Specific covalent binding of a NF-κB decoy hairpin oligonucleotide targeted to the p50 subunit and induction of apoptosis

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Abstract The NF-κB transcriptional factor regulates various functions such as immune responses, cellular growth and development, and is frequently activated in tumor cells. Thus, inhibition of NF-κB could suppress tumor cell growth. Using a decoy synthetic hairpin-shaped oligodeoxyribonucleotide (ODN) containing the κB site with an integrated single diphosphoryldisulfide linkage, we demonstrate its covalent binding to the p50 subunit of NF-κB. Furthermore, this decoy ODN induces apoptosis in a lymphoblastoma cell line. Thus, such chemically modified decoys could be valuable tools for blocking nuclear factors and tumor cell growth.

Key words: Nuclear factor-κB; p50 subunit; Hairpin DNA duplex; Decoy; Disulfide bond

1. Introduction

The transcription factor decoys (TFDs) are modified synthetic DNA molecules which block binding of transcription factors to the target gene by mimicking the specific DNA binding sites. Such decoys are efficient and specific inhibitors of transcription. For instance, decoy oligodeoxyribonucleotides (ODNs) of the transcription factors E2F, NF-κB, CREB or AP-1 have been shown to efficiently cure vascular proliferation [1], myocardial infarction [2], or to prevent tumor growth and invasiveness [3,4]. Initially shown to be implicated in host defense and inflammatory response [5], NF-κB is associated with induction of apoptosis in some cells but it is also involved in protection against apoptosis in Epstein–Barr virus (EBV)-transformed B cells [6]. EBV can induce the proliferation of resting B cells in vitro yielding immortalized cell lines. In these cell lines the viral protein latent membrane protein-1 induces NF-κB by mimicking a constitutively active receptor of the tumor necrosis factor receptor family [7], and the specific inhibition of NF-κB promotes apoptosis [8,9] demonstrating its fundamental role. Chemical modifications of defined positions in a DNA probe have been previously shown to allow crosslinking of the corresponding DNA binding region of the transcription factor. Sets of DNA or RNA duplexes with chemically active internucleotide linkages were successfully used for affinity modification and probing the nucleic acid binding regions of restriction–modification enzymes [10], of RNA recognizing TAT peptide [11] and of transcription factors [12]. Recently, a new class of reactive double-stranded (ds) DNA derivatives which can crosslink with proteins recognizing specific nucleic acid sequences was investigated [13]. In this study we analyzed a new hairpin-shaped modified TFD comprising a NF-κB binding sequence and an integrated diphosphoryldisulfide linkage replacing the native phosphodiester bond (Fig. 1A). According to our previous data [13], this modification can react with nearby cysteine residues by a thiol–disulfide exchange reaction within the specific dsDNA–protein complex. Indeed, X-ray analysis of the NF-κB–DNA complex has shown that the cysteine 59 is located at a distance of 3.98 Å from the internucleotide phosphate group that we chose to chemically modify [14]. This proximity allows covalent entrapment of the decoy ODN with the NF-κB p50 subunit. The new hairpin decoy ODN thus synthesized was shown to efficiently bind the NF-κB p50 subunit and to inhibit cell growth and induce apoptosis in a lymphoblastoid cell line (LCL).

2. Materials and methods

2.1. Synthesis of the NF-κB decoy oligonucleotide

Oligonucleotides with 3′- and 5′-phosphorothioate groups used for preparing the hairpin-shaped decoy, 5′-TGGGAATT-3′-OP(=O)-(OH)(SH) (1) and 5′-(HS)(HO)P(=O)O-CCCCTCGAAGAGGG-GAATTCCCA (2), and the scramble control, 5′-CAGTCACTACG-CAAAGCGTAG-3′-OP(=O)(OH)(SH) (3) and 5′-(HS)(HO)P(=O)-O-TGACTGCACGGT (4), were prepared as described [13]. They were kept in boiled water under argon to avoid their oxidation to disulfide-linked dimers by solved oxygen. The oligonucleotide 5′-phosphorothioate (2) was converted in 95% yield to the 2-pyridyldisulfide derivative by treatment with 0.03 M 2,2′-dipyridyldisulfide in buffer 1 pH 4.5–5 (0.015 M Na-citrate, 0.15 M NaCl, 0.02 M $MgCl_2$), 50% dimethylformamide for 40 min at room temperature and was isolated from the reaction mixture by ethanol precipitation. The other duplex-forming component (1) was then added with a 1.3 molar excess to the 2-pyridyldisulfide derivative in buffer 1 for 40 min at room temperature. The decoy hairpin ODN was isolated from the reaction mixture by ethanol precipitation and analyzed on 20% polyacrylamide, 7 M urea denaturing gel electrophoresis. The reaction product was extracted from gel slices with 10 mM Tris–HCl, pH 7.6, 0.3 M NaCl, 1 mM EDTA overnight at room temperature and isolated by ethanol precipitation.

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Abbreviations: TFD, transcription factor decoy; EBV, Epstein–Barr virus; ODN, oligodeoxyribonucleotide; LCL, lymphoblastoid cell line

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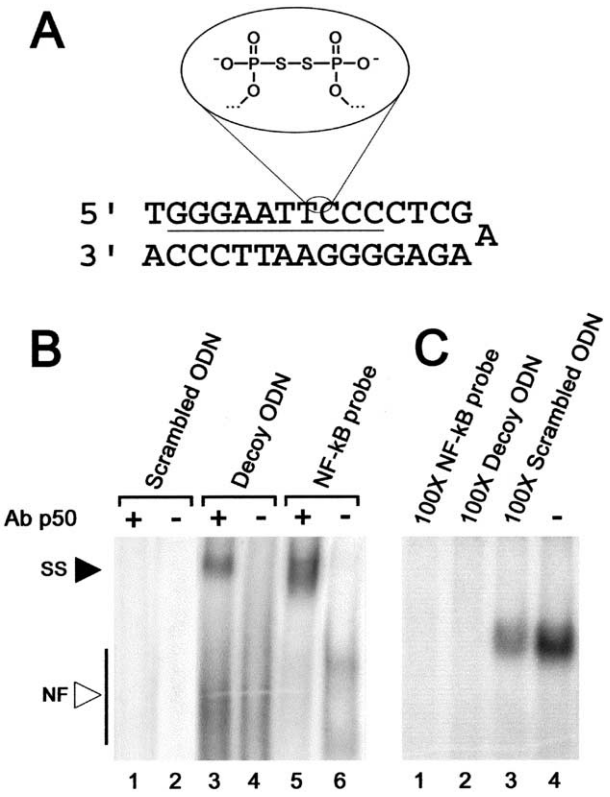

Fig. 1. Binding of the NF-κB decoy ODN to NF-κB detected by EMSA. A: Sequence of the modified hairpin decoy ODN showing the position of the diphosphoryldisulfide link (the κB binding site is underlined). B: Binding of ^{32}P-labeled oligonucleotide probes containing κB sites to the p50 subunit of NF-κB from LCL nuclear extracts. EMSA for scrambled ODN (lanes 1 and 2), decoy ODN (lanes 3 and 4), and control NF-κB probe (lanes 5 and 6) in the absence (−) or in the presence (+) of p50 antibody. The NF-κB–ODN complex (NF) and supershifts (SS) are indicated by white and black arrowheads respectively. C: Competition experiments: lane 1: excess (100×) unlabeled κB probe; lane 2: excess (100×) decoy ODN; lane 3: excess (100×) scrambled ODN; lane 4: κB probe.

2.2. Cell culture and cell transfection

The EBV-transformed lymphoblastoid cell line LCL (Priess) was grown in RPMI 1640 (Eurobio, Les Ulis, France) supplemented with 10% decomplemented fetal calf serum (Invitrogen, Cergy Pontoise, France), 100 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine at 37°C in a humidified 5% CO_2 atmosphere. For transfection, 5×10^6 cells were resuspended in RPMI without fetal calf serum and electroporated with the decoy ODN or the scrambled ODN at 250 V and 960 μF (Bio-Rad, Vitry/Seine, France). In some experiments daunorubicin (Cerubidine 20 mg, Rhône Poulenc, Lyon, France) was added at 0.25 μM 5 h after transfection.

2.3. Western blotting

The cells were washed twice in phosphate-buffered saline, counted and lysed in 2×sodium dodecyl sulfate (SDS) sample buffer (Bio-Rad) followed by brief sonication to break the DNA. The samples were separated on SDS–polyacrylamide gels (8 and 10%) and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Following transfer, the membranes were stained in Ponceau red to verify equal protein loading. The membranes were then washed in water for 30 min and incubated for 2 h with Tris-buffered saline (10 mM Tris, pH 8, 30 mM NaCl, TBS) supplemented with dried skimmed milk (3%). Specific antibodies were added for 2 h. After three washes of 30 min each with TBS, the secondary antibodies conjugated with horseradish peroxidase (1:5000 in TBS) were added for 1 h, followed by another three washes of 30 min in TBS. Bands were visualized on X-Omat R films (Kodak, Rochester, NY, USA) by enhanced chemiluminescence following the supplier's procedure (Amersham Biosciences, Saclay, France). The antibodies against human p50 subunit (rabbit immune serum #1157) used for Western blotting (1:2000), immunoprecipitation (1:30) (rabbit immune serum #1141) and supershifts were a generous gift of Dr. N. Rice (NCI, Frederick, MD, USA). The cleaved poly(ADP-ribose) polymerase (PARP) (Asp214) human specific antibody (Cell Signaling, Beverly, MA, USA) was diluted at 1:1000.

2.4. Electrophoretic migration shift assay (EMSA)

Two oligonucleotides, 5′-AGTTGAGGGGACTTTCCCAGGC and 5′-GCCTGGGAAAGTCCCCTCAACT (Proligo, France), were annealed to give a control NF-κB probe. Nuclear extracts were prepared as described [15]. Total proteins were assayed using the Bradford procedure (Bio-Rad). The control NF-κB probe, scrambled ODN and TFD (100 ng) were labeled at the 3′ end using terminal deoxynucleotidyl transferase (MBI, Euromedex, Mundolsheim, France). ^{32}P-labeled ODNs were purified on a column (Qiaquick removal nucleotide kit, Qiagen, Courtaboeuf, France). The DNA binding reaction mixture contained: 20 μg of nuclear extract and 3–10 ng of ^{32}P-labeled ODN. The reaction was performed in a final volume of 30 μl in a buffer containing 20 mM HEPES, 20% glycerol v/v, 0.1 M KCl, 0.2 mM EDTA and 714 μg/ml of salmon sperm DNA (Sigma-Aldrich, Saint Quentin Falavier, France). For competition a 100-fold excess of the non-radiolabeled competitor ODN was added to the sample before addition of the ^{32}P-labeled ODN. To identify the proteins, antibodies to NF-κB p50 subunit at 1/10 of the final volume were added to the mixture. The samples were analyzed on a 6% non-denaturing polyacrylamide gel run at 150 V for 4 h. The gels were dried and exposed on a phosphor storage screen (Packard, BD, France).

2.5. Covalent binding assay

Nuclear extracts were incubated with the radiolabeled ODN overnight at 4°C. In some experiments 2-mercaptoethanol was added (5, 6 or 10 μM) for 1 h at 37°C, before or after binding. The samples were loaded on SDS–polyacrylamide gel (8%). After electrophoresis and Western blotting, the dried nitrocellulose membranes were exposed

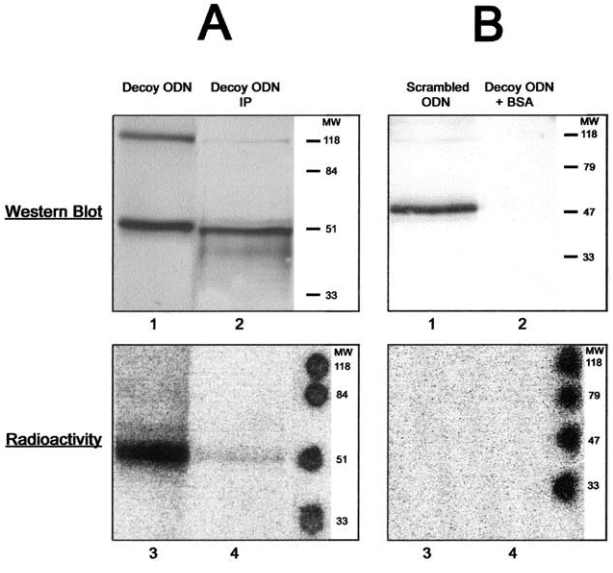

Fig. 2. Demonstration of a covalent bond between the decoy ODN and the p50 subunit of NF-κB. A: Nuclear extracts from LCL cells were incubated with radiolabeled decoy ODN. Western blotting was performed directly from extracts (lane 1) or following immunoprecipitation with anti-p50 antibody (lane 2). The same nitrocellulose membrane was revealed for radioactivity of direct extract (lane 3) or following immunoprecipitation (lane 4). Radioactive ink (5000 cpm) was spotted on membranes to indicate molecular weights and as a standard. B: Controls: a radiolabeled control scrambled ODN was incubated with nuclear extract (lanes 1 and 3) and the decoy ODN was incubated with bovine serum albumin instead of nuclear extract (lanes 2 and 4).

on phosphor storage screen, radioactive intensities were determined using the screen system controlling software (Packard, BD). After exposure, membranes were blocked and processed as above.

3. Results

The binding of the decoy ODN to NF-κB was analyzed by EMSA with nuclear extracts from LCL in which NF-κB is known to be activated [8]. The results (Fig. 1B) show that the decoy ODN binds a component which comigrates with NF-κB. To identify the protein bound to the radiolabeled decoy ODN, supershifts were performed with antibody to the NF-κB p50 subunit. The supershift observed with the decoy ODN was similar to that observed using the control NF-κB probe (Fig. 1B), thus demonstrating specific binding to the p50 subunit of NF-κB. No specific binding was observed with the modified scrambled ODN, moreover, competition with an excess of unlabeled decoy ODN prevented binding of the labeled p50 NF-κB probe (Fig. 1C) demonstrating that the NF-κB decoy binds specifically to the NF-κB p50 subunit. To show that the decoy binds through a covalent bond, the nuclear extracts were incubated with the decoy ODN then treated with SDS and analyzed by SDS–polyacrylamide gel electrophoresis and Western blotting. After transfer, a radioactive band migrated at the same level as the NF-κB p50 subunit and was revealed on the same membrane with a specific antibody (Fig. 2A). Nuclear extract incubated with the decoy ODN and immunoprecipitated with anti-p50 antibody revealed a radioactive 50 kDa band. No binding was detected using either bovine serum albumin or a control scrambled ODN containing the same chemical modification as the decoy ODN (Fig. 2B). Since the covalent bond between the decoy ODN and NF-κB must be a disulfide bond, we treated the crosslinked complex with 2-mercaptoethanol after binding, releasing over 50% of the radioactivity (Fig. 3A). Moreover, addition of the 2-mercaptoethanol before binding completely prevented crosslinking of the modified decoy (Fig. 3B). Altogether, these observations demonstrate that the decoy ODN binds the p50 subunit of NF-κB through a covalent disulfide bond. As NF-κB has been reported to protect cells from apoptosis in the LCL [8,9] we examined whether the decoy affected cell proliferation or apoptosis. Cells were transfected with the decoy ODN and also treated with daunorubicin, a DNA-intercalating chemotoxic drug known to induce apoptosis. Twenty-four hours after transfection with the decoy ODN, cell proliferation was reduced (Fig. 4A) with an amplitude equivalent to that observed with daunorubicin-treated cells. This effect was enhanced when cells were treated with daunorubicin and the decoy ODN. Transfection of the scrambled ODN did not affect cell proliferation. Apoptosis in the cells transfected with the decoy ODN was analyzed using an antibody raised against the cleaved form of PARP [16]. As little as 0.1 μg of the ODN decoy was sufficient to

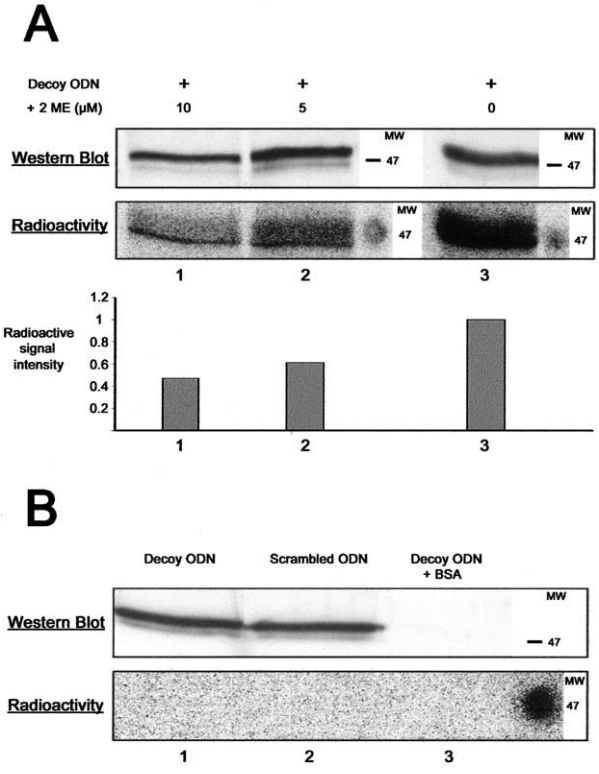

Fig. 3. The bond between the p50 subunit of NF-κB and the decoy ODN is cleaved by 2-mercaptoethanol. A: After binding of the radiolabeled decoy ODN, nuclear extracts were incubated with no (lane 3), 5 μM (lane 2) or 10 μM (lane 1) of 2-mercaptoethanol (2 ME). After gel separation, Western blotting was performed with p50 antibody following which the same membrane was analyzed for radioactivity. Histograms indicate the intensities of the radioactive bands detected. B: The crosslinking between the radiolabeled decoy ODN and the NF-κB p50 subunit is completely prevented by 2-mercaptoethanol. Cell nuclear extracts were incubated with 2-mercaptoethanol before addition of decoy ODN (lane 1) or control scrambled ODN (lane 2). Western blotting was performed with p50 antibody. The decoy ODN was incubated with bovine serum albumin instead of nuclear extract (lane 3).

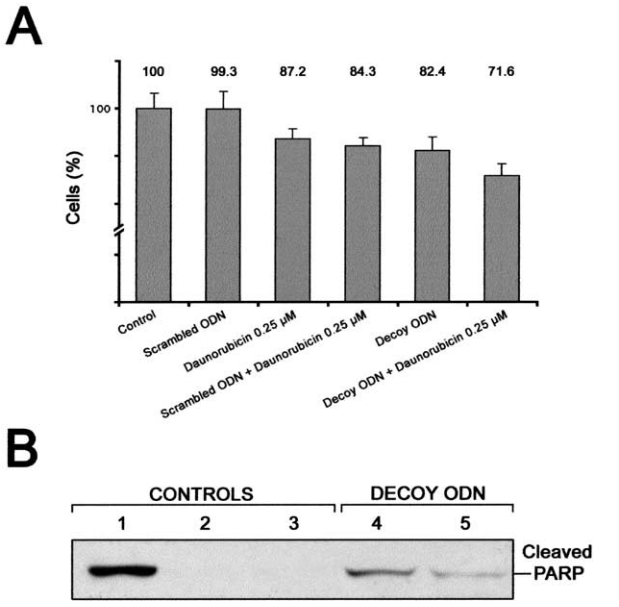

Fig. 4. Effects of the decoy ODN on cell growth and apoptosis. A: Inhibition of the proliferation of LCL cells transfected with decoy ODN (1 μg) or scrambled ODN (1 μg) with or without daunorubicin. Cell proliferation was expressed as a percentage of the level obtained with control electroporated cells. B: Induction of cell apoptosis by decoy ODN as determined by measuring PARP cleavage. LCL cells transfected with 1 μg (lane 4) and 0.1 μg (lane 5) decoy ODN. Control electroporated cells (lane 2). Cells transfected with 1 μg of control scrambled ODN (lane 3). Burkitt's lymphoma cell line BL2 as a cleaved PARP control (lane 1).

induce apoptosis in the LCL and this was increased with 1 μg of decoy ODN (Fig. 4B). Cleavage of PARP was not detected in control electroporated cells or in cells electroporated with control scrambled ODN, although it contained the reactive linkage designed to specifically bind NF-κB. Burkitt's lymphoma cells showing a high level of spontaneous apoptosis were used as a PARP cleavage control. Altogether these results suggested that the modified NF-κB decoy ODN reduces the growth and induces apoptosis in cells.

4. Discussion

The aim of this work was to determine the specificity of a chemically modified decoy ODN towards its target, and analyze its effect in cells. Based on X-ray analysis [14], the modified moiety was introduced at a selected position of the decoy ODN to permit covalent bond formation with NF-κB p50.

Using nuclear cell extracts which represent a complex mixture of hundreds of DNA binding proteins, we demonstrated that the decoy ODN binds only to the NF-κB p50 subunit. Our EMSAs and supershifts show identical migration characteristics of the control NF-κB probe and the decoy ODN. We further verified that the decoy ODN binds the p50 subunit covalently, and found that the decoy ODN and the p50 comigrate following SDS treatment and that the bond is reversed by 2-mercaptoethanol thereby demonstrating that it is a disulfide bond.

Crystallographic studies have shown that the p65 subunit can also interact via a cysteine residue with a phosphate group of the κB binding site [14] suggesting that the decoy ODN could bind the p65. However, in our experiments we did not detect any binding of the decoy ODN with the p65 subunit. As only p50 hetero- or homodimers bind DNA, the crosslinking of the decoy ODN may reduce the amount of NF-κB dimers consisting mostly of p65/p50 heterodimers [17], thereby resulting in a dominant negative effect. Since inhibition of NF-κB in the LCL induces apoptosis [8], the enhanced apoptosis and reduced cell growth of LCL cells can be attributed to the blocking of NF-κB dimers by the binding of the decoy ODN.

Overall, the present study demonstrates that a chemically modified decoy can specifically and covalently bind the NF-κB p50 subunit, induce apoptosis and slow down cell growth. Such a decoy may have therapeutic applications and the technical approach of covalent blocking could also apply to other DNA binding factors.

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