Sequence-selective carbohydrate–DNA interaction: Dimeric and monomeric forms of the calicheamicin oligosaccharide interfere with transcription factor function

(transcription/minor groove binder)

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ABSTRACT The synthetic oligosaccharide moiety of the antibiotic calicheamicin and the head-to-head dimer of this oligosaccharide are known to bind to the minor groove of DNA in a sequence-selective manner preferring distinct target sequences. We tested these carbohydrates for their ability to interfere with transcription factor function. The oligosaccharides inhibit binding of transcription factors to DNA in a sequence-selective manner, probably by inducing a conformational change in DNA structure. They also interfere with transcription by polymerase II *in vitro*. The effective concentrations of the oligosaccharides for inhibition of transcription factor binding and for transcriptional inhibition are in the micromolar range. The dimer is a significantly more active inhibitor than is the monomer.

The antibiotic calicheamicin γ_1^1 belongs to the enediyne class of antibiotics and binds to double-stranded DNA in a sequence-selective manner (refs. 1–3; Fig. 1). Upon binding it cleaves the sugar phosphate backbone of the DNA (4–6). The major DNA contact surface of the antibiotic is an aryltetrasaccharide moiety that interacts with the minor groove of the DNA duplex (refs. 2 and 7–9; Fig. 1). The enediyne portion of the antibiotic appears to contribute to the drug–DNA interaction by increasing binding energy and widening the spectrum of the target sequence (10, 11). The carbohydrate portion of calicheamicin has been obtained by total synthesis, and its interaction with DNA has been studied extensively (12–14). One of the preferred target sequences is the tetranucleotide TCCT (3); others include TCTC and TTTT (2, 10, 15). Structural and chemical analysis has shown that the carbohydrate tail of calicheamicin is oriented toward the 3' end of the TCCT surface and that the iodine of the aryltetrasaccharide plays a critical role in the sequence-selective recognition of DNA (2, 5, 10). Recently, Ho and coworkers (16) reported that the aryltetrasaccharide of calicheamicin can inhibit the binding of transcription factors to sequences containing the TCCT tetranucleotide and that the carbohydrate can interfere with *in vivo* DNA transcription that is dependent on such transcription factor DNA interaction. In order to explore the biological consequences of carbohydrate–DNA interactions, our group has synthesized a head-to-head dimer of the calicheamicin oligosaccharide that preferentially binds to the decanucleotide AGGAXTCCT, where X can be any nucleotide. The dimer has the potential of greater target specificity than does the monomer (refs. 3 and 10; Fig. 1). We have used this dimer as well as the monomer to analyze functions of transcription factors, interaction with their cognate DNA target, and activation of transcription. Our results confirm and extend the observations of Ho and coworkers and demonstrate that

carbohydrates can interfere with the sequence-specific binding of transcription factors to DNA and with the activation of transcription. We find that the dimer of the calicheamicin oligosaccharide is a much more effective inhibitor of biological activity than is the monomer.

MATERIALS AND METHODS

Calicheamicin Carbohydrates. The aryltetrasaccharide of calicheamicin was obtained by total synthesis as described (12, 17). The synthesis of the head-to-head dimer of this compound has also been reported (18). Structure and purity of the carbohydrates were verified by thin-layer chromatography and ^1H NMR spectroscopy.

Electrophoretic Mobility Shift Assays. The oligonucleotides used in electrophoretic mobility shift assays are shown in Table 1. The AP-1 binding site is derived from the human collagenase gene promoter. It does not contain a TCCT sequence and serves as a control for nonspecific binding of the carbohydrate. In the AP-1(TCCT) and the AP-1(TCCT)₂ oligonucleotides, TCCT sequences (doubly underlined in Table 1) were constructed near the AP-1 consensus. The AP-1(TCCT) oligonucleotide contains one TCCT site, five nucleotides 5' to the consensus, and the AP-1(TCCT)₂ oligonucleotide contains two TCCT sites, one immediately 5' to the consensus, and a second one six nucleotides upstream. AP-1(DM) contains the optimal dimer DNA binding sequence AGGAAGTCCT immediately upstream of the AP-1 site. The consensus binding site for the transcription factor NFI (singly underlined in Table 1) contains a central random sequence trinucleotide that separates the two complementary half sites (19). In our experiment this trinucleotide was chosen to yield the sequence TCCT. The APRE is located in the 5' flanking region of several acute-phase protein genes of liver cells, such as the genes encoding α_2 -macroglobulin, fibrinogen, and α_2 -acid glycoprotein (20–22). APRE-dependent transcription is stimulated by interleukin 6 (IL-6) via phosphorylation and binding of the STAT-3 (signal transducer and activator of transcription 3) transcription factor (23, 24). The rat α_2 -macroglobulin promoter used in the present experiments contains two TCCT sites 5' of the STAT-3 binding site (singly underlined in Table 1) (20, 25). The TCCT sequence is also present in the PU.1 binding site derived from the promoter of the major histocompatibility complex class II gene 1A β (26). PU.1 is a B-cell and macrophage-specific transcription factor that shows homology to the extra transforming sequence (Ets) (27). Nuclear extracts were prepared according to published techniques (28).

Abbreviations: TK, thymidine kinase; IL-6, interleukin 6; APRE, acute-phase response element; AP-1, activator protein 1; NFI, nuclear factor I; PU.1, purine-rich sequence binding protein; Ets, extra transforming sequence; STAT-3, signal transducer and activator of transcription 3.

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FIG. 1. Structures of calicheamicin γ_1^1 and its monomeric and dimeric oligosaccharides.

For AP-1 assays, nuclear extracts were made from HeLa cells; for NFI, the source was chicken embryo fibroblasts. APRE binding activity was obtained from the human hepatoma cell line HepG2 that had been stimulated with IL-6 for 15 min before extraction (24). PU.1 was obtained from HeLa cells transfected with the PU.1 expression vector pECE, a kind gift of Richard Maki (27). For electrophoretic mobility shift assays, ≈ 1 ng of radiolabeled oligonucleotide (10^4 cpm) was first incubated with different concentrations of carbohydrates for 5 min at room temperature. Nuclear extract was incubated for 10 min in the presence of 2 μ g of poly (dI-dC) in binding buffer containing 20 mM Hepes (pH 7.6), 1 mM EDTA, 75 mM NaCl, 1 mM dithiothreitol (DTT), and 5% glycerol and was then added, giving a total of 20 μ l for the reaction mixture. After 30 min of incubation at room temperature, the reaction mixtures were loaded onto 5% polyacrylamide gels buffered with Tris/boric acid electrophoresis buffer (22.5 mM Tris-borate/1 mM EDTA). After electrophoresis, gels were dried and exposed to Kodak x-ray film at -70°C or to a Phosphor-Imager for quantitation.

In Vitro Transcription. Two tandem repeats of the APRE from the rat α_2 -macroglobulin promoter sequence were synthesized with flanking restriction sites for *Hind*III and *Bam*HI at the 5' and 3' terminus, respectively. This DNA fragment was then cloned into the TKL plasmid, which is a thymidine kinase (TK) promoter-directed luciferase expression vector (29). The resulting plasmid TKL33 was linearized with the restriction enzyme *Spl* I. The gel-purified plasmid DNA served as template for transcription. APRE-dependent transcriptional activation is mediated by STAT-3 (24). The STAT-3 expression

construct pRC/CMV.STAT-3 [a kind gift from James Darnell (30)] was transfected into HepG2 cells. The cells were then treated with IL-6 for 15 min. A cell extract supporting *in vitro* transcription was prepared according to published techniques (31). Approximately 50 μ g of the extract was first incubated with the DNA template and carbohydrate for 10 min at room temperature in reaction buffer that contains 10 mM Hepes (pH 7.6), 75 mM NaCl, 1 mM DTT, and 5% glycerol. Then 0.6 mM of rATP, rCTP, and rGTP, 0.02 mM UTP, and 2 μ l of [^{32}P]UTP were added, giving a total of 30 μ l for the reaction. The reaction mixture was incubated for 60 min at 30°C . RNA was extracted using the STAT-60 total RNA isolation reagent according to the manufacturer's instruction (Tel-Test, Friendswood, TX) (32). The RNA was then precipitated, resuspended in 80% formamide, and loaded on a 6% denaturing polyacrylamide gel. After electrophoresis the gel was dried and autoradiographed.

RESULTS

Dimeric and Monomeric Forms of the Calicheamicin Oligosaccharide Inhibit the Binding of Transcription Factors to Their DNA Target Sequences. Fig. 2 shows electrophoretic mobility shift assays with target oligonucleotides for four transcription factors: AP-1, NFI, PU.1, and STAT-3. They are tested in the presence and absence of various concentrations of calicheamicin carbohydrates. Binding of AP-1 to the unaltered oligonucleotide from the promoter of the human collagenase gene was affected only at relatively high carbohydrate concentrations. This oligonucleotide does not contain the TCCT sequence. In contrast, binding to the AP-1(TCCT) and to AP-1(TCCT)₂ constructs was inhibited at substantially lower concentrations of the monomeric form of the calicheamicin oligosaccharide. For the AP-1(TCCT)₂ target, we also tested the dimeric form of the calicheamicin oligosaccharide, and it showed greater inhibitory activity than the monomer. On AP-1(DM) with the decamer target site for the dimeric calicheamicin carbohydrate, inhibition of AP-1 binding was observed at even lower carbohydrate concentrations. Jun antibody-induced supershift identified the retarded bands as containing AP-1. The binding of the oligosaccharide to its target was reversible. Inhibition of AP-1 binding by the oligosaccharide could be abolished by incubation with cold APRE oligonucleotide (data not shown). Inhibition by calicheamicin carbohydrates was also observed for the DNA

Table 1. Oligonucleotide targets for calicheamicin oligosaccharides

AP-1	5'-CTAGTGATGAGTCAGCCGGATC-3'
AP-1(TCCT)	GATCCTAGTGATGAGTCAGCCG
AP-1(TCCT) ₂	GATCCTAGTCCTGAGTCAGCCG
AP-1(DM)	GATCAGGAAGTCCTGAGTCAGCCG
NFI	GATCTCTGGCATCCTGCCACG
APRE	GATCGTCCTTAATCCTTCTGGGAATTC
PU.1	GATCCGTCCCAAGTGAGGAACCAATCAGCA-TTG

AP-1, activator protein 1; NFI, nuclear factor I; APRE, acute-phase response element; PU.1, purine-rich sequence binding protein. See text for underlining distinctions.

FIG. 2. Electrophoretic mobility shift assays. Electrophoretic mobility shift assays are shown with oligonucleotides for four transcription factors: AP-1, NFI, STAT-3, and PU.1. The oligosaccharides were first incubated with target DNAs before appropriate nuclear extracts were added, giving a total volume of 20 μ l (see text). (A) AP-1 marks a 22-mer with the AP-1 binding site from the collagenase promoter. It serves as negative control. AP-1(TCCT) contains one oligosaccharide binding site 5 bp upstream of the AP-1 consensus sequence. AP-1(TCCT)₂ contains two TCCT sites upstream of the AP-1 consensus. Concentrations for the monomeric oligosaccharide are indicated at the top (in μ M). The shifted complexes were identified as AP-1 by supershift with anti-Jun antibody. (B) Interference of the dimeric oligosaccharide with AP-1 binding. The left panel represents the effect of dimeric oligosaccharide on targets with the TCCT sequence; the right panel shows the dimeric oligosaccharide with a target containing AGGAAGTCCT. (C) The NFI oligonucleotide contains one TCCT sequence within the NFI binding site. The effects on NFI binding were tested with monomer and dimer oligosaccharides. The shifted complex was confirmed as NFI by supershift with anti-NFI antibody. (D) Both APRE and PU.1 contain oligosaccharide binding sites in the contexts of their natural promoters. APRE binds STAT-3, and PU.1 binds the Ets family transcription factor PU.1. The concentrations are for the monomeric oligosaccharide. Nuclear extracts from IL-6-stimulated HepG2 cells were used for APRE. Nuclear extracts from HeLa cells transfected with the PU.1 expression vector pECE were used for PU.1. No binding to the PU.1 site was seen with normal HeLa cell extracts.

interaction of PU.1, NFI, and STAT-3. Again the dimer was much more effective than the monomer. NFI in the retarded complex was identified by specific antibody supershift, and the PU.1 complex was seen only in cells transfected with the PU.1-expressing plasmid. The results of these electrophoretic mobility shift assays are summarized in Table 2. The effective inhibitory concentrations for the calicheamicin carbohydrates are in the micromolar range; the dimer is 5–12 times more

active on a concentration basis than is the monomer. However, comparisons can be made only between values for a given transcription factor because they must be based on equal transcription factor–DNA affinities. Among the AP-1 targets, AP-1(TCCT)₂, the one with the TCCT sequence in duplicate and closer to the AP-1 consensus, is more readily interfered with than the one with the single TCCT site five nucleotides away from the consensus. Particularly effective interference was seen with the binding of STAT-3 to the rat α -macroglobulin promoter; this promoter contains two TCCT sequences upstream of the STAT-3 consensus. These results show that calicheamicin carbohydrates can interfere with the binding of transcription factors to these DNA targets not only if the binding sequence itself includes a TCCT site but also if a TCCT sequence is located near the transcription factor binding site.

The Calicheamicin Oligosaccharides Inhibit STAT-3-Dependent Transcription. Transcription factors must bind to DNA before they can regulate transcription. Any inhibition of transcription factor–DNA interaction can be expected to reduce the ability of the factor to alter rates of transcription. This assumption was tested with an *in vitro* transcription system using whole cell extracts prepared from HepG2 cells, a human

Table 2. Concentrations of calicheamicin oligosaccharides that induced 50% inhibition of transcription factor–DNA interaction in electrophoretic mobility shift assays

Oligonucleotide	Monomer, μ M	Dimer, μ M
AP-1	500	ND
AP-1(TCCT)	125	ND
AP-1(TCCT) ₂	25	6
AP-1(DM)	ND	0.2
APRE	6	1
NF-1	12.5	1
PU.1	12.5	1

ND, not done.

hepatoma cell line. HepG2 cells were transfected with the STAT-3 expression vector pRC/CMV. STAT-3 and treated with IL-6 to activate the transcription factor STAT-3. The DNA template construct used in these experiments contained two copies of the STAT-3 responsive element from the rat α -macroglobulin promoter (cf. APRE, Table 1) upstream of a TK promoter driving a truncated luciferase gene. This template contains the two TCCT sequences of the α -macroglobulin promoter. The TK promoter sequence does not contain any TCCTs. As a control we used the same construct with the TCCT sequences replaced by TGGT (plasmid TKL33M). Run-on labeling with [32 P]UTP in this system generated distinct mRNAs visible after electrophoresis and radioautography (arrows in Fig. 3). Positive controls consisting of cell extracts from IL-6-treated HepG2 cells and the template construct showed active transcription (Fig. 3A, lane 2). Omission either of IL-6 treatment or of the DNA template abolished transcriptional activation, and these combinations served as negative controls (data not shown). Addition of monomeric calicheamicin oligosaccharide at concentrations in the micromolar range to the active system from IL-6-stimulated cells transcribing the template DNA construct resulted in inhibition of the reaction (Fig. 3A, lanes 3–7). Addition of the dimer oligosaccharide to this system caused an at least 10-fold greater inhibition than the monomer (Fig. 3A, lanes 8–12). Inhibition of the mutated template control (plasmid TKL33M) was less effective by a factor of approximately 2–4 (Fig. 3B).

DISCUSSION

The oligosaccharide moiety of calicheamicin is the prototype of a new class of DNA binding molecules. It targets TCCT.AGGA sites, binding to the minor groove of double-stranded DNA. This sequence-selective interaction has obvious implications for the functions of DNA, notably for serving as template of *de novo* DNA synthesis and for messenger RNA transcription. We have studied elements of the latter, using a chemically synthesized monomer and a dimer of the cali-

cheamicin oligosaccharide. We chose DNA target molecules that contain TCCT sequences within or closely adjacent to the binding sites of transcription factors. Exposure of the DNA targets to monomeric or dimeric calicheamicin oligosaccharide at micromolar concentrations inhibits transcription factor binding. These results are in agreement with the report by Ho and coworkers (16). The interference with transcription factor binding was sequence-selective for the presence of TCCT sites, but at increased carbohydrate concentrations it showed a broader target spectrum, including diverse sequences. It is known that TCTC and TTTT sequences are also bound by the monomer. Other sites in DNA can interact with the calicheamicin carbohydrate moiety at higher concentrations of the reactants (15). This fact is also reflected in the results of the *in vitro* transcription test where a template with the TCCT sites mutated to TGGT shows comparable inhibition at a 2- to 4-fold higher carbohydrate concentration. Therefore, the oligosaccharides used in this study target certain preferred DNA sequences, but they lack the kind of sequence specificity that can be observed with protein transcription factors. The inhibitory effect of carbohydrates on transcription factor binding was seen with TCCT sites located within or near the transcription factor binding site. This observation suggests that the carbohydrates do not prevent transcription factor binding by competing with the protein for the same DNA region but rather by inducing a conformational change in the DNA that interferes with transcription factor recognition. Such a conformational change has already been suggested by NMR studies (9). However, further structural investigations will be needed to characterize the postulated altered conformation.

The head-to-head dimer of the calicheamicin carbohydrate was much more effective than the monomer as an inhibitor of transcription factor binding. Surprisingly, this difference was seen even when there was only one TCCT site in the target, which then does not provide the optimal 10-nucleotide binding sequence for the dimer. This observation could be explained if by attaching to a single TCCT, the dimer induced a greater conformational change in the DNA than does the monomer. When the full dimer binding sequence is provided, interference with transcription factor binding is seen at more than 10-fold lower carbohydrate concentrations.

The interference with transcription factor binding by calicheamicin carbohydrates also manifests itself in an inhibition of transcriptional stimulation. Again, the dimeric oligosaccharide was more effective than the monomeric oligosaccharide. This inhibition of transcription was observed in a defined *in vitro* system in which the template DNA is readily accessible to the carbohydrate inhibitor. In contrast to Ho and coworkers, who reported calicheamicin carbohydrate-induced repression of transcription in live cells (16), we have not been successful in demonstrating such repression with an *in vivo* system using HepG2 cells and the STAT-3-dependent TKL33 plasmid as a template. The calicheamicin oligosaccharide is somewhat lipophilic; it would be expected to pass the plasma membrane and enter the cell. Failure to inhibit transcription *in vivo* could be caused by insufficient nuclear concentrations of the inhibitor or possibly by the presence of numerous competing TCCT sites in the eukaryotic genome. Recently, other minor groove binders have been studied for their effects on the transcriptional machinery (33, 34). For instance, distamycin A, Hoechst 33258, netropsin, and CC-1065 were found to interfere with the interaction of the TATA box binding protein (TBP) and DNA at submicromolar concentrations.

The sequence-selective interaction of calicheamicin carbohydrates with DNA may open new possibilities for the chemical control of genetic information. In principle, it may be possible to regulate individual genes or entire gene programs of viruses or of cells. Although transcriptional control comes to mind first, any function of DNA could be targeted by carbohydrate intervention, including replication. It is also now tempting to

FIG. 3. *In vitro* transcription assays. The linearized plasmid TKL33 or TKL33M containing two copies of APRE sequences upstream of the TK promoter was used as a DNA template. Nuclear extracts were made from HepG2 cells transfected with the STAT-3 expression vector pRC/CMV. STAT-3 and stimulated with IL-6 for 15 min. (A) Effect of the oligosaccharides on transcriptional activity with plasmid TKL33. Lane 1, negative control without DNA template; lane 2, positive control with DNA template but without oligosaccharide; lanes 3–7, reaction mixtures containing various concentrations of monomeric oligosaccharide—50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, and 1 μ M, respectively; lanes 8–12, reaction mixtures containing various concentrations of dimeric oligosaccharide—50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, and 1 μ M. (B) Effect of the monomeric oligosaccharide on transcriptional activity with plasmid TKL33M. Lane 1, positive control without oligosaccharide; lanes 2–6, reaction mixtures containing various concentrations of oligosaccharide, 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, and 1 μ M.

search for biologically significant interactions between carbohydrates and RNA.

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