Characterization of *cis*-regulatory elements of the vascular endothelial growth inhibitor gene promoter

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VEGI (vascular endothelial growth inhibitor), a member of the tumour necrosis factor superfamily, has been reported to inhibit endothelial cell proliferation, angiogenesis and tumour growth. We identified and cloned approx. 2.2 kb of the VEGI promoter from mouse cerebral endothelial cells. The promoter contained an atypical TATA-box-binding protein sequence TAAAAAA residing at −32/−26 relative to the transcription initiation site (+1), 83 bp upstream from the ATG start codon. To investigate critical sequences in the VEGI promoter, a series of deleted and truncated segments were constructed from a 2300 bp promoter construct (−2201/+96) linked to a luciferase reporter gene. Transient transfection of cerebral microvascular cells (bEND.3) and rat C6 glioma cells demonstrated that a 1700 bp deletion from the −2201 to −501 did not significantly affect promoter activity; however, a truncated construct (−501/+96) lacking the region between −312 and −57 resulted in nearly 90% loss of promoter activity. A consensus NF-κB (nuclear factor κB) and several SP1 (specificity protein-1)-binding sequences were identified within the deleted segment. Supershift analysis revealed that NF-κB subunits, p50 and p65, interacted with the VEGI promoter. Exposure of cerebral endothermic cells to the pro-inflammatory cytokine, tumour necrosis factor-α, increased VEGI mRNA levels and DNA-binding activities, whereas an NF-κB inhibitor attenuated this increase. In addition, p65 overexpression enhanced, whereas p50 overexpression decreased, the luciferase activity. Furthermore, mutation of the NF-κB DNA binding site blocked this p65- and tumour necrosis factor-α-induced luciferase activity. These findings suggest that the transcription factor NF-κB plays an important role in the regulation of VEGI expression.

Key words: *cis*-regulatory element, electrophoretic mobility-shift assay, gene promoter, gene regulation, transcription factor, vascular endothelial growth inhibitor.

INTRODUCTION

VEGI (vascular endothelial growth inhibitor, also named TL1) was initially identified as a specific inhibitor of angiogenesis [1,2]. The human VEGI gene encodes a type II transmembrane protein with 20–30% sequence identity to TNF (tumour necrosis factor) [1]. Unlike other members of the TNF family, VEGI is expressed predominantly in endothelial cells [1,3,4]. The constitutive expression of VEGI in many adult tissues suggests a physiological role in maintaining a stable vasculature. VEGI has been shown to cause growth arrest and apoptosis in HUVEC (human umbilical-vein endothelial cells), adult bovine aortic endothelial cells and bovine pulmonary artery endothelial cells [1,2,5,6]. VEGI also inhibits the proliferation of breast carcinoma (MCF-7), epithelial (HeLa) and myeloid (U-937 and ML-1a) tumour cells [7]. Three alternatively spliced isoforms in humans, VEGI-174, VEGI-192 and VEGI-251, differ in their N-terminal regions but share a C-terminal 151-residue segment [4]. VEGI-251, the full-length and most abundant isoform, was demonstrated to be an endothelial cell-secreted inhibitor of angiogenesis and could retard human xenograft tumour growth *in vivo* [4]. Overexpression of secreted VEGI, but not VEGI-174, in murine colon cancer cells resulted in a decrease in tumour growth [1,5], indicating that secretion is essential for VEGI activity. These results underscore the potent anti-angiogenic activity of VEGI, and suggest potential applications in tumour suppression.

Although transcriptional regulation has not been well studied, a previous report has shown that VEGI expression is induced by TNFα [8]. TNFα is a major mediator of inflammation, immunity, apoptosis and angiogenesis. The interaction of TNFα with its receptors activates several signal transduction pathways [9]; TNFα-induced NF-κB (nuclear factor κB) activation is one of the best characterized among these pathways. The transcription factor, NF-κB, is composed of homo- and heterodimers of Rel family proteins including p65/RelA, RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2) [10]. The mature p50 and p52 proteins are derived from their precursors, p105 and p100, after proteolytic processing respectively [11–13]. NF-κB, which binds to DNA as a dimer mostly composed of p65 and p50, is usually sequestered in the cytoplasm by a family of inhibitory proteins termed the IκBs [14,15]. In human endothelial cells, TNFα induces the phosphorylation of IκBs by the IκB kinase complex resulting in the rapid degeneration of this molecule, leading to the release and translocation of NF-κB into the nucleus to activate gene expression [16–18]. Promoter elements that bind NF-κB have been identified in numerous genes, including cytokines and adhesion molecules, some of which influence the process of angiogenesis [19–23].

Abbreviations used: AP-1, activator protein-1; CEC, cerebral endothelial cell; CREB, cAMP-response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA-1, GATA-binding protein 1; GR, glucocorticoid receptor; HUVEC, human umbilical-vein endothelial cells; MA, mithramycin A; NE, nuclear extract; NF-κB, nuclear factor κB; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; RT, reverse transcriptase; SP1, specificity protein-1; TBP, TATA-box-binding protein; TNF, tumour necrosis factor; VEGI, vascular endothelial growth inhibitor.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY761148 and AY764130.

Although there is accumulating evidence indicating that angiogenesis is regulated by both positive and negative regulatory factors, the regulatory mechanisms balancing the angiogenic versus angiostatic drive remain poorly understood. VEGI has been shown to be a potent inhibitor of angiogenesis [1,2], and its expression in HUVEC was inducible by TNFα and interleukin-1α [8]. Thus studying transcriptional regulation of VEGI may help elucidate how physiological and pathological angiogenesis is regulated. In the present study, we investigated the full-length mouse VEGI promoter, and examined regulatory elements that were involved in basal and TNFα-induced VEGI expression.

EXPERIMENTAL

Cell culture and treatment

Primary cultures of murine CECs (cerebral endothelial cells) were prepared as described previously [24] and grown in DMEM (Dulbecco's modified Eagle's medium) with 10 % (v/v) FBS (fetal bovine serum). Rat C6 glioma cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.) and maintained in Ham's F-12K medium with 15 % (v/v) horse serum and 2.5 % FBS. The transformed mouse CEC line, bEND.3 (A.T.C.C.), was grown in DMEM with 10 % FBS. Media were supplemented with 100 units/ml penicillin/streptomycin mixture. All of the aforementioned cells were grown at 37 °C in a 5 % CO_2, 95 % air incubator. For TNFα treatment, CECs were maintained in DMEM with 0.5 % FBS and treated with 20 ng/ml recombinant mouse TNFα (R & D Systems, Minneapolis, MN, U.S.A.) for different time periods. To investigate the effect of NF-κB and SP1 (specificity protein-1) inhibitors, cells were pretreated with 50 μg/ml of the NF-κB inhibitor, SN50 (Biomol Research Labs, Plymouth Meeting, PA, U.S.A.) for 3 h, and/or with 10 nM of the SP1 inhibitor, MA (mithramycin A) (Sigma-Aldrich, St. Louis, MO, U.S.A.) for 6 h, then exposed to 20 ng/ml mouse TNFα for an additional 3 h. Subsequently, cells were used for RNA isolation or nuclear protein extraction.

5′-RLM-RACE

5′-RLM-RACE (RNA ligase-mediated rapid amplification of cDNA ends) was performed to identify the mouse VEGI transcription initiation site by using First Choice™ RLM-RACE kit (Ambion, Austin, TX, U.S.A.) according to the manufacturer's instructions. Briefly, 10 μg of RNA from mouse CECs was dephosphorylated to remove the free 5′-phosphate group. Tobacco acid pyrophosphatase was then used to specifically remove the cap structure from mRNA molecules. An RNA oligonucleotide was next ligated to the newly decapped mRNA using T4 RNA ligase and the resulting RNA was reverse-transcribed using Superscript III Platinum (Invitrogen, Carlsbad, CA, U.S.A.) and oligo$(dT)_{15}$ primers. Outer and nested hot-start PCRs were conducted using Platinum *Taq* High Fidelity DNA polymerase (Invitrogen) with adaptor and gene specific primers. The gene-specific antisense primer 5′-GGCAGCACTTCCACTGGGACTCCTTCT-3′ and the nested PCR primer 5′-GCTCCTCTGCCATCCTTCTGCTGTCT-3′ were designed for RACE based on the sequence of mouse VEGI cDNA. The nested PCR products were then cloned into pGEM-T easy vector (Promega, Madison, WI, U.S.A.). For sequencing, 15 independent clones were selected.

Quantitative real-time PCR and RT (reverse transcriptase)–PCR analysis

Total RNA was isolated from CECs using TRI Reagent (Molecular Research Center, Cincinnati, OH, U.S.A.). Real-time PCR was performed using an ABI Prism 7000 Sequence Detection System following the manufacturer's instructions. Reactions in a final volume of 25 μl contained, 1 μl of sample cDNA (40 ng of total RNA), 0.5 μM primers, 12.5 μl of 2 × PCR SYBR Green master mix (ABI) and 0.25 unit of uracil DNA glycosylase (Invitrogen). The amplification programme included an initial step at 50 °C for 2 min, the denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and finally annealing and extension at 60 °C for 1 min. SYBR Green fluorescence was measured after each cycle. The specific amplification was subjected to melting curve analysis. The relative VEGI mRNA level was normalized to the mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or cyclophilin internal control in the same sample. Primer sequences to amplify the mouse VEGI, GAPDH and cyclophilin cDNAs were the following: VEGI forward, 5′-TCCCCGGAAAAGACTGTATGC-3′; and VEGI reverse, 5′-CTGGGAGGTGAGTAAACTTGCT-3′; GAPDH forward, 5′-GCATGGCCTTCCGTGTTCCTA-3′; and GAPDH reverse, 5′-CCTTCAGTGGGCCCTCAGATG-3′; cyclophilin forward, 5′-GGTGAGCGCTTCCCAGATGAGA-3′; and cyclophilin reverse, 5′-ACCCAGCCAGGCCCGTAGTG-3′. Each PCR assay was run in triplicate.

For RT–PCR analysis, VEGI was amplified with a forward primer 5′-AGAAACAAACCCCAGCACCAC-3′ and a reverse primer 5′-AAGGAGATGTCACTGACG-3′. β-Actin was used as internal control.

Construction of reporter plasmids and cell transfection

The 2.3 kb fragment of mouse VEGI promoter was generated by PCR using Platinum *Taq* High Fidelity DNA polymerase (Invitrogen) with mouse genomic DNA as a template. This approach used a forward primer from the 5′-upstream of genomic sequence with an EcoRV site (underlined) (5′-TAGGATATCGCCCAACAGTGCCGCTC-3′) and a reverse primer from the downstream of the initiator methionine codon with a BamHI site (underlined) (5′-CGCGGATCCGCTCCTCTGCAATCCT-3′). The start codon ATG was mutated to ATT to generate non-fusion expression construct. This fragment was cloned into pGL3 Basic vector (Promega) located upstream of the luciferase reporter gene. A series of 5′-deleted constructs were generated by PCR using the following forward primers with an EcoRV site (underlined): F2 (5′-GATATCGCTTCTCTGGTGTCACCCCTCC-3′), F3 (5′-GATATCGTAGGGCGGGTACCTTTCTGAC-3′), F4 (5′-GATATCCTCCTCCTCCAGGGACTTTCCT-3′), F5 (5′-GATATCGTTTCTGTTGTAGGCGGCGCATT-3′), F6 (5′-GATATCCTCAGTGTGACAGCTGCTCTCT-3′). Truncated construct was generated by PstI restriction digestion. Site-directed mutagenesis of the NF-κB element was performed using a mutagenic primer as follows: wild-type, 5′-CCTCCTCCAGGGACTTTCCTGACTTC-3′, and mutation, 5′-CCTCCTCCAGAGCTCTTCCTGACTTC-3′ (mutated nucleotides are underlined). For co-transfection analysis, the mouse NF-κB p65 and p50/p105 cDNA were cloned into pcDNA3.1 (Invitrogen) respectively. All constructs were confirmed by sequence analysis.

C6 glioma cells were transfected in six-well plates with 1 μg of reporter plasmid, or co-transfected with 1 μg of pcDNA3.1 or pcDNA3.1-based expression vectors p65 or p50/p105 along with 1 μg of reporter plasmid. Transient transfection was performed using Effectene (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's instructions. Transfection of bEND.3 cells was performed using calcium phosphate co-precipitation following the manufacturer's instructions (BD Biosciences, Palo Alto, CA, U.S.A.). As an internal control, the pRL-SV40 vector was introduced into each transfection and leads to constitutive

expression of *Renilla* luciferase. Furthermore, a pEGFP vector was used to monitor the transfection efficiency by fluorescence microscopy. Cells were harvested 48 h post-transfection, and assayed for reporter gene activity with the Dual-Luciferase Reporter Assay System (Promega).

NE (nuclear extract) preparation and EMSA

NEs were prepared from mouse CECs as described in [25] with modifications [24]. Protein concentrations were determined using the Lowry method. PAGE-purified double-stranded oligonucleotides were end-labelled with [γ-^{32}P]ATP (PerkinElmer, Boston, MA, U.S.A.) by T4 polynucleotide kinase (Promega). EMSA (electrophoretic mobility-shift assay) was performed in a total volume of 20 μl with 20 μg of nuclear protein, 1 μg of poly(dI-dC) and 10000–20000 c.p.m. of labelled probe for 20 min at room temperature (22–25 °C) [25]. For competition experiments, unlabelled oligonucleotides at 50-fold molecular excess were added 10 min before the addition of the radiolabelled probe. For supershift assays, the NEs were incubated with 2 μg of anti-p65 or anti-p50 antibody [NF-κB p65 (A) sc-109x and NF-κB p50 (NLS) sc-114x; Santa Cruz Biotechnology, CA, U.S.A.] respectively, for 15 min before the addition of the labelled probe. After incubation with the labelled probe for an additional 20 min, samples were subjected to native 6% PAGE and visualized by exposing the dry gel to a Kodak film. The following oligonucleotides were used for the EMSA: NF-κB, 5′-TCCTCCAGGGACTTTCCTGACTTC-3′ (which corresponds to the predicted NF-κB site at $-246/-223$), and SP1, 5′-TAGGCGGCGCATTCCCTAGCCGGGCAGGGCAGGCTG-3′ ($-95/-60$).

Statistical analysis

Results are expressed as the means $\pm$ S.D. for at least three independent experiments. Statistical differences between means were determined using one-way ANOVA followed by Bonferroni's *post hoc* test or two-tailed Student's *t* test when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

TNFα induction of VEGI in mouse CECs

To investigate the regulation of VEGI gene expression, we examined the induction of gene expression in CECs treated with 20 ng/ml TNFα for 1, 3, 6, 12 or 24 h. Total RNA was extracted from the cells and used to perform RT–PCR and quantitative real-time PCR. TNFα rapidly increased the expression of mouse VEGI mRNA in a time-dependent manner, without affecting the transcription of the β-actin gene (Figure 1A). Peak expression (12-fold higher than basal expression) occurred within 3 h of treatment (Figure 1B). These results are in agreement with a previous study that demonstrated TNFα-induced VEGI expression in HUVEC [8].

Genomic structure of mouse VEGI

To amplify the full-length mouse VEGI coding sequence from reverse-transcribed mouse CEC cDNAs, a pair of primers was synthesized according to previously published sequences [8]. Sequence analysis of the cloned cDNA revealed a 759 bp open reading frame encoding a peptide with 252 amino acids (GenBank® accession number AY764130). This cDNA contained 5 bp that were different from published sequences [8], resulting in a change in two amino acids. All the nucleotides were found within exon 4, including bp537T > C, bp538G > A, bp543C > T, bp653T > C

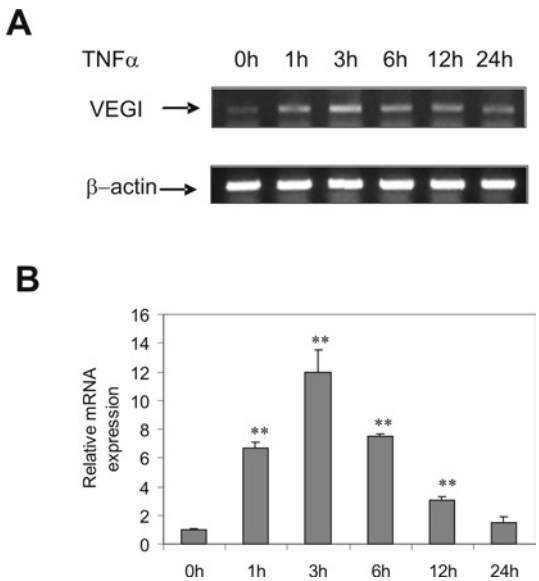

Figure 1 TNFα-induced VEGI gene expression in murine CECs

RNA derived from CECs treated with 20 ng/ml TNFα for times indicated was subjected to RT–PCR using VEGI and actin primers (**A**). The same RNA was analysed for VEGI mRNA by real-time PCR, normalizing to GAPDH mRNA (**B**). Results are representative of experiments performed in triplicate with similar results. $^{**}P < 0.01$ compared with 0 h using ANOVA and Bonferroni's *post hoc* test.

and bp672G > A. (Mistakes were found in the published cDNA sequence of VEGI, e.g., at bp537, we found C instead of T.) A GenBank® database search by BLASTN analysis using the cloned mouse VEGI cDNA was performed. We noted a sequence from a mouse PAC clone, RP23-20K13 (AL691468) and another from a genomic contig (NT_039262, strain C57BL/6J) identical with our cloned sequence. Detailed sequence alignment demonstrated that mouse VEGI is encoded by four exons, which span 15.4 kb from bp 61139953–61155395 on chromosome 4 (excluding the 5′-untranslated sequence) (Figure 2A). Mouse VEGI-252 contains a hydrophobic transmembrane region near the 5′-end (amino acids 40–60) and the carboxyl domain shows high homology to TNF (amino acids 96–252). The N-terminal cleavage site of soluble VEGI is between the amino acids 61 and 62. Several potential sites for post-translational modification exist, including an N-glycosylation site (amino acids 137–140), cAMP-dependent protein kinase phosphorylation site (amino acids 103–106) and a protein kinase C phosphorylation site (amino acids 101–103).

The 5′-RLM-RACE was employed to map the 5′-end of the mouse VEGI transcript, resulting in the amplification of a major PCR product of approx. 200 bp (Figure 2B). The PCR product was gel-purified, cloned into pGEM-T easy vector and sequenced, then aligned to the mouse PAC clone, RP23-20K13 (containing complete VEGI cDNA). From the sequence of 15 independent clones, a major transcription initiation site was identified corresponding to an adenosine nucleotide located 83 bp upstream from the ATG start codon (Figure 2C).

Sequence analysis and functional mapping of the VEGI promoter

A GenBank® database search using BLASTN analysis of the 5′-untranslated region and cDNA coding sequence of mouse VEGI was performed to identify the 5′-flanking region by sequence overlap. The proximal 2.2 kb 5′-flanking region of mouse VEGI was first isolated by PCR from CEC genomic DNA, cloned into the pGEM-T easy cloning vector, and sequenced by genomic

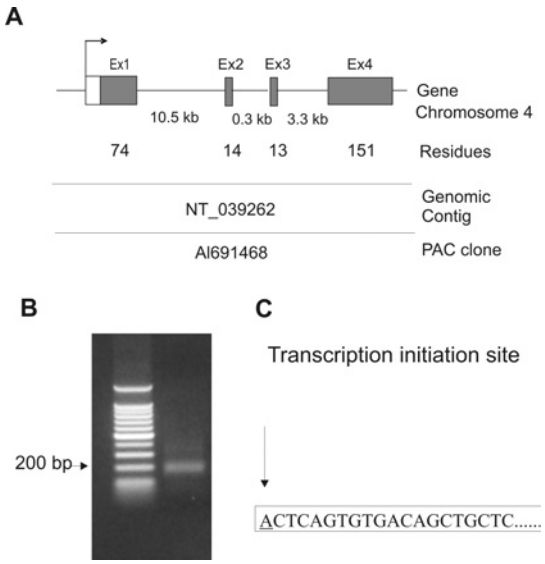

Figure 2 Genomic organization of the mouse VEGI gene

(**A**) Schematic representation of the mouse VEGI genomic structure. The transcription initiation site is indicated by the →, and approx. sizes of the introns are indicated. The number of residues encoded by each exon, the genomic contigs and PAC clones that contain the genomic DNA are also shown. (**B**) Gel analysis of the nested PCRs from 5′-RLM-RACE with RNA derived from CECs. (**C**) Sequence of the transcription initiation site (underlined) of mouse VEGI cDNA.

walking using the designed primers (Figure 3, GenBank® accession number AY761148). The 5′-flanking sequence of mouse VEGI was then analysed using the database software TESS (Transcription Element Search System) and Signal Scan. This genomic fragment did not contain the typical TATA and CAAT boxes in close proximity to the transcription start site. However, an atypical TBP (TATA-box-binding protein)-binding sequence TAAAAAA, located at −32/−26 relative to the transcription initiation site (+1), was identified. Potential binding sites for transcription factors, such as NF-κB, SP1, CREB (cAMP-response-element-binding protein), AP-1 (activator protein-1), NF-1, GR (glucocorticoid receptor) and GATA-1 (GATA-binding protein 1) were also identified in the 5′-flanking region of mouse VEGI gene (Figure 3).

To demonstrate the activity of the VEGI promoter, the 2297 bp (−2201/+96) fragment was subsequently cloned into a pGL3 basic vector and transfected into C6 glioma cells and bEND.3 transformed CECs. These cells were utilized because of superior transfection efficiencies compared with primary CECs, and were not activated by any stimuli (e.g. TNFα). Luciferase activity in cells transfected with the −2201/+96 reporter construct was 27–35-fold higher than that in cells transfected with the promoterless control plasmid pGL3 (Figure 4A), indicating that *cis*-acting elements were present in the VEGI promoter construct. To map further the regions responsible for transcriptional regulation, a series of deletions were constructed from the 5′-terminus of the promoter fragment (from bp −2201 to bp +2). These constructs were then transfected into the above cells, and luciferase activity was normalized to control *Renilla* luciferase activity.

Results of the transfection experiments are shown in Figure 4. The luciferase activity of the longest cloned promoter fragment (−2201/+96) was arbitrarily defined as 100 %. A short promoter fragment spanning bp −501 to +96 showed 120 % of this activity; further deletion to −105 bp significantly decreased the promoter activity to 40 % (C6) and 55 % (bEND.3) (Figure 4A). Two PstI sites at −312 and −57 permitted the creation of a truncated construct derived from −501 to +96, lacking the region between −312 and −57. Both C6 and bEND.3 cells transfected with this construct exhibited <15 % of the activity of that of the −501/+96 promoter fragment (Figure 4B), indicating that the −312/−57 region was critical in the regulation of basal VEGI expression.

Identification of transcription factors that regulate VEGI gene expression

Computer prediction analysis (TESS and Signal Scan) of putative regulatory elements located in the critical 256 bp region (from −312 to −57) suggested binding sites for NF-κB and SP1 transcription factors. The NF-κB binding site was located at −239/−230. In addition, five SP1 binding sites were identified, three in a cluster located at −93 to −60 (Figure 3). To examine which of these potential transcription binding sites might play a role in VEGI gene regulation, we performed experiments using pharmacological inhibitors. Murine CECs were pretreated with the NF-κB inhibitor SN50 or the SP1 inhibitor MA in the presence or absence of TNFα, and VEGI mRNA levels were determined using real-time PCR. Basal VEGI expression was significantly decreased by SN50, but not by MA (Figure 5A). Furthermore, the TNFα-induced expression was markedly attenuated by SN50, but not by MA, and combination of both inhibitors had no further effect suggesting that NF-κB is an important regulator of basal and induced VEGI expression (Figure 5A). To confirm the activities of the two inhibitors, EMSAs were performed to determine the factor binding to the specific DNA sequences. SN50 markedly attenuated the TNFα-induced binding activity of NF-κB to the VEGI promoter binding site (Figure 5B); likewise, MA decreased TNFα-induced binding to the SP1 consensus sequence (Figure 5C), confirming the inhibitory activity of these agents. Excess unlabelled oligonucleotide competed with both protein–DNA complexes efficiently, suggesting that proteins bound specifically to the consensus sequence. Consistent with the above results, SN50 also inhibited basal and TNFα-stimulated luciferase activity in C6 and bEND.3 cells transfected with the VEGI promoter construct (Figure 5D).

NF-κB transcription factors are protein dimers of several possible subunits, including p65, p50, p52, RelB and c-Rel [26–29]. These proteins dimerize to form hetero- or homodimers, depending on the combination, may act as transcriptional activators or repressors [30–34]. To investigate further the specific NF-κB subunits involved in DNA binding, anti-p65 and anti-p50 antibodies were incubated with NEs before EMSA. Under basal and TNFα-induced conditions, two major bands of DNA binding complexes were identified. Incubation with the p50 antibody strongly interfered with the binding of both bands, resulting in a single supershifted band; in contrast, the p65 antibody selectively decreased binding activity of the upper band, with little effect on the lower band (Figure 6A). These results are consistent with the contention that the upper band predominantly represents a p50:p65 heterodimer, although the lower band represents a p50:p50 homodimer, and suggest that TNFα increased the binding of both complexes. These findings are consistent with previous reports that have demonstrated two similar bands of DNA binding complexes [35–38].

To investigate the functional role of NF-κB in the transcriptional regulation of VEGI expression, we cloned the p65 and the p50/p105 genes into pcDNA3.1. These plasmids were co-transfected with the reporter construct containing the −501/+96 VEGI promoter segment into C6 glioma and bEND.3 cells, and luciferase activity was monitored. Co-transfection of the reporter gene with the p65 plasmid increased transcriptional activity of

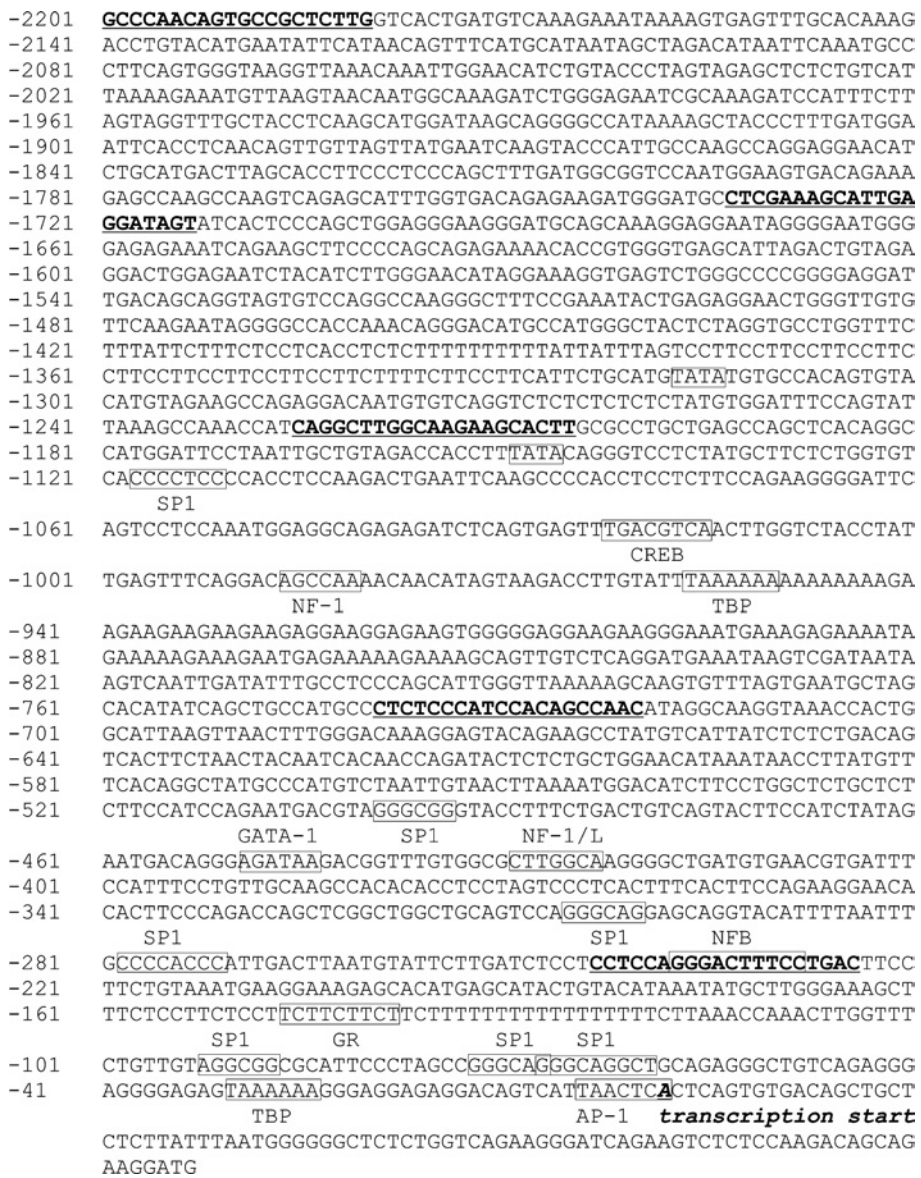

Figure 3 Sequence of the 5′-flanking region of the mouse VEGI gene

Sequence analysis of the 2201 bp 5′-flanking region of mouse VEGI reveals several potential factor binding sites, including consensus sequences for TBP, NF-κB, SP1, NF-1, AP-1, CREB, GR and GATA-1 (shown in boxes). Underlined segments in boldface were used to sequence the cloned 5′-flanking region fragment by genomic walking. The transcription initiation site (A) is indicated in boldface italics and marked as +1. Numbers to the left show bases upstream from the start of transcription.

the VEGI promoter by almost 4-fold, whereas p50/p105 co-transfection had little effect. Furthermore, co-transfection of both p65 and p50/p105 also increased promoter activity, but to a lesser extent than p65 alone (Figure 6B). These results suggest that p65 homo- and/or heterodimers (with p50) may enhance VEGI gene expression, whereas p50 homodimers lack this activity. However, in primary CECs, only p50:p65 heterodimers and p50 homodimers were detected (see above).

Mutation of the NF-κB binding site and VEGI gene induction

To determine if NF-κB DNA binding was required for VEGI gene expression, the NF-κB consensus sequence was mutated in the VEGI promoter-reporter construct (Figure 7A). This mutation resulted in a significant decrease in basal luciferase activity compared with the wild-type VEGI promoter construct. Co-transfection of the mutated reporter gene with p65 failed to induce luciferase activity (Figures 7B and 7C). Furthermore, treatment of cells transfected with the NF-κB mutated reporter with TNFα also failed to induce VEGI promoter activity (Figures 7B and 7C). Consistent with earlier results, C6 and bEND.3 cells carrying the wild-type VEGI promoter demonstrated a 3–5-fold increase in luciferase activity after co-transfection with p65 or treatment with TNFα. These results suggest that NF-κB DNA binding is important for VEGI up-regulation.

DISCUSSION

We have cloned and sequenced a 2.3 kb mouse genomic DNA fragment containing 2201 bp of VEGI gene promoter region. Using 5′-RLM-RACE in mouse CECs, we have identified the

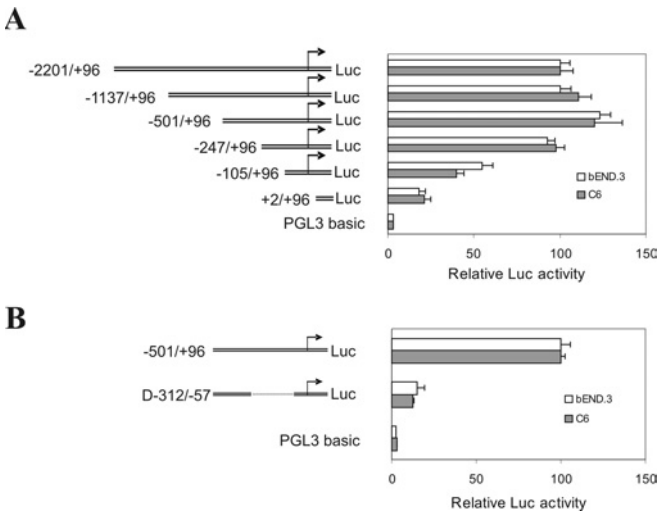

Figure 4 Functional analysis of the mouse VEGI promoter

Fragments containing different lengths of the 5′-upstream sequences of the mouse VEGI gene were cloned into a luciferase reporter construct pGL3. All deletion constructs (left panel of **A**, numbered relative to the transcription initiation site) contained 96 bp of the exon I sequence. Arrows indicate the transcription initiation site. A truncated reporter plasmid was constructed by deleting a 256 bp fragment (−312/−57) with PstI (**B**). C6 glioma and bEND.3 cells were transfected with reporter constructs and luciferase activity was normalized to *Renilla* luciferase activity encoded by co-transfected control plasmid, pGL3-VEGI (**A** and **B**, right panels). Transfections were performed in triplicate in each experiment, and results are expressed as the means ± S.D. for four experiments.

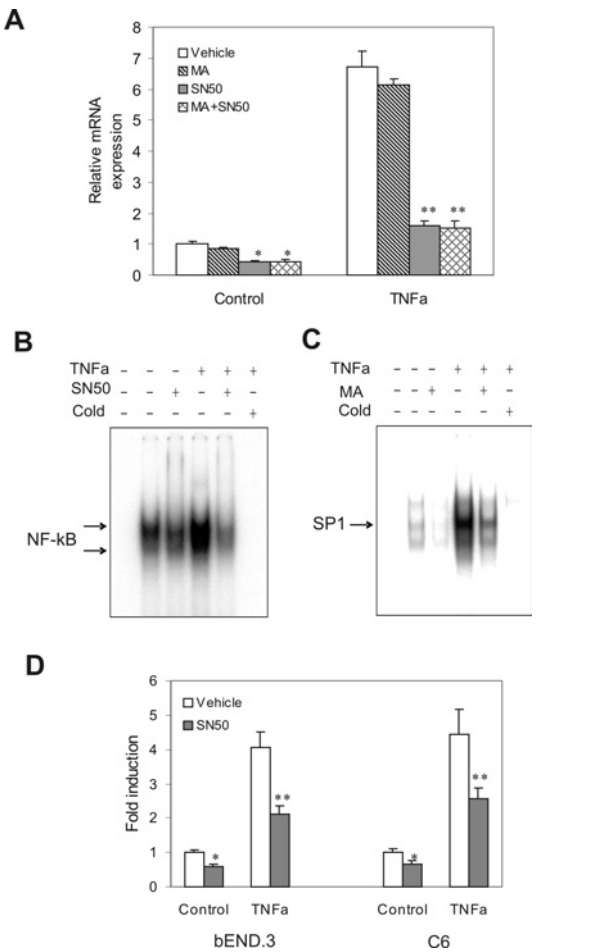

Figure 5 NF-κB and SP1 inhibitors and VEGI gene expression

CECs grown in the presence or absence of 20 ng/ml TNFα were treated with or without MA (10 mM) and/or SN50 (50 ug/ml), as indicated. VEGI mRNA was quantified by real-time PCR from cell extracts, and results are expressed as means ± S.D. for three experiments (**A**). NEs from CECs treated as indicated were subjected to EMSA analysis using labelled NF-κB (**B**) or SP1 (**C**) probes. Arrows indicate the positions of NF-κB (**B**) or SP1 (**C**) specific complexes. Some oligonucleotide competition experiments were performed in the presence of 50-fold excess of unlabelled NF-κB or SP1 probe (as indicated in **B** and **C**). Shown are representative EMSAs performed in triplicate with similar results. (**D**) C6 glioma and bEND.3 cells were transfected with the −501/+96 reporter construct, and grown in the presence or absence of TNFα and/or SN50 as indicated. Luciferase activity was determined and normalized to *Renilla* luciferase activity as described in Figure 4. Results are expressed as means ± S.D. for three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle control using ANOVA and Bonferroni's *post hoc* test.

transcription initiation site of the mouse VEGI gene as an adenine at +1, located 83 bp upstream of ATG. In this genomic fragment, several potential factor binding sites were predicted, including consensus binding sequences for NF-κB, SP1, CREB, AP-1, NF-1, GR, GATA-1, and an atypical TBP-binding sequence TAAAAAA at −32/−26 (Figure 3). Deletion analysis revealed that a relative short promoter region −501/+96 was as active as the full-length promoter; however, further deletion of the −312/−57 segment caused nearly 90% promoter activity loss, suggesting that this segment was critical for transcription regulation of VEGI expression. A consensus NF-κB and five potential SP1 binding elements were identified in this region.

To further investigate the regulation of VEGI gene induction, we examined cells treated with TNFα, a known inducer of the gene [8]. TNFα rapidly increased VEGI expression in CECs with peak expression at 3 h after treatment and decreasing thereafter. This induced expression was completely blocked by treatment with the NF-κB inhibitor SN50, but not the SP1 inhibitor MA, suggesting the importance of NF-κB DNA binding. EMSA studies demonstrated that TNFα increased NF-κB binding activity to the cognate sequence in the VEGI promoter. Supershift experiments using antibodies directed against the p50 and p65 subunits suggest that TNFα specifically increased binding of the p50:p50 homodimer, as well as the p50:p65 heterodimer, to the NF-κB binding site. Transfection of C6 and bEND.3 cells with the p65 gene increased VEGI promoter activity, whereas transfection with p50 did not. In addition, co-transfection with both p50 and p65 genes increased promoter activity, suggesting that the p65:p65 homodimer and/or the p50:p65 heterodimer can promote VEGI gene expression, whereas p50 homodimers lack this activity. These results are consistent with literature demonstrating that p50 homodimers lack a transactivation domain, whereas heterodimers consisting of p65 and p50 represent the primary activated form of NF-κB [10,35,39,40]. Although the p65 homodimer increased VEGI promoter activity in the transfection experiments, its binding to cognate DNA sequences was not detected in primary CECs, suggesting that this form may not play a role in endogenous VEGI gene expression.

The importance of the NF-κB binding site for VEGI expression was further supported by experiments involving the mutation of the NF-κB binding site in the VEGI promoter. Reporter constructs carrying a mutated NF-κB site decreased the basal promoter activity and failed to increase promoter activity after transfection with p65 or treatment with TNFα. Consistent with this finding, SN50 attenuated basal and TNFα-stimulated promoter activity in C6 and bEND.3 cells transfected with the VEGI promoter construct. Furthermore, SN50 inhibited basal and TNFα-induced VEGI mRNA expression in primary CECs. Collectively, these results suggest that NF-κB binding is important for basal and induced expression of the VEGI gene.

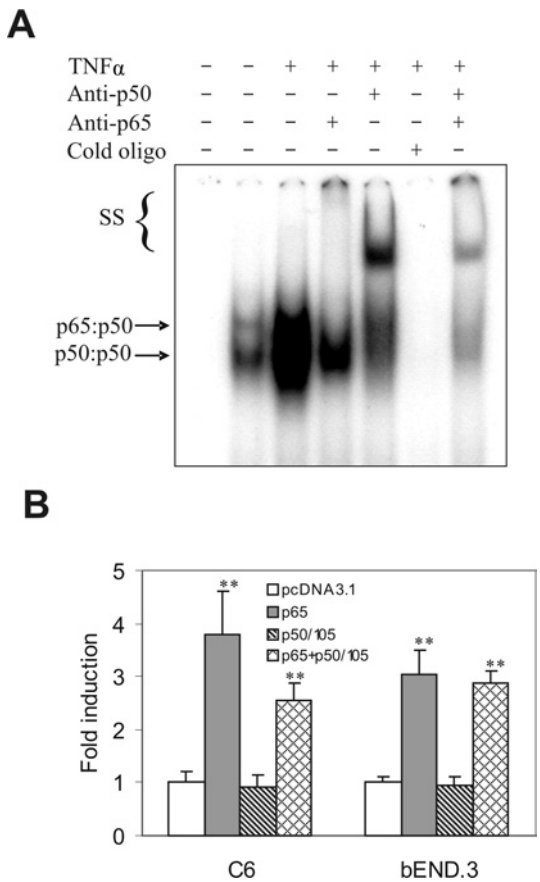

Figure 6 NF-κB subunits and VEGI promoter activity

(**A**) CECs grown in the presence or absence of TNFα were subjected to NF-κB EMSA. Some of the NEs were preincubated with 2 μg of polyclonal anti-p50 or anti-p65 antibody for 15 min at room temperature before the addition of the labelled NF-κB probe. Arrows indicate the location of NF-κB specific complexes and supershifted (SS) bands. Shown is a representative EMSA performed in triplicate with similar results. (**B**) C6 glioma and bEND.3 cells were co-transfected with the $-501/+96$ reporter constructs and pcDNA3.1-derived p65 or p50/p105 expression constructs. Each transfection was performed with 1 μg of reporter construct and 1 μg of pcDNA3.1-based expression construct. (pcDNA3.1 was used as the control.) Luciferase activity was determined and normalized to *Renilla* luciferase activity as described in Figure 4. Results are expressed as means $\pm$ S.D. for three experiments. $^*P < 0.05$ compared with pcDNA3.1 controls using ANOVA and Bonferroni's *post hoc* test.

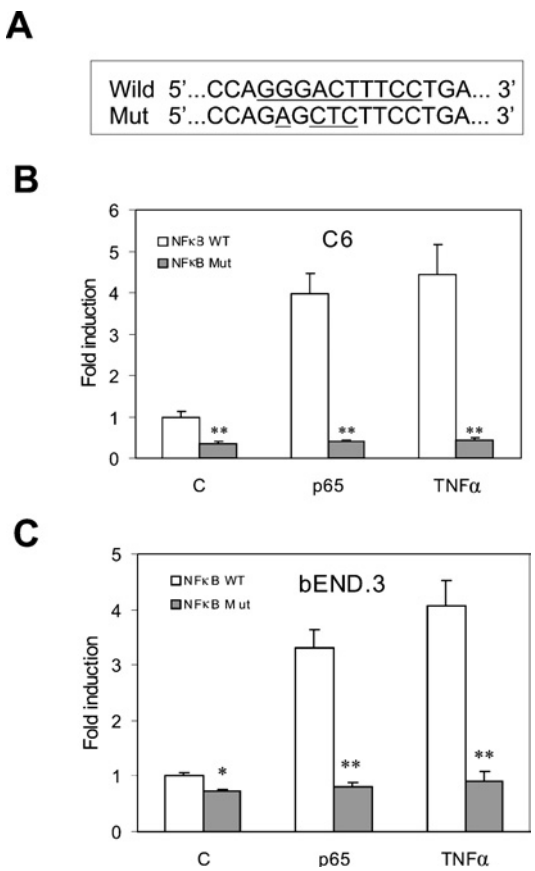

Figure 7 Mutation of the NF-κB binding site in the VEGI promoter

The NF-κB binding site was mutated (as indicated in **A**) in the $-501/+96$ reporter construct. Wild-type or mutated constructs were transfected into C6 (**B**) or bEND.3 (**C**) cells, which were either co-transfected with the p65 plasmid or treated with TNFα, as indicated. Luciferase activity was determined and was normalized to *Renilla* luciferase activity as described in Figure 4. Results are expressed as means $\pm$ S.D. for three experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with NF-κB wild-type controls using two-tailed Student's *t* test.

Although its role in angiogenesis remains unclear, VEGI appears to be a potent inhibitor of endothelial cell growth as well as an inducer of endothelial cell apoptosis [1,2,5,6]. Both actions are against angiogenic drive [1,5,6], in agreement with the *in vivo* effects of VEGI in inhibiting angiogenesis [1,4,5]. It is of interest that several pro-angiogenic proteins, including vascular endothelial growth inhibitor and its receptors, interleukin-8, basic fibroblast growth factor, platelet-activating factor, angiopoitin-1 and its receptor Tie-2 are induced by TNFα [41–46]. Moreover, expression of many of these genes appears to be under the influence of NF-κB activity [41,45,47]. It has been suggested that TNFα-induced angiogenesis may represent pathological angiogenesis, similar to that seen in rheumatoid arthritis [48]. That anti-angiogenic VEGI is also up-regulated by TNFα through NF-κB activity raises the possibility that pathological angiogenesis may involve the dual activation of both pro- and anti-angiogenic factors, initiating a cycle of dysregulated angiogenesis.

The present study is the first to characterize functional control of the VEGI promoter. Our findings suggest that the full-length mouse VEGI gene appears to be transcribed from a major transcription initiation site. Critical control of VEGI expression resides in a segment within the promoter that carries NF-κB and SP1 binding sites, and binding of NF-κB to this site is important for basal and TNFα-induced VEGI expression. Further characterization of the promoter region of this gene may help to elucidate mechanisms of VEGI regulation and angiogenesis under physiological and pathological conditions.

This work is supported by National Science Council grant 92-2321-B038-003 (to C. Y. H.) and NIH grants K08 NS002190 and R01 NS51625 (to J. M. L.) and American Heart Association grant 00460066Z (to J. M. L.).

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Received 13 October 2004/20 January 2005; accepted 10 February 2005
Published as BJ Immediate Publication 10 February 2005, DOI 10.1042/BJ20041739