, PAO1 strain. Total RNA was extracted 4 h post-infection and IL-8 mRNA was quantified as described in the Materials and Methods section. Data are mean ± SEM. Representative of three separate experiments. (B) Effects of 1 μg/ml of NFkappaB and NF-IL6 decoy on IL-8 mRNA production in IB3-1 CF cells infected PAO-1. (C) Chromatin Immunoprecipitation (ChIP) assay showing the effects of NFkappaB ODN decoy on the recruitment of NFkappaB transcription factor on the promoter of the IL-8 gene. Values represent the ratios between the IL-8 promoter Q-PCR performed on NFkappaB specific ChIP and negative IgG ChIP. Each value was derived employing chromatin isolated from untreated IB3-1 cells (gray bar) or cells infected with PAO-1 in the absence (white bar) or in the presence (black bar) of pre-treatment with the NFkappaB decoy ODN. Significance in Student's t test between PAO-1 infected and uninfected cells and between PAO-1 infected, NFkappaB decoy treated and PAO-1 infected cells: **p < 0.01.

The second set of results presented in this paper (**Figs. 4 and 5**) demonstrated that PDP/PDP chimeras targeting NFkappaB are strong inhibitors of IL-8 gene expression even in the absence of protection with lipofectamine (**Fig. 5**) and mimic the biological activity of ODN-based lipofectamine-delivered decoys (insert of **Fig. 4B**). The extent of inhibition of IL-8 gene expression using naked NFkappaB decoy ODN (**Fig. 5C**) approaches that found in lipofectamine-delivered NFkappaB decoy ODN (**Fig. 5B**). This is the first research article from our group reporting this information, which is of great impact, in our opinion, for the development of stable molecules to be used in non-viral gene therapy. Despite the fact that these results should be considered as a proof-of-principle that PNA-DNA chimeras targeting NFkappaB retain selective biological functions, we like to remark that no translation to clinical settings can be proposed based only on data reported in the present paper. On the other hand, we like to underline that IL-8 is one of the master genes in pro-inflammatory processes affecting cystic fibrosis. Accordingly, inhibition of its functions might have a clinical relevance and research efforts on this issue deserve great attention.

Materials and Methods

Synthetic oligonucleotides and peptide nucleic acids. The synthetic oligonucleotides used in this study were purchased from Pharmacia.

PNA-DNA-PNA oligomer assembly. Chimeras were assembled on solid phase, by sequential elongation of the PNA fragment, to whom DNA first and PNA were attached. The chimeras were obtained using Mmt(Bz)PNA monomers, as reported in previously published papers.[29,36] ESI-MS for gcg-ACC CCT GAA AGG T-gcc: $[M + 4\ H]^{4+}$: 1,438.2, $[M + 5\ H]^{5+}$ 1,150.5, calculated for $C_{193}H_{245}N_{90}O_{95}P_{13}$ 5,748.26. ESI-MS for cgc-TGG GGA CTT TCC A-cgg: $[M + 4\ H]^{4+}$: 1,443.6, $[M + 5\ H]^{5+}$: 1,154.9, calculated for $C_{194}H_{247}N_{86}O_{99}P_{13}$ 5,770.26.

Cell cultures and bacteria. IB3-1 cells (LGC Promochem) are human bronchial epithelial cells immortalized with adeno12/SV40, derived from a CF patient with a mutant F508del/W1282X genotype.[37] Cells were grown in LHC-8 basal medium (Biofluids) supplemented with 5% fetal bovine serum (FBS). All culture flasks and plates were coated with a solution containing 35 μg/ml bovine collagen (Becton-Dickinson), 1 μg/ml BSA (Sigma-Aldrich) and 1 μg/ml human fibronectin (Becton-Dickinson). *P. aeruginosa*, PAO1 laboratory strain, was kindly provided by A. Prince (Columbia University). Bacteria were grown in trypticase soy broth (TSB) or agar (TSA) (Difco).

Electrophoretic mobility shift assays. The double-stranded oligonucleotides (ODN) used in the EMSA are reported in

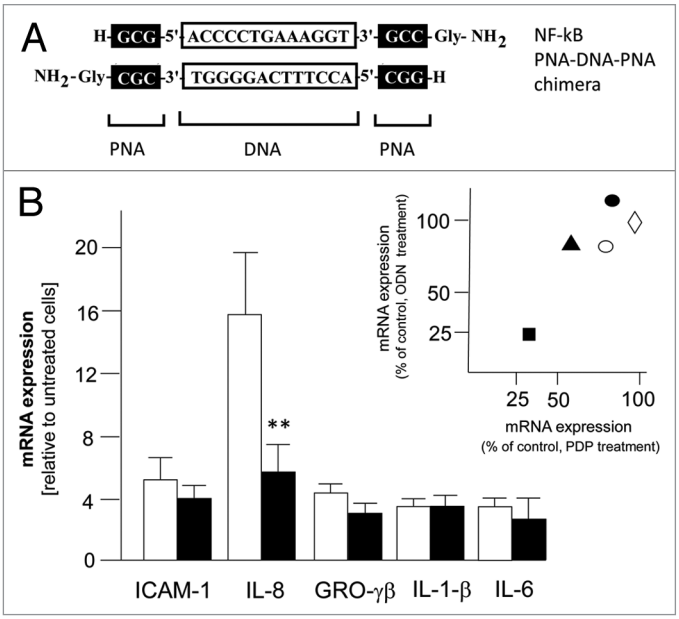

Figure 4. (A) Sequences of the double stranded NFkappaB PNA-DNA-PNA chimeras. DNA sequences are within white boxes; PNA sequences are within black boxes. (B) Effects of PDP/PDP NFkappaB decoy molecules on induction of different pro-inflammatory mRNAs. The PDP/PDP NFkappaB decoy chimera was tested on transcription of ICAM-1, IL-8, GRO-γβ, IL-1β and IL-6 genes in IB3-1 bronchial cells after infection with *P. aeruginosa*. Total RNA was extracted and processed for quantification of transcripts as described in Materials and Methods. White bars: PAO-1 infected IB3-1 cells; black bars: IB3-1 cells infected with PAO-1 and treated with NFkappaB decoy PDP/PDP chimeras. Values are mean ± SEM of four separate experiments. Significance in Student's t test between each scrambled and decoy ODNs: **p < 0.01. Insert. Relationship between the effects of PDP based and ODN-based NFkappaB decoys on accumulation of IL-8 (■), GRO-γβ (▲), ICAM-1 (○), IL-1β (◇) and IL-6 (●) in PAO-1 infected IB3-1 cells. Data represent mRNA content in respect to control decoy-untreated PAO-1 infected cells.

Table 1. ODN (3 pmol each) were ^{32}P-labeled using 10 U T4 polinucleotide kinase (MBI Fermentas) annealed to an excess of complementary ODN and purified from [γ-^{32}P]ATP (Perkin Elmer). Binding reactions were performed by incubating 2.5 μg of nuclear extract, obtained from IB3 cells as previously described,[12] and 16 fmol of ^{32}P-labeled double-stranded ODN, with or without competitor in a final volume of 20 μL of binding buffer (20 mM TRIS-HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 0.01% TritonX100, 0.05 μg·μL^{-1} of poly dI-dC, 0.05 μg·μL^{-1} of a single-stranded ODN). Competitor (100-fold excess of unlabeled ODNs) and nuclear extract mixture were incubated for 15 min and then probe was added to the reaction. After a further incubation of 30 min at room temperature samples were immediately loaded onto a 6% nondenaturing polyacrylamide gel containing 0.25× Tris/borate/EDTA (22.5 mM Tris, 22.5 mM boric acid, 0.5 mM EDTA, pH 8) buffer. Electrophoresis was performed at 200 V. Gels were vacuum heat-dried and subjected to autoradiography.

IB3-1 transfection with decoy ODNs and PDP/PDP chimeras. IB3-1 cells were seeded in 24-wells plates at a density of 30,000 cells/cm^2 and transfected with ODNs using cationic liposome vector Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Lipofectamine 2000 (4 μl) was diluted in 1 ml of serum-free LHC-8 basal medium (Biofluids) and double-stranded decoy or PDP/PDP chimeras were added and incubated for 10 min to generate liposome:DNA complexes. In parallel, a scrambled ODN was employed as negative control (**Table 1**). Then, the complexes were added to IB3-1 cells and incubated for 6 h. After this time of incubation cells were washed twice with serum-free culture medium and left at 37°C and 5% CO_2 for 20–24 h before pro-inflammatory challenge with *P. aeruginosa* (100 CFU/ml), IL-1β (10 ng/ml) or TNFα (50 ng/ml) for a further 4 h.

RNA extraction and quantitative RT-PCR. Total RNA from IB3-1 cells was purified using High Pure RNA Isolation Kit (Roche) and 2.5 μg RNA were reverse-transcribed to cDNA using the High Capacity cDNA Archive Kit and random primers (Applied Biosystems) in a final reaction volume of 100 μl. cDNA (5 μl) and primers (15 nM each) (**Table 1**) were mixed with Fast SYBR Green Master Mix (Applied Biosystems). GAPDH gene expression was determined as normalizer gene. Primer sets were purchased from Sigma-Genosys. Real Time PCR was performed in duplicate for both target and normalizer genes using the ABI PRISM 7900 HT Fast Real Time PCR System (Applied Biosystems) as follows: 50°C for 2 min, 95°C for 20 sec and 40 two-step cycles: 95°C for 1 sec and 60°C for 20 sec. Results were collected with SDS 2.3 Software (Applied Biosystems) and relative quantification was performed using the comparative threshold (Ct) method. Data were analyzed with RQ Manager Software 1.2 (Applied Biosystems). Changes in mRNA expression levels were calculated after normalization to calibrator gene. The ratios obtained after normalization are expressed as negative fold change over untreated samples.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation assays were performed by using the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology). A total of 5 × 10^6 IB3 cells were treated, for 10 min at room temperature, with 1% formaldehyde culture medium. Cells were washed in phosphate-buffered saline, and then glycine was added to a final concentration of 0.125 M. The cells were then suspended in 0.5 ml of lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-Cl, pH 8.1) plus protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride) and the chromatin subjected to sonication (using a Sonics Vibracell VC130 sonicator with a 2 mm probe). Fifteen 15-sec sonication pulses at 30% amplitude were required to shear chromatin to 200–1,000 bp fragments. Aliquots of 0.2 ml of chromatin were diluted to 2 ml in ChIP dilution buffer containing protease inhibitors and then cleared with 75 μl of salmon sperm DNA/protein A-agarose 50% gel slurry (Upstate Biotechnology) for 1 h at 4°C before incubation on a rocking platform with either 10 μg of NFkappaB p65 specific antiserum (sc-372X, Santa Cruz Biotechnology) or normal rabbit serum as negative control IgG (Upstate Biotechnology). Twenty microliters of diluted chromatin was saved and stored for later PCR analysis as 1% of the input extract. Incubations occurred

overnight at 4°C and continued an additional 1 h after the addition of 60 µl protein A-agarose slurry. Thereafter the agarose pellets were washed consecutively with low salt, high salt and LiCl buffers. DNA/protein complexes were recovered from the pellets with elution buffer (0.1 M $NaHCO_3$ with 1% SDS), and cross-links were reversed by incubating overnight at 65°C with 0.2 M NaCl. The samples were treated with RNase A and proteinase K, extracted with phenol/chloroform and ethanol-precipitated. The pelletted DNAs were washed with 70% ethanol and dissolved in 40 µl of Tris/EDTA. Two µl aliquots were used for each real-time PCR reaction to quantitate immunoprecipitated promoter fragments. For quantitative real-time PCR reaction, a pair of primers that amplify a 301 bp region on the IL-8 promoter, containing the NFkappaB binding site, was designed (IL-8 ChIP f, IL-8 ChIP r, **Table 1**).

PCR reactions were also performed using negative control primers that amplify a 255 bp genomic region about 5 kb upstream of the IL-8 promoter, lacking NFkappaB binding sites (Neg ChIP f, Neg ChIP r, **Table 1**). Each Real-time PCR reactions were performed in 25 µl of final volume, using 2 µl of template DNA (from chromatin immunoprecipitations), 10 pmol of primers and 1 × iQ™ SYBR® Green Supermix (Bio-Rad) for a total of 45 cycles (96°C for 15 sec, 66°C for 30 sec and 72°C for 20 sec) using an iCycler IQ® (Bio-Rad). The relative proportions of immunoprecipitated promoter fragments were determined based on the threshold cycle (Tc) value for each PCR reaction. Real time PCR data analysis were obtained using the comparative cycle threshold method.[38] The data represent the ratios between the IL-8 promoter Q-PCR performed on NFkappaB specific ChIP and negative IgG (preimmune rabbit serum) ChIP. Each value was derived employing chromatin isolated from untreated IB3-1 cells or cells infected with PAO-1 in the absence or in the presence of pre-treatment with the NFkappaB decoy ODN. Each sample was performed in duplicate on at least three separate experiments.

Statistics. Results are expressed as mean ± standard error of the mean (SEM). Comparisons between groups were made by using paired Student's t test and a one-way analysis of variance (ANOVA). Statistical significance was defined with $p < 0.05$ (*significant) and $p < 0.01$ (**highly significant).

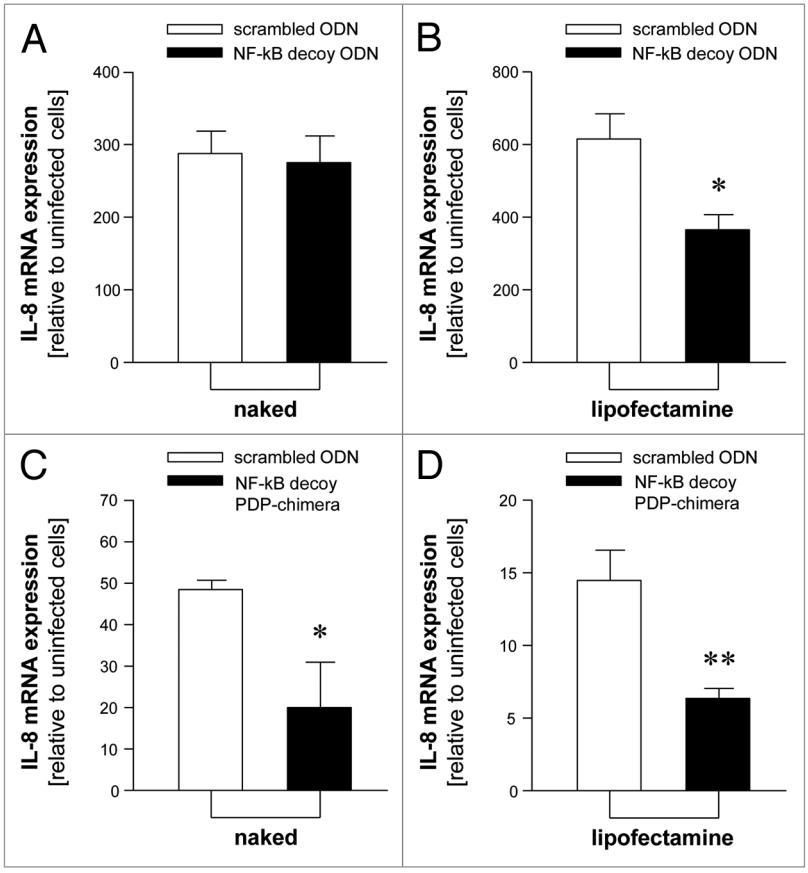

Figure 5. Comparison of the effects on IL-8 mRNA expression of ODN (A and B) and PDP-based decoys (C and D) in the absence (naked molecules) or in the presence of lipofectamine. Values are mean ± SD of three separate experiments. Significance in Student's t test between each scrambled and decoy ODNs: *$p < 0.05$; **$p < 0.01$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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