CD40-induced transcriptional activation of vascular endothelial growth factor involves a 68-bp region of the promoter containing a CpG island

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Lapchak, Peter H., Michael Melter, Soumitro Pal, Jesse A. Flaxenburg, Christopher Geehan, Markus H. Frank, Debabrata Mukhopadhyay, and David M. Briscoe. CD40-induced transcriptional activation of vascular endothelial growth factor involves a 68-bp region of the promoter containing a CpG island. Am J Physiol Renal Physiol 287: F512–F520, 2004. First published May 12, 2004; 10.1152/ajprenal.00070.2004.—Vascular endothelial growth factor (VEGF) is produced by several cell types in the kidney, and its expression is tightly regulated for the maintenance of normal renal physiology. Increases or decreases in its expression are associated with proteinuria and renal disease. Recently, we found that the expression of VEGF is markedly induced following interactions between CD40 ligand (CD40L) and CD40. Here, endothelial cells (EC) or Jurkat T cell lines were transiently transfected with luciferase reporter constructs under the control of the human VEGF promoter and were treated with human soluble CD40L (sCD40L). We identified a CD40-responsive 68-bp region (bp –50 to +18) of the promoter and 43 bp within this region (bp –25 to +18) that have 97% homology to a sequence of CpG dinucleotides. A computerized search revealed that the CpG region has putative binding domains for the transcriptional repressor protein methyl CpG binding protein-2 (MeCP2). In EMSA, we found that the 43-bp methylated sequence formed four complex(es) with nuclear extracts from untreated EC and reduced binding of at least one complex when nuclear lysates from sCD40L-activated EC (30 min) were used. Supershift analysis using anti-MeCP2 demonstrated that most of the complex(es) in both untreated and sCD40L-activated EC involved interactions between the 43-bp DNA and MeCP2. In addition, we found that other CpG binding proteins may also interact with this region of the promoter. Taken together, this is the first demonstration that CpG binding transcriptional repressor proteins including MeCP2 may be of importance in VEGF biology.

base pair; angiogenesis; vascular endothelium; angiogenesis factors; transactivation

VASCULAR ENDOTHELIAL GROWTH factor (VEGF), a major angiogenesis factor, is also a multifunctional cytokine that has been shown to be of importance in several developmental, physiological, and inflammatory processes in the kidney (8, 18, 30, 39, 51, 58). It is expressed in human cells as a polypeptide of either 206, 189, 165, 145, and 121 amino acids (aa), arising from the alternative splicing of a single gene (8, 18, 35, 63). All the activities of VEGF are thought to be mediated mainly through three high-affinity receptor tyrosine kinases, KDR/Flik-1, Flt-1, and neuropilin, which are expressed by the vascular endothelium as well as other cell types including renal epithelial cells (14, 21, 31, 55, 58, 69).

In the normal human kidney, VEGF is expressed mainly by podocytes, tubular epithelial cells, and endothelial cells (EC) (7, 17, 21, 59). Increases in VEGF expression are well established to be functional for renal vasculogenesis and normal renal development in the embryo (30, 39, 64). However, recent data also suggest that VEGF has a major role in glomerular diseases (25, 46, 61). In an experimental model of nephritis, blockade of VEGF in vivo was found to increase intrarenal apoptosis, impair glomerular capillary repair, and result in proteinuria (48). In humans, blockade of VEGF with anti-VEGF for the treatment of metastatic renal cancer was found to result in proteinuria (67), and it has been proposed that the use of high concentrations of anti-VEGF may be limited by this “renal” side effect.

Additional studies further confirmed that glomerular capillary function is under strict control by VEGF within the glomerulus (17, 38). When intraglomerular VEGF levels decrease in a transgenic mouse, capillary EC were found to swell, capillary loops collapsed, and proteinuria developed (60). Also, consistent with this observation, conditional VEGF isoform knockout mice are reported to have impaired glomerular filtration (39). Furthermore, in humans with preeclampsia it was found that circulating levels of a VEGF antagonist rise (36, 40). This molecule was identified, in part, to be a soluble form of the VEGF receptor Flt-1 that binds to circulating VEGF and results in a decrease in local concentrations of VEGF in the kidney (40). Decreasing local intrarenal concentrations of VEGF was demonstrated to be associated with glomerular capillary leak and proteinuria, a characteristic finding in preeclampsia (40). Nevertheless, it is also notable that overexpression of intrarenal VEGF can also be associated with proteinuric diseases including HIV nephropathy as well as allograft rejection (2, 26, 34, 51, 57). Thus it is likely that the maintenance of normal glomerular capillary function is dependent on some threshold level(s) of intrarenal expression of VEGF.

Recently, we reported that the interaction between CD40 ligand (CD40L) and CD40 induces the expression of VEGF by EC (41, 53). CD40, a type 1 transmembrane-glycoprotein member of the tumor necrosis factor receptor (TNF-R) gene family, is expressed by many cell types, including renal epithelial cells and EC (1, 24, 37, 65, 66, 68). CD40L is expressed

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as a cell-surface molecule by activated CD4+ T cells and platelets (23, 47) and also exists in plasma in a soluble circulating form of unknown function. Thus it is possible that ongoing interactions between CD40L and CD40 may play a role in the maintenance of normal EC VEGF expression. Moreover, it is also possible that CD40L-CD40 interactions are functional for the increased levels of VEGF that occur in inflammation (13, 51, 53).

In the present study, we identified the CD40-responsive region of the VEGF promoter and we show that this region contains a CpG island overlying the major transcription start site. Recent evidence suggests that CpG dinucleotides within a promoter play a significant role in gene silencing via an ability to bind transcriptional repressor proteins (3, 29). We demonstrate that the CpG island within the CD40-responsive region of the VEGF promoter binds the transcriptional repressor protein methyl CpG binding protein-2 (MeCP2) and perhaps other CpG binding proteins, and we show that MeCP2 is removed following CD40-dependent activation of EC. We suggest that these observations for the first time define an important role for CpG islands and transcriptional repressors in the regulation of VEGF in EC.

EXPERIMENTAL PROCEDURES

Reagents. Soluble human CD40L was used as a recombinant protein (Ancell, Bayport, MN and a gift of Yen-Ming Hsu of Biogen, Cambridge, MA). Anti-CD40 (G28.5) was a gift from D. Hollenbaugh (Bristol-Myers Squibb, Princeton, NJ). Cytokines used in these studies were human IFN-γ, human IL-4 (Genzyme, Cambridge, MA), human IL-1 (PharMingen, San Diego, CA), and human TNF-α (a gift from Biogen). Recombinant human MeCP2 was a gift from A. Wolfe [National Institute of Child Health and Human Development, National Institutes of Health (NIH), Bethesda, MD]. For EMSA supershift studies, anti-MeCP2 was also obtained from A. Wolfe, and control IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell isolation and culture. Single donor human umbilical vein endothelial cells (HUVEC; Clonetics, Walkersville, MD) were cultured in complete endothelial medium (EGM BulletKit, Clonetics) as supplied and according to the recommended instructions. HUVEC were subcultured and used at passage 4-7 for all studies. Jurkat T lymphoma cell lines with variable levels of expression of CD40 were used for transfection experiments (a gift from E. Kurt-Jones, UMass lymphoma cell lines with variable levels of expression of CD40 were subcultured and used at

Flow cytometry. Cell suspensions were incubated with primary antibody, were washed, and were incubated with a secondary FITC-conjugated antibody at 4°C for 30 min as described (53, 54). The stained cells were washed and fixed in 1% paraformaldehyde and analyzed by FACScan (Becton-Dickenson, Oxnard, CA).

Transfection and luciferase assay. HUVEC were seeded at 2.5 \times 10^4 cells/well in 3.0 ml of EBM basal medium (Clonetics) containing 5% FBS in six-well tissue culture plates. VEGF promoter-luciferase constructs used for transfection included either a 2.6-kb full-length VEGF promoter fragment (bp −2361 to +298, relative to the major transcription start site) or one of the following truncated promoter-reporter constructs: 350 bp (bp −194 to +157), 200 bp (bp −50 to +157), or 68 bp (bp −50 to +18) as described previously (42, 63). All constructs were used in transient-transfection assays. Transfection was performed by electroporation using the GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's instructions. Brieﬂy, HUVEC were ﬁrst incubated overnight in 0.5% FBS containing EBM basal medium but no other supplements. Subsequently, 1–2 μg of DNA and 3 μl of GenePORTER for each 1 μg of DNA were separately diluted in 500 μl of EBS and then rapidly mixed and incubated for 30 to 45 min at room temperature. Culture medium was then aspirated, and 1 ml of the DNA-GenePORTER mix was carefully added to each well and incubated at 37°C. After 3 h, 1 ml of EBS containing 1% FBS was added to each well, and the incubation was continued up to 24 h in the absence or presence of sCD40L at 37°C in 5–10% CO_2, using duplicate wells for each condition. Subsequently, cells were lysed in lysis buffer and protein concentration was determined. With the use of equal amounts of total cellular protein, luciferase activity was analyzed.

Transfection of Jurkat cells was performed by electroporation. Briefly, Jurkat cells were washed and resuspended in serum-free RPMI 1640; 4 \times 10^4 cells were mixed with or without 3–5 μg of DNA and incubated for 10 min at room temperature. Cells were then subjected to a single pulse (250 V, 975 μF). After 10-min incubation at room temperature, cells were made up in 4 ml medium and divided into four wells of a 24-well tissue culture plate. After 24 h of culture in the absence or presence of sCD40L at 37°C in 5–10% CO_2, cells were lysed in lysis buffer and protein concentration was determined. With the use of equal amounts of total cellular protein, luciferase activity was analyzed as described above. Transfection efficiency was internally controlled by cotransfection of a β-galactosidase gene under control of the cytomegalovirus immediate early promoter using a standard β-gal assay kit (Promega, Madison, WI).

Western blot analysis. Protein samples were mixed with 2× sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, 4% SDS, and 0.0025% bromophenol blue), boiled, and run on 7.5% polyacrylamide gel with Tris-glycin-SDS running buffer (Bio-Rad) as described (49). Size-separated proteins were transferred to a PVDF membrane (New England Nuclear Life Science Product, Boston, MA). For immunodetection, the membranes were blocked with 5% milk in PBS-Tween 20 and then coated with primary antibody. After being washed, the membranes were incubated with peroxidase-linked secondary antibody and the reactive bands were detected by chemiluminescent substrate (49).

Nuclear extract preparation and EMSAs. HUVEC grown to confluence were incubated in the presence of EBM basal medium (Clonetics) containing 0.5% FBS but no other supplements for 24 h. Subsequently, the cells were cultured for 30 to 180 min in the absence or presence of sCD40L at 37°C in 5–10% CO_2. Crude nuclear extracts were prepared and EMSAs were performed as described (42, 50). Briefly, the following components were mixed and preincubated at room temperature for 20 min: 1–2 μg nuclear extract protein were incubated with 1 μg of poly(dA-dT) in a buffer containing 8 mM Tris (pH 7.5), 10 mM KCl, 1% glycerol, 1 mM EDTA, and 10 mM dithiothreitol (Sigma). Antibodies for supershift studies (1 or 2 μg) were added at this time. In some experiments, recombinant human MeCP2 protein (a gift from A. Wolfe) was added in place of the nuclear extracts. After 20 min at room temperature, the labeled probe (~0.1 ng) was added, and incubation was continued for 20 min at room temperature. The samples were resolved by electrophoresis on a 7% polyacrylamide gel, and expression was quantified by densitometry using an Alpha Imager 2000 system (Alpha Innotech, San Leandro, CA). For quantification by densitometry, signals were standardized to the intensity of the free probe.

The radiolabeled oligonucleotide used in EMSAs was the PCR-generated fragment (bp −25 to +18, relative to the transcription start site) of the VEGF promoter. Methylation was performed in vitro with Sss I (New England Biolabs, Beverly, MA) in the presence or absence of the methyl donor S-adenosylmethionine, according to the manufacturer’s instructions and as described (10). Methylated oligos were labeled by employing a Klenow fill-in reaction and purified for EMSA.
RESULTS

Ligation of CD40 with sCD40L induces VEGF promoter activity. To confirm that CD40 signals induce VEGF transcription, we initially transiently transfected HUVEC with a full-length (2.6 kb) VEGF promoter-reporter construct using equal amounts of total cellular protein for each sample. We found a four- to sixfold increase in luciferase activity following treatment with sCD40L compared with untreated cells or mock controls (Fig. 1A). In contrast, activation of HUVEC with the cytokines IFN-γ, IL-1, IL-4, and TNF-α did not mediate any VEGF transcriptional activation in concentrations that markedly induced the expression of E-selectin, ICAM-1, and VCAM-1 (data not shown).

CD40 signals are reported to regulate the transcription of many genes, predominantly via NF-κB- or AP-1-dependent mechanisms. Analysis of the VEGF promoter sequence revealed a single major transcription start site, containing several binding sites for HIF-1, Sp-1, AP-1, and AP-2, but no putative NF-κB binding sites as previously described (42, 63). To define the region of the VEGF promoter responsive to CD40 signaling, we next transfected HUVEC with deletion constructs developed by sequential 5′-deletions of the 2.6-kb promoter to exclude potential CD40-regulatory sites. We generated a 0.35-kb construct that contains four Sp-1 binding sites but no AP-1 binding site and a 0.2-kb construct that does not contain any putative NF-κB, AP-1, or Sp-1 binding sites (42). As shown in Fig. 1B, like the 2.6-kb VEGF promoter, there was a strong induction of transcriptional activity of both 0.35- and 0.2-kb VEGF promoter-reporter constructs in response to sCD40L treatment of EC.

CD40-regulatory element(s) resides in a 68-bp sequence of the human VEGF promoter. We next addressed the specificity of CD40 ligation in the process of VEGF transcriptional activation, using Jurkat T cells that produce VEGF and either express cell-surface CD40 (CD40+ or do not express CD40 (CD40−; Fig. 2A). Both Jurkat cell lines were transiently transfected with the 2.6-kb VEGF promoter-luciferase construct and were cultured untreated or treated with sCD40L for 24 h. Consistently, we found enhanced luciferase activity following sCD40L treatment only in the CD40+ Jurkat cells (Fig. 2B). These data suggest that sCD40L induces VEGF transcription by a signal specifically associated with CD40 ligation.

CD40+ Jurkat cells were also transiently transfected with all the VEGF promoter constructs as well as a 68-bp VEGF promoter-luciferase construct (−50 to +18 bp, relative to transcription start site) (42), which we were unable to efficiently transfect into human EC (above). We found that ligation of CD40 enhanced luciferase activity in CD40+ Jurkat cells transfected with all of the constructs (data not shown) including the 68-bp VEGF promoter sequence (Fig. 2C). In contrast, the pGL2-promoter vector containing the simian virus 40 (SV40) promoter upstream of the luciferase gene (which served as a negative control) was not inducible by CD40 ligation. Furthermore, sCD40L treatment did not enhance the luciferase activity in CD40− Jurkat cells transfected with the constructs. Thus the CD40-regulatory region of the VEGF promoter resides within a 68-bp region of the VEGF promoter that does not contain any binding sites for known CD40-responsive transactivators.

MeCP2 associates with a CpG island within the CD40-regulated region of the VEGF promoter. Through a computerized search of genomic DNA libraries (Blast), and by using MatInspector V2.2 (Genomatrix) (52), it was found that the identified 68-bp sequence does not contain any putative binding sites recognized by known CD40-regulatory proteins. However, we identified the consensus binding sites for several transcription factors, including Sp-1, STAT A, AP-4, and IK2 within this region. Furthermore, we defined a 43-bp region (bp −25 to +18) within this sequence that has a 97% homology (42/43 nucleotides) in reverse read to a sequence of a particular

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**Fig. 1.** Ligation of CD40 results in enhanced vascular endothelial growth factor (VEGF) promoter activity in human umbilical vein endothelial cells (HUVEC). VEGF promoter-luciferase constructs were transfected into the HUVEC by lipofection as detailed in EXPERIMENTAL PROCEDURES. A: luciferase activity in lysates of endothelial cells (EC) transfected with a 2.6-kb full-length VEGF promoter-luciferase construct, either unstimulated or following stimulation for 24 h with sCD40L, TNF-α, IFN-γ, IL-1, or IL-4. B: relative increase in luciferase activity in 24-h sCD40L-treated EC or untreated EC (Mock) transfected with VEGF promoter deletion constructs. A and B: luciferase activity was analyzed using equal amounts of total cellular protein for each sample. The fold-increase in luciferase activity was calculated as the relative luciferase counts from each group of cells compared with untreated cells. Values represent means ± 1 SE of 3 (A) and 6 (B) independent experiments performed in duplicate cultures.
CpG island: Homo sapiens CpG island DNA genomic Mse1 fragment, clone 188b10 (15) (illustrated in Fig. 3). It has been previously demonstrated that this particular CpG island, if methylated, is a binding site for the abundant chromosomal protein MeCP2, which has been described as a potent transcriptional repressor (11, 15).

We performed EMSA using the 43-bp sequence as a probe and found that the unmethylated 43-bp probe did not form a complex with nuclear proteins in extracts of either untreated or sCD40L-treated EC. In contrast, after methylation, the same probe consistently formed complex(es) with nuclear extracts derived from EC, illustrated as four bands in EMSA (Fig. 4, lane 5). We next treated EC with sCD40L, and nuclear extracts were used in EMSA with the methylated 43-bp probe. Compared with untreated EC, we found that treatment with sCD40L (for 30 min) caused a reduction in at least in one of the four bands that interact with the methylated DNA probe (Fig. 4, lanes 5-6). Thus CD40 signals in EC regulate the binding of protein(s) to the methylated VEGF promoter.

Although it is possible that several methyl CpG binding proteins interact with the methylated 43-bp region of the VEGF promoter, we found that this CpG island has the capacity to interact with MeCP2. To determine whether the nuclear protein-DNA complex(es) involves MeCP2-CpG interactions, we
first performed supershift analysis using a polyclonal anti-MeCP2 antibody in EMSA. Extracts from EC again formed complexes with the methylated 43-bp probe, and there was a major supershift of one of the three bands with the anti-MeCP2 antibody (Fig. 5A). The other two bands were also supershifted but to a lesser extent. In contrast, there was no supershift of the complexes using a control Ig antibody. We also treated EC with sCD40L and again evaluated the binding and supershift of nuclear extracts using the anti-MeCP2 antibody. As illustrated in Fig. 5B, treatment with sCD40L resulted in a reduction in the intensity of at least one band (vs. untreated EC; Fig. 5A), and anti-MeCP2 resulted in a partial supershift of the other remaining bands. We interpret this finding to indicate that MeCP2 in sCD40L-treated cells may still interact with the VEGF promoter as a part of a complex. In addition, there is a weak band that is perhaps not supershifted by anti-MeCP2 (Fig. 5B), indicating that not all the complex(es) in sCD40L-treated cells that bind the VEGF promoter construct interact with MeCP2. By Western blot analysis, we confirmed that MeCP2 is an abundant nuclear protein in extracts from both untreated and sCD40L-treated EC (Fig. 5C). Furthermore, we found that purified recombinant human MeCP2 protein formed a single complex with the methylated (but not the unmethylated) 43-bp DNA probe in EMSA (Fig. 5D). This 43-bp MeCP2 protein-DNA complex was supershifted following incubation with the polyclonal anti-MeCP2 antibody (Fig. 5D). Thus MeCP2 is an abundant nuclear protein in untreated and sCD40L-treated EC and has the capacity to bind to the methylated 43-bp VEGF promoter sequence alone or as a complex with other nuclear proteins.

Taken together, these data suggest that methylated CpG dinucleotides within a 43-bp region of the VEGF promoter bind MeCP2 and perhaps other nuclear CpG binding proteins. Moreover, ligation of CD40 may result in the release of CpG binding proteins from the methylated CpG dinucleotides and thus may regulate VEGF transcriptional activation. Because this region of the VEGF promoter overlaps the major transcription start site, these findings suggest that transcriptional repressors or/and the methylation status of CpG dinucleotides within the promoter are likely to be of importance in VEGF biology.

DISCUSSION

The expression of CD40 is prominent on many cell types in pathological processes known to be associated with high levels of VEGF, including renal EC and tubular epithelial cells at sites of inflammation (16, 37, 51, 54, 66). CD40L-CD40 interactions result in several intracellular signals in EC that
Fig. 5. Methyl CpG binding protein-2 (MeCP2) binds to the 43-bp CpG island within the VEGF promoter. EC were untreated or treated with sCD40L for 30 min as described in EXPERIMENTAL PROCEDURES. Supershift gel mobility assays were performed with nuclear cell extracts from untreated HUVEC (A) or sCD40L-treated HUVEC (B), using the methylated 43-bp VEGF promoter fragment as the probe and 2 different concentrations of anti-MeCP2 antibody [1 μg (+) and 2 μg (++)]. No antibody (lane 1) and rabbit IgG (2 μg; A, lane 2) served as negative controls. C: Western blot analysis was performed with nuclear extracts from untreated or sCD40L-treated cells using the anti-MeCP2 polyclonal antibody. Antibody binding to recombinant MeCP2 protein (rhMeCP2) served as a positive control (not shown). D: rhMeCP2 was incubated with the 43-bp methylated probe in EMSA, and supershift gel mobility assays were performed using two concentrations of anti-MeCP2 antibody (1 μg [+]) and 2 μg [++]). Illustrated is a representative blot without (lane 1) or with (lane 2) rhMeCP2 showing binding of the protein to the construct. Supershift is evident with anti-MeCP2 (lanes 3 and 4). Autoradiographs are representative of 3 independent experiments.

Fig. 6. Cartoon illustrating the interaction between CD40 signaling in EC and the binding of transcriptional repressor proteins to the VEGF promoter. In quiescent EC MeCP2, and perhaps other methyl CpG binding proteins, interacts with the 43-bp region of the VEGF promoter. After CD40-dependent activation, transactivators (T) translocate into the nucleus and interact with complexes of methyl CpG binding proteins. This results in their release from the promoter. The removal of transcriptional repressor proteins may enable transactivators to bind to the promoter as has been demonstrated in other cell types (28).
promote activation responses including the expression of the angiogenesis factor VEGF (12, 41, 53, 62). In this study, we identified a CD40-responsive element for VEGF expression in EC and we show that it resides within a 68-bp region of the VEGF promoter. Furthermore, we established that CD40 signals regulate the binding of transcriptional repressor CpG binding proteins to a 43-bp region of the promoter overlying the major transcription start site. We suggest that our findings define a potential role for CpG motifs and/or the binding of transcriptional repressors in VEGF biology.

DNA methylation of the C-5 position of cytosine within CpG dinucleotides is established to suppress transcription through interactions with repressor molecules (4, 5, 9, 11, 43, 44). This process commonly called “gene silencing” has been found to be dysregulated following cellular activation. For many genes, hypermethylation of CpG islands is associated with delayed replication, condensed chromatin, and gene silencing (4, 5, 20, 43). Transcriptional repressor proteins that have been shown to be functional in this process include the methyl-CpG-binding proteins-1 and -2 (MeCP1, MeCP2) and methyl-CpG binding domain family members (MBD1, MBD2a, MBD2b, and MBD4) (6, 22, 44). MeCP2 is a relatively abundant chromosomal protein, whose localization in the nucleus is primarily dependent on CpG methylation (32, 44). It contains an 80-aa long methyl-CpG-binding domain (that binds to a methylated-CpG dinucleotide) and a transcriptional repressor domain. In the methylated state, CpG islands form nuclease-resistant chromatin structures enriched in hypoacetylated histones (45). MeCP2 is reported to be involved in recruiting histone deacetylases to methylated DNA promoters and facilitating a bridge between the hypoacetylated histone and the methylated-DNA dinucleotides (29, 45). However, to date, its role in VEGF gene transcription, VEGF function, physiological or pathological angiogenesis is not known.

Removal of transcriptional repressors from CpG islands may result in the removal of gene repression and via this reported simple mechanism also enables the binding of transcriptional activators to related regions of the promoter (5, 28). Furthermore, it is reported that transcriptional activators can facilitate the direct removal of transcriptional repressors from a promoter. For instance, although the transactivator Gata-3 is necessary for the expression of IL-4 in T cells, in the absence of the transcriptional repressor MBD-2, Gata-3 is unnecessary for IL-4 gene activation (28). Thus, for T cell activation, it is proposed that there is an interplay between activating signals (Gata-3; transcriptional activators) and gene-silencing signals (MBD-2; transcriptional repressors) (28). Gata-3 binds to MBD-2 resulting in its removal and thus the removal of transcriptional repression of the IL-4 gene. Here, we propose that a similar mechanism may function in the regulation of VEGF (illustrated in Fig. 6). MeCP2, which is an abundant nuclear protein, has the ability to bind to the CpG island within the identified critical region of the VEGF promoter. Ligation of CD40 is well established to activate NF-κB and NF-kB-like transcription factors, including the NF-κB family members p50, relA, and c-Rel (19, 56). In addition, CD40 ligation results in the activation of the inducible transcription factor complex, AP-1, typically consisting of heterodimers between Jun and Fos proteins (19, 27). Thus any one of several CD40-regulated transactivators may interact with MeCP2, resulting in its removal from the VEGF promoter. Indeed, the identified 68-bp CD40-responsive region of the VEGF promoter does not have binding sites for these CD40-regulated transactivators, but MeCP2 has potential binding domains for both NF-kB and AP-1 (Melter M, Mukhopadhyyay D, and Briscoe DM, unpublished observations). However, in preliminary studies using competitive oligonucleotides to NF-kB and AP-1 binding sites, we failed to alter interactions between methylated 43-bp DNA and nuclear lysates of HUVEC (Melter M, Mukhopadhyyay D, and Briscoe DM, unpublished observations). Another candidate is the transactivation protein Sp-1, which has been implicated in the regulation of VEGF transcription (49). MeCP2-methyl-CpG DNA interactions have been shown to be associated with the transactivator Sp-1; and it has been suggested that Sp-1 is linked to the methylation status of CpG islands as well as the formation of active chromatin structures (32, 33). However, to date, it is not known whether ligation of CD40 regulates Sp-1 interactions or that Sp-1 is functional in CD40-dependent VEGF transcription.

Finally, another possibility is that CD40 signals may regulate the methylation status of the VEGF gene to alter the binding of MeCP2 (and other MBPs) to the methylated CpG region of the promoter. Although we did not test this possibility, our studies demonstrate that CD40-regulated binding of MeCP2 to the VEGF promoter is independent of methylation. Of note, however, in our EMSA studies, we found that the incubation of nuclear lysates from HUVEC with the methylated sequence of the VEGF promoter resulted in four distinct complexes, and supershift analysis suggests that these bands may represent either MeCP2 alone or MeCP2 as a complex with other proteins. In addition, following CD40-dependent activation of EC, we found a marked reduction in one complex, and all but one of the remaining complex(es) were partially supershifted by anti-MeCP2. Thus CD40 signals result in the removal of MeCP2 (or MeCP2 as a complex with other proteins) from the VEGF promoter. We also interpret our findings to indicate that other CpG binding protein(s) may interact with this region of the promoter independently of MeCP2 and CD40 signaling. We suggest that this finding will lead to further avenues of experimentation to fully understand the implication of VEGF gene methylation for activation responses.

In conclusion, our findings define for the first time that CD40 signals may regulate the binding of the transcriptional repressor CpG binding molecule(s) to a 43-bp region of the VEGF promoter. We believe these data are of importance in CD40-dependent regulation of many genes and provide a framework for the further understanding of transcriptional repressors and gene silencing in VEGF biology and renal disease.

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DISCLOSURES

D. Mukhopadhyyay and D. M. Briscoe are co-senior authors.
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