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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′-CTCC-3′). 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. Overexpression 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 tumorogenesis (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 Cα, -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

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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 (FBf), and aortic endothelial cells (BAEC) were isolated from fresh slaughterhouse tissues by homogenization and a series of filtration steps as described previously (14). BREC were cultured in Endothelial Basal Medium (Clonetics, San Diego) with 10% plasma-derived horse serum (Weanthon Scientific), heparin (50 mg/liter), and 50 μg/ml endothelial cell growth factor (Boehringer Mannheim). BRPC were cultured in Dulbecco's modified Eagle's medium with 5.5 mM glucose and 20% fetal bovine serum (HyClone). BAEC, BRPE, BSMC, FBf, 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—The plG2 Basic plasmid (Promega) contains the firefly luciferase gene with no promoter or enhancer. Plasmid pSV40 β-gal 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). The DNA sequence of the general region conveying endothelial cell-specific expression is also presented with nuclear protein consensus sequences underlined (bottom). The human DNA sequence that retains endothelial cell-specific activity is presented with classic Sp1 and NFκB nuclear-binding protein consensus sequences underlined. Nucleotides that are important (*) and critical (V) for nuclear protein-DNA binding as determined by mutational electrophoretic mobility shift analysis described in the text are labeled. The DNA sequences for mutations 1–19 are presented with the modified nucleotides marked in bold type. Nucleotide numbering is in relation to the transcriptional start site defined as +1.

XhoI site of the coding area. The amplified polymerase chain reaction fragment was gel-purified and digested with XhoI at nucleotide +296 and cloned directly into the plG2 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 pSV40 β-gal to normalize for transfection efficiency in the 1.5-3.0 × 105 cells used. Cells were harvested 48 h after transfection, and luciferase activity was measured using the Luciferase Assay System (Promega) and an ML3000 microtiter plate luminometer (Dynatech Laboratories). β-Galactosidase activity was assayed as described previously (46). For each transfection, luciferase activity was normalized 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 p5Sp1-pCMV4, and 1 μg of pCMV4, 1 μg of p5Sp3-pCMV4, and 1 μg of p5Sp1-pCMV4 and 1 μg of p5Sp3-pCMV4. All cells were also co-transfected with 1 μg of pSV40 β-gal 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 analysis described in the text. The DNA sequences for mutations 1–19 are presented with the modified nucleotides marked in bold type. Nucleotide numbering is in relation to the transcriptional start site defined as +1.
Sp1- and Sp3-mediated Expression of VEGF Receptor KDR

Enzymatic Cell-specific Expression of KDR mRNA and KDR Promoter Luciferase Reporter Construct Activity

RESULTS

Enzymatic 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 in fibroblasts (BFibro), smooth muscle cells (BSMC), retinal pericytes (BRPC), fibroblasts (BFibro), 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). 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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 \textit{2101 to 1296} region of the KDR promoter (data not shown). These findings demonstrate that KDR mRNA expression is restricted to endothelial cells and that the endothelial cell specificity of the KDR promoter is retained within the \textit{285 to 1296} region.

Previous reports have shown a near complete loss of KDR promoter activity when the region \textit{59 to nucleotide 260} is deleted (39). These data and those described above suggest that the region regulating endothelial specific activity resides between \textit{85 to 260}. To determine if nuclear proteins could specifically bind this region, EMSA was performed using nuclear extract from BREC and radiolabeled \textit{101 to 56} fragment 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 \textit{solid} and \textit{open} arrowheads, respectively. After competition, specific DNA-protein complexes were evident as a major slower migrating band.
(immediately above a weak nonspecific band) and a faster migrating doublet band. The slower migrating band was usually resolved as a closely migrating doublet on careful examination. These bands were competed by 100-fold molar excess of unlabeled –101/–56 promoter fragment but not by the nonspecific –225/–164 fragment. Unlabeled promoter fragments with 5’ deletions within nucleotide –85 (–81/–64) and 3’ deletions beyond residue –64 (–85/–68) were unable to compete with nuclear protein binding to region –101/–56, whereas fragments containing the –85/–64 region –95/–56, –85/–56, –101/–64, and –85/–64 competed well.

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 NFkB 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’-CTCCT-3’) 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 pSV40βgal 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 NFkB 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 NFkB, and five potential Sp1-binding sites, and since the region conveying endothelial cell specificity both potential Sp1 and NFkB 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 NFkB, 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 NFkB, 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 NFkB, 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 NFkB, Sp1, and AP2 DNA consensus sequences.
The specific DNA-protein binding complexes were only competed by unlabeled 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 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′-AGCCCCCTCTCCCT-3′) which is located immediately 5′ to a classic Sp1 consensus sequence at −69 to −64 (5′-CCGCC-3′) (39) and is unlike previously reported related Sp1 binding motifs (GCCGCGCCCC or CTCCCCAGCAG) (58) or CT elements (CCCTCCCCCA) (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 were again 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 cells 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. Decoy 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 transcription (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 nM Sp1 decoy, and 85% inhibition was obtained at 100 nM. 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 86% at 30, 100, and 300 nM, respectively. NFκB decoy reduced KDR mRNA expression only 13, 24, and 27% at 30, 100, and 300 nM, 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 Sp1 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.
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 as 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.

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
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-E2, NS-1, and Pit-1 (58, 69, 70–73). Expression of KDR is partially mediated by Sp1 binding to a novel recognition sequence within the 68 region of the KDR promoter which retains endothelial cell specificity and attenuates Sp1-mediated activation. This 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.

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