Nucleic Acids Research, 1995, Vol. 23, No. 21 **4239–4245**

Binding of phosphorothioate oligodeoxynucleotides to basic fibroblast growth factor, recombinant soluble CD4, laminin and fibronectin is P-chirality independent

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Received September 11, 1995; Revised and Accepted October 4, 1995

ABSTRACT

Antisense oligodeoxynucleotides can selectively inhibit the expression of individual genes and thus have potential applications in anticancer and antiviral therapy. A critical prerequisite to their use as therapeutic agents is the understanding of their non-specific interactions with biological structures, e.g. proteins. In this study we examined the interactions of P-chiral phosphorothioate oligodeoxynucleotides with several proteins. The Rp- and Sp- diastereomers, and racemic machine-made mixtures, or M-oligodeoxynucleotides were used independently as competitors of the binding of a probe, phosphodiester oligodeoxynucleotide bearing a 5′ alkylating moiety, to rsCD4, bFGF and laminin. These oligodeoxynucleotides were also used as competitors of the binding of a non-alkylating probe M-phosphorothioate oligodeoxynucleotide, 5′-^{32}P-SdT$_{18}$ to fibronectin. The average values of and quantitative estimates for the IC_{50} of competition and the constant of competition (K_c) of Rp-, Sp- and M- stereoisomers of several homo- and heteropolymer oligodeoxynucleotides were determined and compared. Surprisingly, in the proteins we studied, the values of IC_{50} and K_c for the Rp-, Sp- and M-oligodeoxynucleotides were essentially identical. Thus, the ability of the phosphorothioate oligodeoxynucleotides we employed, to bind to the proteins studied in this work, is virtually independent of P-chirality. Our results also imply that the role of the purine and pyrimidine bases in oligodeoxynucleotide–protein interactions, as well as the nature of the contact points (sulfur versus oxygen) between the oligomer and the protein, may be relatively unimportant.

INTRODUCTION

Phosphorothioate oligodeoxynucleotides, first synthesized by Stec *et al.* (1), are isoelectronic congeners of phosphodiester oligonucleotides that retain the property of solubility in aqueous solvents and Watson–Crick base pair hybridization, but which are more nuclease-resistant than the corresponding phosphodiester oligodeoxynucleotides (2). These compounds have the ability to bind avidly to cellular proteins (3). Such proteins, for example, include rsCD4 (4), gp 120 (5), heparin binding growth factors such as bFGF (6), and laminin and fibronectin (7). Furthermore, phosphorothioate oligodeoxynucleotides tend to bind to proteins with lower dissociation constants (*K*d) than those of phosphodiester oligodeoxynucleotides (4,8). The avidity of binding appears to be relatively independent of sequence, but is directly and highly dependent upon oligodeoxynucleotide length.

Phosphorothioate oligodeoxynucleotides contain a sulfur atom at each phosphorus atom; each replaces a single non-bridging oxygen atom. A complication of sulfur substitution is that an asymmetry is introduced at each internucleotide bond. Each *n*mer phosphorothioate oligodeoxynucleotide has $(n - 1)$ centers of asymmetry at phosphorus because each linkage can occur as either the Rp- or Sp-diastereomer. It is unclear what effect this property may have on the interactions of phosphorothioate oligodeoxynucleotides with biological systems (e.g. with cellular proteins). The recent, automated synthesis of stereoregular phosphorothioate oligodeoxynucleotides by Stec *et al.* (9) has permitted the production of sufficient quantities of the all-Rp and all-Sp forms so that determination of the protein binding capabilities can be made, and compared to that of a phosphorothioate oligodeoxynucleotide that contains a random mixture of diastereomers (M-oligodeoxynucleotide).

In this report, we have determined the average values of the constant of competition (K_c) for stereoregular and M-phosphoro-

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thioate oligonucleotides. In this work, these reagents are used as competitors of the binding of a probe, alkylating phosphodiester oligodeoxynucleotide to recombinant soluble (rs) CD4, basic fibroblast growth factor (bFGF) and laminin. Quantitative estimates of the IC_{50} for the binding of the stereoregular oligodeoxynucleotides to fibronectin have also been made; these oligodeoxynucleotides were used as competitors of the non-alkylating probe random mixture phosphorothioate oligodeoxynucleotide, 5′-^{32}P-SdT_{18}. To our great surprise, we have demonstrated that the values of K_c and IC_{50} of competition of binding for Rp-, Sp- and M-oligodeoxynucleotide are, for each protein, essentially identical. This result is at variance with commonly held notions concerning the effects on protein binding of stereoregularity at phosphorus. Thus, the binding of phosphorothioate oligonucleotides that we have studied to rsCD4, bFGF, laminin and fibronectin is not only relatively independent of base sequence, but apparently independent of P-chirality as well.

MATERIALS AND METHODS

Synthesis of oligodeoxynucleotides

Random-mixture oligodeoxynucleotides. Phosphodiester oligonucleotides were synthesized by standard phosphoramidite chemistry on an Applied Biosystems (Foster City, CA) 380B synthesizer. Phosphorothioate oligos were also synthesized by standard methods, and sulfurization was performed using tetraethylthiuram disulfide/acetonitrile (TETD; Applied Biosystems, Foster City, CA). Following cleavage from the controlled pore glass support, oligodeoxynucleotides were base deblocked in ammonia hydroxide at 60°C for 8 h and purified by reversed phase HPLC (0.1 M triethylammonium bicarbonate (TEAB)/acetonitrile, PRP-1 support). Oligomers were detritylated in 3% acetic acid and precipitated with 2% lithium perchlorate/acetone, dissolved in sterile water and reprecipitated as the sodium salt from 1 M NaCl/ethanol (2). Oligodeoxynucleotide concentrations were determined by spectroscopy. When used in native gels (no SDS present), 5′-^{32}P-labeled phosphorothioate oligodeoxynucleotides were synthesized using T4 polynucleotide kinase by the method of Sambrook *et al.* (10).

Stereoregular phosphorothioate oligodeoxynucleotides. The method for the stereocontrolled synthesis of phosphorothioate oligodeoxynucleotides is based on the concept of 1,3-diazabicyclo(5.4.0)undec-7-ene (DBU) promoted, nucleophilic substitution at the phosphorus of the 2-thio-1,3,2-oxathiaphospholane ring system (9,11). The synthesis was performed on an ABI 391 synthesizer using separated pure diastereomers of 5′-*O*-DMT-(*N*-protected)-nucleoside-3′-*O*-(2-thio-4,4–*spiro*-cyclohexylidene-1,3,2-oxathiaphospholanes) (12). The first nucleoside was anchored to the solid support via a DBU-resistant sarcosinyl–succinoyl linker (-$COCH_2CH_2CON(Me)CH_2CO$-LCA-CPG). For the 1 μmol scale synthesis, a mixture of 450 μl 0.5 M DBU in CH_3CN (300-fold molar excess) and 150 μl 0.1 M monomer in CH_3CN (20-fold excess) was used. The solution of DBU in CH_3CN was delivered from the position of the 1H-tetrazole. Standard solutions of dichloroacetic acid in methylene chloride and DMAP/Ac_2O/lutidine in THF were used for the detritylation and capping steps, respectively. Critical parameters of this protocol are shown in Table 1, and the detailed protocol for this synthesis is available on request from W. Stec.

Table 1. Parameters for the synthesis of stereoregular phosphorothioate oligodeoxynucleotides

Step	Reagent or solvent	Volume (ml)	Purpose	Time (sec)
1	a) dichloroacetic acid in CH_2Cl_2	2.3	detritylation	50
	b) acetonitrile	7.0	wash	150
2	a) activated monomer in CH_3CN	0.6	coupling	220
	b) methylene chloride	3.3	wash	200
	c) acetonitrile	7.0	wash	150
3	a) DMAP/Ac_2O/lutidine in THF	0.3	capping	20
	b) acetonitrile	7.0	wash	150

The oligonucleotides were cleaved from the solid support, deprotected and purified via two-step reverse phase HPLC (DMT-on and DMT-off). Concentrations were determined spectrophotometrically and the chain length integrity was assessed by polyacrylamide gel electrophoresis. In order to assess diastereomeric purity of the synthetic oligonucleotides, the purified compounds (as the triethylammonium salts) were labeled with ^{32}P at the 5′-terminus and treated with nucleases: the [all-Rp]-diastereomer with nuclease P1, and the [all-Sp]-isomer with snake venom phosphodiesterase (svPDE). Under all conditions tested to date, svPDE recognizes and hydrolyzes dinucleoside phosphorothioates of the Rp-configuration. Conversely, nuclease P1 promotes the hydrolysis of internucleotide Sp-phosphorothioates, while internucleotide Rp-phosphorothioates are inert. We treated stereoregular phosphorothioate oligomers with svPDE, and independently with nuclease P1, in order to determine their diastereomeric purity. The Rp-SdT_{19} prepared from the FAST (pro-Rp monomer with a diastereomeric purity of 99%) was partially digested with nuclease P1 and the content of 'undigested' Rp-SdT_{19} (75% of the total product) corresponded with a calculated diastereomeric purity of $(0.99)^{18} = 80\%$. Similar results were obtained after digestion of Rp-$Sd(TC)_9T$ which was synthesized from FAST (pro-Rp) monomers with a diastereomeric purity of 99%. After a 24 h incubation, ~70% of the substrate remained intact, which is in reasonable agreement with the calculated diastereomeric purity of 80%, assuming 99% diastereomeric purity at each phosphorus. Independently, using the Rp-specific enzyme (svPDE) 70% of the Sp-SdT_{19} oligomer and 65% of the Sp-$Sd(TC)_9T$ oligomer (calculated 80%) remained undigested, i.e. full length.

Synthesis of alkylating, radioactive phosphodiester oligodeoxynucleotide 5′-*N*-methyl-*N*-(2-chloroethyl) aminobenzylamine-^{32}P-OdT_{18} or -OdT_{12} (RClNH^{32}P-OdT_{18} or RClNH^{32}P-OdT_{12})

These compounds were synthesized by a modification of the method of Knorre *et al.* (13). Briefly, after 5′-phosphorylation of OdT_{18} or OdT_{12} by Chemical Phosphorylation Reagent (Glen Research, Herndon, VA), a reaction exchanging the ^{32}P of [γ-^{32}P]ATP was carried out using T4 polynucleotide kinase with ADP as the phosphate acceptor (10). Then, *N*-methyl-*N*-(2-chloroethyl)aminobenzylamine was coupled to the 5′ terminal ^{32}P by reaction with triphenylphosphine/dipyridyl disulfide. The final product was stored at –70°C.

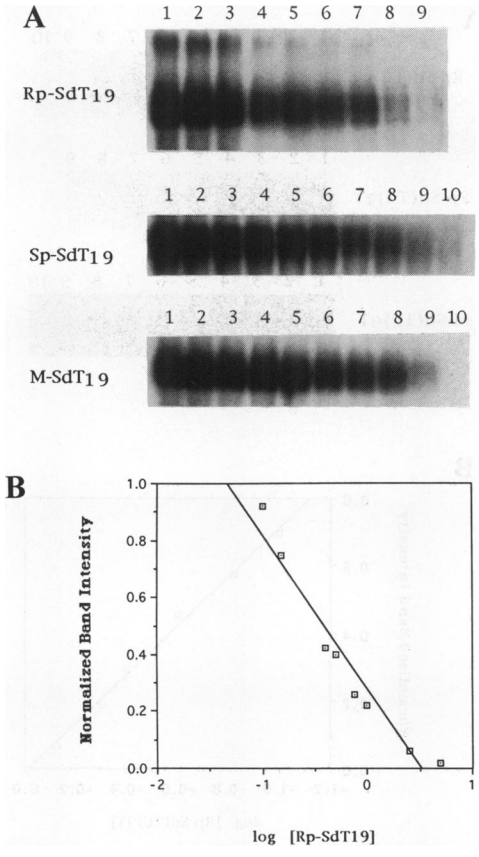

Figure 1. (**A**) Competition by Rp-, Sp- and M-SdT_{19} for binding of $ClRNH^{32}P\text{-}OdT_{18}$ to rsCD4. Rp-, Sp- and M-SdT_{19} were used as competitors of $ClRNH^{32}P\text{-}SdT_{18}$ (3 μM) binding to rsCD4 (0.9 μM) as described in the text (8% PAGE). Bands represent the 55 kDa subunit of rsCD4 modified by the oligodeoxynucleotide. The concentration of Rp-SdT_{19} was (lanes 1–9, respectively) 0, 0.1, 0.15, 0.4, 0.5, 0.75, 1, 2.5 and 5 μM. The concentration of Sp-SdT19 was (lanes 1–10, respectively) 0, 0.1, 0.15, 0.25 0.4, 0.5, 0.75, 1, 2.5 and 5 μM. The concentration of M-SdT_{19} was (lanes 1–10, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.5, 0.75, 1, 2.5 and 5 μM. (**B**) Determination of the value of K_c for Rp-SdT_{19} by the Cheng–Prusoff equation (Equation 1). The data from (A) was quantitated by excision of the gel bands and β-counting. Shown is a plot (r^2 = 0.97) of the normalized band intensity versus the log of the Rp-SdT_{19} concentration.

Modification of rsCD4 , bFGF and laminin by $ClRNH^{32}P\text{-}OdT_{18}$ or $ClRNH^{32}P\text{-}OdT_{12}$

This was accomplished by the method of Yakubov *et al.* (4). Initially, rsCD4 (0.9 μM), bFGF (0.3 μM) or laminin (0.25 μM) was incubated in 0.1 M Tris–HCl, pH 7.4, containing 3 μM $ClRNH^{32}P\text{-}OdT18$. Nine phosphorothioate oligodeoxynucleotides, including Rp-SdC_{19}, Sp-SdC_{19}, M-SdC_{19}, Rp-SdT_{19}, Sp-SdT_{19}, M-SdT_{19} and Rp-$Sd(TC)_9T$, Sp-$Sd(TC)_9T$, M-$Sd(TC)_9T$ were used at the stated concentrations as competitors of the binding of the probe phosphodiester oligodeoxynucleotide to the proteins. After 1 h at 37°C, 0.5 vol of a buffer containing 10% glycerol, 4% 2-mercaptoethanol, 4% SDS and 0.2% bromophenol blue was added and SDS–PAGE was performed. The gels were dried and allowed to expose Kodak X-ray film until bands were visualized. The film was developed, and band densities were quantitated by excision of the gel bands and β counting.

Binding of 5′-$^{32}P\text{-}SdT_{18}$ to fibronectin

This was accomplished by a electrophoretic mobility shift assay in native 6% polyacrylamide gels. Fibronectin (5 μM) was incubated in 10 μl 0.1 M Tris–HCl, pH 7.4, containing 3 μM $^{32}P\text{-}SdT18$. Six compounds, Rp-SdT_{19}, Sp-SdT_{19}, M-SdT_{19} and Rp-$Sd(TC)_9T$, Sp-$Sd(TC)_9T$, M-$Sd(TC)_9T$ were used at the stated concentrations as competitors of the binding of the probe, ^{32}P-labeled oligodeoxynucleotide to fibronectin. After 1 h at 37°C, 0.5 vol of a buffer containing 10% glycerol, 4% 2-mercaptoethanol and 0.2% bromophenol blue was added and PAGE was performed. After electrophoresis, the gel was dried, and allowed to expose Kodak X-ray film at –70°C. The data were quantitated by excision of the gel bands and β-counting.

RESULTS

Determination of the competition constant (K_c) of stereoregular phosphorothioate oligodeoxynucleotides binding to rsCD4, bFGF and laminin

We have previously determined the values of K_c for competitors of modifying oligodeoxynucleotide binding to rsCD4 (4), to bFGF (6) and to laminin (7). The value of K_c may be calculated from Equation 1 as described by Cheng and Prusoff (14):

Equation 1 $$K_c = IC_{50}/(1 + [ClRNH^{32}P\text{-}OdT_{18}]/K_d)$$

Alternatively, the value of K_c may be calculated by Equation 2, which is a limiting case of Equation 1:

Equation 2 $$K_c/K_d = L_2/L_1(1/(R_0/L_1R) - 1)$$

where L_2 = [competitor stereoregular phosphorothioate oligodeoxynucleotide] = 0.1 or 0.5 μM; L_1 = $[RClNH^{32}P\text{-}OdT_{12}]$ = 5 μM; R_0 = bound counts in the absence of competitor; L_1R = bound counts in presence of competitor.

The average value of K_d for rsCD4 is 0.5 μM, as determined by Yakubov *et al.* (4), 0.5 μM for bFGF, as determined by Guvakova *et al.* (6), and 14 μM for laminin (7).

In Figure 1 competition for binding to rsCD4 is shown. As per Equation 1, a plot of bounds counts, as determined by β-counting, versus competitor concentration, was linear (r^2 values for Rp-SdT_{19}, Sp-SdT_{19} and M-SdT_{19} = 0.97, 0.93 and 0.95, respectively). Similar r^2 values were obtained for Rp-$Sd(TC)_9T$, Sp-$Sd(TC)_9T$ and M-$Sd(TC)_9T$. A representative plot (shown for Rp-SdT_{19}) of the normalized band intensity versus oligodeoxynucleotide concentration is shown in Figure 1B. The IC_{50} of competition of binding for Rp-SdT_{19} and for all the oligodeoxynucleotides tested was determined by inspection. The values of K_c for phosphorothioate oligodeoxynucleotide binding to rsCD4, as determined by the Cheng–Prusoff equation, are given in Table 2. These values are essentially identical, given the error of the experiment.

In the initial experiments examining competition of probe oligodeoxynucleotide binding to bFGF, the values of K_c were calculated by Equation 2 at two concentrations (0.1 and 0.5 μM) of stereoregular competitor phosphorothioate oligodeoxynucleotide. The values are essentially concentration independent, i.e. identical for either 0.1 or 0.5 μM oligodeoxynucleotide ($RClNH^{32}P\text{-}OdT_{12}$). For Rp-$SdC_{19}$, Sp-$dC_{19}$ and M-dC_{19}, the averaged values of K_c are 17, 36 and 18 nM, respectively; these are also essentially identical. Competition by Rp-$Sd(TC)_9T$, Sp-$Sd(TC)_9T$ and M-$Sd(TC)_9T$ for binding of $CIRNH^{32}P\text{-}OdT_{18}$

to bFGF is shown in Figure 2. Two competed bands, as described by Guvakova *et al.* (6) are clearly visible on the gel which migrate at the approximate position of bFGF (M_r = 17.4 kDa). A representative plot [for Rp-Sd(TC)$_9$T] is shown in Figure 2B, of the normalized band intensity versus the log of the oligomer concentration. The value of IC_{50} and of K_c were calculated as above. Competition for probe binding to bFGF by the Rp-, Sp- and M forms of SdT$_{19}$ is shown in Figure 3.

Table 2. Values of IC_{50} and K_c (from Equation 1)

Competitor	IC_{50} (μM)	K_c (μM)
rsCD4		
Rp-SdT$_{19}$	0.37	0.053
Sp-SdT$_{19}$	0.30	0.043
M-SdT$_{19}$	0.62	0.089
Rp-Sd(TC)$_9$T	0.28	0.040
Sp-Sd(TC)$_9$T	0.47	0.067
M-Sd(TC)$_9$T	0.23	0.033
bFGF		
Rp-SdT$_{19}$	0.39	0.056
Sp-SdT$_{19}$	0.55	0.079
M-SdT19	0.44	0.063
Rp-Sd(TC)$_9$T	0.20	0.029
Sp-Sd(TC)$_9$T	0.54	0.077
M-Sd(TC)$_9$T	0.46	0.066
Rp-SdC19		0.017a
Sp-SdC19		0.036[a]
M-SdC19		0.018[a]
Laminin		
Rp-SdT$_{19}$	0.44	0.36
Sp-SdT$_{19}$	0.68	0.56
M-SdT19	0.74	0.61
Rp-Sd(TC)$_9$T	0.90	0.74
Sp-Sd(TC)$_9$T	0.88	0.73
M-Sd(TC)$_9$T	0.64	0.53
Fibronectin		
Rp-SdT$_{19}$	144	
Sp-SdT$_{19}$	135	
M-SdT$_{19}$	74	
Rp-Sd(TC)$_9$T	72	
Sp-Sd(TC)$_9$T	118	
M-Sd(TC)$_9$T	117	

[RClNH^{32}P-OdT18] = 3 μM.
K_d for CD4 = 0.5 μM; for bFGF = 0.5 μM; for laminin = 14.3 μM.
[a]Calculated from Equation 2. [RCl^{32}P-OdT$_{12}$] = 5 μM.

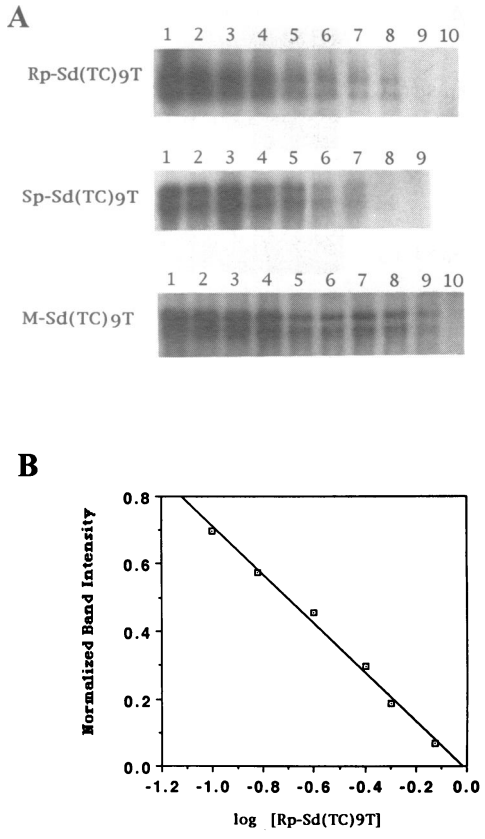

Figure 2. (**A**) Competition, by Rp-, Sp- and M-Sd(TC)$_9$T for binding of ClRNH^{32}P-OdT$_{18}$ to bFGF. Rp-, Sp- and M-Sd(TC)$_9$T were used as competitors of ClRNH^{32}P-OdT$_{18}$ (3 μM) binding to bFGF (0.3 μM) as described in the text (13.6% PAGE). Bands represent the 17.4 kDa subunits of bFGF modified by the oligodeoxynucleotide. The concentration of Rp-Sd(TC)$_9$T was (lanes 1–10, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.5, 0.75, 1, 2.5 and 5 μM. The concentration of Sp-Sd(TC)$_9$T was (lanes 1–9, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.75, 1, 2.5 and 5 μM. The concentration of M-Sd(TC)$_9$T was (lanes 1–10, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.5, 0.75, 1 and 5 μM. (**B**) Determination of the value of K_c for Rp-Sd(TC)$_9$T by the Cheng–Prusoff equation (Equation 1). The data from (A) was quantitated by excision of the gel bands and β-counting. Shown is a plot (r^2 = 0.99) of the normalized band intensity versus the log of the Rp-SdT$_{19}$ concentration.

Competition for probe, alkylating oligodeoxynucleotide RClNH^{32}P-OdT18 binding to the 400 kDa A subunit of laminin is shown in Figure 4. The approximate values of K_c for Rp-, Sp- and M-phosphorothioates are essentially identical here also.

Determination of the value of IC_{50} of stereoregular phosphorothioate oligodeoxynucleotide binding to fibronectin

Competition for protein binding by stereoregular phosphorothiate oligonucleotides occurs not only when the probe phosphodiester oligodeoxynucleotide bears an alkylating substituent, but also occurs when the alkylating group is not present. In Figure 5A we show competition for ^{32}P-SdT$_{18}$ binding to fibronectin by Rp-SdT$_{19}$, Sp-SdT$_{19}$ and M-SdT$_{19}$. A plot of the normalized band intensity (for a representative example, M-SdT19) versus log oligomer concentration, is shown in Figure 5B. However, the

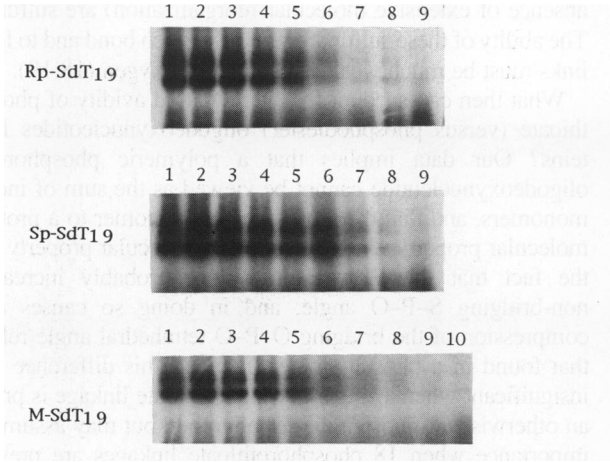

Figure 3. Competition by Rp-, Sp- and M-SdT_{19} for binding of $ClRNH^{32}P\text{-}OdT_{18}$ to bFGF. Rp-, Sp- and M-SdT_{19} were used as competitors of $ClRNH^{32}P\text{-}OdT_{18}$ (3 μM) binding to bFGF (0.3 μM) as described in the text (13.6% PAGE). Bands represent the 17.4 kDa subunits of bFGF modified by the oligodeoxynucleotide. The concentration of Rp-SdT_{19} was (lanes 1–9, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.5, 0.75, 2.5 and 5 μM. The concentration of Sp-SdT_{19} was (lanes 1–9, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.5, 0.75, 2.5 and 5 μM. The concentration of M-SdT_{19} was (lanes 1–10, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.5, 0.75, 1 and 5 μM.

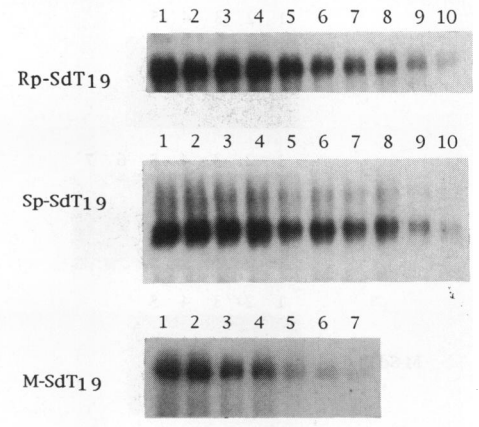

Figure 4. Competition by Rp-, Sp- and M-SdT_{19} for binding of $ClRNH^{32}P\text{-}OdT_{18}$ to laminin. Rp-, Sp- and M-SdT_{19} were used as competitors of $ClRNH^{32}P\text{-}OdT_{18}$ (3 μM) binding to laminin (0.25 μM) as described in the text (6% PAGE). Bands represent the 400 kDa subunit of laminin modified by the oligodeoxynucleotide. The concentration of Rp-SdT_{19} was (lanes 1–10, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.5, 0.75, 1, 2.5 and 5 μM. The concentration of Sp-SdT19 was (lanes 1–10, respectively) 0, 0.1, 0.15, 0.25, 0.4, 0.5, 0.75, 1, 2.5 and 5 μM. The concentration of M-SdT_{19} was (lanes 1–7, respectively) 0, 0.1, 0.25, 0.5, 0.75, 2.5 and 5 μM.

determination of K_c is not possible. This is because the value of K_d for binding of $^{32}P\text{-}SdT_{18}$ to fibronectin has not been determined as this binding is complex and does not fit a single-site or a two-site model (7). Regardless, the approximate values of IC_{50} of competition for the P-chiral oligomers and the M-oligomer are also essentially identical (Table 2).

DISCUSSION

The ability of phosphorothioate oligodeoxynucleotides to bind non-specifically to a variety of proteins has been well documented (3). This ability is due, at least in significant part, to the fact that these compounds act as heparin mimetics (4,6,7,15). All of the proteins studied in this work have heparin-binding sites, and it has been demonstrated that phosphorothiate oligodeoxynucleotides most likely bind to each of these proteins at or near the heparin binding site. For rsCD4, this site lies on the basic amino-acid rich D1 (N-terminal) domain on both the CDR-2 like and CDR-3 like loops. Each of these loops is known to contain a polyanion binding site (4). We have also demonstrated (L. Benimetskaya and C.A. Stein, unpublished observations) that oligodeoxynucleotides bind, in a manner independent of Ca^{2+}, Mg^{2+} and Mn^{2+}, to fibronectin; this binding is competitive with several discrete, synthetic persulfated heparin analogs. One of the effects of the binding of phosphorothioate oligodeoxynucleotides to laminin is inhibition of the binding of laminin to one of its ligands, galactosylceramide sulfate (sulfatide). Similarly, the binding of phosphorothioate oligodeoxynucleotides to fibronectin, though significantly weaker than their binding to laminin, is also inhibited by the discrete persulfated synthetic heparin analogs. Further, despite the relatively low affinity of phosphorothioate oligodeoxynucleotides for fibronectin, they can still cause blockade of binding of fibronectin to its cell surface receptors on phorbol-12,13-myristate acetate treated Jurkat T-cells. In this case, blockade of binding appears to be caused by events occurring at the cell surface.

The binding of phosphorothioate oligodeoxynucleotides to bFGF may be significantly sequence dependent (6), especially when the G-quartet motif is present in the sequence. At least in some cases, the presence of the G-quartet appears to produce an oligomer with a higher affinity for protein than non-G quartet containing species, even in the absence of frank oligomer tetraplex formation. This increased affinity may be due to the formation of non-Watson–Crick hydrogen bonds between adjacent guanosines, (J. Wyatt, personal communication), leading to the formation of relatively more rigid species.

Here, we have examined the ability of phosphorothioate oligodeoxynucleotides to compete for protein binding with a probe phosphodiester thymidine homopolymer oligodeoxynucleotide. The phosphorothiote oligodeoxynucleotides employed are of quite high stereopurity. The Sp-$Sd(TC)_9T$ may be slightly less pure than the others (65 versus 80%). However, this may be due to the fact that the svPDE is not completely stereoselective and to a limited extent will also hydrolyze dinucleoside phosphorothioates of the Sp-configuration. Furthermore, the nucleases digest phosphorothioates more slowly than phosphodiester oligomers, and the long total incubation times and the high concentration of the enzyme necessary may promote additional degradation.

The competition studies described in this work are virtually independent of P-chirality. The fact that the all-Rp and all-Sp forms of the oligomers have virtually identical values of K_c seems to imply that the role of the nitrogenous bases (i.e. their ability to interact directly with the proteins) is minimal. This assertion is in part confirmed by data (16) demonstrating that phosphorothiote polymers without the nitrogenous bases ('abasic' DNA) can inhibit CD4-dependent HIV replication. However, these compounds, at equimolar concentrations, are not as potent inhibitors of HIV-replication as identical-length phosphorothioate oligodeoxynucleotides

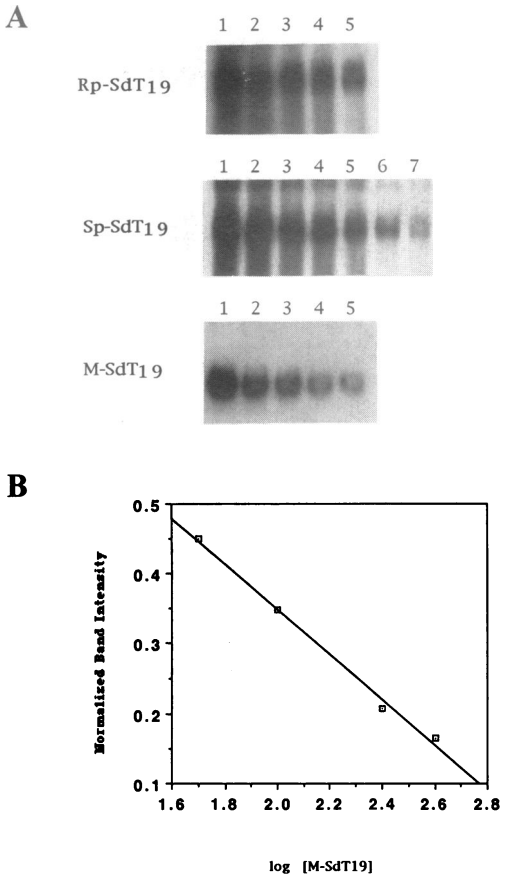

Figure 5. (**A**) Competition by Rp-, Sp- and M-SdT$_{19}$ for binding of 5′-^{32}P-SdT$_{18}$ to fibronectin. Rp-, Sp- and M-SdT$_{19}$ were used as competitors of 5′-^{32}P-SdT$_{18}$ (3 μM) binding to fibronectin (5 μM) as described in the text (6% PAGE). Bands represent the M_r = 220 kDa subunit of fibronectin modified by the oligodeoxynucleotide. The concentration of Rp-SdT19 was (lanes 1–5, respectively) 0, 25, 75, 250 and 400 μM. The concentration of Sp-SdT19 was (lanes 1–7, respectively) 0, 25, 50, 75, 100, 250 and 400 μM. The concentration of M-SdT19 was (lanes 1–5, respectively) 0, 50, 100, 250 and 400 μM. (**B**) Determination of the value of K_c for M-SdT$_{19}$ by the Cheng–Prusoff equation (Equation 1). The data from (A) was quantitated by excision of the gel bands and β-counting. Shown is a plot (r^2 = 0.99) of the normalized band intensity versus the log of the M-SdT$_{19}$ concentration.

with pendant bases. However, the nature and sequence of the bases (if they are not G-quartets) is still of relatively minimal importance.

Nevertheless, the finding that the values of K_c (Table 2) for the machine-made, and thus presumably stereorandom oligomers, were virtually identical to those of the P-chiral oligomers, was entirely unexpected. These data imply that not only are protein-base interactions minimal, but reveals something about nature of the contact points (sulfur versus oxygen) between the oligomer and protein. Apparently, in oligomers containing phosphorothioate linkages of defined absolute configuration, the phosphates play the role of contact points with the protein by means of hydrogen-bonding between the amide hydrogens of the protein and the P=O double bond, and by 'salt-links' between the positively charged amino acid functions and the ionized P–O bonds of phosphates (17). In the M-phosphorothioate oligodeoxynucleotides used in this study, it is likely that at least some of the contact points (in the absence of extensive molecular reorganization) are sulfur atoms. The ability of these sulfur atoms to hydrogen bond and to form salt links must be much weaker than that of oxygen (18,19).

What then can account for the increased avidity of phosphorothioate (versus phosphodiester) oligodeoxynucleotides for proteins? Our data implies that a polymeric phosphorothioate oligodeoxynucleotide cannot be viewed as the sum of individual monomers, and that the binding of the oligomer to a protein is a molecular property. The origin of this molecular property may be the fact that the larger sulfur atom probably increases the non-bridging S–P–O angle, and in doing so causes a slight compression of the bridging O–P–O tetrahedral angle relative to that found in a phosphodiester linkage. This difference may be insignificant when a single phosphorothioate linkage is present in an otherwise all-phosphodiester oligomer, but may assume larger importance when 18 phosphorothioate linkages are present per molecule, as in this work. The proposed diminished tetrahedral angle may reduce the number of rotational degrees of freedom of the phosphorothioate polymer, leading to a conformationally more rigid molecule. This, in turn, may result in a diminished component of the entropic cost of binding to protein. This hypothesis predicts that, on the basis of entropic considerations, the rate of dissociation of a protein–phosphorothioate complex would be much slower than that of a similar complex with a phosphodiester oligomer. Such a rate difference has been observed by Stein *et al.* and by D. Hare *et al.* (unpublished observations) for complexes of phosphorothioate oligodeoxynucleotides with bFGF.

Thus, when viewed in aggregate, the data suggest that the affinity of phosphorothioate oligodeoxynucleotides for heparin-binding proteins is (i) dependent on the presence of nitrogenous bases, but relatively independent of their sequence, except when hydrogen-bonding guanosine bases are present; and (ii) independent of P-chirality, possibly because each phosphorothioate monomer regardless of stereochemistry at phosphorus, contributes equally to a reduction in the entropic cost of binding. This reduction, in the case of the M-oligomers, more than compensates for oxygen to sulfur changes in the contact points. However, verification of this hypothesis will require formal determination of the association and dissociation rates of protein–oligodeoxynucleotide complexes as a function of temperature, as well as determination of the crystal structure of a phosphorothioate oligodeoxynucleotide.

Finally, Cosstick and Eckstein (20) have shown that when an Rp-phosphorothioate is placed 5′ to a deoxycytidine residue in the oligodeoxynucleotide d[G(p(S)CpG$_3$p(S)C] the B–Z transition is potentiated in comparison to the Sp- and the all-phosphodiester oligomer. Conversely, an Rp-phosphorothioate placed 5′ to deoxyguanosine strongly inhibits the transition. The oligodeoxynucleotides employed in this study are most likely always in the B configuration, and not morphologically affected by chirality at phosphorus. In future studies, the interactions of oligodeoxynucleotides with proteins will be examined in which phosphorothioate modification of known sense of chirality may influence the conformation of the overall oligodeoxynucleotide molecule.

ACKNOWLEDGEMENTS

This work was partially funded by NCI 60639 and an Irving Scholarship (to C.A.S.). Generous support from the Maria Sklodowska-Curie Fund, MZ/HHS-95-228 is greatly appreciated, as are helpful discussions with J. Wyatt.

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