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Transcription Factors Sp1 and Sp3 Alter Vascular Endothelial Growth Factor Receptor Expression through a Novel Recognition Sequence*

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Kinase domain receptor (KDR) is a high affinity, endothelial cell-specific, autophosphorylating tyrosine kinase receptor for vascular endothelial growth factor. This transcriptionally regulated receptor is a critical mediator of endothelial cell (EC) growth and vascular development. In this study, we identify a DNA element modulating KDR promoter activity and evaluate the nuclear binding proteins accounting for a portion of the cell-type specificity of the region. KDR promoter luciferase activity was retained within −85/−296 and was 10–30-fold higher in EC than non-EC. Electrophoretic mobility shift assays demonstrated specific nuclear protein binding to −85/−64, and single point mutations suggested important binding nucleotides between −79/−68 with five critical bases between −74/−70 (5’-CTCCT-C3’). DNA-protein complexes were displaced by Sp1 consensus sequence oligodeoxynucleotides and supershifted by Sp1- and Sp3-specific antibodies. Sp1 and Sp3 protein in EC nuclear extracts bound the −79/−68 region even when all surrounding classic Sp1 recognition sites were removed. Sp1 protein in nuclear extracts was 4–24-fold higher in EC than non-EC, whereas Sp3 was 3–7-fold higher. Sp1/Sp3 ratios in EC were 2–10-fold higher. Over-expression of Sp1 protein increased KDR promoter activity 3-fold in both EC and non-EC, whereas simultaneous co-expression of Sp3 attenuated this response. An Sp1 consensus sequence cis element “decoy” reduced EC KDR promoter activity and mRNA expression by 85 and 69%, respectively. An antisense phosphorothioate oligodeoxynucleotide to Sp1 inhibited Sp1 and KDR protein expression by 66 and 68%, respectively, without changing Sp3 protein expression.

These data illustrate that Sp1 and Sp3 modulate KDR promoter activity through a novel recognition binding sequence. However, since Sp1-mediated promoter activation is attenuated by Sp3, endothelial selective KDR promoter activity may be partially regulated by variations in the Sp1/Sp3 ratio.

Vascular endothelial growth factor (VEGF)1 (1), also known as vascular permeability factor (2) or vasculotropin (3), is a hypoxia-inducible, endothelial cell-selective mitogen and potent vasopermeability factor. VEGF is thought to play a central role in mediating the formation of new blood vessels during fetal development (4) and during other physiologic and pathologic conditions associated with angiogenesis such as wound healing (5), collateral vascular formation (6), and tumorigenesis (7). The role of VEGF in mediating pathologic neovascularization (8, 9) and vascular permeability (10–13) in the eye has been well documented. Numerous retinal cell types produce VEGF, and expression can be increased up to 30-fold under hypoxic conditions (14, 15). Intraocular VEGF concentrations are highly correlated with neovascularization within the human eye resulting from common sight-threatening conditions (9, 16–22). Inhibition of VEGF activity can suppress retinal (23, 24) and iris (25) neovascularization as well as tumor growth in animals (26, 27).

VEGF exerts its action through two high affinity, tyrosine phosphorylating, transmembrane receptors named KDR/flk-1 and flt-1 (28, 29). KDR/flk-1 expression is selective for endothelial cells in vivo (29, 30), whereas flt-1 is expressed both in endothelial cells and in some cells of non-endothelial origin such as retinal pericytes (31–32), renal glomerular mesangial cells (33), and mononuclear phagocytes (34). Both receptors possess seven extracellular immunoglobulin-like domains and an intracellular tyrosine kinase region containing a kinase insert (29, 35). However, KDR is much more efficiently phosphorylated in vitro in response to VEGF stimulation than is flt-1 (36). VEGF binding to either receptor can also activate numerous intracellular signaling molecules including phosphatidylinositol 3-kinase, phospholipase Cγ, and protein kinase Ca, -β, and -δ isoforms although the signal transduction pathways may differ between the receptors (36–38).

Recent analysis of the human KDR promoter has demonstrated that maximal activity resides in the −225 to +127 5’-flanking region of the KDR gene relative to the transcriptional start site and that deletions of −95 to −37 result in complete loss of promoter activity (39, 40). This region of the KDR promoter contains putative AP2-, Sp1-, and NFκB-binding sites. DNase I footprinting experiments by Patterson et al. (40) suggest that transcription factor Sp1 binds the human KDR promoter specifically.

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; KDR, kinase domain receptor; BREC, bovine retinal endothelial cells; BRPC, bovine retinal pericytes; BRPE, bovine retinal pigment epithelial cells; BSMC, bovine aortic smooth muscle cells; BFibro, bovine fibroblasts; BAEC, bovine aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; kb, kilobase pair(s); Rb, retinoblastoma protein; EMSA, electrophoretic mobility shift assays.
KDR promoter in this region and that Sp1 binding is endothelial cell-specific in vivo, potentially due to cell-specific alterations in chromatin structure. Nevertheless, it has not been documented that Sp1 binding actually alters either KDR promoter activity or KDR expression, and the involvement of other nuclear proteins modulating expression of this gene have not been fully defined.

Due to the critical role of KDR in mediating important physiologic and pathologic processes and the incomplete understanding of the mechanisms of endothelial cell-specific KDR gene expression, we have performed a detailed functional characterization of the DNA-binding proteins regulating KDR promoter activity in both endothelial and non-endothelial cells. In this article we provide evidence that transcription factor Sp1 increases KDR promoter activity in both endothelial and non-endothelial cells through binding to a novel recognition sequence, whereas simultaneous co-expression of Sp3 attenuates this response. These data suggest that portions of KDR promoter activity and endothelial selective expression of KDR may in part be regulated by variations in the Sp1/Sp3 ratio. In addition, our data demonstrate that the majority of KDR promoter activity, mRNA expression, and protein synthesis require Sp1 activity in endothelial cells.

MATERIALS AND METHODS

Cell Culture—Primary cultures of bovine retinal endothelial cells (BREC), retinal pericytes (BRPC), retinal pigment epithelial cells (BRPE), smooth muscle cells (BSMC), fibroblasts (BFibro), and aortic endothelial cells (BAEC) were isolated from freshly slaughtered tissue by homogenization and a series of filtration steps as described previously (14). BREC were cultured in Endothelial Basal Medium (Clonetics, San Diego) with 10% fetal bovine serum (FBS; Life Technologies, Inc.). HUVEC were cultured in the Ethcul b/galactosidase activity to obtain a 1999 media with 10% fetal bovine serum. All cells were cultured at 37 °C in 5% CO₂, 95% air, and media were changed every 2–3 days. BAEC, BRPC, BSMC, BFibro, COS, Chinese hamster ovary, and 3T3 cells were cultured in Dulbecco's modified Eagle's medium with 5.5 mM glucose and 20% fetal bovine serum (HyClone). BAEC, BRPC, BSMC, BFibro, COS, Chinese hamster ovary, and 3T3 cells were cultured in Dulbecco's modified Eagle's medium with 10% calf serum (Life Technologies, Inc.). HUVEC were cultured in M199 media with 10% fetal bovine serum. All cells were cultured at 37 °C in 5% CO₂, 95% air, and media were changed every 2–3 days. BAEC, BRPC, BSMC, BFibro, COS, Chinese hamster ovary, and 3T3 cells were cultured in Dulbecco's modified Eagle's medium with 10% calf serum (Life Technologies, Inc.). HUVEC were cultured in M199 media with 10% fetal bovine serum. All cells were cultured at 37 °C in 5% CO₂, 95% air, and media were changed every 2–3 days.

Plasmids—pSV40 b/gal (Promega) contains the β-galactosidase gene driven by the SV40 promoter and enhancer. A 100-kb human genomic DNA fragment encompassing the KDR gene was obtained by P1 phage cloning (42) and confirmed by sequence comparison to previously published data (39). A luciferase reporter construct containing 4.3 kb of the KDR 5′-flanking region was created by restriction digestion of the purified P1 phage DNA with BamHI and XhoI, corresponding to the −4-kb and +296 sites of the KDR gene, respectively. In this article, all KDR gene nucleotides are expressed relative to the KDR transcriptional start site which is defined as +1. The fragment was inserted into the pGL2 Basic plasmid. Similar luciferase reporter constructs were created from human KDR regions −101/+296 and −85/+296 (Fig. 1A). These were obtained by polymerase chain reaction amplification of KDR P1 phage DNA and were inserted into plasmid pGL2 Basic. All constructs were sequenced from both the 5′ and 3′ ends to confirm orientation and sequence. The Sp1 and Sp3 expression vectors were generously provided by Dr. Jonathan M. Horowitz (Duke University Medical Center) and were confirmed by sequencing from BES and Sp3 cDNA cloned into pcMV-4, an expression vector containing the cytomegalovirus immediate early promoter, as described previously (43–45).

Mutagenesis—Nineteen different double-stranded 22-mer oligodeoxynucleotides representing the KDR promoter region −85/−64 were constructed with each incorporating 1–6 nucleotide substitutions by synthesis of complementary single-stranded fragments (Oligo Etc.) and subsequent annealing at room temperature after heating to 85 °C (Fig. 1B). Site-directed mutagenesis of nucleotides within the human KDR −85/+296 luciferase reporter construct was performed using polymerase chain reaction and the human KDR P1 phage DNA as template. The nucleotides mutated (−85 to −80 and −76 to −74, or −73 to −71) were identical to those changed in oligodeoxynucleotide substitutions 1, 3, and 4, respectively, as shown in Fig. 1B. Internal mismatches in the 5′ primer were used in conjunction with a 3′ primer matched to the XhoI site of the coding area. The amplified polymerase chain reaction fragment was gel-purified and digested with XhoI and cloned directly into the pGL2 Basic plasmid. All mutation constructs were sequenced from the 5′ and 3′ ends to confirm orientation and sequence.

Transfections—Plasmid DNA was introduced into all cell types with the LipofectAMINE reagent (Life Technologies, Inc.) as instructed by the manufacturer. The appropriate luciferase reporter construct (1 μg) was always co-transfected with 1 μg of pSV40βgal to normalize for transfection efficiency in the 1.5–3.0 × 10⁵ cells. Cells were harvested 48 h after transfection, and luciferase activity was measured using the Luciferase Assay System (Promega) and an M1L3000 microtitre plate luminometer (Dynamet Laboratories). β-Galactosidase activity was assayed as described previously (46). For each transfection, luciferase activity was displayed by β-galactosidase activity to obtain normalized luciferase activity.

Co-transfections of Sp1 and Sp3 Expression Plasmids—The Sp1 and Sp3 expression plasmids and transfection conditions are described above. All cells were transfected with a total of 2 μg of expression plasmids for 48 h in one of the following combinations: 2 μg of pCMV4 (control), 1 μg of Sp1-pCMV4, and 1 μg of pCMV4, 1 μg of Sp3-pCMV4, and 1 μg of pCMV4 and 1 μg of Sp1-pCMV4 and 1 μg of Sp3-pCMV4. All cells were also co-transfected with 1 μg of pSV40βgal to normalize for transfection efficiency.

Electrophoretic Mobility Shift Assays—Preparation of nuclear extracts was performed as described previously (47). Protein concentrations were determined by the method of Bradford (48), and the same amount of nuclear protein was subjected to electrophoretic mobility shift assays. The DNA fragment containing the known KDR promoter region was labeled. The DNA sequences for the fragments used for electrophoretic mobility shift assay are presented with classic Sp1 and Sp3 binding consensus sequences underlined. Nucleotides that are important (*) and critical (V) for nuclear protein-DNA binding as determined by site-directed mutagenesis are indicated with classic Sp1 and Sp3 binding consensus sequences.
Sp1- and Sp3-mediated Expression of VEGF Receptor KDR

Endothelial Cell-specific Expression of KDR mRNA and KDR Promoter Luciferase Reporter Construct Activity. A, RNA extracted from cultured bovine retinal endothelial cells (BREC), aortic endothelial cells (BAEC), retinal pigment epithelial cells (BRPE), smooth muscle cells (BSMC), retinal pericytes (BRPC), fibroblasts (BFibro), and human umbilical vein endothelial cells (HUVEC) was evaluated by Northern blot analysis using 20 μg of total RNA per lane and human KDR cDNA probe. Human 36B4 cDNA probe was used to demonstrate equal lane loading (55). Migration positions of ribosomal 28 S and 18 S RNA are indicated. B, a construct composed of the −85 to +296 region of the human KDR promoter and the pGL2 luciferase reporter plasmid was transfected into the indicated cell types as described in the text. All cells were co-transfected with pSV40βgall to normalize for transfection efficiency. The horizontal axis is expressed as fold increase over pGL2 control plasmid luciferase activity (mean ± S.E.). Values located at the end of each bar indicate promoter activity as a percent of that observed for BREC.

shift assays as described previously (49). Double-stranded oligodeoxynucleotides were constructed by annealing commercially synthesized single-stranded oligodeoxynucleotides corresponding to published human sequences (39, 41, 50–52). The KDR sequences used are detailed in Fig. 1. Oligodeoxynucleotide sequences corresponding to DNA binding consensus sites were as follows: Sp1, 5′-TTTGCGTTGGTATGGTTTGG-3′; Sp2, 5′-GGGGTGGTATGGTTTGG-3′; Sp3, 5′-GGGGTGGTATGGTTTGG-3′; Sp4, 5′-CCCTGTTATGATCCTAAT-3′; c-Fos, 5′-CGTTTCTGGCTGAAGGAAAG-3′; c-Jun, 5′-ATCGTGGCTGGCTGAAGGAAAG-3′; AP2, 5′-GATCGAATTCCGTGATAGCG-3′; NF1, 5′-AGTTGAGGG-3′; NF2, 5′-GGGGTGGTATGGTTTGG-3′; NF3, 5′-GGGGTGGTATGGTTTGG-3′; NF4, 5′-GGGGTGGTATGGTTTGG-3′; kappa, 5′-ATTTAGGG-3′; and JUN, 5′-AGTTGAGGG-3′. Each oligodeoxynucleotide contained neither a promoter nor an enhancer. A similar vector incorporating a construct composed of the −85 to +296 region of the human KDR promoter was transfected into a variety of cell types along with pSV40βgall to normalize for transfection efficiency. The horizontal axis is expressed as fold increase over pGL2 control plasmid luciferase activity (mean ± S.E.). Values located at the end of each bar indicate promoter activity as a percent of that observed for BREC.

RESULTS

Endothelial Cell-specific Expression Is Retained within the −85 to +296 Region of the Human KDR Promoter.—To confirm endothelial specific expression of KDR in the primary cell cultures of interest in this study, Northern blot analysis using a human KDR cDNA probe was performed on total RNA (20 μg/lane) extracted from various cell types of both endothelial and non-endothelial origin (Fig. 2A). KDR mRNA was detected from all endothelial cell cultures (BREC, BAEC, and HUVEC), but no signal was observed from non-endothelial cells (BSMC, BRPC, BRPE, or BFibro). KDR expression was also undetectable using polyadenylated RNA in non-endothelial cells (data not shown). The hybridization signal was greatest for HUVEC probably because both cDNA probe and cell type were of human origin. Between the two bovine endothelial cell types, KDR expression was 2-fold greater in BREC than in BAEC. This finding is consistent with our previous report that KDR receptor number is 3-fold higher in BREC than in BAEC (56).

A previous study suggested that positive regulatory elements exist in the KDR promoter region −95 to −60 (39). To evaluate this area further, we isolated a 100-kb human genomic fragment including the KDR gene by P1 phage cloning. A fragment containing the −101/+296 region was cloned into the pGL2 basic firefly luciferase reporter plasmid (Promega) which contains neither a promoter nor enhancer. A similar vector incorporating the −85 to +296 region of the KDR promoter was constructed. The luciferase reporter constructs were co-transfected into a variety of cell types along with pSV40βgall to normalize for transfection efficiency. Luciferase activity was measured after 48 h. Results using the −85/+296 construct are shown (Fig. 2B). Normalized KDR promoter luciferase activity was 500- and 200-fold greater than that of control plasmid in BREC and BAEC, respectively. KDR promoter activity was...
2.8-fold greater in BREC than in BAEC. Promoter activity was 8–30-fold lower in the non-endothelial cell types tested. Similar results were observed using the −101 to −296 region of the human KDR promoter as a probe. Competition was performed in the presence of 100-fold molar excess of the nonradioactive KDR promoter fragments indicated. Specific and nonspecific DNA-protein complexes are indicated with solid and open arrowheads, respectively. B, electrophoretic mobility shift analysis was performed as indicated above. Competition was performed in the presence of 100-fold molar excess of the nonradioactive KDR promoter fragments or mutated promoter fragments indicated. All mutations were located within the −85/−64 sequence as shown in Fig. 1B. C, the −85/−296 human KDR promoter luciferase reporter construct, or similar constructs incorporating the mutations (Mut) indicated, were transfected into BREC. All cells were co-transfected with pSV40βgal to normalize for transfection efficiency. Results are expressed as fold increase over pGL2 control plasmid luciferase activity (mean ± S.E.). All statistical comparisons refer to the unmutated −85/−296 construct.

**KDR Promoter Nucleotides −79 to −68 Permit Specific Nuclear Protein Binding within the Endothelial Cell-selective Region**—Previous reports have shown a near complete loss of KDR promoter activity when the region 5′ to nucleotide −60 is deleted (39). These data and those described above suggest that the region regulating endothelial specific activity resides between −85 and −60. To determine if nuclear proteins could specifically bind this region, EMSA was performed using nuclear extract from BREC and radiolabeled KDR promoter fragment −101/−56 (Fig. 3A). Specific DNA-protein binding complexes were evident as a major slower migrating band.
both potential Sp1 and NF sites, and since the region conveying endothelial cell specificity beled mutant (Fig. 3 2 101/56, and competition with 100-fold molar excess of each using BREC nuclear extracts, radiolabeled promoter fragment...

To map the −85 to −64 binding area in greater detail, mutations were created that spanned the region with 2–6 sequential nucleotide substitutions (mutants 1–7) or with single point mutations (mutants 8–19) as shown in Fig. 1B. The −85 to −64 region contains theoretical binding sites for both transcription factors NFxB and Sp1. EMSA was performed using BREC nuclear extracts, radiolabeled promoter fragment −101/−56, and competition with 100-fold molar excess of each mutant (Fig. 3B). The three major DNA-protein binding complexes were competed by 100-fold molar excess of either unlabeled −101/−56 or −85/−64 promoter fragment but not by the nonspecific −225/−164 fragment. Mutants 1, 6, and 7 each retained the ability to displace nuclear protein binding, suggesting that regions −85 to −80 and −67 to −64 were not critical for the DNA-protein interaction. EMSA evaluation of the point mutation fragments revealed a reduction in the ability to compete for nuclear protein binding that was minimal for mutants 8, 14, and 18, modest for mutants 9–12 and 19, and marked for mutants 13 and 15–17. These data suggest that specific nuclear protein binding to the KDR promoter within the region which retains endothelial cell specificity primarily involves nucleotides −79 to −68 and that each of residues −74 to −70 (5′-CTCC-T3′) are essential. The point mutation in mutant 14 creates a new Sp1 recognition sequence (5′-CCCGCC-3′) within the −85/−64 fragment and allows complete competition of nuclear protein binding.

To determine if nuclear protein binding to this region was necessary for endothelial specific KDR promoter activity, luciferase reporter plasmids were constructed to incorporate the −85+/296 region of the KDR promoter. Mutations in these constructs were created that corresponded to mutations 1, 3, and 4. The luciferase reporter constructs were co-transfected into BREC along with pSV40gal to normalize for transfection efficiency, and luciferase activity was measured after 48 h (Fig. 3c). Normalized −85+/296 KDR promoter luciferase activity was 400-fold greater than that of control plasmid. This activity was not significantly altered by introduction of mutation 1 which eliminates the NFxB consensus sequence but does not interfere with formation of the DNA-protein complexes. However, introduction of either mutation 3 or 4, both of which suppress nuclear protein binding to this KDR promoter region, suppressed KDR promoter activity by 3-fold. In contrast, these mutants did not further suppress the already low promoter activity observed in BRPC, BRPE, and Chinese hamster ovary cells (data not shown). These data suggest that the nuclear protein binding to the −85/−56 KDR promoter region is important for approximately two-thirds of the promoter activity observed in the endothelial cells.

**Nuclear Proteins Sp1 and Sp3 Bind to a Novel Recognition Sequence in the KDR Promoter**—Since the 150 base region 5′ to the initiation start site of the KDR gene contains three potential AP2, two potential NFxB, and five potential Sp1-binding sites, and since the region conveying endothelial cell specificity both potential Sp1 and NFxB sites, we evaluated whether DNA consensus sequences for various transcription factors could compete for nuclear protein binding to the KDR promoter. EMSA was performed using BREC nuclear extracts, radiolabeled promoter fragment −101/−56, and competition with 100-fold molar excess of nonradioactive KDR promoter fragments (−225/−164, −101/−56, and −85/−64) or the NFxB, Sp1, AP2, or AP1 consensus binding sequences. Identification of the nuclear proteins bound to the probe was performed by preincubating nuclear extracts for 30 min with antibodies (Ab) specific for the proteins indicated. Specific DNA-protein complexes are indicated with solid arrowheads. Bands supershifted by preincubation with antibodies are marked (<). B, electrophoretic mobility shift analysis was performed as above except Sp1 consensus sequence was used as the probe. Competition was accomplished in the presence of 100-fold molar excess of nonradioactive KDR promoter fragments, the −85/−64 region mutations indicated, or NFxB, Sp1, and AP2 DNA consensus sequences.

**Fig. 4. Identification of nuclear proteins binding to the region of the human KDR promoter which retains endothelial cell selectivity.** A, electrophoretic mobility shift analysis was performed using nuclear extract (NE) isolated from BREC and the radiolabeled −101/−56 fragment of the human KDR promoter. Competition was performed in the presence of 100-fold molar excess of the nonradioactive KDR promoter fragments (−225/−164, −101/−56, and −85/−64) or the NFxB, Sp1, AP2, or AP1 consensus binding sequences. Identification of the nuclear proteins bound to the probe was performed by preincubating nuclear extracts for 30 min with antibodies (Ab) specific for the proteins indicated. Specific DNA-protein complexes are indicated with solid arrowheads. Bands supershifted by preincubation with antibodies are marked (<). B, electrophoretic mobility shift analysis was performed as above except Sp1 consensus sequence was used as the probe. Competition was accomplished in the presence of 100-fold molar excess of nonradioactive KDR promoter fragments, the −85/−64 region mutations indicated, or NFxB, Sp1, and AP2 DNA consensus sequences.
The specific DNA-protein binding complexes were only competed by unlabelled promoter fragments which included the intact binding region (−101/−56, −85/−64) and by the Sp1 consensus sequence. These data suggest that members of the Sp family of transcription factors known to bind the Sp1 consensus sequence may be binding to the −101/−56 region of the KDR promoter.

To identify the proteins in BREC nuclear extract which bind to this portion of the native KDR promoter, EMSA supershift experiments using the radiolabeled −101/−56 KDR promoter fragment and antibodies specific for various transcription factors were performed (Fig. 4A). Antibodies specific for Sp1 and Sp3 resulted in 1 or 3 supershifted DNA-protein complexes, respectively. The fastest migrating DNA-protein complex doublet was nearly completely shifted by anti-Sp3 antibody alone. The slower migrating complex was nearly completely shifted by combining anti-Sp1 and Sp3 antibodies, but only partially shifted by either antibody alone, suggesting that this band represents binding of both Sp1 and Sp3. In addition, 4 supershifted bands were observed when both antibodies were used, which migrated in a manner corresponding to the single Sp1 shifted band and the three Sp3 shifted bands. No supershifted complexes were observed with antibodies specific for Sp2, Sp4, AP2, c-Fos, c-Jun, retinoblastoma protein, or the p50 or p65 subunits of NFκB. Anti-Sp1 and Sp3 antibodies also supershifted DNA-protein complexes from BAEC nuclear extracts in the same manner (data not shown). These results demonstrate that both Sp1 and Sp3 are capable of binding the region of the KDR promoter which retains endothelial cell-selective expression.

EMSA using radiolabeled Sp1 consensus sequence as a probe was also performed (Fig. 4B). The binding and competition profile was exactly the same as that observed for nuclear protein binding to the original −101/−56 KDR promoter fragment. In addition, the Sp1 consensus sequence, but not AP2 or NFκB consensus sequences, was able to displace binding.

The data presented above suggest that transcription factors Sp1 and Sp3 bind the −101/−56 region of the KDR promoter. However, the mutation analysis suggests that the critical binding sequence is between −79 and −68 (5′-AGCCCTCTCCCTCCTCC-3′) which is located immediately 5′ to a classic Sp1 consensus sequence at −69 to −64 (5′-CGCCGCC-3′) (39) and is unlike previously reported Sp1 binding motifs (GCCGGCCCCC or CTCCCCACAGC) (58) or CT elements (CCCTCCCTCA) (43). To determine if Sp proteins from BREC nuclear extract are actually capable of binding this novel recognition sequence, EMSA supershift experiments using radiolabeled mutant 7 (which lacks the classic Sp1 consensus sequence) and antibodies specific for transcription factors Sp1−4 and AP2 were performed (Fig. 5). The same major DNA-protein complexes were observed that once again were not displaced by fragments containing mutations in the −79 to −68 region (mutants 2−5) but were displaced by fragments containing the native −79/−68 region (mutants 1, 6, and 7). Antibodies specific for Sp1 or Sp3 each resulted in supershifted DNA-protein complexes similar to those observed with the native promoter. The fastest migrating doublet was again completely shifted by the anti-Sp3 antibody. In contrast to the native promoter, when binding mutant 7 the nonspecific band immediately under the major slow migrating complex was not observed. The slower migrating specific complex was shifted more by both Sp1 and Sp3 antibodies than by either antibody alone. The major slower migrating complex appeared as a closely migrating doublet. Anti-Sp1 almost completely shifted the upper portion of this doublet, whereas anti-Sp3 nearly completely shifted the lower portion. No supershift was noted with antisera specific for Sp2, Sp4, or AP2. In addition, recombinant human Sp1 protein was able to specifically bind and shift radiolabeled mutant 7, and this binding was competed only by nucleotide sequences containing the intact −74 to −70 region (data not shown).

These data indicate that Sp1 and Sp3 are capable of binding a novel recognition sequence on the human KDR promoter even in the absence of a classic Sp1-binding site. Furthermore, it appears that Sp3 binding accounts for the faster migrating doublet, whereas the slower migrating complex represents both Sp1 and Sp3 binding.

Nuclear Protein Levels and Binding to the KDR Promoter Correlate with KDR Expression—If the nuclear protein-DNA interactions in this region are important for KDR promoter activity, then the DNA-protein binding might be expected to be higher in endothelial cell nuclear extracts. EMSA was performed using the radiolabeled −101/−56 promoter fragment and nuclear extracts from a variety of endothelial (BREC, BAEC, and HUVEC) and non-endothelial cell types (BSMC, BRPC, BRPE, and BFibro) (Fig. 6A). The specific DNA-protein complexes were evident in all endothelial cell nuclear extracts tested but were >9-fold less prevalent in extracts from non-endothelial cells.

If Sp1 and Sp3 affect the endothelial cell-specific expression of KDR, then nuclear protein concentrations of these proteins might also be altered in endothelial cells as compared with cells of non-endothelial origin. Western blot analysis using Sp1- and Sp3-specific antibodies was performed on nuclear extracts derived from a variety of cells (Fig. 6B). Nuclear protein levels of both Sp1 and Sp3 were 4−24-fold higher in cells of endothelial origin (BREC, BAEC, and HUVEC) than in non-endothelial cells (BSMC, BRPC, BRPE, and BFibro) under the culture conditions studied. Nuclear protein levels of Sp1 were moderately higher in BREC than in BAEC, whereas the converse was true for Sp3. The Sp1/Sp3 ratios in all three endothelial cell
types were higher than in all four non-endothelial cells types. The Sp1/Sp3 ratio in BREC was 2.6-, 5.3-, 2.0-, and 9.6-fold greater than the Sp1/Sp3 ratio in BSMC, BRPC, BRPE, and BFibro, respectively.

**Nuclear Proteins Sp1 and Sp3 Partially Regulate Endothelial Cell-specific Expression of KDR**—To determine if elevated Sp1 expression is sufficient to increase KDR promoter activity, BREC and BSMC were transiently transfected with the −101/+296 human KDR promoter luciferase reporter construct and expression plasmids for either Sp1, Sp3, neither, or both (Fig. 7). Overexpression of Sp1 increased KDR promoter activity nearly 3-fold in both BREC and the non-endothelial BSMC. Expression of Sp3 alone reduced basal KDR promoter activity in BREC; however, co-transfection of Sp3 along with Sp1 attenuated the Sp1 activation of the KDR promoter in both cell types. Similar results were obtained in BRPC, BRPE, and BFibro (data not shown).

To determine if Sp1 and/or Sp3 binding is required for KDR promoter activity in BREC, phosphorothioate-modified cis element decoys to the Sp1 and NFκB consensus sequences were constructed. Decay cis elements block the binding of nuclear factors to promoter regions of genes by specific high affinity binding to the targeted transcription factors resulting in inhibition of gene transactivation (59–62). When BREC were transfected with both the −101/+296 KDR luciferase promoter construct and increasing concentrations of Sp1 decoy, KDR promoter activity was reduced in a dose-dependent manner (Fig. 8A). Statistically significant attenuation in KDR promoter activity (35%) was evident with 10 μM Sp1 decoy, and 85% inhibition was obtained at 100 μM. Transfection with NFκB decoy did not significantly alter KDR promoter activity.

To determine if Sp1 and Sp3 binding to the KDR promoter is required for cellular KDR mRNA expression, BREC were transfected with increasing concentrations of either Sp1 or NFκB decoy, and RNA was isolated after 24 h (Fig. 8b). Sp1 decoy reduced KDR mRNA expression 38%, 68%, and 83% at 30, 100, and 300 μM, respectively. NFκB decoy reduced KDR mRNA expression only 13%, 24%, and 27% at 30, 100, and 300 μM, respectively.

To determine specifically if Sp1 is required for KDR protein expression, phosphorothioate-modified sense and antisense oligodeoxynucleotides were made against the Sp1 translational start site. BREC were transfected with either sense, antisense, or no oligodeoxynucleotide, and total protein was isolated after 48 h (Fig. 9). Total protein was employed for Western blot analysis using antisera specific for KDR, Sp1, or Sp3. Sp1 antisense and sense oligodeoxynucleotides reduced Sp1 by 66 and 30%, respectively. Sp3 protein concentrations were unaffected. KDR protein expression was reduced 68% by Sp1 antisense but only 10% by Sp1 sense oligodeoxynucleotides. These data indicate that Sp1 is required for the majority of KDR mRNA and protein expression in BREC even when Sp3 is present.

**DISCUSSION**

In this study, we demonstrate that expression of KDR is induced by transcription factor Sp1 and attenuated by Sp3 binding to a novel recognition sequence located within the −79 to −68 region of the human KDR promoter. To our knowledge, this is the first demonstration of Sp3 binding to the human KDR promoter. Furthermore, we show that Sp3 is necessary for maximal expression of both KDR mRNA and protein in endothelial cells and that overexpression of Sp1 in non-endothelial cells activates the KDR promoter. These data suggest that a portion of the endothelial cell-selective expression of KDR is mediated by the relative binding of Sp1 and Sp3 to a novel recognition sequence.
Our findings demonstrate that both Sp1 and Sp3 bind to the −79/-64 promoter sequence 5′-AGCCC[CTCCT]CC(GCCC)-3′ where the 5 residues in the square brackets are the most critical for binding, the 7 unmarked bases partially affect binding, and the 4 nucleotides in parentheses are required for binding but do not need to form a classic Sp1 consensus site. The −79/-64 KDR promoter sequence has some similarity to the positively acting homopyrimidine CT element (5′-CCCTC-CCTCA-3′) found upstream of the c-myc gene which is known to bind Sp1 and related factors (43, 63). However, the CT region usually consists of 4 imperfect direct repeats of the CT element and a fifth downstream repeat separated by 9 base pairs. At least two nonadjacent units are required for transcription factor binding. Such repeats are not present in the KDR promoter (39).

Although Sp1 has recently been shown to bind to the KDR promoter by EMSA (40), it has not been previously demonstrated that Sp1 binding actually alters either KDR promoter activity or KDR expression. The critical role of Sp1 and Sp3 binding to the KDR promoter on KDR promoter activity and KDR mRNA expression was demonstrated using phosphorothioate oligodeoxynucleotide modification. Although Sp1 has recently been shown to bind the KDR promoter, there is considerable evidence that Sp1 participates in the positively acting homopyrimidine CT element Sp1 consensus sequence decoys. However, there could also be other factors contributing to the endothelial selectivity of KDR expression.

The Sp1/Sp3 stimulatory ratio was 48% greater in BREC than in BAEC, a result consistent with the increased KDR promoter activity observed in BREC. The Sp1/Sp3 ratios for the various cells of endothelial origin were greater than for all the non-endothelial cell types studied. This observation combined with the markedly lower total Sp1 expression in non-endothelial ocular cells could partially account for the endothelial cell-specific expression of KDR. However, there could also be other factors contributing to the endothelial selectivity of KDR expression.

Although Sp1 is generally considered a ubiquitous transcription factor, there is considerable evidence that Sp1 participates in cell type-specific gene expression (16, 40, 58, 64, 65), is developmentally (66) and functionally regulated (67, 68), and is highly expressed during vasculogenesis (66). These attributes are similar to those observed for KDR (30, 31). The mechanism of the Sp1-mediated increase in KDR promoter activity is discussed in the text. RNA was extracted from the cells and evaluated by Northern blot analysis (20 μg of RNA/lane) performed using radiolabeled KDR cDNA and 36B4 cDNA as a lane loading control.

These results demonstrate that proteins which bind the Sp1 consensus sequence are important for KDR promoter activity and mRNA expression in endothelial cells. Since both Sp1 and Sp3 bind the same sequence, these results do not help differentiate the relative contributions of Sp1 and Sp3. However, an Sp1 antisense phosphorothioate oligodeoxynucleotide markedly reduced both Sp1 and KDR protein expression without affecting Sp3 protein levels (Fig. 9), thus demonstrating the critical role of Sp1 in mediating KDR protein expression in endothelial cells.

Several observations in the current study suggest that Sp1 may modulate a portion of the endothelial selectivity of KDR expression. Overexpression of Sp1 protein by transient transfection in both endothelial and non-endothelial cells resulted in a 3-fold increase in KDR promoter activity. Although Sp1 expression in non-endothelial cells did not increase KDR promoter activity to the same level of BREC, it did increase it to roughly that of BAEC. BREC have nearly 3-fold higher KDR promoter activity than BAEC, which correlates with the 2-fold higher KDR mRNA (Fig. 2A) and 3-fold higher protein levels in BREC (56). It is not possible to determine from these experiments whether additional elevation of Sp1 protein over that obtained with the expression vector would further increase non-endothelial cell KDR promoter activity.

Co-expression of Sp1 and Sp3 protein resulted in attenuation of the Sp1-mediated increase in KDR promoter activity. Sp3 has previously been shown to produce repression of Sp1-mediated activation of numerous Sp1-responsive promoters by competition with Sp1 for their common binding sites (45, 57). This finding might also account for the high KDR promoter activity in BREC since protein levels of Sp1 were 35% greater in nuclear extracts from BREC than those from BAEC, whereas levels of Sp3 were 10% greater in BAEC (Fig. 6B). Thus, the Sp1/Sp3 stimulatory ratio was 48% greater in BREC than in BAEC, a result consistent with the increased KDR promoter activity observed in BREC.
by which Sp1 preferentially binds to some promoters in specific cell types is not clear (40, 58, 65). Most studies have not observed differences in the ability of nuclear extracts from different cell types to protect Sp1-binding sites in DNase I footprinting experiments in vitro. Similarly, EMSA results and evaluation of nuclear protein levels of Sp1 in some cell types have not demonstrated significant differences in Sp1 levels. It has been proposed that another protein may be binding near the Sp1 site, thus preventing Sp1 binding in cells where the promoter is inactive. However, in vivo DNase I footprinting has confirmed that DNA-protein interactions occur within Sp1 elements in HUVEC but not in other non-endothelial cells, and changes in nucleosomal positioning are present (40). This has led to the hypothesis that distant elements of the KDR promoter alter the chromatin structure and thus permit specific Sp1-mediated expression of KDR in endothelial cells (40). Sp1 activity can be influenced by other transcription factors such as API, Egr-1, GATA, NF-E1, NS-1, and Ptx-1 (58, 69, 70–73).

Footprinting experiments directly bound to this region of the KDR promoter nor tightly bound to either Sp1 or Sp3 while they are bound to the promoter. Sp1 can also be glycosylated with multiple O-linked N-acetylgalosamine monosaccharide residues which may alter its transcriptional activation (72, 75).

Although these mechanisms for Sp1 cell-specific activity could occur in the cell types we evaluated in this study, our results differ somewhat from those presented above in that they suggest that either the absolute levels of nuclear Sp1 and Sp3 and/or the Sp1/Sp3 ratio might be partially responsible for differences in promoter binding and cellular KDR expression. This possibility is supported by the findings that in endothelial cells both Sp1 and Sp3 levels (as determined by Western blot analysis) were up to 24-fold higher, and the Sp1/Sp3 ratios were up to 10-fold higher as compared with those of non-endothelial cells. In addition, the specific KDR promoter DNA-protein interactions were 9-fold more prevalent using endothelial cell-derived nuclear extracts than when using those from non-endothelial cells. Finally, overexpression of Sp1 in non-endothelial cells resulted in increased KDR promoter activity.

Our results might reflect the use of ocular bovine cells which have not been evaluated in previous studies. These cell types are biologically relevant since VEGF plays a critical role in ocular development and in mediating a wide variety of intraocular neovascular disorders such as diabetic retinopathy, central retinal vein occlusion, retinopathy of prematurity, age-related macular degeneration, and numerous others. Biochemical results obtained from the ocular bovine cell types used in this study have been very similar to those observed in humans with regard to both VEGF action, glucose reactivity, and biochemical responses such as protein kinase C activation, endothelin production, etc. (8, 10, 14, 23, 25, 38, 55, 76).

In addition, it has been previously suggested that Sp1 promoter specificity could be the result of both the abundance of Sp family members and the relative ratios of Sp1 and Sp3 (45). Finally, our results could be explained by the unlikely presence of novel Sp1 and Sp3-like molecules which have different binding specificity and cell type expression but share the specific Sp1 and Sp3 antigenic epitopes recognized by the antisera used in this study.

In summary, our studies are the first to demonstrate that expression of KDR is partially mediated by Sp1 binding to a novel recognition sequence within the −79 to −68 region of the KDR promoter. We show that Sp3 binds to the region of the KDR promoter which retains endothelial cell specificity and attenuates Sp1-mediated activation. These data also demonstrate that Sp1 is critical for maximal expression of both KDR mRNA and protein in endothelial cells and that increased expression of Sp1 is sufficient to partially increase KDR promoter activity in non-endothelial cells. The exact mechanism by which Sp1 exerts its endothelial selectivity on the expression of KDR remains unclear and is the focus of future studies.
Sp1- and Sp3-mediated Expression of VEGF Receptor KDR
