Selective inhibition of the human tie-1 promoter with triplex-forming oligonucleotides targeted to Ets binding sites

Selective Inhibition of the Human *tie*-1 Promoter with Triplex-Forming Oligonucleotides Targeted to Ets Binding Sites

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The Tie receptors (Tie-1 and Tie-2/Tek) are essential for angiogenesis and vascular remodeling/integrity. Tie receptors are upregulated in tumor-associated endothelium, and their inhibition disrupts angiogenesis and can prevent tumor growth as a consequence. To investigate the potential of anti-gene approaches to inhibit *tie* gene expression for anti-angiogenic therapy, we have examined triple-helical (triplex) DNA formation at 2 tandem Ets transcription factor binding motifs (designated E-1 and E-2) in the human tie-1 promoter. Various tie-1 promoter deletion/mutation luciferase reporter constructs were generated and transfected into endothelial cells to examine the relative activities of E-1 and E-2. The binding of antiparallel and parallel (control) purine motif oligonucleotides (21-22 bp) targeted to E-1 and E-2 was assessed by plasmid DNA fragment binding and electrophoretic mobility shift assays. Triplex-forming oligonucleotides were incubated with tie-1 reporter constructs and transfected into endothelial cells to determine their activity. The Ets binding motifs in the E-1 sequence were essential for human tie-1 promoter activity in endothelial cells, whereas the deletion of E-2 had no effect. Antiparallel purine motif oligonucleotides targeted at E-1 or E-2 selectively formed strong triplex DNA (K_d ~10^{-7} M) at 37 °C. Transfection of tie-1 reporter constructs with triplex DNA at E-1, but not E-2, specifically inhibited tie-1 promoter activity by up to 75% compared with control oligonucleotides in endothelial cells. As similar multiple Ets binding sites are important for the regulation of several endothelial-restricted genes, this approach may have broad therapeutic potential for cancer and other pathologies involving endothelial proliferation/dysfunction.

Online address: http://molmed.org
doi: 10.2119/2005-00046.Hewett

INTRODUCTION

Tumor growth and the development of hematologic metastases depend on angiogenesis, and the inhibition of this process represents a target for therapy (1). Several endothelial receptor tyrosine kinases (RTKs) play key roles in coordinating vascular proliferation, differentiation, and maintenance/integrity (2,3). These include the vascular endothelial growth factor (VEGF) family of receptors (Flt-1, Flt-4, and KDR/Flk-1) and Tie (Tie-1 and Tie-2/Tek) RTKs (2,3). The Tie receptors are essential for embryonic development, and targeted disruption of the *tie* genes in mice highlights their importance in angiogenesis and vascular remodeling (4). The angiopoietins (Ang-1 through -4) were identified as ligands for Tie-2 (3). Tie-1 was recently shown to bind Ang-1 and Ang-4 and increase its association with Tie-2 in endothelial cells (5). Proteolytic cleavage of the Tie-1 ectodomain following protein kinase C activation produces a signaling-competent membrane-bound truncated Tie-1 receptor (6). As with Tie-2, the activation of Tie-1 signaling has been reported to promote endothelial cell survival through the phosphoinositol-3-kinase/Akt pathway (7).

In the adult, Tie-1 and Tie-2 are upregulated in endothelium during both physiological and pathological angiogenesis (3,8). Increased Tie receptor expression has been reported in diabetic retinopathy (9), psoriasis (10), and arthritis (11), in the vasculature of human tumors including brain and breast cancers, and in melanoma metastases (3,8,12-16). The soluble extracellular domain of Tie-2 was reported to act in a dominant-negative manner to block tumor-stimulated angiogenesis in the rat cornea and subcutaneous window chamber models (17). Moreover, when delivered systemically, an adenovirus expressing soluble Tie-2 inhibited the growth of primary murine tumors and their metastases (18). Similarly, sTie-1 or anti–Tie-1 antibodies have been shown to inhibit the growth of tumors by disrupting the tumor neovasculature.

Various therapeutic approaches are being investigated to inhibit pathological angiogenesis, including the use of

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Submitted December 21, 2005; accepted for publication March 13, 2006.

blocking antibodies, small molecule inhibitors, antisense oligonucleotides, and ribozymes (1). A novel alternative strategy is to employ anti-gene approaches to inhibit the expression of angiogenesis-associated genes. To test this paradigm, we are using triple helical (triplex) DNA formation, which relies on the sequence-specific binding of oligonucleotides to duplex DNA (19,20), to validate targets in the endothelial-restricted RTK genes. The ideal triplex DNA target is a homopurine-homopyrimidine sequence that allows the triplex-forming oligonucleotide (TFO) to bind in the major groove of double-stranded DNA, forming Hoogsteen hydrogen bonds with the purine strand (reviewed in 21-23). Triplex DNA formation represents a promising approach to selectively inhibit gene expression. It offers potential advantages over the use of antisense oligonucleotides and ribozymes, as generally only two sequences (alleles) need to be blocked per cell to prevent the generation of all RNA species arising from the target gene (21,22). TFO can be directed to inhibit gene expression by competing with the binding of activating transcription factors to regulatory sites or by disrupting transcriptional elongation (21-32). Advances in this approach have been achieved through the development of oligonucleotide analogs such as N3′ → P5′ phosphoramidates (28), peptide nucleic acid (PNA) (33), and 2′-aminoethoxy–substituted riboses (30) that are resistant to intra- and extracellular nucleases and form strong DNA triplexes (21-23). Whereas the close association of condensed DNA with chromatin can serve as a barrier to DNA triplex formation, this does not appear to present a problem when targeting transcriptionally active regions of DNA (21). Indeed, triplex DNA formation has been successfully targeted to inhibit endogenous gene expression (28). The recent demonstration of triplex-mediated gene modification in a murine model represents a major step toward the use of TFO for gene-based therapies (34). In addition, the current development of novel sequence-specific DNA binding agents (35) may provide alternative agents for the clinical application of anti-gene approaches.

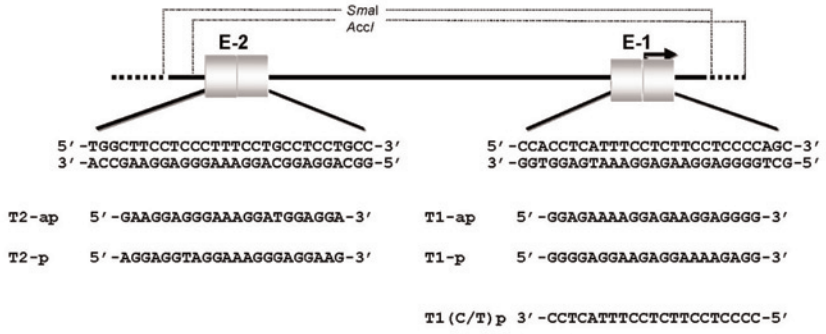

Figure 1. Diagram showing the relative positions of the tandem ets transcription factor duplex target sequences (E-1 and E-2), *Sma*I and *Acc*I restriction enzyme sites, and reported transcription start site (arrow) of the human tie-1 promoter (37). The sequences of the oligonucleotides encoding the E-1 and E-2 target sites that were annealed and used for electrophoretic mobility shift assays are indicated. The sequences of the antiparallel (relative to the purine strand of the target DNA sequence) triplex-forming oligonucleotides, T1-ap and T2-ap, targeted against E-1 and E-2, respectively, parallel control oligonucleotides (T1-p and T2-p), and parallel pyrimidine-rich (T1-(C/T)-p) oligonucleotide are shown.

We have identified several potential duplex target sequences in the 5′ regulatory regions of the *tie* genes (36,37), including three conserved sequences that encode multiple Ets transcription factor core (5′-GGAA/T-3′) DNA binding motifs (38,39). Members of the Ets family of transcription factors are key regulators of many genes associated with differentiation and tissue-specific and homeostatic processes, including angiogenesis (38-45). In this study, we demonstrate that two near-perfect homopurine-homopyrimidine sequences (E-1 and E-2) containing tandem Ets binding sites in the human tie-1 promoter represent good targets for triplex DNA formation with purine motif TFO. By promoter-reporter analysis, we demonstrate that one of these sequences (E-1) is essential for tie-1 promoter activity in cultured endothelial cells, and the selective formation of DNA triplex at the E-1 site leads to significant inhibition of tie-1 promoter activity in endothelial cells.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides synthesized with phosphodiester and phosphorothioate linkages and HPLC-purified were purchased from MWG-Biotech (Germany). The sequences of the antiparallel oligonucleotides, T1-ap and T2-ap targeted to E-1 and E-2, respectively, control parallel oligonucleotides (T1-P and T2-P), and together with parallel pyrimidine motif oligonucleotide, T1(C/T)-P, are shown in Figure 1. The duplex sequences used for electrophoretic mobility shift assays (EMSAs) consist of the E-1 (21 bp) and E-2 (22 bp) near homopurine-homopyrimidine targets with three additional base pairs of the tie-1 flanking sequences at each end (Figure 1).

Generation of tie-1 luciferase reporter constructs

The human tie-1 (~830 bp) gene promoter (37) was cloned into the pGL3 basic luciferase reporter vector (Promega, WI, USA) (p*tie*-1*luc*) as described previously (36). In addition, we cloned a larger 880-bp fragment of the human tie-1 promoter using an alterna-

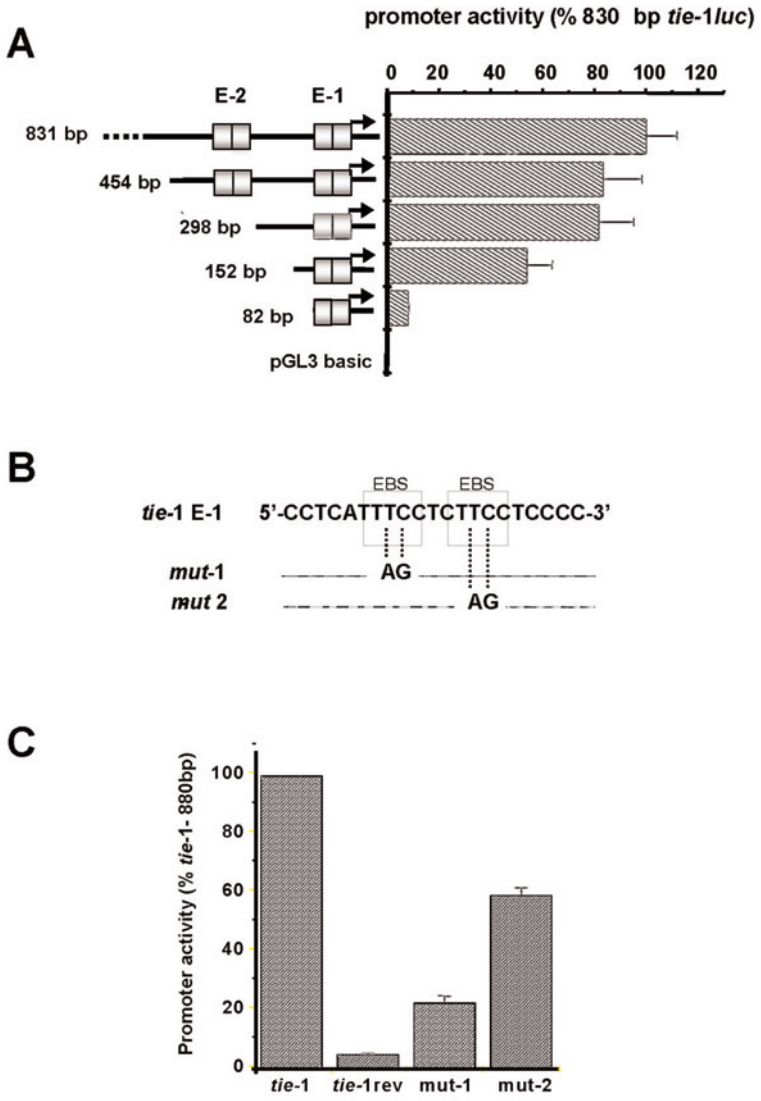

Figure 2. The activity of the two tandem Ets transcription factor core DNA binding sites, E-1 and E-2, in the human tie-1 promoter. (A) The effect of 5′ deletion on the activity of tie-1 luciferase reporter constructs (p*tie*-1*luc*) in endothelial cells. p*tie*-1*luc* constructs were cotransfected with a CMV promoter–driven Renilla luciferase control (pRL-CMV) plasmid into bovine aortic endothelial cells (BAECs). After a 72-h expression period, promoter activity was quantified by dual luciferase assay and normalized to pRL-CMV activity. Results are the means (± 1 SE, bars) of duplicate samples from six separate experiments (n = 12) expressed as a percentage of the activity of the 830-bp p*tie*-1*luc* construct. (B) Mutations (mut-1 and mut-2) were introduced into the putative ets core DNA binding motifs (EBS, 5′-GGAA/T-3′) in E-1 by overlap PCR as described in "Materials and Methods." The top strand of the E-1 sequence from the human tie-1 promoter is shown. (C) The effect of Ets binding site mutations in E-1 on the activity of the human tie-1 promoter. Wild-type (tie-1), mutated (mut-1 and mut-2), and reverse tie-1 control (*tie*-1*rev*) luciferase constructs were cotransfected with pRL-CMV into BAECs. Promoter activity was determined by dual luciferase assay after a 72-h expression period and represented as a percentage of wild-type 880-bp p*tie*-1*luc* plasmid (tie-1) following normalization to pRL-CMV activity. Results are means (± 1 SE, bars) of duplicate samples from at least 3 separate experiments ($n \geq 6$).

tive 3′ PCR primer (5′-TAC TCC AGA GGC CGA CCC AGG CCA G-3′) under the conditions described previously (36). p*tie*-1*luc* (830 bp) 5′ deletion constructs were generated using the Erase-a-base system according to the manufacturer's instructions (Promega). Briefly, p*tie*-1*luc* was subjected to progressive exonuclease-III digestion, blunt ended, religated, and sequenced. Additional 5′ deletions were generated by PCR using p*tie*-1*luc* as a template. Specific p*tie*-1*luc* Ets binding site mutants were produced by overlap PCR using pfu DNA polymerase (Stratagene, Amsterdam, Netherlands) under the following conditions: 94 °C for 30 s, 60 °C for 1 min, and 76 °C for 3 min for 30 cycles. Mutations were introduced into each of the two Ets binding motifs in E-1 (see Figure 2B) by PCR using the following primers (mut-1 sense 5′-AGC CAC CTC ATT AGC TCT TCC TCC CCA-3′, mut-1 antisense 5′-TGG GGA GGA AGA GCT AAT GAG GTG GCT-3′; and mut-2 sense 5′-AGC CAC CTC ATT TCC TCT AGC TCC CCA-3′, mut-2 antisense 5′-TGG GGA GCT AGA GGA AAT GAG GTG GCT-3′) in combination with primers flanking the tie-1 promoter sequence described previously (36). Mutant E-1 tie-1 PCR products were gel-purified and cloned into pCR-BLUNT (Invitrogen-BV, Netherlands), restriction-digested with *Sac*I and *Xba*I, and subcloned into the *Sac*I and *Nhe*I sites of pGL-3 basic. All DNA plasmids were prepared using Qiagen maxi-prep columns (Qiagen, Sussex, UK) and resuspended in sterile deionized water.

Plasmid DNA binding assay

Plasmid DNA binding assays were performed essentially as described by Vasquez et al. (46). Briefly, TFO (8 pM) was labeled with 10 μCi [γ^{32}P]dATP (Amersham Pharmacia Biotech UK, Bucks, UK) using 10 U T4-polynucleotide kinase (New England Biolabs, Boston, MA, USA) in a total volume of 10 μL and purified on Chromaspin + STE-10 columns (Clontech, Basingstoke,

UK). The p*tie*-1*luc* plasmid was restriction-digested with *Sma*I and *Acc*I (New England Biolabs), phenol-extracted, ethanol-precipitated, and resuspended in deionized water. The digested plasmid DNA (~1 μg) was incubated with radiolabeled TFO (~0.2 pM) in 10 μL binding buffer (10 mM Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$ and 10% sucrose) at 37 °C for 16-18 h. Loading buffer (10% Ficoll and 0.1% bromophenol blue) was added (2 μL), and the samples were separated immediately on 1% agarose gels containing ethidium bromide (EtBr) in running buffer (90 mM Tris-Borate buffer, pH 8.0, containing 10 mM $MgCl_2$) at 65 V for 5 h. The DNA was visualized under UV light, and the gels were dried under vacuum at 80 °C on Hybond-N membrane (Amersham Pharmacia Biotech UK) and exposed to film.

Electrophoretic mobility shift assays

For EMSAs, we used oligonucleotides encoding the duplex target sites corresponding to E-1 and E-2 (see Figure 1). The pyrimidine-rich oligonucleotides were labeled with [γ^{32}P]dATP, purified as described above, and annealed with a 1.5-fold excess of the complementary purine-rich oligonucleotide at 95 °C for 10 min and allowed to cool slowly to room temperature. Radiolabeled duplex (2×10^{-9} to 10×10^{-9} M) was incubated with increasing quantities of TFO (0.01 to 500 pmol) in a total volume of 10 μL binding buffer for 16 to 18 h at 37 °C. Loading buffer (2 μL) was added, and the samples were separated immediately on 6% nondenaturing polyacrylamide gels in running buffer at 100 V for ~ 2-4 h, dried under vacuum at 80 °C, and exposed to film. The intensity of the bands on the autoradiographs was determined by scanning densitometry (Image-Pro Plus; Media Cybernetics, MD, USA). The dissociation constant (K_d) was estimated by determining the fraction of triplex DNA at each concentration of TFO and calculating the midpoint of the titration when the intensities of the target duplex and triplex bands were equivalent.

Endothelial cell transfection and dual luciferase assays

Bovine aortic endothelial cells (BAECs) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% iron-supplemented calf serum, 25 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate (Sigma). Luciferase reporter constructs were transfected into BAECs at 50% to 60% confluence on 6-well culture dishes using ExGen 500 (Euromedex, France) as described previously (36). Briefly, ExGen 500 (10 μL) was mixed with 2 μg p*tie*-1*luc* or SV40 promoter control reporter plasmid (pSV40*luc*) (Promega) and 8 ng of the CMV-driven Renilla luciferase plasmid (pRL-CMV) in a total volume of 100 μL serum-free RPMI 1640 for 20 min. The cells were incubated with the transfection mixture in a total volume of 1 mL full growth medium at 37 °C. After 6 h, the medium was replaced and the cells incubated for a further 72 h and harvested and the luciferase activity was determined using the Dual Luciferase Assay (Promega) as described previously (36). To examine the activity of TFO on tie-1 promoter activity, various concentrations of TFO and control oligonucleotides were incubated with p*tie*-1*luc* or pSV40*luc* in a total volume of 10 μL binding buffer for 16 to 18 h at 37 °C. Before transfection, these samples were then mixed with 8 ng of pRL-CMV and ExGen 500 (10 μL) in a final volume of 100 μL RPMI 1640 serum-free medium, incubated for 20 min at room temperature, and added to BAECs as described above. Results obtained from duplicate samples were normalized to pRL-CMV activity.

RESULTS

Selection of triplex target sites in the human tie-1 promoter and design of TFOs

Based on our recent characterization of the human tie promoters (36) (P.W.H., unpublished data), we have identified several purine-rich potential triplex DNA target sites in these genes. We selected two sites (E-1 and E-2) in the human tie-1 promoter sequence (37) comprising conserved tandem Ets binding sites as targets for our initial investigations (Figure 1). The E-1 homopurine-homopyrimidine sequence is interrupted by a single T:A inversion and located between 756 and 796 bp; the E-2 sequence containing one G:C inversion lies between 505 and 526 bp of the human tie-1 promoter sequence (37) (GenBank accession no. S79347).

Triplex DNA formation has been described using three oligonucleotide motifs (21,22). Pyrimidine motif (C/T) TFOs bind in a parallel orientation to the purine strand of the target duplex to form C^+*G:C (TFO*homopurine:homopyrimidine) and T*AT triplets and require low pH to facilitate the protonation of cytosine bases, whereas purine (G/A) motif TFOs bind in antiparallel orientation to the purine strand of the target duplex forming G*G:C and A*A:T triplets. Mixed (G/T) TFOs may bind in either orientation forming G*G:C and T*AT triplets. We used purine motif TFOs in preference to pyrimidine and mixed motif TFOs, as they form DNA triplex at neutral pH, and as T residues may promote guanine quartet formation by providing the points for stable hairpins in G/T TFOs, respectively (47). We designed specific antiparallel G/A motif oligonucleotides against E-1 and E-2 (Figure 1) using standard rules of triplex DNA formation (21,22). To minimize the destabilizing effect of the G:C to C:G inversion in E-2, we used thymine in T2-ap, as it is reported to weakly associate with C:G inversions (T*C:G) in purine motif TFOs (20,48). However, there is no natural base that will accommodate a T:A inversion in the purine motif, so adenine was used in T1-ap in preference to guanine, as runs of contiguous guanines promote self-association and the formation of multimers (20). Oligonucleotides (T1-P and T2-P) were synthesized in parallel orientation to the purine-rich strand of E-1 and E-2 as controls (Figure 1). To improve the resistance of TFOs to intracellular and extracellular nucleases, we also generated oligonucleotides with phosphorothioate linkages (21,22). As fully phosphorothioate-linked oligonucleotides have

been reported to exhibit reduced target specificity and triplex DNA formation (22,49), oligonucleotides were synthesized with phosphorothioate linkages substituted for the last three phosphodiester bonds at the 3′ end.

Activity of the tandem Ets binding sites in the human tie-1 promoter

The tie-1 promoter contains several Ets binding sites (36), including the tandem Ets motifs contained within E-1 and E-2, and mutation of these Ets motifs in the murine tie-1 promoter dramatically reduces its activity in transgenic mice (50). Whereas Ets factors have recently been shown to bind to the E-1 and E-2 Ets sites (51), the relative contribution of these two tandem Ets sites to human tie-1 promoter activity has not been reported. To determine whether the Ets binding motifs in E-1 and E-2 are critical for the regulation of tie-1 and therefore suitable targets for triplex-mediated gene inhibition, we generated 5′ deletions of the human tie-1 promoter by progressive exonuclease-III digestion (Figure 2A) and PCR. The p*tie*-1*luc* human tie-1 promoter–driven firefly luciferase reporter constructs were transiently transfected into BAECs, and the activity was measured by dual luciferase assay (36). The progressive 5′ deletion of p*tie*-1*luc* beyond the E-2 site demonstrated that this tandem Ets binding site does not affect tie-1 promoter activity in cultured endothelial cells, whereas E-1 lies in the minimal promoter region (Figure 2A). The activity of the E-1 site was further investigated through the introduction of specific mutations into the E-1 Ets motifs of p*tie*-1*luc* by overlap PCR (Figure 2B). Mutant E-1 p*tie*-1*luc* reporter activity was reduced to 20% (mut-1) and 60% (mut-2) of wild-type p*tie*-1*luc* activity, demonstrating that these Ets binding sites are critical for tie-1 regulation (Figure 2C) and highlighting the potential of blocking the E-1 site.

Triplex DNA formation with the human tie-1 promoter

Plasmid binding assays were used as a rapid method to screen for specific triplex DNA formation (46). TFO binding was initially examined against a range of p*tie*-1*luc* and human tie-2 promoter (p*tie*-2*luc*) luciferase reporter plasmid restriction-fragments (Figure 3A) at a TFO-to-target DNA ratio of ~1:1 (TFO:DNA duplex). *Sma*I digestion of p*tie*-1*luc* produces a 770-bp fragment that contains the E-1 and E-2 sites (Figure 1). Both the antiparallel oligonucleotides, T1-ap and T2-ap, form triplex DNA on the 770-bp tie-1 promoter fragment following overnight incubation at 37 °C in the presence of 10 mM Mg^{2+}, which promotes purine motif triplex

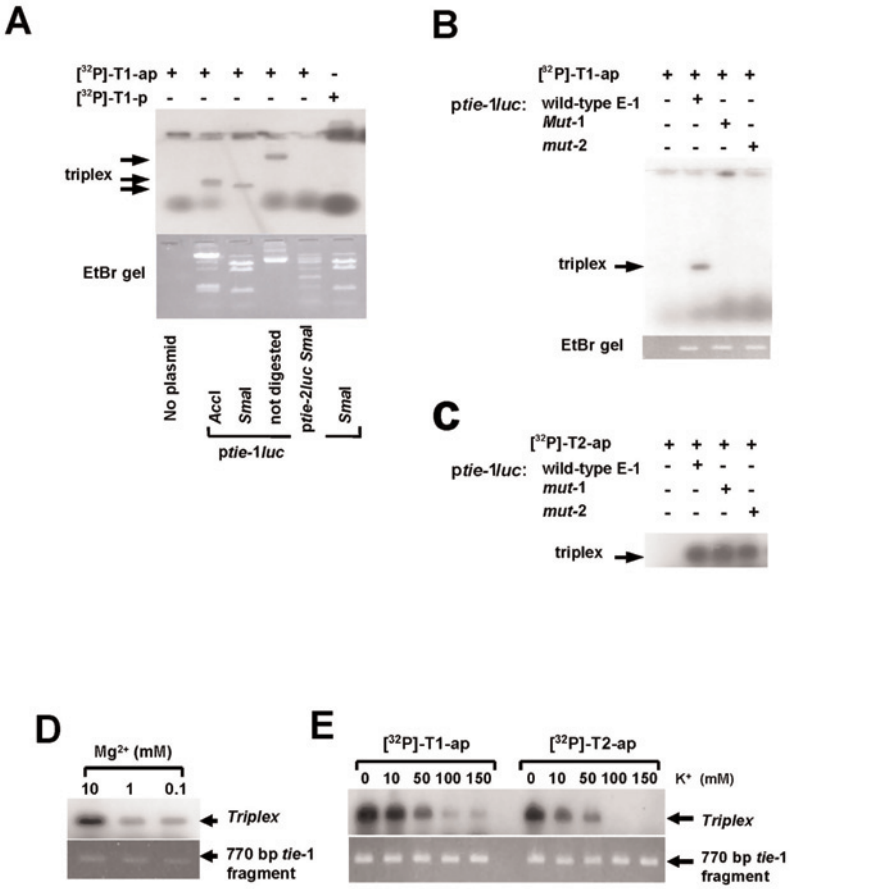

Figure 3. Demonstration of specific triplex DNA formation with purine motif oligonucleotides (T1-ap and T2-ap) at the E-1 and E-2 tandem Ets transcription factor binding sites of the human tie-1 promoter by plasmid DNA binding assay. Plasmids containing the tie-1 promoter (p*tie*-1*luc*) (see Figure 2B) or 900 bp of the human tie-2 promoter (p*tie*-2*luc*) (37) were digested with *Sma*I alone or in combination with *Acc*I (see Figure 1) and incubated overnight with radiolabeled oligonucleotides (T1-ap or T2-ap) at 37 °C in the presence of 10 mM Mg^{2+}. Samples were separated on 1% agarose gels containing ethidium bromide (EtBr) and the DNA visualized under UV light (EtBr gel). The gels were dried and autoradiographed to detect triplex DNA formation. (A) Triplex DNA formation of the T1-ap oligonucleotide with intact p*tie*-1*luc* plasmid and *Sma*I or *Acc*I p*tie*-1*luc* restriction fragments containing the E-1 site. No triplex DNA is observed with T1-ap against p*tie*-2*luc* *Sma*I restriction fragments or with the parallel control oligonucleotide T1-P. (B) Selective binding of T1-ap only to the 770-bp *Sma*I p*tie*-1*luc* restriction fragment containing wild-type E-1 sequence, but not the E-1 mutants (mut-1 and mut-2, see Figure 2B). (C) T2-ap binding to wild-type E-2 present in the same series of wild-type and E-1 mutant (mut-1 and mut-2) *Sma*I-digested p*tie*-1*luc* plasmid fragments. Effect of Mg^{2+} (D) and K^+ (E) ion concentration on triplex formation with radiolabeled T1-ap and T2-ap.

formation/stability (20-22). Figure 3A shows a typical result from these experiments, with triplex DNA formation of radiolabeled T1-ap against a range of restriction-digested tie-1 and tie-2 (p*tie*-2*luc*) promoter plasmids. Selectivity of T1-ap and T2-ap for the tie-1 promoter was demonstrated by a lack of triplex DNA formation with irrelevant plasmid DNA fragments, including the human tie-2 promoter (p*tie*-2*luc*, Figure 3A), which contains a similar multiple Ets binding site (39,52). No triplex DNA formation was detected with the control parallel G/A oligonucleotides (T1-P and T2-P) under these conditions. To confirm the specificity of T1-ap and T2-ap, they were incubated with *Sma*I-digested wild-type and E-1 mutant p*tie*-1*luc* plasmids (Figure 2B). There was no apparent triplex DNA formation with T1-ap against the p*tie*-1*luc* E-1 mutant (mut-1 and mut-2) *Sma*I fragments, demonstrating its selectivity for the E-1 site (Figure 3B). In contrast, the T2-ap oligonucleotide formed triplex DNA at the wild-type E-2 site present in these p*tie*-1*luc Sma*I fragments, as expected (Figure 3C). Similar results were obtained with a partially phosphorothioate-linked T1-ap ($_{PSO}$-T1-ap), which contains three phosphorothioate bonds at the 3′ end.

High Mg^{2+} ion concentrations increase the stability of purine motif triplex DNA by decreasing the repulsion between the negatively charged TFO and DNA duplex (21,22). Reducing the Mg^{2+} ion concentration from 10 mM to ≤1 mM strongly decreased the amount of triplex DNA formed by T1-ap and T2-ap TFO in plasmid binding assays (Figure 4E). Physiological levels of monovalent ions generally decrease the formation of DNA triplex with purine motif TFO by promoting self-association (21,22). As expected, increasing concentrations of K^+ ions decreased T1-ap and T2-ap triplex DNA formation in the presence of 10 mM Mg^{2+} (Figure 3D). At physiological potassium ion concentrations (~150 mM), however, DNA triplex was still detected with T1-ap against the 770-bp *Sma*I fragment of the tie-1 promoter.

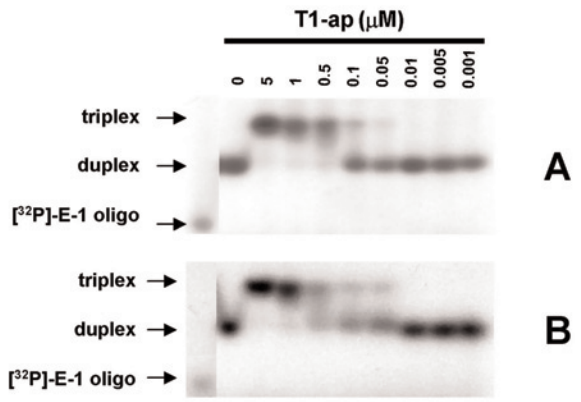

Figure 4. Electrophoretic mobility shift assays showing triplex DNA formation at the E-1 tandem Ets binding motif sequence of the human tie-1 promoter. Radiolabeled oligonucleotide encoding the C/T-rich strand of the tie-1 promoter E-1 site (^{32}P)-E-1 oligo) was annealed to the cold complementary G/A strand (see Figure 1), and incubated with increasing concentrations of phosphodiester (A) and phosphodiester-phosphorothioate–linked (B) cold T1-ap (Figure 1), in binding buffer containing 10 mM MgCl and 150 mM KCl.

The binding characteristics of TFO were also examined quantitatively by EMSA. Radiolabeled E-1 or E-2 duplex target sites (2×10^{-9} to 10×10^{-9} M) were incubated with increasing concentrations of TFO for 16 to 18 h at 37 °C in binding buffer and separated on 12% nondenaturing polyacrylamide gels. Typical results of experiments with T1-ap are shown in Figure 4. The 27-bp E-1 duplex was shifted to a distinct, more slowly migrating triplex band with increasing concentrations of T1-ap. The relative amount of duplex and triplex DNA was determined from the autoradiographs by scanning densitometry. The concentration of TFO at the midpoint of the titration was taken to be equivalent to the dissociation constant (K_d). Strong triplex DNA formation was observed with both T1-ap and T2-ap, with dissociation constants of 2×10^{-7} to 4×10^{-7} M determined at physiological pH and 37 °C. No triplex DNA formation was observed with the control parallel oligonucleotides (T1-P and T2-P) at concentrations of up to 5×10^{-5} M. The introduction of three phosphorothioate bonds at the 3′ end of TFO ($_{PSO}$T1-ap) did not significantly affect triplex DNA formation: the K_d of $_{PSO}$T1-ap was ~3×10^{-7} M (Figure 4B). With both T1-ap and $_{PSO}$T1-ap, complete formation of triplex DNA was observed at 1×10^{-6} M in EMSA (Figure 4).

Effect of triplex DNA formation on p*tie*-1*luc* luciferase reporter activity in endothelial cells

To determine the effect of targeted triplex formation on the tie-1 promoter, TFO and control oligonucleotides were incubated with p*tie*-1*luc*, or the SV40 viral promoter-driven firefly luciferase plasmid pSV40*luc* as a control, in the presence of 10 mM Mg^{2+} at 37 °C (16-18 h) to allow the formation of triplex DNA. The entire DNA complex was then transfected into BAECs, and the activity was determined by dual luciferase assay after a 72-h expression period. T1-ap resulted in a concentration-dependent inhibition of up to 60% of p*tie*-1*luc* activity at 20 μm in comparison with T1-P–treated controls (Figure 5A). As predicted from our 5′ deletion study of the tie-1 promoter (Figure 2B), T2-ap, which forms strong triplex DNA at E-2, did not affect tie-1 promoter activity (Figure 5A). Tie-1 promoter activity was also not affected by a parallel pyrimidine-rich TFO [T1-(C/T)-P] (see Figure 1) targeted to E-1, which does not form DNA triplex at neutral pH. When T2-ap and T1-ap in combination were preincubated with p*tie*-1*luc*, as expected no further inhibition of tie-1 promoter activity was observed (data not shown). The oligonucleotides had no significant effect on the pSV40*luc* control plasmid activity.

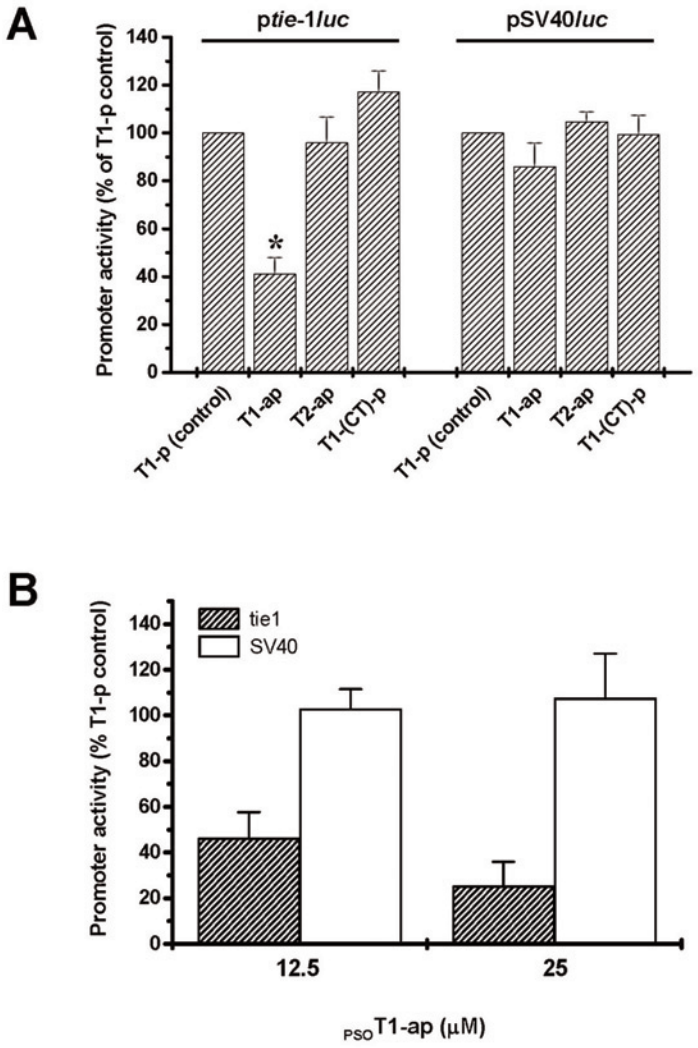

Figure 5. Selective inhibition of tie-1 promoter activity with the T1-ap triplex-forming oligonucleotide. Tie-1 or SV40 promoter-driven luciferase reporter constructs (p*tie*-1*luc* and pSV40*luc*, respectively) were incubated with phosphodiester (20 μM) (A) or partial phosphorothioate (PSO)-modified T1-ap (B), T2-ap and control oligonucleotides (T1-P, T2-P, and T1-(C/T)-P; see Figure 1) in binding buffer for 18 h and transfected into bovine aortic endothelial cells. After 72 h, reporter activity was determined by dual luciferase assay and normalized to the co-transfected pRL-CMV control plasmid. Results are means (± 1 SD, bars) of three to five experiments (n = 6-10) expressed as a percentage of the T1-P–treated control reporter plasmid.

It should also be noted that the oligonucleotides did not affect the activity of the cotransfected CMV promoter–driven Renilla luciferase control plasmid (pRL-CMV) used to monitor cell transfection efficiency, even at ≤ 10,000-fold excess.

The replacement of the last three 3′ phosphodiester bonds with phosphorothioate linkages produced oligonucleotides with similar binding characteristics to those of phosphodiester TFO. Preformed $_{PSO}$T1-ap:E-1 DNA triplex led to a concentration-dependent inhibition of the activity of p*tie*-1*luc* of up to 74% at 25 μM in BAECs in comparison with $_{PSO}$T1-P–treated controls (Figure 5B).

DISCUSSION

Triplex DNA formation has been investigated as a highly selective means of regulating many clinically relevant targets such as c-myc (25,31), cyclin D1 (24), mdr-1 (27), HIV (28), insulin-like growth factor-1 receptor (29), platelet-derived growth factor (32), rhodopsin (53), and the IgE germline gene promoter (30). In addition to the disruption of transcriptional elongation (21,22,27-29,53), inhibition of gene expression has been achieved through triplex DNA formation at sequences encoding transcription factor binding sites including SP-1, SRF, CNBP, PuF, MAZ, Pax5, PU.1, STAT6, and NF-κB (21,22,24-26,31,32). Here we demonstrate the ability of antiparallel purine motif TFOs to selectively form DNA triplex at two sequences encoding tandem Ets core DNA binding motifs in the tie-1 promoter and the triplex-mediated inhibition of promoter activity through TFO binding to one of these sequences (E-1). Although the inhibition of the IgE germline proximal gene promoter through triplex DNA formation at a sequence regulated by STAT6, NF-κB, and the Ets factor PU.1 was reported recently (30), to our knowledge this is the first demonstration of selective triplex DNA formation at sequences containing multiple Ets binding sites.

Ets transcription factors are key regulators of blood vessel growth (40-45). Tel-1 and Fli-1 have been shown to be essential for yolk-sac angiogenesis and maintenance/integrity of the vasculature beyond embryonic days 11.5 and 12.5, respectively, in transgenic mice (40,41). Ets-1 is upregulated in endothelium in wound healing, inflammation, and the supporting vasculature of tumors, and in cultured endothelial cells following stimulation with the highly potent angiogenic factor, VEGF (43-45). Ets proteins are important regulators of many endothelial-restricted genes including thrombomodulin (54), von Willebrand factor (55), ICAM-2 (56), vascular endothelial (VE)-cadherin (57), and VEGF receptor-1 (Flt-1) (58). The murine tie-1 and tie-2 promoters are transactivated by Ets-1, Ets-2, Elf-1, and Nerf-2 in vitro, and mutation of the Ets binding motifs results in reduced tie-1 promoter activity in mice (50-52). Recently, Elf-1 was shown to bind the tandem Ets motifs

contained in the E-1 and E-2 sequences (51); however, the relative contribution of these two sequences to the regulation of the human tie-1 promoter has not been reported. Here we demonstrate that the E-1 Ets binding motifs in the minimal tie-1 promoter are essential for activity in vitro, whereas deletion of the E-2 tandem Ets sites does not affect human tie-1 promoter activity. Moreover, these results are supported by our findings that only TFOs (T1-ap) targeted to E-1 inhibit the activity of the tie-1 promoter in endothelial cells. As similar multiple Ets binding sites are present in several endothelial genes, such as Tie-2 (36,52), thrombomodulin (54), ICAM-2 (56), and VE-cadherin (57), that play key roles in the regulation of endothelial proliferation, differentiation, and function, this approach may have broad applicability to cancer and other pathologies involving endothelial proliferation/dysfunction.

Antiparallel purine motif TFOs were used in this study, as they are generally reported to form triplex DNA with greater stability than unmodified G/T and C/T oligonucleotides (22,53) and do not require low pH (5.0-5.5) to facilitate the protonation of cytosines for triplex DNA formation (21,22). As expected, reduction of Mg^{2+} ion concentration resulted in a loss of triplex DNA formation/stability. This effect is due to the reported stabilizing effect of divalent cations on DNA triplexes through the neutralization of the repulsive forces between the negatively charged phosphodiester backbones of the TFO and target DNA duplex (21,22,59,60). Conversely, monovalent ions, and in particular K^+, are inhibitory to triplex DNA formation/stability with guanine-containing oligonucleotides. This may reflect the ability of monovalent ions to coordinate and stabilize TFO self-association (through G-quartets and related structures) or displace divalent cations and positively charged factors that serve to stabilize DNA triplex (21,60). Physiological levels of K^+ ions (~150 mM) in the presence of 10 mM Mg^{2+} resulted in a strong decrease of triplex DNA formation. The destabilizing effect of physiological monovalent ion concentrations may be reduced by the use of oligonucleotide analogs such as N5′ → P3′ phosphoramidates and 2′-aminoethoxy–substituted riboses that result in positively charged TFO (28,30,47).

As phosphodiester oligonucleotides are susceptible to both endo- and exonucleases and subsequently may have a relatively short half-life in cells and tissue culture medium, we attempted to increase TFO stability by using phosphorothioate analogs that are highly resistant to degradation (61). Phosphorothioate oligonucleotides have been reported to produce marked alteration in cellular function in vitro and in vivo (22,31). However, the propensity of phosphorothioates to bind nonnuclear targets means that much of their activity may be due to non–DNA-specific effects, and they may also exhibit reduced triplex DNA formation (21,22,49). Indeed, the replacement of phosphodiester with phosphorothioate bonds resulted in a loss of specificity of $_{PS}$T1-ap for E-1, and binding of the control oligonucleotide ($_{PS}$T1-P) to E-1 was detected at 1×10^{-5} M. Similarly, we found that phosphorothioate-linked TFOs and control oligonucleotides ($_{PS}$T1-ap and $_{PS}$T1-P) nonselectively decreased the relative activity of p*tie*-1*luc* and pSV40*luc* in endothelial cells compared with the activity of the reporter constructs alone (data not shown). However, limiting the number of phosphorothioate linkages to the last three 3′ bases produced oligonucleotides with binding characteristics similar to those of unmodified phosphodiester TFOs and selectively inhibited tie-1 promoter activity in endothelial cells (Figures 3-5). Our results highlight the potential of antigene approaches to specifically modulate the transcriptional activity of genes regulated by Ets transcription factors, and may form the basis of an alternative strategy to inhibit angiogenesis.

ACKNOWLEDGMENTS

We are extremely grateful to Prof. Malcom Stevens, Cancer Research Laboratories, Department of Pharmaceutical Sciences, University of Nottingham, Nottingham, UK, for helpful discussions. This work is supported by the British Heart Foundation and the Birmingham Women's Hospital NHS Trust.

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