

RTEF-1, a Novel Transcriptional Stimulator of Vascular Endothelial Growth Factor in Hypoxic Endothelial Cells*

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Jue-Lon Shie, Guifu Wu, Jiaping Wu, Fen-Fen Liu, Roger J. Laham, Peter Oettgen, and Jian Li‡

From the Division of Cardiology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor known to be up-regulated in ischemic heart and hypoxic endothelial cells. However, the transcriptional regulation of VEGF in hypoxia-induced angiogenesis is not fully understood. Transcriptional enhancer factor-1 (TEF-1) is a transcriptional factor family that can regulate many genes expressed in cardiac and skeletal muscle cells by binding to myocyte-specific chloramphenicol acetyltransferase heptamer elements in the promoters of these genes. In this study, we demonstrated that related TEF-1 (RTEF-1), a member of the TEF-1 family, is up-regulated in hypoxic endothelial cells. Overexpression of RTEF-1 increases VEGF promoter activity and VEGF expression. Sequential deletion and site-directed mutation analyses of the VEGF promoter demonstrated that a GC-rich region containing four Sp1 response elements, located between –114 and –50, was essential for RTEF-1 function. This region is beyond the hypoxia-inducible factor-1 α binding site and does not consist of M-CAT-related elements. By electrophoretic mobility shift assay, RTEF-1 was found to interact with the first Sp1 residue (–97 to –87) of the four consecutive Sp1 elements. Binding activity of RTEF-1 to VEGF promoter is also confirmed by chromatin immunoprecipitation. In addition, induction of VEGF promoter activity by RTEF-1 results in an increase of angiogenic processes including endothelial cells proliferation and vascular structure formation. These results indicate that RTEF-1 acts as a transcriptional stimulator of VEGF by regulating VEGF promoter activity through binding to Sp1 site. In addition, RTEF-1-induced VEGF promoter activity was enhanced in a hypoxic condition, indicating that RTEF-1 may play an important role in the regulation of VEGF under hypoxia.

amplifies the expression of angiogenesis-related genes such as vascular endothelial growth factor (VEGF) (1) and VEGF receptor-1 (VEGFR-1) (2), basic fibroblast growth factor 2 (3), and angiopoietin-1 (4), suggesting that hypoxia is an important stimulator for the formation of new blood vessels in coronary artery disease (5), tumor angiogenesis (6, 7), and diabetic neovascularization (8). The effect of hypoxia-associated gene regulation in angiogenesis is dependent on several transcription factors for the activation of targeting genes. HIF-1 α is one of the transcriptional factors that have been shown to regulate VEGF expression. HIF-1 α can up-regulate VEGF *in vitro* (7, 9, 10) and *in vivo* (6–9,11) with the responding element located between –975 and –968 on VEGF promoter (related to the 5' transcriptional starting site) (12). Recent studies have reported that oxidative stress (13), oxidants (H₂O₂) (14), UVB radiation (15), and transforming growth factor β 1 (16) may also regulate the expression of VEGF by binding to a GC-rich region (–114 to –50) on the VEGF promoter sequence.

Related transcriptional enhancer factor-1 (RTEF-1) is a member of the transcriptional enhancer factor-1 (TEF-1) family. All TEF-1 members share a highly conserved amino acid DNA-binding domain capable of binding to M-CAT elements CATN(T/C)(T/C) (17–19) in the promoters of many genes expressed in cardiac and skeletal muscle cells including skeletal troponin T (20–22), skeletal α -actin (23, 24), β -myosin heavy chain (25–30), and α -myosin heavy chain (31, 32). Elimination of the TEF-1 expression in the mouse causes cardiac defects and embryonic lethality (33). Stewart *et al.* (34) cloned a TEF-1-related cDNA, encoding RTEF-1, from the human heart library. Although TEF-1 is responsible for more than 85% of M-CAT binding activity in rat cardiac myocytes, it has been reported that only RTEF-1 has the potential to mediate the reactivation of α_1 -adrenergic response to induce hypertrophy and reactivate cardiac and skeletal genes, such as β -myosin heavy chain and skeletal α -actin, through the M-CAT element in cardiac myocytes (35). However, whether this transcription factor family is involved in regulation of other genes beyond muscle cells has not been fully studied.

In the present study, we have determined that the expression of RTEF-1 was increased in endothelial cells under hypoxic conditions. We demonstrated that hypoxia-induced RTEF-1 expression in endothelial cells is linked to VEGF expression regulated by hypoxia. Interestingly, RTEF-1 is able to bind to the Sp1 site located in the GC-rich region of the VEGF promoter but not to M-CAT elements, although there are many M-CAT-like sequences existing in the VEGF promoter. Moreover, the location of the RTEF-1 binding to VEGF promoter is independent of the HIF-1 α -binding site. We report here, for the first time, that RTEF-1 is a novel transcriptional factor target-

Hypoxia changes many endothelial cell properties including directly regulating many genes, especially by increasing cellular levels of hypoxia-inducible factor (HIF)-1 α ,¹ which in turn

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‡ To whom correspondence should be addressed: Div. of Cardiology, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-8423; Fax: 617-975-5201; E-mail: jli@caregroup.harvard.edu.

¹ The abbreviations used are: HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; TEF-1, transcriptional enhancer factor-1; RTEF-1, related TEF-1; BAEC, bovine aortic endothelial cell(s); EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation; CAT, chloramphenicol acetyltransferase; M-CAT, myocyte-specific CAT heptamer; Sp1, stimulating protein 1; Egr, early growth

response; AP2, activator protein 2; PAS, Per-acyl hydrocarbon receptor nuclear translocator-Sim.

ing VEGF promoter and stimulating VEGF expression in endothelial cells, which contributes to the induction of VEGF expression in hypoxia.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfection—The bovine aortic endothelial cells (BAEC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 units/ml penicillin at 37 °C in a 95% air, 5% CO_2 atmosphere. The cells were transfected with DNA (1 $\mu\text{g}/10^5$ cells) using the LipofectAMINE method according to the manufacturer's protocol (Invitrogen) and as described previously (36). RTEF-1 stably expressed BAEC were selected with zeocin (300 $\mu\text{g}/\text{ml}$). VEGF promoter luciferase constructs (0.5 μg) and control vector pcDNA3.1/GS (1.0 μg) or equal amount of RTEF-1 cDNA were transfected. pcDNA/lacZ (0.01 μg) was cotransfected to determine the transfection efficiency of approximately 70–80%. After transfection, the cells were incubated for an additional 48 h before analysis.

Hypoxic Incubation—Hypoxia was induced using a Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA). The hypoxia chamber was filled with artificial atmosphere, and the concentration of oxygen (<1%) was determined before and after incubation by using an Oxygen Analyzer (Vascular Technology, Bradford, MA). The hypoxia chamber containing cell culture dishes was transferred to a culture incubator according to the time schedule of the studies.

Construction of the RTEF-1 Expression Vector and VEGF Promoter-Luciferase Plasmids—The full-length of RTEF-1 cDNA was obtained from Invitrogen and subcloned into a pcDNA3.1/GS expression vector in frame. The VEGF reporter constructs, containing sequences derived from the human VEGF promoter-driven expression of the firefly luciferase gene, were kindly provided by Dr. Debabrata Mukhopadhyay (37) (Pathology, Beth Israel Deaconess Medical Center, Boston, MA). Briefly, the sequences were inserted into pGL2-Basic vector (Promega) and named according to the length of the fragment from the transcription start site in the 5' and 3' directions: VEGF 2.6 (–2361/+298), VEGF 1.5 (–1226/+298), VEGF 0.35 (–194/+157), VEGF 0.2 (–50/+15), and VEGF 0.07 (–50/+18). The encompassing sequence for the VEGF 0.22 (–71/+157) construct has been amplified by a PCR technique with flanking 5'-XhoI and 3'-HindIII enzyme restriction sites to facilitate directional cloning into the pGL2-Basic vector. The Sp1-I, and III mutant constructs, derived from the 0.35-kb VEGF promoter fragment using polymerase chain reaction, were inserted into pGL-2 basic luciferase expression vector (Promega) as described (38). The constructs carrying two-nucleotide mutations (CC to tt) within the core Sp1 consensus sites (–104 to –50) were generated identically. Sp1-II and IV mutant constructs in pT81 vector, kindly gifted from Dr. Michael Höcker (13) (Berlin, Germany), were resubcloned into pGL-2 basic luciferase expression vector. All of the constructs were sequenced from the 5' and 3'-ends to confirm orientation and sequence correctness.

^3H Thymidine Incorporation—For ^3H thymidine incorporation, BAEC and BAEC stably overexpressing RTEF-1 DNA were incubated with 1 $\mu\text{Ci}/\text{ml}$ ^3H thymidine (20 Ci/mmol) at 37 °C for 4 h before harvesting. After washing twice with cold phosphate-buffered saline, the cells were fixed with 10% trichloroacetic acid at 4 °C for 30 min, rinsed with 10% trichloroacetic acid, solubilized with 1 N NaOH, and neutralized with HCl. Aliquots equal to 0.1 volume of the solubilized material were counted in triplicate by liquid scintillation. Dishes that contained no cells were labeled and counted to provide background counts.

Northern Blot Hybridization Analysis—Total RNA from BAEC was extracted using TRI Reagent (Sigma) according to the manufacturer's protocol and was electrophoresed on a 1.3% agarose, 6% formaldehyde gel. Hybridization was analyzed under stringent conditions with human RTEF-1 cDNAs radiolabeled with ^{32}P dCTP, using the Klenow fragment of DNA polymerase I and random oligonucleotides as primers (Promega). The blots were washed, and autoradiograms were developed after exposure to x-ray film at –70 °C, using a Cronex intensifying screen (DuPont, NY).

Western Blot Analysis—BAEC were washed twice with cold phosphate-buffered saline, lysed in cold RIPA buffer (Boston BioProducts Inc.) containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and cocktail protease inhibitors (Roche Applied Science). The protein concentrations were determined with the DC Protein Standard Assay (Bio-Rad). The samples were subjected to 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) and subsequently blocked in phosphate-buffered saline-Tween 20 containing 7.5% nonfat milk. The membranes were incubated with the indicated primary an-

tibodies (monoclonal anti-VEGF antibody, Santa Cruz; polyclonal anti-RTEF-1 antibody, Genemed Synthesis, Inc.), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit IgG, Calbiochem, La Jolla, CA). The blots were developed using the ECL detection system according to the instructions of the manufacturer (Amersham Biosciences).

Matrigel Analysis—BD Matrigel™ Matrix Growth Factor Reduced (BD Biosciences, Bedford, MA) was diluted (1:1) with cold serum-free Dulbecco's modified Eagle's medium (total concentration, 100 $\mu\text{l}/\text{well}$) and coated on prechilled 24 well culture dish on ice. After Matrigel solidification for 30 min in an incubator, 5×10^4 BAEC with pcDNA 3.1/GS or RTEF-1 transfection was cultured in serum-free Dulbecco's modified Eagle's medium onto Matrigel-coated wells. 24–48 h later, the extent of network formation was observed and photographed.

In Vitro Transcription-Translation—The full length of human RTEF-1 cDNA encoding the entire open reading frames were inserted downstream of the T7 promoter into the pcDNA3.1/GS vector. Coupled *in vitro* transcription-*in vitro* translation reactions were performed with 1 μg of plasmid DNA using the TnT reticulocyte lysate kit (Promega) and T7 RNA polymerase as recommended by the manufacturer. The plasmid vector pcDNA3.1/GS without an insert was used as a control.

Electrophoretic Mobility Shift Assays—EMSA were performed to identify the protein binding to the regulatory elements on the VEGF promoter. *In vitro* translated RTEF-1 products or cell extracts were prepared as described above. Double-stranded oligonucleotides, corresponding to the sequence of regulatory elements, were synthesized by Invitrogen. Double-stranded oligonucleotides (500 ng) were radiolabeled by the 5'-end-labeled reaction in a buffer consisting of 50 mM Tris-HCl, pH 7.6, 10 mM MgCl_2 , 10 mM 2-mercaptoethanol, 100 mM $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ (PerkinElmer Life Sciences), and 30 U T4 polynucleotide kinase (United States Biochemical, Cleveland, OH). EMSA was carried out by incubating 5 μl of *in vitro* translated products or nuclear extracts with 25,000 cpm of $\gamma\text{-}^{32}\text{P}$ -labeled oligonucleotide DNA probe in a 20- μl binding reaction containing 25 mM Tris-HCl, 100 mM KCl, 0.2 mM EDTA, 10% glycerol, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. After incubation at room temperature for 30 min, the samples were loaded onto a 5% polyacrylamide (acrylamide-bisacrylamide, 29:1), 0.25 \times Tris borate gel and electrophoresed at 25mA for 4 h. The gel was dried and exposed to x-ray film (Marsh Bio Product, Rochester, NY) at –70 °C for 18 h. For competition experiments, the *in vitro* translated RTEF-1 products or nuclear extract were preincubated with excess unlabeled wild type or mutated double-stranded oligonucleotides before the addition of the $\gamma\text{-}^{32}\text{P}$ -labeled oligonucleotide DNA probe. Also, a supershift assay was performed by the incubation of RTEF-1 protein/nuclear extracts and oligonucleotide mixture with RTEF-1 antiserum (Genemed Synthesis, Inc., South San Francisco, CA) at room temperature for 30 min before electrophoresis.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays were performed according to the protocol from Dr. Farnham's laboratory (39–41) with the following modifications. Briefly, immunoprecipitation (IP) of transfected and formaldehyde-cross-linked BAEC were performed overnight at 4 °C using certain amount of RTEF-1 antiserum. 50% of the supernatant from the RTEF-1 antiserum-free IP was saved as "total input" chromatin and processed with the eluted IPs beginning with the reverse of formaldehyde cross-linking. After final ethanol precipitation, the TE (10 mM Tris and 1 mM EDTA) resuspended IP products were used as templates for PCR using two primers (5'-GCTGAGGCTCGCCTGTCCCCGCC-3' and 5'-CAAAT-TCCAGACCGAGCGCCCTGG-3'). These two primers were designed according to the sequences 5' and 3', respectively, to the putative Sp1-binding domain on the proximal portion of the VEGF promoter. 1 \times dilution buffer was used as a negative control (mock) for PCR.

Statistics—The results are expressed based on triplet experiments as the means \pm S.E. Statistical analysis was performed using analysis of variance and Student's *t* test. A *p* value of <0.05 was considered to be statistically significant.

RESULTS

RTEF-1 Is Inducible in Response to Hypoxia—From the results of a DNA microarray experiment on normoxic and hypoxic endothelial cells, we found that there is a 3-fold increase of RTEF-1 expression in endothelial cells under hypoxia compared with that in normoxia (data not shown). To confirm this observation, we performed a Northern blot analysis of RTEF-1 using total RNA derived from BAEC stimulated by a series of time points in hypoxia (<1% O_2). RTEF-1 is induced by hy-

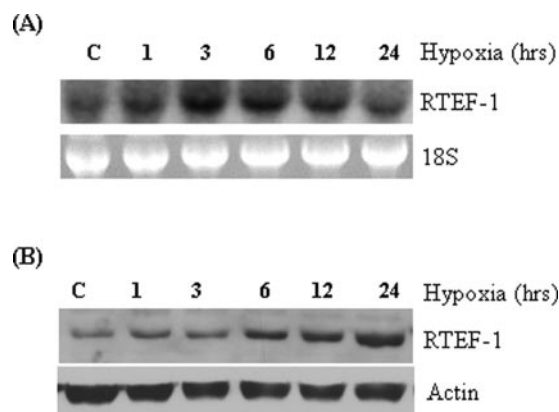


FIG. 1. Hypoxia up-regulates RTEF-1 expression in BAEC. *A*, Northern blot analysis shows that hypoxia induces RTEF-1 mRNA expression in BAEC. *B*, Western blots show that RTEF-1 protein expression increased in 6 h of hypoxia when it is compared with normoxia as control (*lane C*). The increase of RTEF-1 proteins remains up to 24 h after hypoxia.

poxia, and its expression peaks at approximately 6 h in hypoxic endothelial cells (Fig. 1A). The effect of hypoxia on cellular RTEF-1 protein levels was also observed in a time-dependent manner. As Fig. 1B shows, changes in RTEF-1 protein expression by hypoxia demonstrate an early increase of expression at 6 h and remains significantly elevated above base-line values up to 24 h.

RTEF-1 Induces VEGF Expression in Hypoxic Endothelial Cells—To further understand function of RTEF-1 in hypoxia and to determine the target genes of RTEF-1 in endothelial cells, overexpression of RTEF-1 in BAEC was generated by transfection of RTEF-1 cDNA. Among hypoxia-induced factors, we have found that the expression of the VEGF protein level was increased in RTEF-1 overexpressed cells as shown in Fig. 2A. The increase of VEGF expression was significantly enhanced additionally by RTEF-1 in hypoxic conditions (Fig. 2B). These results suggested that induction of RTEF-1 in hypoxia plays an important role in increasing the expression of VEGF. As a transcriptional factor, we hypothesize that RTEF-1 may have some ability in regulating VEGF promoter.

VEGF Is a Potential Target Gene for RTEF-1—To determine that RTEF-1 stimulates VEGF on its transcriptional level, the activities of a luciferase construct under the control of a VEGF promoter were measured. As shown in Fig. 3A, exposure of BAECs cotransfected with this construct with RTEF-1 cDNA led to a 2.2 ± 0.3 -fold increase in luciferase activity, and also RTEF-1 transacted VEGF promoter in a dose-dependent manner. In addition, compared with normoxia, exposure of BAECs cotransfected with VEGF promoter construct and RTEF-1 cDNA to hypoxia revealed a 2.7 ± 0.2 -fold increase (Fig. 3B). These results imply that RTEF-1 regulates VEGF expression and that the increased VEGF promoter activity is enhanced under hypoxic conditions.

Localization of Sequences Required for RTEF-1 on VEGF Promoter—It has been shown previously that the RTEF-1 regulating target gene is through the M-CAT element with the sequence CATN(T/C)(T/C) (18). By searching the transcriptional factor-binding elements on the VEGF promoter region, it was found that many M-CAT-like elements exist, indicating that M-CAT elements on the VEGF promoter may be a binding site interacting with RTEF-1. To investigate this hypothesis, the region of the VEGF promoter required for RTEF-1 regulation was determined in BAEC by using a series of truncated VEGF promoter constructs. Overexpression of RTEF-1 in BAEC resulted in over 2-fold activation of the VEGF promoter activities in three different length of sequences: 2.6 kb (−2361/

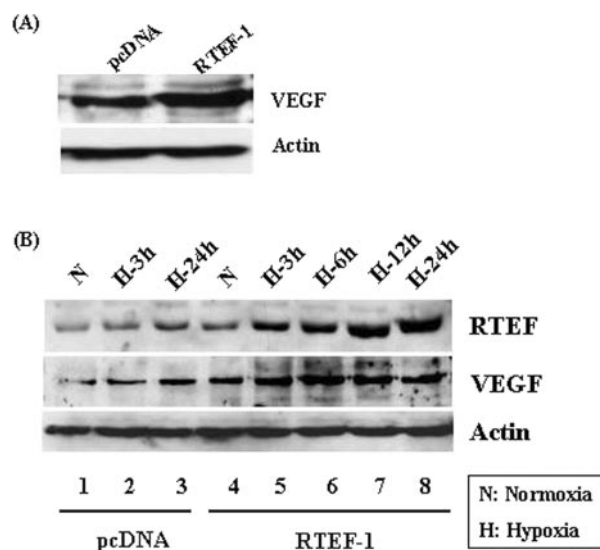


FIG. 2. Effect of RTEF-1 on VEGF expression in hypoxia. *A*, BAEC were transfected with control vector pcDNA3.1/GS or equal amounts of RTEF-1. Western blot analysis shows that VEGF expression is increased in RTEF-1 transfected cells. *B*, RTEF-1 and VEGF protein expression at various time points of hypoxia in BAEC with or without RTEF-1 overexpression. As shown, expression of VEGF is stimulated in hypoxia. VEGF expression was induced synchronously with RTEF-1 expression and enhanced by hypoxia. Note, hypoxia *per se* increases VEGF expression. With overexpression of RTEF-1, VEGF expression is up-regulated.

+298), 1.5 kb (−1226/+298), and 0.35 kb (−194/+157) (Fig. 4A). Deletion of the VEGF promoter sequence from 2.6 to 0.35 kb did not affect the stimulation effect of RTEF-1. In contrast, deletion of the region between VEGF 0.35 kb (−194/+157) and VEGF 0.22 kb (−66/+157) abolished the RTEF-1-mediated effect (Fig. 4A). These findings suggest that the possible gene-regulatory elements on VEGF promoter necessary for RTEF-1 induction are located between −194 and −66 of VEGF promoter sequence. Interestingly, none of the M-CAT elements in the VEGF promoter is found in this region. Moreover, this region is far beyond the HIF-1 α -binding site on VEGF promoter (12) (Fig. 4B). However, there are three consensus Sp1 (stimulating protein 1) elements, one transcription factor Egr-1 (early growth-response 1 gene product) elements, and two AP2 (activator protein 2) elements found in the VEGF promoter sequence of −194 to −66 (Fig. 4B).

Sp1-I Is the Key Element to Induce VEGF Promoter Activity by RTEF-1—To demonstrate RTEF-1-binding sites on GC-rich area of the VEGF promoter, the potential impact on transcriptional activation of mutant-luciferase constructs were created and transfected into BAEC. The constructs comprised VEGF 0.35 mutant (Sp1-I, −97 to −90), VEGF0.35 mutant (Sp1-II, −86 to −79), and VEGF 0.35 mutant (Sp1-III, −75 to −68), in which critical two-nucleotide mutations were incorporated within the core Sp1 sites, and the shorter luciferase construct VEGF 0.22, which contains Sp1-IV and Egr1-II sites. As shown in Fig. 5A, analyses of the VEGF 0.35 mutant constructs VEGF 0.22 and VEGF 0.2 indicated that only VEGF 0.35 mutant (Sp1-I) could not retain the activity when stimulated by RTEF-1. These data suggest that VEGF transactivated by RTEF-1 is Sp1-dependent, and the Sp1-I-binding domain (−97 to −87) of the VEGF promoter is required for the stimulation of RTEF-1.

To identify the specific regulatory element on the VEGF promoter binding by RTEF-1, two procedures were carried out. First, *in vitro* transcription/translation labeled with [³⁵S]methionine to express RTEF-1 product from RTEF-1 cloned construct was performed. As shown in Fig. 5B, RTEF-1 cDNA

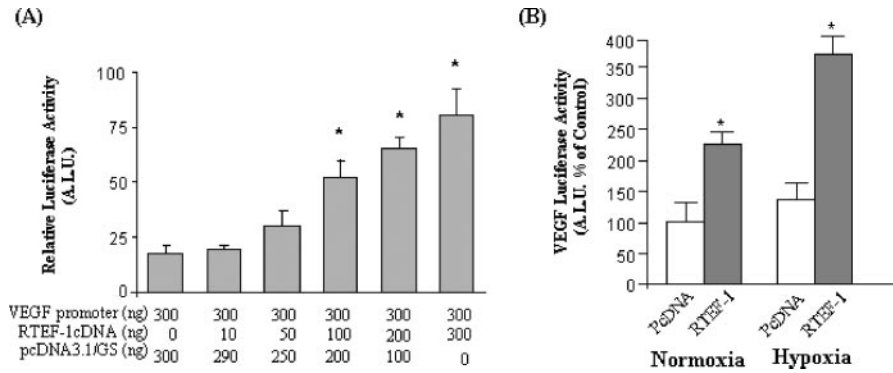
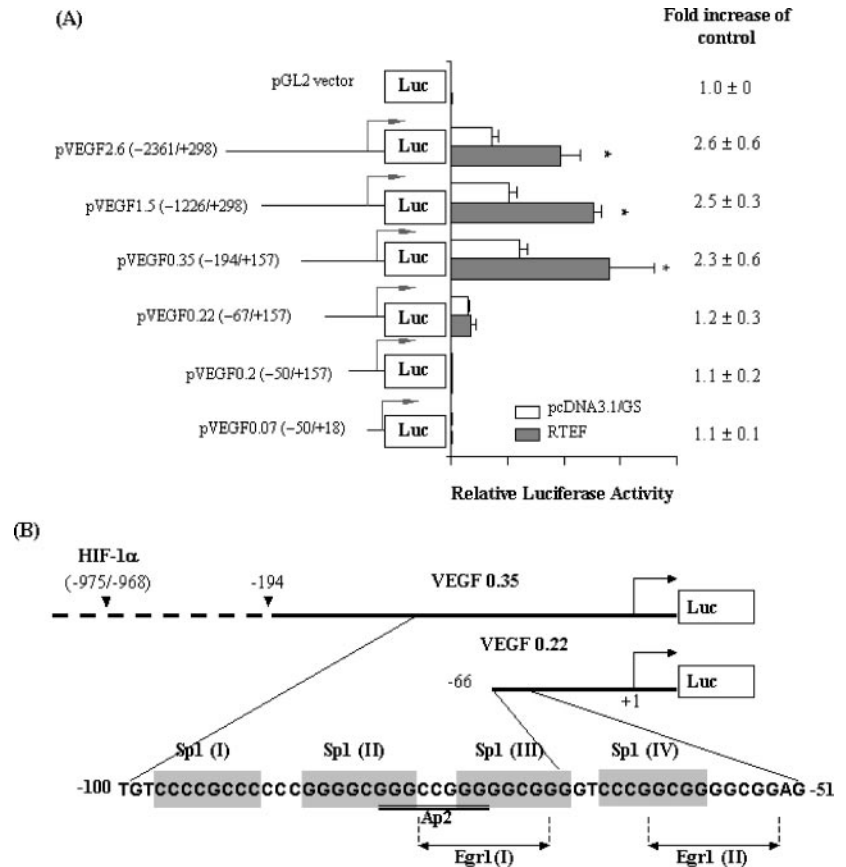


FIG. 3. Up-regulation of VEGF promoter mediated by RTEF-1 is dose-dependent. *A*, 300 ng of VEGF promoter construct was cotransfected with various combination of pcDNA 3.1/GS and RTEF-1 but made total amount to be 300 ng into BAEC. Luciferase activity was determined after 48 h of transfection. Note that VEGF promoter activity increases as amount of RTEF-1 increases. The data are expressed as the means \pm S.E. of three independent experiments. *B*, BAEC were transfected with control vector 1.0 μ g of pcDNA3.1/GS (open bar) or an equal amount of RTEF-1 (black bar) and VEGF promoter constructs (0.5 μ g). Transfected cells were fasted and either incubated normoxia or hypoxia for 6–8 h after 16 h of transfection, and then luciferase activity was determined. As indicated, RTEF-1 significantly stimulated VEGF promoter activity in hypoxia. The data are expressed as percentages of pcDNA3.1/GS transfected cells as control using the means \pm S.E. of three independent experiments. *, $p < 0.05$.

FIG. 4. Functional analysis of VEGF promoter sequence in response to RTEF-1. *A*, transient transfection assays using a set of truncated VEGF promoter luciferase constructs (0.5 μ g) and control vector pcDNA3.1/GS (1.0 μ g, open bar) or equal amount of RTEF-1 cDNA (black bar). pcDNA4.0TO/lacZ (0.01 μ g) was cotransfected to correct the transfection efficiency. Note that RTEF-1 activated VEGF promoter activity is located at the sequence between VEGF 0.35 and VEGF 0.22. The data are expressed as the means \pm S.E. of three separate experiments. *, $p < 0.05$, compared with pcDNA3.1/GS transfected cells in each individual construct. *B*, schematic representation of the VEGF promoter sequence between –100 and –50, which comprises Sp1, Egr1, and AP2 elements. Note that VEGF 0.35, which is responsible for RTEF-1 stimulation, is far beyond the HIF-1 α -binding element.



translated to a 54-kDa protein, which is the same expected size of RTEF-1. Second, a series of double-stranded oligonucleotide probes corresponding to the VEGF promoter Sp1 sequences were created and performed in EMSAs. The Sp1-I, -II, and -III oligonucleotides in the VEGF promoter were used to generate radiolabeled probes. In addition, two mutated double-stranded oligonucleotides, in which the Sp1-I consensus whole sequence was substituted with **tttttttttt** (Mut I, –97 to –87) or **CC** of the core Sp1 sequence was substituted with **tt** (Mut II, –92 to –91), were used to determine the specificity of RTEF-1-Sp1-I binding. As shown in Fig. 5C, RTEF-1 was found to bind to the Sp1-I motif on the VEGF promoter by EMSA (specified by anti-RTEF-1 antiserum; *band SS*); there was no band at same

position found in Sp1-II, Sp1-III, AP2, and Egr1-I-labeled complex. This complex from Sp1-I-labeled was specific because it was repressed by excessive (100 \times) unlabeled Sp1-I oligonucleotide. The DNA-protein complex (Fig. 5C, *band C*) was not eliminated by mutant Sp1-I consensus oligonucleotide (Mut I). However, the mutated core Sp1 oligonucleotide (Mut II) has an ability to compete with radiolabeled Sp1-I oligonucleotide to bind to RTEF-1 product, which competed away some of the DNA-protein complex. No DNA-protein complex was identified when mutated Sp1-I consensus oligonucleotide (Mut I) was used as a probe (data not shown). Supershifts were conducted with antiserum to RTEF-1. The addition of the RTEF-1-specific antiserum supershifted the complex (*band SS*) binding to the

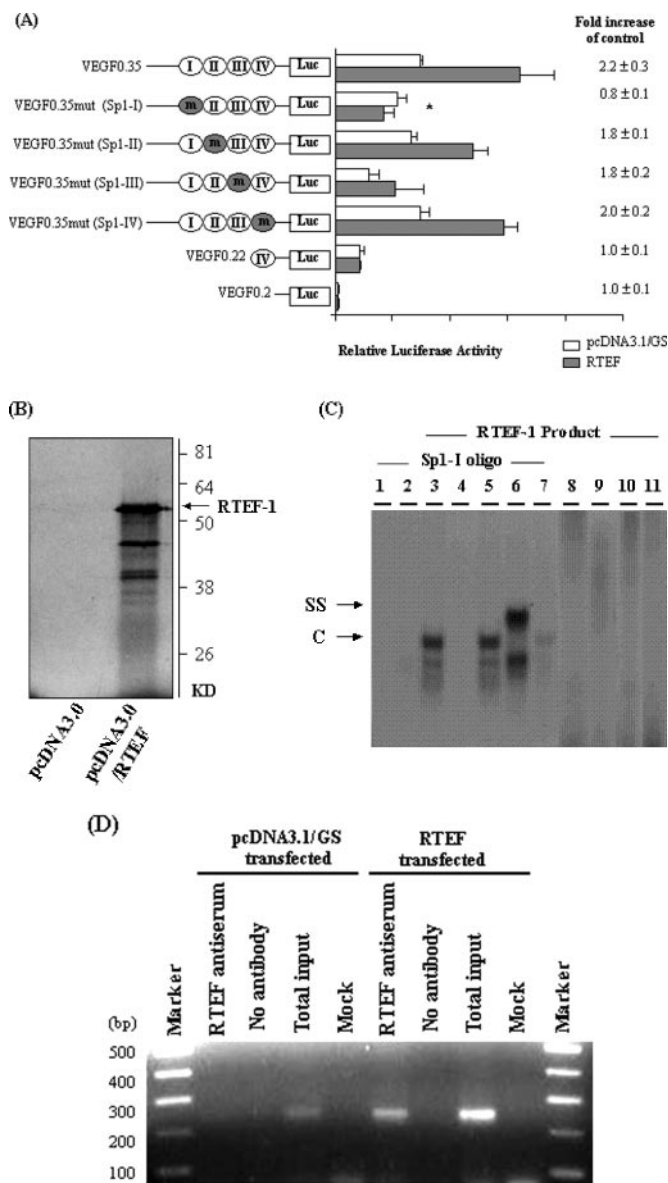


FIG. 5. RTEF-1 binds to SP1-I site in GC-rich area of VEGF promoter. A, mutation and deletion of the potential Sp1 site within -100 to -50 for RTEF-1 response. 0.5 μ g of wild, mutated, or deleted VEGF 0.35 constructs were cotransfected with 1.0 μ g pcDNA3.1/GS or RTEF-1 cDNA into BAEC. Luciferase activity was determined after 48 h of transfection. Transfection efficiency was corrected with the amount of expressing input pcDNA4.0TO/LacZ. Note the significant reduction of RTEF-1-induced VEGF promoter activity was observed only in the mutation of Sp1-I sequence. The data are expressed as the means \pm S.E. of three separate experiments. *, $p < 0.05$, compared with pcDNA3.1/GS transfected cells in each individual construct. B, product of RTEF-1 cDNA was obtained from *in vitro* transcribed/translated with [³⁵S]methionine. The arrow indicates the position of the 54-kDa RTEF-1 product. C, EMSAs were performed to identify the specific binding activity between RTEF-1 and Sp1-I site. *In vitro* translated pcDNA3.1/GS (lane 1) and RTEF-1 products (lane 3) were incubated with radiolabeled VEGF Sp1-I oligonucleotide. RTEF-1 product was preincubated with excessive (100 \times) unlabeled Sp1-I oligonucleotide (lane 4), mutated whole Sp1-I like sequence (Mut I, lane 5), mutated core Sp1-I sequence (Mut II, lane 7), or RTEF-1 antiserum (lane 6) and then was incubated with radiolabeled VEGF Sp1-I oligonucleotide. Lane 2 was RTEF-1 antiserum alone incubated with Sp1-I labeled oligonucleotide. Note complex (band C) indicates the complex of RTEF-1 product and Sp1-I oligonucleotide. There was no significant binding activity found when RTEF-1 product incubated with radiolabeled Sp1-II (lane 8), Sp1-III (lane 9), AP2 (lane 10), and Egr1-I (lane 11) oligonucleotide. The supershift (SS) band indicates the super complex combination of RTEF-1, Sp1-I oligonucleotide, and RTEF-1 antiserum. D, An 1.2% agarose gel analysis of PCR products from chromatin immunoprecipitation assay. Chromatin immunoprecipitation was per-

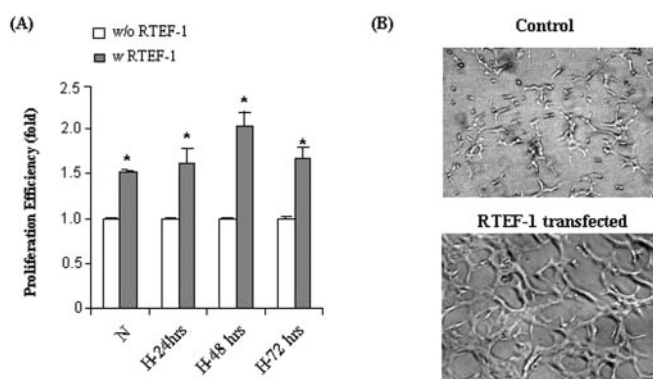


FIG. 6. Effect of RTEF-1 in angiogenic process. A, [³H]thymidine incorporation in BAEC with and without stably transfected with RTEF-1 cDNA. The data are expressed as percentages of change of RTEF-1 overexpression cells over BAEC and represented as the means \pm S.D. *, $p < 0.05$ based on five separate experiments. B, *in vitro* Matrigel assays. Wild type or RTEF-1 stably transfected BAEC were plated on growth factor-reduced Matrigel and cultured with serum-free Dulbecco's modified Eagle's medium. Note that ring and cord formation were observed in RTEF-1 stably transfected BAEC but not in control cells after 48 h of culture.

Sp1-I oligonucleotide, indicating that the DNA-protein complex contained RTEF-1 product.

The physical interaction of RTEF-1 on the VEGF promoter was further examined by a chromatin immunoprecipitation assay. Chromatin fragments from BAEC transfected with pcDNA3.1/GS or RTEF-1 DNA were immunoprecipitated with or without RTEF-1 antiserum. DNA from the immunoprecipitant was isolated and subjected to PCR analysis using primers specific to the putative Sp1-binding domain on the proximal portion of VEGF promoter. As illustrated in Fig. 5D, an expected 250-bp DNA fragment was amplified in samples containing total input chromatin or RTEF-1 immunoprecipitant but not in the control sample (mock) containing dialysis buffer, or RTEF-1 antiserum-free sample, suggesting that RTEF-1 binds to this highly GC-rich sequence containing the Sp1 domains on the proximal portion of VEGF promoter to regulate VEGF expression. Furthermore, the intensity of amplified DNA fragment is higher in RTEF-1 than in pcDNA3.1/GS transfected cells; this result is consistent with the possibility that the expression of VEGF increases synchronously with the amount of RTEF-1.

Effect of RTEF-1 in Angiogenesis—After determining of RTEF-1 binding to VEGF promoter increased VEGF expression, we measured the effect of RTEF-1 to accelerate the proliferation of cell growth and formation of vascular structure. This was done to explore the ability of RTEF-1 and its derivatives to induce angiogenesis via VEGF. RTEF-1-overexpressing BAEC demonstrated a markedly faster growth rate compared with BAEC wild type or vector-transfected cells by [³H]thymidine uptake, especially in a hypoxic condition (Fig. 6A). In addition, the measurement of visible ring and cord formation on growth factor-reduced Matrigel illustrated that RTEF-1 has an ability to accelerate the formation of vascular structures in the absence of serum after 24–48 h when compared with the control cells (Fig. 6B).

formed from pcDNA3.1/GS or RTEF-1 transfected BAEC using RTEF-1 antiserum or antiserum-free control. An amplified 250-bp fragment was seen from total input chromatin and RTEF-1 transfected/RTEF-1-antiserum immunoprecipitated samples. A very faint band was seen from the pcDNA3.1/GS transfected/RTEF-1-antiserum immunoprecipitated sample. The quantification of this 250-bp fragment was higher from RTEF-1 transfected sample than from the pcDNA3.1/GS transfected sample.

DISCUSSION

The principal finding of this study is that RTEF-1, as a novel transcription factor in vascular endothelial cells, is induced by hypoxia, and this induction further stimulates VEGF promoter activity. Three discoveries have been made in this study. First, RTEF-1 has, for the first time, been isolated in endothelial cells where it induces the angiogenic gene, VEGF. Second, it has been determined that regulation of VEGF by RTEF-1 is not through the M-CAT element, which is known to act in muscle-related genes. Rather, it is mediated by the Sp1 element in a GC-rich area of the VEGF promoter. Finally, RTEF-1 is involved in hypoxia-induced VEGF expression; the RTEF-1-mediated element in VEGF promoter, however, is independent from the HIF-1 α -binding area.

Hypoxia and ischemia trigger a multitude of responses designed to compensate for reduced oxygen availability; these responses include increased expression of angiogenic growth factors, their receptors as well as specific transcription factors that are capable of promoting the expression of these factors. One of the main classes of transcription factors that promote this process is the basic helix-loop-helix-PAS domain family (42). In this transcription factor family, HIF-1 α and EPAS1 (endothelial PAS domain protein 1) serve as stimulus for the release of angiogenic growth factors in matured endothelial cells (42). We have identified RTEF-1 as a novel up-regulator that not only stimulates VEGF expression through activating its promoter activity but also induces VEGF expression additionally in hypoxia, which elucidates a novel potential family of transcription factors (TEF-1 family) in gene regulation in vascular endothelial cells.

Previous reports indicated that RTEF-1 is a muscle-enriched transcriptional factor that forms a sequence-specific complex on the M-CAT sites of targeting genes in muscles (43). Many genes expressed in cardiac muscle are now known to contain M-CAT elements. Overexpression of the RTEF-1 increases the α_1 -adrenergic response of the skeletal muscle α -actin promoter through the M-CAT element (44), and RTEF-1 is a direct target of α_1 -adrenergic signaling in hypertrophic cardiac myocytes (44). After myocardial infarction, RTEF-1 modulates S100B gene expression induced by α_1 -adrenergic signaling through the protein kinase C signaling pathway (45), indicating that the RTEF-1 regulation is linked to cardiac disorder altered by ischemia. We observed that the increase of RTEF-1 expression in hypoxic endothelial cells results in induction of VEGF promoter activity, suggesting a novel role for RTEF-1 in the regulation of hypoxia/ischemia-related angiogenesis.

We chose to investigate the VEGF gene because VEGF has been identified as a key mediator in formatting new blood vessels in ischemia (46) *in vivo* and stimulating endothelial cell proliferation by hypoxia *in vitro*. In addition, there are more than five M-CAT-like sequences existing in VEGF promoter region. In the present study, RTEF-1 increases VEGF expression by stimulating VEGF promoter activity. Overexpression of RTEF-1 induces endothelial cells proliferation in culture and accelerates the vascular structure formation on Matrigel gel. The M-CAT sites on VEGF promoter supposedly serve as targets for RTEF-1, although it is not clear what RTEF-1 targets in nonmuscle cells. Interestingly, although the luciferase assay on the interaction of RTEF-1 and serial deletion of VEGF promoter constructs showed that the target residue on VEGF promoter for RTEF-1 function is located between -194 and -66, there is no M-CAT element exhibiting at this sequence. This finding differs from the reported data on the target of TEF-1 transcription factor family, indicating that RTEF-1 is not only a novel transcriptional factor able to stimulate the

expression of VEGF but also binds to a non-M-CAT element to regulate VEGF gene expression.

We report here that the sequence between -114 and -50 is a GC-rich (89%) fragment within VEGF promoter is in response to RTEF-1. Several studies have reported that this is an important area for which various factors will regulate VEGF expression by binding to this GC-rich sequence. Reactive oxygen species have been reported to promote wound angiogenesis by increasing VEGF expression in wound-related cells (47, 48). Sen *et al.* (14) found that the responsible sequence on VEGF promoter for H₂O₂ induction is located at -194 to -50; this 145-base pair sequence contains a GC-rich region. The GC-rich region -87 to -65 in the VEGF promoter sequence contains an AP2/Sp1 cluster; this AP2/Sp1 cluster was responsible for the mediation of UVB radiation to increase VEGF promoter activity in HaCaT keratinocytes, and this UVB-dependent VEGF stimulation may be critical for the skin angiogenesis (15). Transforming growth factor β 1 has the capability of regulating cell proliferation, differentiation, angiogenesis, and metastasis (49-51) via the GC-rich area (-85 to -50) of the VEGF promoter sequence, where three Sp1 consensus binding sites are essential for transforming growth factor β 1 responsiveness (16). Consistent with these investigations, our study found that RTEF-1 has the capacity to stimulate VEGF promoter activity by targeting this -114 to -50 GC-rich region. We also specifically determined that the first Sp1 element, -97 to -87, in this GC-rich region is the RTEF-1 response element on the VEGF promoter.

There are four Sp1 elements in the GC-rich region (-114 to -50) on the VEGF promoter. Using the Transcription Element Search System (University of Pennsylvania), there are three consensus Sp1 sites, one Egr1-I element, and one AP2 element, which are located between the sequence of -114 and -66 and partially overlapped. We also found that RTEF-1 product can recognize the two-nucleotide-mutated core Sp1-I site (Mut II) but not the whole muted Sp1 like sequence (Mut I) in EMSAs. However, luciferase assay detected no stimulation activity on the two nucleotide-mutated construct (VEGF 0.35mut (Sp1-I)), indicating that the Sp1-I site (-97 to -87) is the right RTEF-1 response element on VEGF promoter, and the core Sp1 sequence (CGCC) might be necessary but not essential for the mediation of the VEGF promoter to RTEF-1 function. However, specifically identifying the function of individual Sp1 in VEGF promoter has not been fully studied. From the results of EMSAs, neither the Sp1-II, Sp1-III, AP2, nor Egr1 showed any binding activity with the RTEF-1 product. Furthermore, chromatin immunoprecipitation assays also confirmed that a specific 250-bp fragment, which comprised -114 to -50 GC-rich region, was PCR-amplified from the RTEF-1 transfected and/or RTEF-1 antiserum-precipitated samples. This result indicates that RTEF-1 protein binds to this GC-rich region to regulate VEGF expression in endothelial cells. PCR products using pairs of primers specific to Sp1-I were strongly amplified, whereas those specific to Sp1-III show only a little amount of product amplified. These studies indicated that the second and third Sp1 elements as well as AP2 and Egr1 in this GC-rich region might not be the predominant elements for RTEF-1 function. However, the precise ability of RTEF-1 binding to these Sp1 elements will need further investigation.

VEGF expression is regulated by a variety of factors including platelet-derived growth factor, tumor necrosis factor- α , transforming growth factors α and β 1, and interleukins 1 β and 6 (52-59). Specifically, in hypoxic conditions, VEGF expression was mediated by the induction of HIF-1 α (12). As we report in this study, VEGF promoter activity induced by RTEF-1 was enhanced by hypoxia. It has been widely studied that VEGF

can be stimulated in hypoxia through the stimulation of HIF-1 α . The response element that mediates VEGF expression for HIF-1 α function is located at -985 to -939 (12), but the RTEF-1 response Sp1-I element is located at -97 to -87 on the VEGF promoter sequence, indicating that HIF-1 α was not related to RTEF-1-induced VEGF stimulant. The hypoxia-induced amount of RTEF-1 might be responsible for the complementary effect of VEGF expression in hypoxia.

RTEF-1 inducing VEGF expression and derivatively accelerating neovascularization was demonstrated both by the rapid vessel-like structure formation and thymidine uptake cell proliferation in our study. Whereas RTEF-1 has a role in cardiac gene regulation, its ability to induce angiogenic process in endothelial cells, especially in the hypoxic conditions, may play a particularly important role in mediation of VEGF-associated angiogenesis in myocardial ischemia and requires further investigation.

In summary, our studies demonstrate that RTEF-1 is a novel VEGF stimulator via binding to the Sp1 element in a GC-rich region of VEGF promoter. In particular, RTEF-1 expression can be induced by hypoxia in endothelial cells, and hypoxia strengthens RTEF-1-induced VEGF expression.

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