Inhibition of Interleukin-4- and CD40-induced IgE Germline Gene Promoter Activity by 2′-Aminoethoxy-modified Triplex-forming Oligonucleotides*

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Elevated levels of IgE are intimately associated with a number of allergic diseases, such as allergic rhinitis or asthma. Therefore, prevention of IgE production in human B-cells represents an attractive therapeutic target. IL-4-induced IgE germline gene transcription represents a crucial early step during IgE isotype switch differentiation. Gene induction is orchestrated by the coordinated action of the transcription factors STAT6 (signal transducer and activator of transcription), NF-κB, PU.1, and C/EBP. This study shows that 2′-aminoethyl-oxo-modified oligonucleotides, which partially overlap with the STAT6 and the adjacent PU.1/NF-κB binding site, inhibit DNA binding of all three proteins with high affinity in a dose- and time-dependent fashion in vitro. Loss of protein binding correlated strongly with increasing DNA triplex formation. Importantly, the oligomers also effectively displaced pre-bound recombinant NF-κB p50 from double-stranded DNA in vitro. Functionally, the oligonucleotides led to a selective inhibition of IL-4-induced reporter gene activity from a construct driven by the IgE germline gene promoter in human B-cells. These data confirm the critical role of this cytokine-responsive regulatory region in IgE germline gene induction and further support the concept of specific modulation of gene expression by DNA triplex formation induced with chemically modified oligonucleotides.

Interleukin-4 (IL-4) is a multifunctional cytokine that plays a critical role in the regulation of immune responses. It is intimately associated with the differentiation of antigen-stimulated naive T-cells into specialized helper T-cells (Th2 cells) (1, 2). In addition, IL-4 increases the expression of genes involved in cell activation and inflammation, such as class II major histocompatibility complex molecules on B cells (3), CD23 (4), the IL-4 receptor (5) and, together with tumor necrosis factor-α, etoxacin (6) and VCAM-1 (7). IL-4 also controls the specificity of immunoglobulin class switching toward the IgE isotype (8–11) by inducing the expression of IgE germline transcripts during the early phase of the switch cascade (12). CD40-mediated signaling synergizes with the IL-4 signal but has no or little effect by itself (13). A number of studies have identified the minimal regulatory region in the IgE germline promoter responsible for mediating IL-4-induced activation of transcription (14–17). The pivotal transcription factor appears to be STAT6 (18, 19), which interacts with DNA upon cytokine induction. Latent STAT6 is activated in the cytoplasm by tyrosine phosphorylation. In its activated state, it can dimerize, translocate to the nucleus, and bind to a specific DNA sequence in the regulatory region of the IgE germline gene (20). There it functionally interacts with other constitutively bound factors such as C/EBP (21), PU.1 (22), and two different sets of NF-κB/Rel family members (23–25). NF-κB proteins are involved in the synergistic function of the costimulatory CD40 signal (26). Based on these data, inhibition of IgE isotype switching and therefore IgE gene synthesis represents an attractive therapeutic target.

Synthetic oligonucleotides are attracting increasing interest as tools for specific manipulation of gene expression and have potential therapeutic applications. The “antigene” strategy is based on inhibition of transcription of the selected gene by oligonucleotide-directed intermolecular DNA triplex formation. A number of studies have demonstrated DNA triplex structures with critical polypurine:polypyrimidine cis-acting DNA elements found in the regulatory region of genes and the prevention of DNA binding of regulatory proteins (30–35). Recently, the synthesis and physicochemical properties of chemically modified oligonucleotides containing 2′-aminoethoxy-substituted riboses was reported (36). Such triplex-forming oligonucleotides (TFO) assemble into very stable DNA-triplexes and display a >1000-fold higher association rate constant compared with their unmodified counterparts (37).

The STAT6 binding site and the composite PU.1/NF-κB1 element in the IgE germline promoter are composed of an almost perfect DNA polypurine stretch. To explore the possibility, if the “antigene” approach can be applied to this regulon, 2′-aminoethoxy-modified oligonucleotides were tested for their ability to form DNA triplex structures with this region. The TFOs formed DNA triplex-helix structures and inhibited the interaction of STAT6, NF-κB, and PU.1 in a dose-dependent fashion with high specificity and selectivity. As a functional consequence, the activity of the IgE germline promoter to drive transcription of a reporter gene was blocked.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The human B-cell line DG75 was carried in Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin. Purified human recombinant IL4

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The abbreviations used are: IL, interleukin; TFO, triplex-forming oligonucleotide; STAT, signal transducer and activator of transcription; ds, double-stranded; EMSA, electrophoretic mobility shift assay; MeC, 5′-methylcytidine.

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was obtained from Novartis AG (Basel, Switzerland) with a specific activity of 0.5 units/ng. The fusion protein composed of soluble mouse CD40 ligand and soluble mouse CD6 (sCD40L-sCD6) (38), a kind gift of Dr. Peter Lane, was purified by immuno-affinity chromatography. The three TFOs were synthesized as described (36) and dissolved in 8% ammonium hydroxide. The Me-C copolymer was synthesized using 5′-methylcytidine (MeC) as building block like in the other three TFOs. It is identical in sequence to the 3654 TFO but does not contain the 2′-aminoethoxy modification. All oligonucleotides were characterized by analytical anion-exchange high performance liquid chromatography and matrix-assisted laser desorption-time of flight.

The DNA region of the IgE germline proximal promoter encompassing the STAT6 site and the composite PU.1/NF-κB element represents an almost perfect polypurine DNA stretch. To investigate the biologic properties of 2′-aminoethoxy modified TFOs on protein binding and gene induction, complementary poly pyrimidine TFOs were synthesized (Fig. 1). The 21-mer 3654 TFO covers the 3′ half of the STAT6 site, the entire PU.1 motif, and the 5′ half of the NF-κB1 element. The 15-mer 3651 oligonucleotide is shorter at the 3′ end and does not cover the NF-κB1 site. Both TFOs contain one mismatch sequence specificity of the 3654 oligonucleotide. Identical results were obtained using a composite STAT6/NF-κB complex, a predominant NF-κB, and a faster migrating PU.1 complex (Fig. 2) (22). Increasing concentrations of both 3654 and 3651 triplex oligonucleotides led to a dose-dependent reduction of all three complexes. A 13-fold molar excess of oligonucleotide 3651 led to almost complete disappearance of the STAT6/NF-κB and the PU.1 complex and a 50% reduction of the NF-κB band. The same effect was observed with a 90-fold molar excess of the longer 3654 oligonucleotide, showing that the increased length of the TFO positively affected its inhibitory behavior. Increasing amounts of TFO led to the appearance of a new band representing the DNA triple helix, which migrated just above the free radiolabeled duplex and became more prominent with increasing TFO concentrations (Fig. 2, lower panel). The intensity of the triple helix band inversely correlated with the intensity of the nucleoprotein complexes, demonstrating a direct relationship between inhibition of protein factor binding and formation of DNA triplex structures. The observed inhibition of protein binding was sequence-specific since the control TFO 3595 had no effect. Similarly, the unmodified polypyrimidine single-stranded oligonucleotide corresponding to position −106 to −55 of the IgE germline promoter sequence had no effect. The simultaneous presence of protein and TFO never led to the appearance of a slow migrating band indicative of a tetrameric complex consisting of ds-probe, TFO, and protein. This suggested that protein and TFO binding occurred in a mutually exclusive fashion.

Further confirmation for the sequence specificity of the TFOs came from experiments in which a different radiolabeled probe was used. Increasing amounts of the oligonucleotides were pre-incubated with another IgE germline promoter probe spanning positions −57 to −3 (41). This DNA (57/3) contains the NF-κB2 element, which is very similar to the PU.1/NF-κB1 sequence in the 106/55 probe (23). This sequence produces a complex banding pattern consisting of at least six different complexes, of which one has been identified to contain NF-κB family members (Fig. 3) (23). Even a very high excess of the TFO did not lead to DNA triplex formation but caused changes in nucleoprotein complex formation compared with the positive 106/55 control, demonstrating high sequence specificity of the 3654 oligonucleotide. Identical results were obtained using a composite STAT6/NF-κB site (42) from the human eotaxin gene promoter (data not shown).

To characterize the triplex-forming reaction in more detail, the time of addition of the TFO relative to the nuclear extract was varied. The 106/55 ds-probe was either pre-incubated with
a 300-fold molar excess of the 3654 TFO on ice for 45 or 15 min or added simultaneously with nuclear extract or was pre-incubated with the proteins for the same time periods before addition of the TFO. After addition of all components, all samples were further incubated for another 30 min on ice. The results demonstrated that triplex formation was strongest when the probe was pre-incubated with the TFO for 45 min and weakest when added 45 min after the nuclear extract (Fig. 4, lower panel). Similar to the data shown in Fig. 2, increasing amounts of triplex DNA correlated with reduced protein binding. It should be noted that, even in the samples in which the TFO was added after the extract, all three nucleoprotein complexes were significantly reduced, suggesting that the TFO could interfere with already formed nucleoprotein complexes (Fig. 4, upper panel). Again, this effect was not observed with the 3595 control TFO.

To corroborate these results, the stability of the triplex structures at two different temperatures was measured. The 106/55 probe was simultaneously incubated with nuclear extract and a 300-fold molar excess of the 3654 TFO. Then the mixtures were incubated for different time periods on ice or 37 °C and subsequently analyzed. Incubation on ice in the absence of TFO led to a gradual increase in NF-κB and STAT6/NF-κB protein binding, while the PU.1 band remained constant. The STAT6/NF-κB complex became detectable only after 60 min, suggesting a slower on-rate than the one of the other two proteins (Fig. 5A, upper panel). In the presence of the TFO, a gradual increase in triplex formation was apparent at the expense of free duplex DNA probe within the first 60 min. Thereafter, no significant amounts of free ds-probe could be detected (Fig. 5A, lower panel). Simultaneously, at the longer time points, a gradual loss of protein binding was observed. At 240 min, no PU.1 and STAT6/NF-κB bands were measurable and the NF-κB complex was strongly reduced. It should be noted that inhibition of protein binding was only observable when all the free available ds-duplex was converted to the triplex form (Fig. 5A).

When the mixtures were incubated at 37 °C in the absence of
TFO, not much difference in NF-κB and PU.1 protein binding was seen with time, suggesting that the interaction with the probe occurred much faster, was nearly complete even after 10 min and remained stable during the experiment (Fig. 5B). The STAT6/NF-κB complex was not formed at the higher temperature possibly due to instability under the experimental conditions used. In the presence of the 3654 oligonucleotide, most of the available duplex DNA was converted to the triplex structure already after 10 min, probably due to an increased association rate at 37 °C. Thereafter, a slight and constant increase of the triplex signal over the observation period could be measured (Fig. 5B, lower panel). Protein binding was severely reduced at 10 min and resembled the one observed at 240 min upon incubation on ice. At later time points, an additional decrease of the remaining NF-κB signal occurred. This suggested that, in the absence of excess free dsDNA, the TFO actively displaced the remaining NF-κB molecules from the DNA probe and thus may explain the increase of triplex DNA at the later time points. In addition, the triplex structures appeared to be stable, since no duplex DNA or PU.1 protein binding could be detected over time. To further substantiate the possibility that the TFOs can displace dsDNA-bound protein, recombinant NF-κB p50 was incubated with the radiolabeled 106/55 probe such that all available DNA was occupied by protein. Then, 3654 TFO was added at a 10-fold higher molar concentration than the protein and incubated for various time.
periods at room temperature (Fig. 6). In the absence of TFO, p50 produced two nucleoprotein complexes. The lower band likely represents a p50 homodimer, based on the finding that it migrated at the same position as the NF-κB complex in crude nuclear extracts, which contains p50 (Ref. 23, and data not shown). The upper complex may consist of a higher order p50 multimer. The presence of 3654 TFO led to increasing formation of triplex DNA in a time-dependent fashion. At the same time, a loss of intensity of protein binding was observed, which was most prominent at the longest time point. This effect was specific since the irrelevant 3595 TFO did not lead to triplex formation. The experiment confirms the importance of the 2-aminoethoxy substitution to mediate triplex formation since an oligonucleotide with the same sequence but lacking this side chain (MeC-3654) was not able to displace p50. Identical results, albeit weaker, were obtained when equimolar amounts of TFO and p50 was used (data not shown). These experiments demonstrated that the specific TFO could efficiently displace DNA-bound NF-κB p50 by forming DNA triplex structures, which are then inaccessible for the protein.

To evaluate the observed inhibitory effect of the TFOs on IL-4/CD40-induced expression of the human IgE germline gene, transient transfection experiments were carried out. A reporter gene plasmid (LUCwt) in which the human IgE germline promoter drives expression of the luciferase gene was used (22). IL-4-induced reporter gene expression in this construct is mediated by a functional cooperation of STAT6 with PU.1 and NF-κB. Cross-linking of CD40 leads to further stimulation in a synergistic fashion. This effect is also mediated by the PU.1/NF-κB1 element (26). The LUCwt DNA was mixed with increasing concentrations of TFOs and then transfected into DG75 cells by electroporation. STAT6 expression vector was also included to maximize IL-4 inducibility (22, 43). The transfectants were induced with a mixture containing IL-4, a fusion protein consisting of soluble murine CD40 ligand and soluble murine CD8 (38), and anti-CD8 antibodies to cross-link the CD40 receptor for 24 h. IgE germline promoter activity was inhibited in a dose-dependent manner by the 3654 TFO compared with the solvent control. The shorter 3651 oligonucleotide also inhibited reporter gene expression but with less efficiency (Fig. 7). This is in line with the results obtained in the EMSA assays, in which the 3651 TFO was also less effective compared with the 3654 oligonucleotide. In contrast, the 3595 control TFO had no effect. These data demonstrated that the loss of transcription factor binding due to triplex formation correlated strongly with the inhibition of gene induction. It also underscores the importance of this regulatory region of the promoter as mediator of IL-4/CD40 triggered activation of IgE germline gene expression.

**DISCUSSION**

An almost perfect polypurine stretch in the promoter region of the IgE germline gene is part of the critical regulatory region involved in activation by IL-4 and CD40 triggering. The transcription factors binding to this sequence after activation have been identified to be STAT6, PU.1, and members of the NF-κB family such as p50 (22–24, 26). 2′-Aminoethoxy-modified oligonucleotides complementary to this polypurine target formed a triplex structure in a dose-dependent fashion. Triplex DNA formation occurred with high sequence specificity since two other similar targets were not recognized. The binding affinity...
of the specific TFOs was more than 800-fold higher than that of an unrelated TFO (3595). These numbers are in good agreement with previous results obtained in surface plasmon resonance experiments (36). In addition, the binding reaction in the EMSA assays was carried out at pH > 7, which does not favor the formation of protonated cytidines, even considering that 5′-methylcytidine was used in the synthesis of the TFOs. These results support previous findings showing that the interaction of the 2′-aminoethoxy side chains of the TFO with phosphate groups of the duplex DNA is a very important contribution to the Hoogsteen hydrogen bonds for the total affinity of the observed DNA triplexes (36, 37). Although the shorter 3651 TFO does not hybridize directly with the NF-κB site, protein binding was inhibited to a similar degree as PU.1 or STAT6. This suggests either that the triplex structure affects the conformation of the neighboring duplex to an extent that precludes binding of NF-κB proteins or that steric hindrance does not allow access of the proteins. Alternatively, the presence of STAT6 or PU.1 may be a pre-requisite for NF-κB nucleus-protein complex formation. This alternative seems unlikely, however, because ds-oligonucleotides containing point mutations that specifically abolished protein binding of STAT6 or NF-κB were still able to bind NF-κB factors (22).

A time kinetic at 37 °C demonstrated that the simultaneous incubation of protein and TFO with dsDNA led to an increase in triplex DNA at the expense of protein binding in the absence of free ds-probe. This effect was even evident when the TFO was added after the nuclear extract. This strongly suggested competition of the TFOs with the transcription factors for duplex DNA binding. The \( K_v \) value for a 15-mer 2′-aminoethoxy-modified oligonucleotide has been determined to be 0.9–4.8 \( \times 10^{-9} \) M (36). This interaction is considerably weaker than the one measured for NF-κB proteins \( (10^{-12} \text{ to } 10^{-13} \text{ M}) (44) \) and thus could argue against a competition mechanism. However, it is difficult to compare these numbers since different methods, conditions, oligonucleotide lengths, and ds-targets were used to determine these values. Therefore, it is possible that, under the conditions used in this study, the 21-mer 3654 TFO has a binding affinity similar or higher than NF-κB, making a dsDNA interaction with the TFO over that with protein more likely. The observed shift from nucleoprotein complexes to DNA-triplex structures with time may also be driven by the large excess of TFO molecules compared with the amount of protein and perhaps by a possible difference in half-lives of the complexes. It has been shown that the half-life of NF-κB protein on its target was 45 min (45). In the absence of free ds-target DNA, shorter-lived dissociating protein-DNA complexes may be converted to DNA-triplexes with a longer half-life. In fact, it has been measured that a 2′-aminoethoxy-modified TFO dissociates at one fortieth the rate from its target duplex than an unmodified oligonucleotide with the same sequence (36). In addition, triplex DNA formed with a 2′-aminoethoxy-modified TFO was stable at 55 °C (37), whereas NF-κB binding was not possible anymore at this temperature (44). It is also possible that the 3654 TFO first binds to the adjacent STAT6 binding site and then “zips” through the NF-κB site, competing for both bases and phosphates binding, resulting in efficient NF-κB displacement. Such a mechanism is not possible with unmodified oligonucleotides, as they can only interact weakly with the bases of the dsDNA target. Although our experiments cannot discriminate between these various possibilities, it is worth noting that the TFOs were capable of displacing DNA-bound p50 protein. To our knowledge this is the first demonstration of an NF-κB binding inhibitor that exerts its biological activity on a pre-formed NF-κB nucleoprotein complex as opposed to an experimental set-up in which the inhibitor was pre-incubated with the dsDNA (35, 46, 47).

The simultaneous transfection of a reporter gene plasmid and TFO led to inhibition of reporter gene activity after stimulation in a dose-dependent fashion. This result demonstrated that loss of DNA-protein interaction resulted in inhibition of IgE germine promoter activation and thus underscores the importance of the targeted promoter region in activating gene expression. Although our data do not prove the existence of triplex structures within the transfected cells, it is worth noting that the amounts of triplex oligonucleotide necessary to inhibit gene expression by 50% was similar to that required to block protein binding to DNA to the same extent. This suggests that the mechanism(s) responsible for the inhibitory effect was due to blockade of critical nucleoprotein complex formation caused by the presence of a local DNA-triplex structure in the IgE germine promoter.

Overall, this first demonstration of the biological effects of 2′-aminoethoxy-modified oligonucleotides represents a step forward to overcome the lack of binding affinity and the slow association rate for the DNA target classically associated with conventional oligonucleotides. With this type of chemically modified oligonucleotides, displacement of already DNA-bound transcription factors may become feasible in a therapeutic setting. This may be important not only for inducible genes such as the IgE germine gene but also for constitutively active genes whose activity is associated with the disease phenotype. Further efforts are aimed to explore the properties of these oligonucleotide derivatives with regard to nuclease resistance and cellular uptake.

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