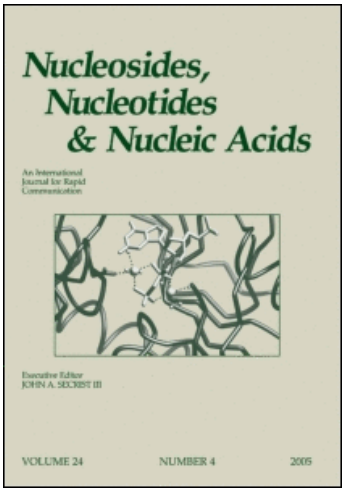

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Selective Attachment of Oligonucleotides to Interleukin-1β and Targeted Delivery to Cells

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SELECTIVE ATTACHMENT OF OLIGONUCLEOTIDES TO INTERLEUKIN-1β AND TARGETED DELIVERY TO CELLS

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Abstract: Conjugates between oligodeoxyribonucleotides and an interleukin-1β mutant protein have been constructed using a heterobifunctional cross-linker. These protein-DNA conjugates had conserved binding activity to the interleukin-1 receptor. The oligonucleotide hybridization properties were unchanged.

INTRODUCTION

There is now well documented evidence that antisense oligonucleotides can be used in vitro for the selective inhibition of gene expression[1)]. While oligonucleotides have been so far targeted to single-stranded mRNA, chromosomal duplex DNA is the ultimate target because the amount of antisense DNA required in the cell for inhibition of mRNA is too high. Two major limitations to the potential use of oligonucleotides as therapeutic agents are the targeting to specific cells and the transport through the cellular membranes. One possible solution to these problems is the use of a cell-specific protein ligand as carrier to deliver the oligonucleotides into whole cells after internalization.

Covalent conjugates between oligodeoxynucleotides and proteins are emerging as useful tools in molecular biology, in medical diagnostics and in biotechnology[2-6)]. There is a need for the development of

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Abbreviations: IL-1, Interleukin-1; IL-1βK138C, Interleukin-1β mutant with cysteine substituted for lysine at position 138; SMCC, succinimidyl 4-(N-maleimidomethyl) cyclohexane-1 carboxylate; SMPB, succinimidyl 4-(p-maleimidophenyl) butyrate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

methods for the preparation of stable protein-DNA adducts. Several recent approaches describe the introduction of thiol-linkers into oligonucleotides, their activation as 2-pyridyldisulfides and their subsequent coupling to a sulphydryl group on the protein surface by disulfide exchange reaction[3,5]. However this methodology has two disadvantages: firstly thiol-linkers are rapidly oxidized by atmosphere or solubilized oxygen to give unwanted side products, thus decreasing the efficiency of the coupling procedure; secondly disulfide-linked protein-DNA conjugates are unstable in cellular systems. I wish to report an efficient method for the preparation of protein-oligonucleotide conjugates via a stable thioether linkage using 5'-maleimido-derivatized oligonucleotide and a thiol-containing IL-1β mutant protein under mild conditions. I show that these DNA-protein conjugates specifically bind thymoma cells bearing IL-1 receptors.

MATERIAL AND METHODS

Oligonucleotides and 5'-aminoalkyl derivatives. Oligodeoxynucleotides were synthesized on an automated Applied Biosystems 380A instrument using phosphoramidite methodology. Oligonucleotides were converted to their 5'-(6-aminohexyl) phosphoramidate derivatives as described earlier [7]. Alternatively, oligonucleotides 5'-(6-aminohexyl)phosphate were synthesized on the automated machine by running an extra cycle with the Aminolink 2 reagent (Applied Biosystems; Foster City, CA) at the end of the synthesis. To a 1.0-1.5 mM solution of 5'-aminoalkyl derivative of oligonucleotide in 0.05 M sodium phosphate pH 7.5 was added an equal volume of 20-30 mM SMPB (or SMCC) (Pierce Chemical Co.; Rockford, IL) in N,N-dimethylformamide and the mixture was allowed to react for 2 h at room temperature. The modified oligonucleotide was dialysed against water at 4°C or purified through a short column of G-25 Sephadex (Pharmacia; Uppsala, Sweden) packed in water and then lyophilized.

Conjugation to Interleukin-1β. Recombinant human IL-1βK138C [8] in 100 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 0.02% sodium azide was dialyzed at 4°C against phosphate buffer saline (PBS) pH 7.2 and diluted to 20 mM. This solution was added to a 10-fold molar excess of lyophilized 5'-maleimidoalkyl oligonucleotide and allow to react at

4°C for 2.5 h. The conjugate was purified on a G-50 Sephadex column packed in PBS buffer pH 7.2.

(125)I-labelling. The IL-1βK138C conjugate with the 17mer oligonucleotide (5 µg) and recombinant human IL-1β were radiolabelled with (125)I-Bolton-Hunter reagent, 1.36 µCi at 2200 Ci/mmol (DuPont-NEN, Dreieich, W.-Germany) according to the manufacturer instructions. Purified recombinant human IL-1α was radiolabelled with (125)I as described [9].

IL-1 receptor binding assay. A murine thymoma cell line, EL4-6.1[10] (a gift from Dr. H.R. McDonald, Lausanne, Switzerland) expressing 6,000 IL-1 receptors per cell, was grown in RPMI 1640 supplemented with 10% fetal calf serum in a 5% CO_2/air humidified atmosphere. Competitive binding assays to EL4-6.1 cells were carried out as described[10], using (125)I-labelled IL-1 and various concentrations of proteins to be tested at 20°C for 2 h.

DNA Hybridization experiments. DNA-protein conjugates were separated by SDS-polyacrylamide gel electrophoresis and transferred by capillarity to a nitrocellulose filter ("Western" blotting). Alternatively, conjugates were spotted in varying amounts to nitrocellulose filters. Filters were prehybridized at 30°C for 6 h in 6 x SSC hybridization buffer[7]. Oligonucleotide probes were (32)P-labelled at the 5′-end as described[11]. Nitrocellulose filters were hybridized with 1 mM probe (17mer, 50°C, 6 h) in 6 x SSC hybridization buffer, washed twice with 2 x SSC 0.1% SDS at 45°C and autoradiographed.

RESULTS

The scheme of Figure 1 was followed to attach oligonucleotides to IL-1. The strategy was: 1) to activate oligonucleotides as 5′-maleimidoalkyl derivatives (1) by using a heterobifunctional cross-linking reagent, SMCC or SMPB, that contains a thiol-selective maleimido group and a N-hydroxysuccinimide activated ester group, specific for primary aminoalkyl groups introduced at the 5′-end of oligonucleotides, and, 2) to couple 1 to IL-1βK138C, an IL-1β mutant protein in which the surface-exposed lysine 138 residue was substituted by a cysteine residue[8]. Maleimide groups react rapidly with

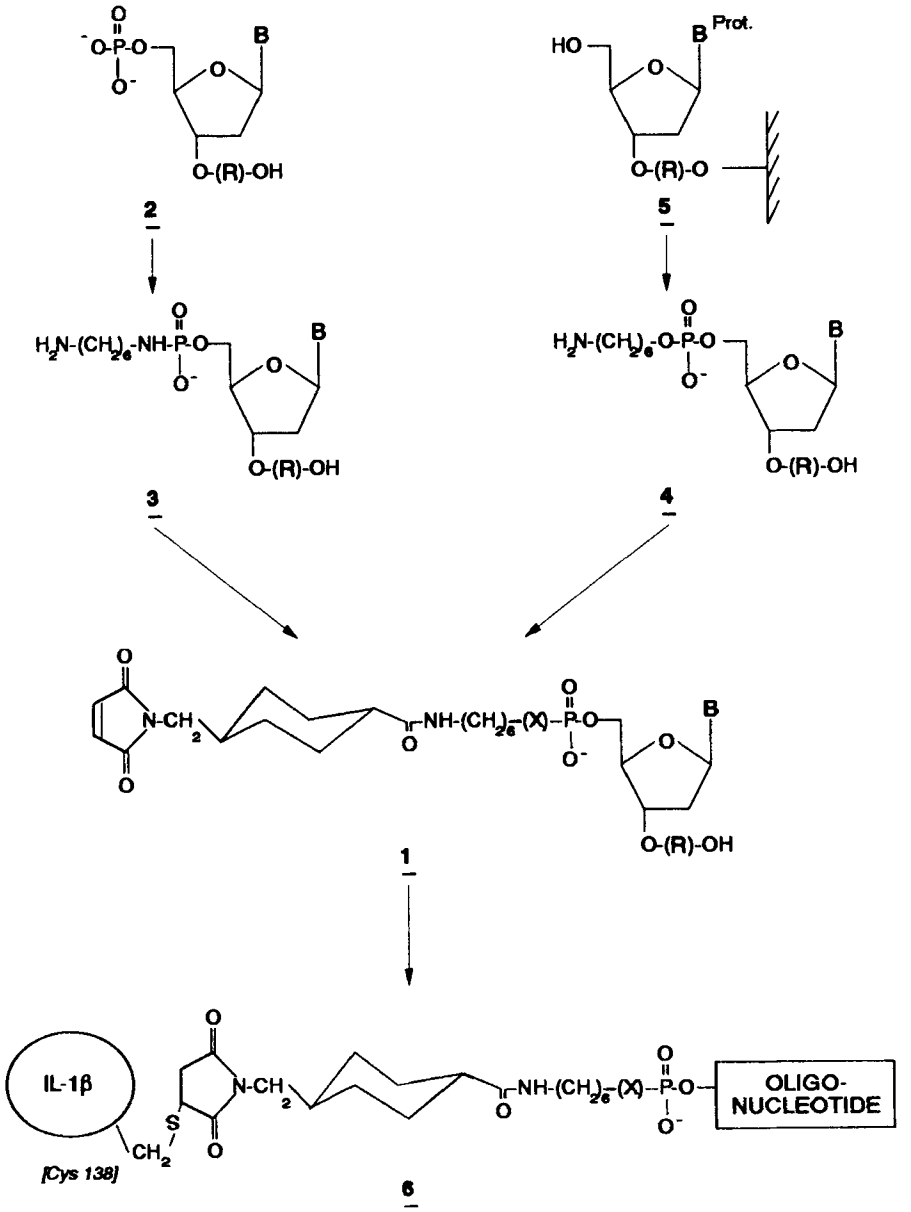

FIGURE 1. Synthesis of oligonucleotide-interleukin-1β mutant conjugates. X = O or NH; B = A,C,G,T; (R) = oligodeoxynucleotidyl; see Table 1 for sequences.

thiol groups, but very slowly with other functional groups of proteins or oligonucleotides.

Two approaches were used to prepare 1 (Figure 1). Firstly, the synthetic 5'-terminal phosphate parent oligonucleotide 2 was converted, in solution, to its stable 5'-(6-aminohexyl)phosphoramidate (3) as described earlier[7]. Alternatively, the 5'-(6-aminohexyl)phosphate analogue 4 was made from the protected and support-bound 5'-OH oligonucleotide 5, on the automated DNA synthesizer, by coupling a phosphoramidite derivative of 6-(N-trifluoroacetyl)aminohexanol using standard phosphoramidite methodology and subsequent deprotection. The phosphoramidite 3 and the phosphate 4 showed no difference in stability or reactivity in the subsequent steps. The route via 4 was preferred for its rapidity and ease to perform.

Then 4 (or 3) was treated with a 20-fold excess of heterobifunctional reagent SMCC to yield 1 (90%). The reactions were insensitive of the sequence and length of the oligonucleotide (Table 1). The oligonucleotides 1 were purified by gel electrophoresis (Figure 2) or ethanol precipitated. In most cases, the crude materials were used directly after dialysis or passage through a short gel filtration column. Activated oligonucleotides 1 were kept at pH 7.

Maleimido oligonucleotides 1 were coupled to IL-1βK138C to produce stoechiometrically defined 1:1 DNA-protein conjugates 6 (Figures 1 and 2) via a stable thioether linkage between the Cys138 position of the protein and the 5'-derivatized oligonucleotide. When wild-type IL-1β was submitted to identical coupling reaction conditions it was recovered unchanged. The conjugates were easily separated from excess unreacted oligonucleotides 1 and traces of unreacted IL-1βK138C by gel filtration chromatography.

The IL-1βK138C-17mer S4 oligonucleotide conjugate was assayed for IL-1 receptor affinity by competitive binding analysis with ^{125}I-IL-1α on murine thymoma EL4-6.1 cells (Figure 3). We found that the conjugate bound to IL-1 receptors with a 12x reduced affinity (D_{50} = 1.0×10^{-9} M) compared to wild-type IL-1β (D_{50} = 0.8×10^{-10} M).

Conversely, the binding of the ^{125}I-labelled conjugate IL-1βK138C-17mer S4 oligonucleotide to IL-1 receptors on EL4-6.1 cells was competitively inhibited by increasing amounts of wild-type IL-1β (Figure 3). Binding of ^{125}I-conjugate on cells treated with trypsin to remove IL-1 receptors was less than 10% the binding to non-

TABLE 1. Sequences of oligodeoxynucleotides

Name	Length	Sequence
S1	13	CCTCACAGTGACC
S2	13	GGTCACTGTGAGG
S3	17	CACAGTGACCTCAAGTC
S4	17	GACTTGAGGTCACTGTG
S5	17	GTCCTTAGAAGATGAAC
S6	17	GTTCATCTTCTAGGCAC

treated cells. Heat-denatured ^{125}I-conjugate did not bind to EL4-6.1 cells. All these observations demonstrated that IL1-βK138C conjugate retained the ability to bind specifically IL-1 receptors on whole cells.

DNA-DNA hybridization studies were carried out on nitrocellulose filters blotted with varying amounts of IL-1βK138C-17mer S4 oligonucleotide conjugate, by transfer from a SDS-polyacrylamide gel or by dot blotting. Filters were hybridized to ^{32}P labelled oligonucleotide probe S3 and DNA-protein conjugate was specifically detected (Figure 2).

DISCUSSION

The method described here allows the preparation of stoechiometrically defined DNA-protein conjugates via a stable linkage. It should be of general use for the attachment of maleimido-derivatized oligonucleotides to any protein. In cases where the protein does not contain a free or accessible thiol, sulfhydryl groups can be introduced by chemical modification with a thiolating agent (iminothiolane or N-succinimydyl-S-thioacetate) or by site-specific mutagenesis of a surface-exposed amino acid to cysteine. This latter approach was followed to prepare the IL-1βK138C-oligonucleotide conjugates. The created cysteine 138 is located in a loop between two antiparallel β-strands as described in the X-ray structure of human IL-1β[12]. The position 138 in IL-1β is not directly involved in the receptor binding and can be derivatized with biotin with full conservation of bioactivity[16]. Our results show that attachment of the polyanionic oligonucleotide to IL-1β does minimally decrease the ability of IL-1β to bind its T-cell receptor. This effect may be

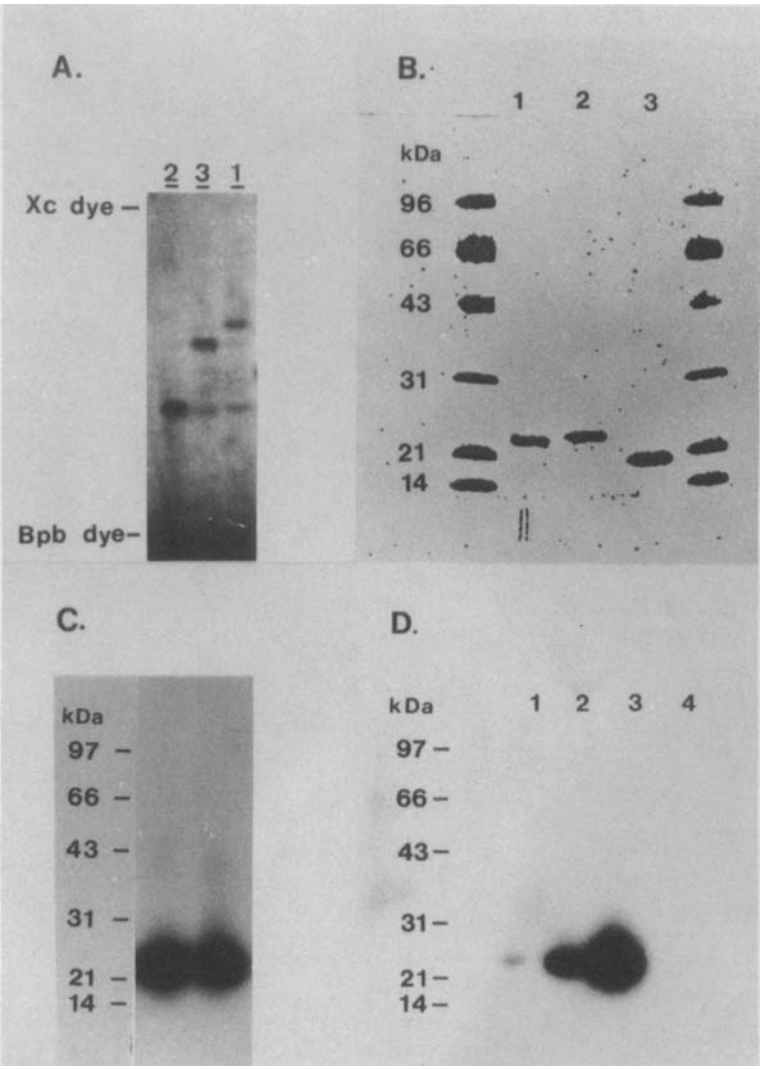

FIGURE 2. Gel electrophoresis analyses and hybridization experiments. A: Polyacrylamide gel electrophoresis (7M urea) of 5′-end derivatized 13-mer S1 oligonucleotides 1, 2 and 3 (see text). B: SDS-polyacrylamide protein gel, lanes 1 and 2 = conjugate 6 (with 13-mer S1) DTT-reduced and non-reduced; lane 3 = IL-1βK138C. C: SDS-polyacrylamide protein gel of (125)I-IL-1βK138C-oligo 17mer S4 conjugate 6 under DTT-reducing and non-reducing conditions (autoradiography). D: Hybridization of [(32)P]oligo 17mer probe S3 to a nitrocellulose filter blotted with IL-1βK138C-oligo 17mer S4 conjugate by transfer from protein electrophoresis gel; lanes 1-3 = IL-1βK138C-oligo 6, 0.1, 0.4 and 2 pmol respectively; lane 4 = IL-1β, 10 pmol.

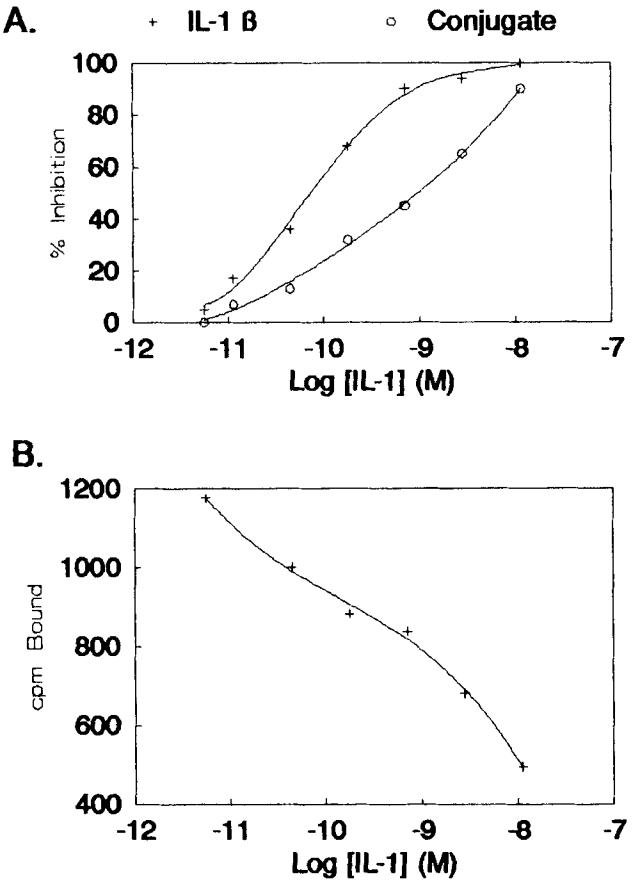

FIGURE 3. IL-1 receptor binding assays. A: Competitive inhibition of (125)I-IL-1 binding to EL4-6.1 cells by IL-1βK138C-oligonucleotide 17mer S4 conjugate. B: Competitive inhibition of (125)I-IL-1βK138C-oligonucleotide 17mer S4 conjugate binding by IL-1β.

caused by steric or electrostatic perturbations of IL-1β. The oligonucleotide moiety retained its ability to hybridize to a complementary sequence in vitro.

In this study, the ability of IL-1β to recognize cell-specific membrane IL-1 receptors is exploited to transport oligonucleotides to target cells. The use of proteins as carriers of small pieces of DNA has recently been reported[6]. Oligonucleotide are becoming increasingly important as reagents to inhibit gene expression on specific RNA

or DNA targets. Little is known about the mechanism by which oligonucleotides are transported into cells, except that the process is rather inefficient. Putative cell receptors may be involved[13]. Earlier attempts to increase the uptake by cells were made by attaching the oligonucleotides to poly-(L)-lysine[14]. Receptor-mediated internalization of IL-1[10,15] or derivatized IL-1[16] into EL4 cells has been demonstrated. Moreover IL-1 has been shown to be translocated to the cell nucleus[15], an important observation in the prospect of targeting DNA. Similar studies with the oligonucleotide-IL-1 conjugates are underway. Therefore the use of DNA-protein conjugates such as the ones described here to specifically deliver oligonucleotides to target cells has a great potential.

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