NF-κB p50 subunit cross-linking to DNA duplexes, containing a monosubstituted pyrophosphate internucleotide bond

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Abstract The new express technique based on the use of BrCN to synthesize DNA duplexes, containing non-substituted or monosubstituted pyrophosphate internucleotide bonds has been proposed. Using this technique, DNA duplexes having modified internucleotide bonds between dT and dC residues in the human NF-κB transcription factor recognition sequence in HIV-1 (5′-GGAAAGTCCC-3′) have been prepared. We demonstrate that these internucleotide bonds within the recognition site do not prevent the formation of NF-κB p50 subunit complex with the corresponding duplexes. The cross-linking of NF-κB p50 subunit to the DNA duplex containing a monosubstituted pyrophosphate internucleotide bond has been successfully performed.

Key words: Transcription factor; Activated internucleotide bond; Covalent binding

1. Introduction

The mammalian transcription factor NF-B was first identified as B-cells protein complex specifically binding with the enhancer of the immunoglobulin k-light chain [1]. Later, NF-κB was shown to recognize and bind to the 10-base pair (bp) consensus site of double-stranded DNA possessing a common structure: 5′-GGPuPuNNPyCCC-3′ (where Pu indicates A or G; Py C or T; and N A,G,C or T). In some cases, it can interact with the sequences differing from the consensus site [2].

NF-κB is activated in many different cell types and regulates expression of a large number of genes involved into the response to infection and stress. This protein also takes part in the transcription activation of the human immunodeficiency virus genes ([3] and references therein). Artificial inhibition of gene expression in the field of cancer or retroviruses is the main purpose of detailed studies of NF-κB functioning. One of the approaches is based on the using of the excess of short synthetic DNA duplexes containing NF-κB recognition sequenses as a decoy for the transcription factor [4–6]. If these DNA duplexes are able to form a covalent linkage with the protein, the inhibition effect would be increased. The method of incorporation of the reactive substituted pyrophosphate groups into the sugar-phosphate backbone of oligodeoxyribonucleotides (oligos) via chemical ligation has been developed [7]. These reactive groups provide a covalent binding of oligos to different nucleophiles, including those that occur in proteins [8]. Therefore, a covalent linkage of a target protein and an oligonucleotide could be obtained as a result of the cross-linking [8–10]. We propose these type of activated DNA duplexes as inhibitors of human NF-κB transcription factor activity. We suppose that the covalent binding of the duplex fragment to NF-κB could inhibit NF-κB activity.

In this report, we have studied interaction of the 32-bp synthetic duplex containing a monosubstituted pyrophosphate bond with p50 subunit of the NF-κB transcription factor. The expression of p50 part of human p105 in *E. coli* is known to yield soluble and active protein which binds specifically to NF-κB recognition sites as a dimer [11]. The sequence of the DNA duplex under study is derived from the long terminal repeat of HIV-1 and has two NF-κB recognition sites. One of them includes the reactive monosubstituted pyrophosphate internucleotide bond (Fig. 1, duplex I). Since p50 subunit of NF-κB preferably binds to the first half-site containing three GC pairs [12], we placed the reactive monosubstituted pyrophosphate group just near this GC cluster.

The novel, very simple and quick method of monosubstituted pyrophosphate group incorporation in DNA duplexes has been developed. This method is based on the usage of BrCN instead of traditional carbodiimide. We show the possibility of p50 NF-κB subunit cross-linking to DNA duplexes with monosubstituted pyrophosphate internucleotide bonds.

2. Materials and methods

2.1. Purification of the fusion protein p50-GST

The p50 subunit of NF-κB coupled to glutathione-*S*-transferase (GST) and expressed in *E. coli* was purified as described previously [4,5]. The plasmid construction was kindly offered by Professor Alain Israel (Institut Pasteur, Paris, France). A p50-GST monomer molecular weight is 76 kDa.

2.2. Oligonucleotides

The 24-membered substrate II which we used as a positive control for testing p50-GST protein activity was synthesized as described in [5]. The oligos ACAAGGGACTTTCCGCTGGGGACTTTCCAGGG (1), CCCTGGAAAGTCCCCAGCGGAAAGTCCCTTGTTT (2), CCCTGGAAAGTp (3), CCCCAGCGGAAAGTCCCTTGTTT (4), which are components of duplexes I, III and IV, were from Genset (Paris, France). 5′-Phosphorylation and ^{32}P-labeling of oligos were carried out using T4-polynucleotide kinase and ATP or [γ-^{32}P]ATP.

Synthesis of the methyl ester of the 3′-phosphorylated oligonucleotide (3) was carried out using 1-ethyl-3′,3′-(dimethylaminopropyl)carbodiimide (CDI)-induced activation of the 3′-end phosphate in the presense of methanol [13].

2.3. Synthesis of DNA duplexes containing modified internucleotide bonds

To obtain oligonucleotide containing a pyrophosphate internucleo-

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Abbreviations: CDI, 1-ethyl-3′,3′-(dimethylaminopropyl)carbodiimide; DTT, dithiothreitol; GST, glutathione-*S*-transferase; MES, 2-morpholinoethane sulfonate

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Table 1
The influence of the buffer components and concentration on the efficiency of the modified internucleotide bond synthesis

Reaction conditions		Ligation product yield (%)	
Buffer	Number of BrCN treatments	Pyrophosphate bond	Monosubstituted pyrophosphate bond
0.25 M MES, 0.02 M $MgCl_2$, pH 7.5	1	17–19	4–6
	2	28–30	7–9
	3	32–34	11–13
1 M MES, 0.02 M $MgCl_2$, pH 7.0	1	38–40	18–20
	2	66–68	28–30
	3	76–78	38–40
1 M *N*-methylmorpholine, 0.02 M $MgCl_2$, pH 7.5	1	62–64	18–20
	2	73–75	26–28
	3	80–82	33–35

tide bond, 5′-phosphorylated oligomer (4), oligonucleotides (1) and (3) were mixed in equimolar ratio, liophilized and dissolved in 100 μl of the 1 M *N*-methylmorpholine buffer (pH 7.5, 0.02 M $MgCl_2$). The duplex concentration was 10^{-4} M per monomer. Then, 10 μl of 5 M BrCN solution in dry acetonitrile were added. The reaction mixture was incubated for 1–3 min at room temperature. Then, DNA fragments were precipitated by adding of 100 μl of 2 M $LiClO_4$ and 1 ml of acetone followed by centrifugation. The oligonucleotide mixture was treated 2× by the same way and the reaction product was isolated by electrophoresis in 20% PAGE, 7 M urea. DNA fragments were visualized by autoradiography.

To obtain oligonucleotide, including a monosubstituted pyrophosphate internucleotide bond, chemical ligation of the 5′-phosphorylated oligomer (4) with the methyl ester of oligonucleotide (3) on the template (1) was carried out in the 1 M 2-morpholinoethane sulfonate (MES) buffer (pH 7.0, 0.02 M $MgCl_2$) as described above.

2.4. DNA–protein binding assay

The binding of p50-GST (3 μg) to the ^{32}P-labeled DNA duplexes (0,1 nM) was carried out in 50 μl of buffer L (7.5 mM Hepes, pH 8.0, 35 mM NaCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM DTT), containing 15% (v/v) glycerol. The reaction mixture was incubated for 10 min at room temperature and loaded onto the 6% polyacrylamide gel (acrylamide/bis acrylamide ratio was 19/1). No marker dye was added to the samples. The electrophoresis was carried out at room temperature for 7 h at 140 V. The electrode buffer included 45 mM Tris-borate, pH 8.0, 1 mM EDTA. The gel was autoradiographed at −20°C.

2.5. Cross-linking assay

Cross-linking of p50-GST to substrate I was performed under the conditions of DNA–protein binding assay for 18 h. Reactions were followed by 0.1% SDS–12% PAGE [14] after heating samples in 0.1% SDS-2-mercaptoethanol solution at 95°C. The formation of enzyme covalent adduct with duplex I was monitored by autoradiography. The same gel was stained with Coomassie blue to vizualize the protein bands.

3. Results and discussion

3.1. Design of the modified duplexes

In this report, we used two types of the modified substrates, viz. DNA duplexes containing pyrophosphate or monosubstituted pyrophosphate internucleotide bonds in the NF-κB recognition site (Fig. 1, duplexes I and IV, respectively). Up to now, the only reagent for the successful synthesis of these modified internucleotide bonds was water-soluble CDI [7]. It should be noted that sometimes the ligation method using CDI is not suitable because of too long reaction time. Moreover, sometimes the ligation product purity is not sufficient because of dG- and dT-residue modification by CDI. To avoid the heterocyclic base modification and to accelerate the modified internucleotide bond synthesis we decided to use BrCN as a condensing reagent. The efficiency of BrCN-induced phosphodiester bond formation is about 90–95%, the reaction time is 1–3 min. But the pyrophosphate bond was formed with the significantly less efficiency [15]. Synthesis of a monosubstituted pyrophosphate internucleotide bond using BrCN was not performed until now and is originally proposed in this report. We studied the influence of the buffer composition and concentration on the efficiency of pyrophosphate and monosustituted pyrophosphate internucleotide bond formation. The effect of single, double and thrice-repeated treatment of the reaction mixture by BrCN was also investigated. The results obtained are summarized in Table 1.

The optimal reaction time was 1–3 min, the further increase of the reaction time did not lead to the more efficient reaction proceeding: the product yield was not increased. The optimal reaction conditions are the following: 1 M *N*-methylmorpholine (for pyrophosphate bond) or 1 M MES (for monosubstituted pyrophosphate bond) buffer, pH 7.5, 20°C (for stable duplexes), thrice-repeated BrCN treatment (with the intermediate precipitation of DNA followed by centrifugation, drying and dissolving in a new portion of the buffer), the time of each BrCN treatment, 1 min.

The method proposed allows one to obtain the desirable substituted or non-substituted pyrophosphate internucleotide bond with the same yield as CDI-induced ligation provides, but the product is much more pure and the reaction rate is remarkably higher: common time of pyrophosphate product

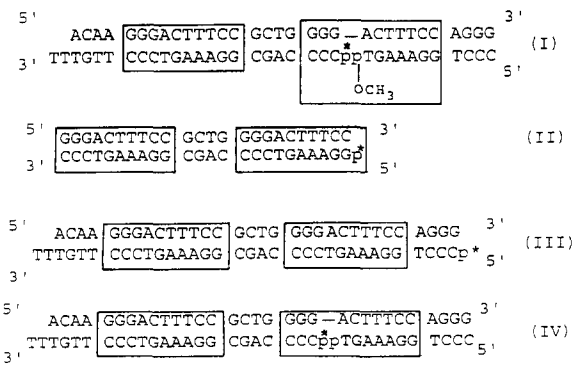

Fig. 1. DNA duplexes, substrate analogs of NF-κB transcription factor: duplex I contains a reactive monosubstituted pyrophosphate internucleotide bond; II and III, control duplexes without modifications; IV, control duplex with a stable non-substituted pyrophosphate internucleotide bond. *The location of ^{32}P label. The recognition sites of NF-κB are pick out by frames. pp, pyrophosphate internucleotide bond in duplex IV.
*$^{pp}_{|}$, monosubstituted pyrophosphate internucleotide bond in duplex I.
$_{OCH_3}$

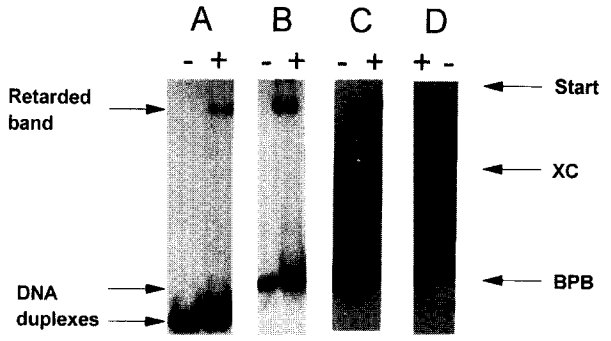

Fig. 2. Gel retardation assay. All the duplexes are ^{32}P-labeled (duplex II, lanes A; duplex III, lanes B; duplex IV, lanes C; duplex I, lanes D). +, incubation products with the purified p50 subunit of NF-κB; −, control incubation without protein; BPB, bromophenol blue; XC, xylencyanol.

preparation is less than 1 h whereas CDI-induced ligation proceeds for 24–48 h.

3.2. *Visualization of p50-GST-DNA complex using retardation assay*

Retardation assays were performed in order to detect if p50-GST is able to recognize the duplexes I–IV. ^{32}P-labeled DNA duplexes I–IV were incubated with the constant amount of p50-GST under conditions of nucleic acid–protein complex formation and analyzed by electrophoresis in 6% non-denaturing gel. Bands with the lower mobility than those of the initial duplexes were observed (Fig. 2). These bands can be reffered to the nucleic acid–protein complexes. The similarity of results obtained in retardation assay for all DNA duplexes allows us to conclude that the introduction of the non-substituted as well as monosubstituted pyrophosphate internucleotide bonds into the recognition site does not prevent formation of the nucleic acid–protein complex.

3.3. *Cross-linking experiments*

The ^{32}P-labeled duplex I was used for affinity cross-linking to p50-GST. ^{32}P-label was in the disubstituted phosphate group of the pyrophosphate bond. This group undergoes the attack of a nucleophile, e.g. an amino group. If the protein contains an amino group adjacent in zero distance to the monosubstituted pyrophosphate internucleotide bond, the disubstituted phosphate is responsible for the covalent bond formation between the oligonucleotide (4) and p50-GST (Fig. 3). Thus, the resulting protein–oligonucleotide covalent complex can be detected by denaturing PAGE owing to the presence of ^{32}P-label in the band corresponding to the protein.

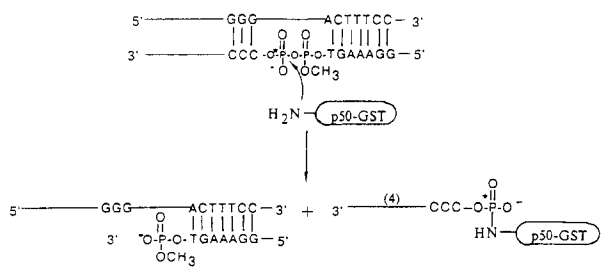

Fig. 3. Scheme of the protein nucleophylic group interaction with duplex I containing a monosubstituted pyrophosphate internucleotide bond.

It was found that the cross-linking of some DNA-binding enzymes to reactive substrate containing a monosubstituted pyrophosphate internucleotide bond has a reaction half-time about 4 h and a saturation time about 8 h ([10] and unpubl. results). So, the ^{32}P-labeled duplex I and p50-GST protein were incubated overnight at room temperature (see section 2 for other details). The reaction mixture was analyzed in SDS-PAGE with protein molecular weight standards to determine the size of the protein subunits attached to the oligonucleotide (4). As seen from Fig. 4a, two ^{32}P-labeled bands (bands A and B) with the less mobility than that of the monosubstituted pyrophosphate containing strand of the duplex I appear. We assume these bands to correspond to the products of NF-κB cross-linking to oligonucleotide (4) because SDS treatment of the reaction mixture at 95°C excludes formation of non-covalent complexes. At the same time, only one band (about 75 kDa) having the same mobility as band A was observed in the gel stained with Coomassie blue (Fig. 4b). Since the protein-containing band in the Coomassie-stained gel could be superposed with the band A of the autoradiograph, we can conclude that the band A corresponds to the ^{32}P-labeled oligodeoxyribonucleotide-p50-GST monomer complex. These results confirm the cross-linking of p50-GST to the radioactively labeled oligonucleotide (4). Nucleic acid–protein complex corresponding to band A has the same mobility as p50-GST monomer in SDS-PAGE gel because of too small weight of the oligonucleotide if compared with the protein.

The slower and more intensive band B in autoradiogram (Fig. 4a) has an apparent molecular weight higher than 100 kDa and approximately twice than that of p50-GST monomer. The protein is not visualized in this band probably because of too small amount of nucleic acid–protein complex. At present, we are studying the structure of this compound.

Our extensive experience in the field of cross-linking of DNA duplexes containing a monosubstituted pyrophosphate internucleotide bond to DNA-binding proteins allows us to conclude that the specificity of cross-linking in this case depends on the specificity of DNA-binding by the protein. We have shown that proteins which do not interact with DNA (e.g. albumin) are not able to cross-link to reactive DNA

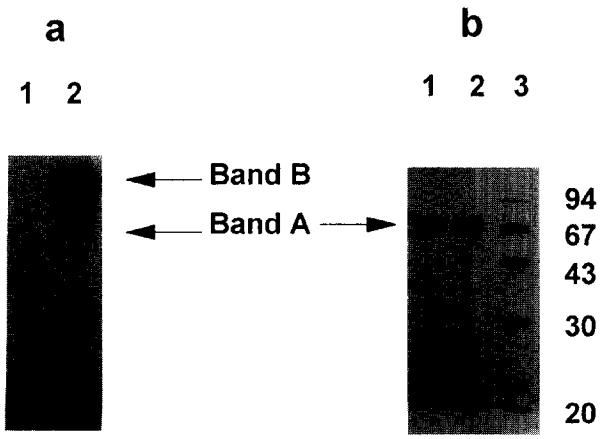

Fig. 4. Laemmli electrophoretic test of NF-κB cross-linking to ^{32}P-labeled duplex I. (a) Autoradiograph of the gel: lane 1, initial duplex I; lane 2, duplex I after incubation with p50-GST. (b) Gel stained with Coomassie brilliant blue R-250: lane 1, p50-GST incubated with duplex I; lane 2, initial p50-GST; lane 3, protein weight markers (molecular weights are indicated on the right).

substrates. On the other hand, *Eco*RII and *Mva*I restriction endonucleases which recognize the definite pentanucleotide fragment of DNA (5′-CC(T/A)GG-3′), are shown to cross-link sucsessfully to the DNA duplex having a monosubstituted pyrophosphate internucleotide bond within the recognition site. The cross-linking specificity in this cases have been demonstrated by competition experiments with the non-modified substrate as well as by the absence of the protein cross-linking to the duplex having the reactive internucleotide bond but lacking a recognition site [8,10]. Since NF-κB is found to interact specifically to the definite DNA sequence [2], we assume that the cross-linking of the duplex I to p50-GST should be specific. The incubation of p50-GST with the control duplex IV in which the monosubstituted pyrophosphate bond is replaced by non-substituted pyrophosphate bond does not result in the covalent conjugate formation (data not shown).

Recently, X-ray data for the structure of mouse p50, bound with the self-complementary 5′-GGGAATTCCC-3′ recognition sequence, were published [16]. The electrostatical contact with the phosphate group located between dT and dC residues is provided by the ε-amino group of the Lys-145 from one mouse p50 homodimer subunit and SH group of the Cys-59 from another one. X-ray data for the complex of human NF-κB p50 with the 11-bp duplex having an dA·dA mismatch at its center were obtained [3]. They also indicate that Lys residues interact with central phosphates of the recognition site. But the electrostatical contact of the Lys-147 in human p50, which corresponds to the Lys-145 in mouse p50, is shifted of one position towards the center of the DNA substrate. The contacts of Cys residues with phosphates are not discussed.

The p50-GST cross-linking to the duplex I confirms the presence of nucleophilic amino-acid residues adjucent to the phosphate group between dC and dT residues. We are going to investigate whether the ε-amino group of the Lys-147 participates in the cross-linking.

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