BTEB2, a Krüppel-Like Transcription Factor, Regulates Expression of the SMemb/Nonmuscle Myosin Heavy Chain B (SMemb/NMHC-B) Gene

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We have recently characterized the promoter region of the rabbit embryonic smooth muscle myosin heavy chain (SMemb/NMHC-B) gene and identified the 15-bp sequence, designated SE1, located at −105 from the transcriptional start site as an important regulatory element for its transcriptional activity in a smooth muscle cell (SMC) line. In this study, we attempted to isolate cDNA clones encoding for the transcription factors that control the expression of the SMemb gene through binding to this *cis*-regulatory element. We screened a λgt11 cDNA library prepared from C2/2 cells, a rabbit-derived SMC line, by using a radiolabeled concatenated oligonucleotide containing SE1 as a probe. Sequence analysis revealed that one of the cDNA clones corresponds to the rabbit homologue of basic transcriptional element binding protein-2 (BTEB2), which has previously been identified as one of the Krüppel-like transcription factor. Gel mobility shift assays and antibody supershift analyses with nuclear extracts from C2/2 cells indicate that BTEB2 is a major component of nuclear factor : SE1 complexes. Furthermore, a glutathione *S*-transferase-BTEB2 fusion protein binds to the SE1 in a sequence-specific manner. In support of the functionality of BTEB2 binding, basal promoter activity and BTEB2-induced transcriptional activation were markedly attenuated by the disruption of the SE1. In adult rabbit tissues, BTEB2 mRNA was most highly expressed in intestine, urinary bladder, and uterus. BTEB2 mRNA levels were downregulated in rabbit aorta during normal development. Moreover, immunohistochemical analysis indicated a marked induction of BTEB2 protein in the neointimal SMC after balloon injury in rat aorta. These results suggest that BTEB2 mediates the transcriptional regulation of the SMemb/NMHC-B gene and possibly plays a role in regulating gene expression during phenotypic modulation of vascular SMC. **(*Circ Res*. 1999;85:182–191.)**

Key Words: SMemb/NMHC-B ■ smooth muscle cell ■ basic transcriptional element binding protein-2

Proliferation and phenotypic modulation of vascular smooth muscle cells (SMCs) play a major role in the pathogenesis of cardiovascular diseases, including atherosclerosis and restenosis after balloon angioplasty.[1] Phenotypic modulation is characterized by the loss of expression of the SMC-specific genes as well as a selective upregulation of fetal/neonatal isoforms of the contractile proteins, extracellular matrix proteins, growth factors, and their receptors.[2–6] The mRNA population particular to either adult- or embryonic-phenotype of SMCs is determined in part by its characteristic array of transcription factors. Alteration of phenotype from embryonic/fetal type to adult type or vice versa may require a change in expression level of a member of these transcription factor subsets. Significant progress has been made in the identification and characterization of receptors for growth factors and cytokines responsible for the proliferation of vascular SMCs and signal transduction pathways to which they are coupled.[7] However, the transcriptional regulation of phenotypic modulation in vascular SMCs is poorly understood.

Among the most interesting genes shown to be associated with the phenotypic change of vascular SMCs is the smooth muscle myosin heavy chain (MHC) gene. We and others have previously shown that normal medial SMCs in rabbit arterial wall predominantly express either SM1 or SM2, which are generated by an alternative splicing mechanism from a single gene, SM1/2 gene.[8–13] A recent study has indicated that the SM1/2 gene is expressed exclusively in smooth muscle-containing tissues throughout development.[14] SM1 is constitutively expressed at all developmental stages, whereas SM2 appears only after birth. On the other hand, the neointima resulting from vascular injury, including fat feeding or

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balloon angioplasty, or in vein graft and transplant atherosclerosis, expresses SMemb/NMHC-B, an embryonic isoform of MHC, the expression of which is prominent in fetal aorta and normally downregulated during development.[10] Studies on the SMC phenotypes in human coronary arteries demonstrated that expression of SM2 is increased with development but decreased in activated SMCs after angioplasty.[15] These studies suggest that the smooth muscle MHC isoforms serve as excellent molecular markers for identifying the diversity of vascular SMC phenotypes in both humans and experimental animals during development and in vascular diseases.

During the last decade, a great deal of progress has been made in understanding the molecular mechanisms that regulate the striated muscle lineage-specific gene expression.[16] Efforts to identify the transcription factors that regulate cell type and lineage-specific gene expression in skeletal muscle have led to the discovery of master regulatory factors such as the MyoD/myogenin/myf-5/MRF4 family.[17,18] In contrast, relatively little has been known about the *cis*-acting sequences and *trans*-acting factors that control smooth muscle-specific gene expression. Recent studies with transient transfection assays of promoter-reporter constructs into cultured vascular SMCs have identified the important *cis*-regulatory sequences within the 5′-flanking region for directing transcription of the SM1/2 gene in a smooth muscle-specific manner.[19–22] More recently, promoter regions of other SMC genes, including SM22α and smooth muscle α-actin have been characterized by both in vitro and in vivo experiments.[23–27] These studies have suggested that the CArG box/serum response factor binding sequence is important for their expression in SMCs.

In contrast to SM1 and SM2 isoforms, expression of 2 nonmuscle MHC isoforms, NMHC-A and SMemb/NMHC-B, is not restricted to SMCs.[28] Notably, distribution of each isoform is quite distinct. NMHC-A is expressed in a variety of cell types and is seen as a constitutively expressed "housekeeping" protein required for the structure of the cytoskeleton, whereas expression of the SMemb/NMHC-B gene appears to be more tightly regulated both in terms of cell-type specificity and developmental timing.[29] In addition to the downregulation of expression levels in aorta and restricted expression to selected tissues, the induced expression of the SMemb/NMHC-B gene in vascular diseases enables this gene to serve as a model system for identifying the mechanisms of phenotypic change of vascular SMCs. Presumably there exist *trans*-acting factors that interact with the SMemb/NMHC-B gene and influence its expression in a specific manner.

We have recently characterized the SMemb/NMHC-B promoter and delineated a *cis*-regulatory element that controls the expression of this promoter in vascular SMCs.[30] We mapped an essential sequences for promoter activity to the 15-bp spanning from −105 to −91, designated SE1. In the present study, we have used the oligonucleotide that contains SE1 to screen an expression cDNA library from C2/2 cells,[31] an SMC line derived from rabbit aorta, and isolated BTEB2, a zinc finger transcription factor.[32] We demonstrate that basic transcriptional element (BTE) binding protein-2 (BTEB2) binds to the SE1 and regulates transcription of the SMemb gene. BTEB2 expression is tissue restricted, with abundant expression in smooth muscle tissues, including intestine, bladder, and uterus, and is scarcely expressed in heart, kidney, spleen, and skeletal muscle. Furthermore, BTEB2 mRNA levels are downregulated in the aorta during development. Immunoreactivity to BTEB2 was clearly increased in the neointimal layer after balloon injury in rat aorta. Thus, BTEB2 defines a transcription factor regulating the SMemb/NMHC-B gene and may play a role in phenotypic modulation of vascular SMCs associated with vascular diseases.

Materials and Methods

Cell Culture

C2/2 cells,[31] an established cell line derived from rabbit aortic SMCs, were grown in DMEM (GIBCO-BRL) supplemented with 5% FCS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cultures were incubated at 37°C in 95% air/5% CO_2. NIH3T3, COS-7, and A10 cells were obtained from the American Type Cell Culture Collection and grown in DMEM supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (GIBCO-BRL).

cDNA Cloning and Sequencing of Rabbit BTEB2

A λgt11 cDNA library from C2/2 cell mRNA was screened with modifications of the methods originally described by Singh et al.[33] Two complementary oligonucleotides, 5′-AATTCATGAGGGCCAGCCTATGAGATTGGGACTTCGGTGG-CCTG-3′ and 5′-ATTCCCAATCTCATAGGCTGGCCCTCATGCC-3′, which contain the SE1 (indicated by underlining), an essential sequence for the rabbit SMemb/NMHC-B promoter,[30] were annealed, phosphorylated, and ligated. Ligated products containing 5 tandem repeats of the oligonucleotide were isolated by acrylamide gel electrophoresis and cloned into the *Eco*RI site of pBluescript II (Stratagene). The DNA fragment containing 5 tandem repeats of the oligonucleotide was isolated from this plasmid, ^{32}P labeled, and used as a probe for screening. Approximately 1 million phage clones were plated and transferred to nylon membranes. Of 11 positive clones isolated, 3 corresponded to BTEB2. The other 3 clones were identified as overlapping cDNAs of another gene. The largest of these 3 was cloned into pBluescript II and used to generate a probe for screening a rabbit fetal aortic cDNA library in the *Eco*RI site of λZAPII (Clontech Laboratories, Inc) to obtain the full-length clone, termed λBTEB2A. Both strands of the entire rabbit BTEB2 cDNA were sequenced on an automated DNA sequencer using the dye termination method.

Preparation of Glutathione *S*-Transferase (GST)-Fused BTEB2

A bacterial expression plasmid encoding a fusion protein between GST and amino acids 1 through 219 of the BTEB2 molecule was prepared by the polymerase chain reaction (PCR). The oligonucleotide 5′-CCCGAATTCATGCCCAGTTCTACAAACCAG-3′ was used as the forward primer, the oligonucleotide 5′-GGGCTCGAGTCAGTTCTGGTGCCTCTTCAT-3′ was used as the reverse primer, and λBTEB2A phagemid was used as the template in the PCR. The PCR product was digested with *Eco*RI and *Xho*I and cloned inframe into pGEX-4T-1 vector (Pharmacia). The resulting construct, pGEX/BTEB2, was used to transform *Escherichia coli* strain BL21, and GST-BTEB2 synthesis was induced with 1 mmol/L isopropylthiogalactopyranoside (IPTG) for 24 hours at 20°C. The fusion protein was purified from bacterial extracts by affinity chromatography on glutathione-agarose.

Plasmid Constructions and Transfections

Plasmids Del-105, Del-99, Del-89, Del-36, pGVm1, pGVm2, pGVm3, and pGVm4 have been described.[30] Eukaryotic expression

vector pcDNA/BTEB2 was constructed by PCR amplification of BTEB2 cDNA obtained from λBTEB2A with the same primers as those used for pGEX/BTEB2 construction. The expression of BTEB2 was directed by cytomegalovirus promoter. For transient transfection assays, COS-7 cells were transfected by a modified calcium phosphate-DNA coprecipitation method as previously described.[30] Each 35-mm-diameter culture dish of 1×10^5 cells was transfected with 1 μg of a luciferase reporter plasmid and 1 μg of either vector alone or pcDNA/BTEB2. Transfected cells were harvested for extract preparation at 48 hours after transfection. Transfections were performed in duplicate in at least 3 independent experiments. Cell extracts were prepared by luciferase assay kit (PicaGene system, Nippon Gene), and levels of luciferase activity were measured with a luminometer (Lumat LB9501, Berthold). Protein concentration was measured by using the BCA protein assay reagent (Pierce) with BSA as standard. The luciferase activity was normalized to protein concentration of the cell lysates.

Electrophoretic Mobility Shift Assays (EMSAs)

The extraction of nuclear proteins was performed with modifications of the methods originally described by Dignam et al.[34] Briefly, cells were washed twice in cold PBS and scraped, and the cellular pellet was resuspended in (in mmol/L) HEPES (pH 7.6) 10, KCl 10, and $MgCl_2$ 1.5 on ice. Cells were homogenized with 10 strokes of Dounce homogenizer (type B pestle) and centrifuged to collect the nuclei, which were subsequently resuspended in 20 mmol/L HEPES (pH 7.6), 0.42 mol/L NaCl, 5 mmol/L $MgCl_2$, and 25% glycerol to allow elution of nuclear proteins by gentle shaking on ice for 30 minutes. Nuclear extracts were pelleted for 15 minutes at 15 000 rpm at 4°C, and supernatant was then dialyzed against (in mol/L) HEPES (pH 7.6) 20, EDTA 0.2, KCl 50, and $MgCl_2$ 5, and 20% glycerol overnight. Nuclear extracts were then pelleted for 30 minutes at 15 000 rpm at 4°C, and the supernatant was then aliquoted in liquid nitrogen and stored at −80°C until use. All solutions contained the protease inhibitors leupeptin and aprotinin at 2 μg/mL, PMSF at 1 mmol/L, and DTT at 1 mmol/L. For gel-shift assays, 10-μg portions of nuclear extracts or 1 μg of GST fusion proteins were incubated in 25 μL of binding reaction that contained (in mmol/L) Tris-HCl (pH 7.5) 10, NaCl 50, and DTT 0.5, and 10% glycerol, 0.05% NP-40, and 2 μg of poly(dI-dC) for 20 minutes at room temperature to titrate out nonspecific binding before the addition of 15 000 to 20 000 cpm of labeled oligonucleotide; the reaction mixture was then further incubated for 20 minutes. When unlabeled oligonucleotides or antibodies were added, nuclear extracts were preincubated for 20 minutes at room temperature or for 30 minutes at 4°C, respectively, before the addition of labeled probe. Samples were loaded on a prerun 5% polyacrylamide gel in 0.5× TBE (45 mmol/L Tris borate and 1 mmol/L EDTA) and electrophoresed at 150 V. The gels were then dried and exposed to Kodak XAR film at −80°C.

RNA Extraction and Northern Analysis

Total cellular RNA was prepared from organ samples and from cultures of the rabbit aortic SMC line C2/2, the rat SMC line A10, and non-SMC lines, including murine NIH3T3 cells and monkey kidney-derived COS-7 cells, by the single-step guanidinium thiocyanate protocol described previously.[35] Total RNA was fractionated in 1.2% agarose/formaldehyde gels. Northern blotting was performed using 20 μg of RNA/sample as described previously.[20] RNA was transferred to nylon membrane (Hybond N^+, Amersham) as described by the manufacturer. After being UV cross-linked, blots were stained with 0.04% methylene blue to permit quantification of the 28S and 18S ribosomal RNA subunits in each lane. Probes included a 664-bp fragment that contains an entire coding region of the rabbit BTEB2 cDNA and a 1.2-kb fragment of human Sp-1 cDNA probe. Filter hybridizations were carried out in a solution of 50% formamide, 5× SSPE, 10× Denhardt's solution, 1% SDS, and 0.1 mg/mL herring sperm DNA at 42°C. Filters were washed to a final stringency of 0.1× SSC at 42°C and exposed to Kodak XAR film at −80°C for 48 hours. Antibody against Sp1 (PEP2) was obtained from Santa Cruz Biotechnologies.

BTEB2 Antibody and Western Blot Analysis

A short peptide specifying the carboxyl terminal end of BTEB2 (Asp-His-Leu-Ala-Leu-His-Met-Lys-Arg-His-Gln-Asn) was synthesized and used for immunization. The synthetic short peptide was conjugated with BSA and injected subcutaneously into rabbits at biweekly intervals. Titers of the antisera were determined by an ELISA. Tissues from either neonatal or adult rats were pulverized under liquid nitrogen. These tissues and cells were incubated in ice-cold sample buffer containing 2% SDS, 0.1 mol/L DTT, and 60 mmol/L Tris-HCl (pH 6.8) for 30 minutes. Equal amounts (20 μg of total protein) of each lysate were boiled for 5 minutes, separated on 8% SDS-PAGE, and then transferred to nitrocellulose (Hybond-ECL, Amersham Corp). The membrane was blocked for 2 hours at room temperature with 10% nonfat dried milk in Tris-buffered saline containing 0.1% (wt/vol) Tween 20 (TBS-T). The blots were incubated for 1 hour at room temperature with anti-BTEB2 antibody and followed by incubation for 1 to 2 hours with horseradish peroxidase-labeled goat anti-rabbit IgG in TBS-T containing 2% nonfat dried milk. The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Life Science, Inc.).

RNase Protection Assay

The 274-nucleotide *Eco*RI-*Sac*I fragment of the rabbit BTEB2 cDNA clone was subcloned into pBluescript II. After linearizing the plasmid DNA with *Hin*dIII, the RNA probe was synthesized with T3 RNA polymerase and [α-^{32}P]UTP according to the manufacturer's protocols as described for the Riboprobe Gemini System II kit (Promega). Total RNA (5 μg) was hybridized with the RNA probe. The RNase protection assay was carried out according to the manufacturer's protocols as described for the ribonuclease protection assay kit (Ambion). The probe and protected fragments were analyzed on a denaturing urea-5% polyacrylamide gel.

Immunohistochemistry

Balloon injury of the aorta was performed in adult male Wister rats (300 to 350 g) under general anesthesia. A 2F balloon embolectomy catheter was introduced via the femoral artery and advanced to the level of the thoracic aorta. The balloon was then inflated and the catheter withdrawn along the length of the thoracoabdominal aorta. The balloon was deflated, and the procedure was repeated 3 times. Rats were euthanized at 2 weeks after injury. The aorta was fixed in 10% formalin and paraffin embedded. Immunohistochemistry using anti-BTEB2 antibody was carried out by using the Vectastain Elite ABC kit (Vector Laboratories).

Results

Isolation of Rabbit BTEB2 cDNA

We have recently shown that the 15-bp DNA sequence located at −105, to which we refer as SE1 (sequence element-1), is important for a high level of expression of the SMemb/NMHC-B gene.[30] To isolate cDNA clones encoding proteins that bind to the SE1 sequence, we constructed an expression cDNA library from C2/2 cells, an SMC line derived from rabbit aorta, in the λgt11 vector. We chose C2/2 cells because our previous experiments suggested that they contain factors that can modulate SMemb/NMHC-B promoter activity.[20] The screening was performed with concatenated oligonucleotides that contain the SE1 sequence. From ~1 million phage plaques, 11 clones that exhibited binding to SE1 were isolated. Sequence analysis revealed that 3 of these clones contain independent, partial cDNA inserts derived from the BTEB2 mRNA. Sequences encoding the missing coding region of the gene were obtained by screening a rabbit fetal aortic cDNA library using this clone as a probe. We finally obtained a 2.3-kb cDNA insert, which contained the

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ATGCCCAGTTCTACAAACCAGACAGCAGTGATGGACACTCTCAATGTTTCTATGTCAGCC 60
MetProSerSerThrAsnGlnThrAlaValMetAspThrLeuAsnValSerMetSerAla 20

GCCATGGCAGGCCTGAACACACACACCTCTGCCGTTCCACAGACTGCAATGAAACAGTTC 120
AlaMetAlaGlyLeuAsnThrHisThrSerAlaValProGlnThrAlaMetLysGlnPhe 40

CAGGGCATGCCCCCTTGCACATACACAATGCCAAGTCAGTTTCTGCCACAACAGGCCACT 180
GlnGlyMetProProCysThrTyrThrMetProSerGlnPheLeuProGlnGlnAlaThr 60

TACTTTCCCCCATCACCACCGAGCTCAGAGCCTGGAAGTCCTGATAGACAAGCAGAGATG 240
TyrPheProProSerProProSerSerGluProGlySerProAspArgGlnAlaGluMet 80

CTCCAGAATTTAACCCCACCGCCATCCTATGCTGCTACCATTGCTTCCAAGCTGGCAATT 300
LeuGlnAsnLeuThrProProProSerTyrAlaAlaThrIleAlaSerLysLeuAlaIle 100

CACAATCCAAATTTACCTGCCACCCTGCCAGTCAATTCGCAAAACATCCAACCCGTCAGA 360
HisAsnProAsnLeuProAlaThrLeuProValAsnSerGlnAsnIleGlnProValArg 120

TACAATAGAAGGAGTAACCCCGACCTGGAGAAACGCCGCATCCATTACTGCGATTACCCT 420
TyrAsnArgArgSerAsnProAspLeuGluLysArgArgIleHisTyrCysAspTyrPro 140

GGCTGCACAAAAGTTTATACAAAGTCTTCTCATTTAAAAGCTCACCTGAGGACTCACACT 480
GlyCysThrLysValTyrThrLysSerSerHisLeuLysAlaHisLeuArgThrHisThr 160

GGTGAGAAGCCGTACAAGTGCACCTGGGAAGGCTGCGACTGGAGGTTCGCGCGCTCCGAC 540
GlyGluLysProTyrLysCysThrTrpGluGlyCysAspTrpArgPheAlaArgSerAsp 180

GAGCTGACTCGCCACTACCGGAAGCACACGGGCGCCAAGCCCTTCCAGTGCGGGGTGTGC 600
GluLeuThrArgHisTyrArgLysHisThrGlyAlaLysProPheGlnCysGlyValCys 200

AACCGCAGCTTCTCACGCTCGGACCACCTGGCCCTGCACATGAAGCGGCACCAGAACTGA 660
AsnArgSerPheSerArgSerAspHisLeuAlaLeuHisMetLysArgHisGlnAsn*** 219
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Figure 1. Nucleotide sequences paired with deduced amino acid sequences of rabbit BTEB2 cDNA clone. For each pair, numbers on the right refer to the nucleotide sequence (upper) and the deduced amino acid sequence (lower). Underlined bold type indicates the three Cys2-His2 zinc finger domains; nonunderlined bold type, the 3 amino acids, 10, 37, and 107, which are different between rabbit and human. Homology levels of the nucleotide and amino acid sequences between rabbit and human are 93.6% and 98.6%, respectively.

full-length coding region of the rabbit BTEB2 cDNA. Analysis of the predicted open reading frame encoding 219 amino acids revealed 3 putative zinc finger domains (Figure 1). Sequence comparisons showed a high homology to human BTEB2 with 93.6% and 98.6% sequence identity at the nucleotide level and amino acid level, respectively.

BTEB2 Is Present in the Nuclear Factor Complex That Binds to the SE1

In our previous EMSAs performed with nuclear extracts from C2/2 cells, SE1 sequence has been shown to form a single complex that was competed by a sequence for CCAAT binding protein but not for Sp1 or Sp1-related proteins.[30] However, C2/2 nuclear extracts prepared by the modified version of the original procedure described by Dignam et al[34] have yielded 2 shifted complexes (C1 and C2, Figure 2), both of which comigrated with the complexes formed by the canonical Sp1 binding site that was used a reference. The formation of these complexes was sequence specific, because a 5-bp substitution mutation within SE1 sequence, from GGGCC to TTTAA located between −105 and −101, lost its ability to compete for the binding with SE1 sequence (Figure 2A). Competition with excess unlabeled oligonucleotides containing Sp1 or BTEB sequences indicated that SE1 is homologous to the binding sequences for Sp1 or BTEB1. Most notably, incubation with the BTEB2 antibody gave rise to a supershifted complex, which indicates that BTEB2 is a component of the complexes C1 and C2 (Figure 2B). The specificity of this supershifted complex was supported by the observations that rabbit preimmune serum has no effects on the factor bindings with SE1 probe, and BTEB2 antibody has no effects on the interaction between Sp1 and its consensus binding sequences. These results unequivocally demonstrate that BTEB2 is present in the nuclear factor:SE1 complexes. In addition, we found that SE1 can also serve as a binding site for Sp1, because addition of Sp1 antibody yielded supershifted bands (Figure 2C).

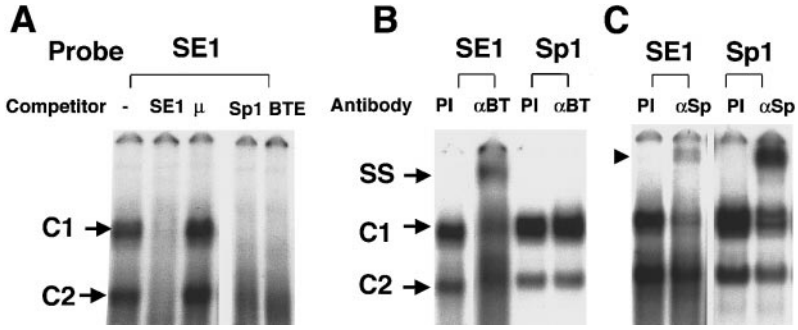

Figure 2. BTEB2 is present in the nuclear factor complexes that bind to the SE1 sequence. A, EMSA. A double-stranded oligonucleotide SE1 was radiolabeled and incubated with the nuclear extracts prepared from C2/2 cells in the absence or presence of 100-fold molar excess of unlabeled competitors. Positions of protein:DNA complexes (C1 and C2) are indicated. The competitors used were the following: SE1, mutated version of SE1 (μ), consensus Sp1 binding sequence (Sp1), and consensus BTEB1 binding sequence (BTE) (see Materials and Methods for sequences). B and C, EMSAs with a radiolabeled oligonucleotide containing SE1 or consensus Sp1 binding sequence and C2/2 nuclear extracts were performed in the presence of either rabbit preimmune serum (PI) or antibody against BTEB2 (αBT) (B) or Sp1 (αSp) (C). After incubation for 30 minutes at 4°C, the binding reaction mixtures were subjected to EMSA, as described in Materials and Methods.

Nucleotide Sequences of Probes and Competitors Used in EMSAs

Probes/ Competitors	Sequences
SE1	5′-ATGGGCATGAGGGCCAGCCTATGAGA-3′
μ	5′-ATGGGCATGA*TTTAA*AGCCTATGAGA-3′
EM0	5′-ATGGGCATGAGGGCCAGCCTATGAGATTGGG-3′
EM1	5′-ATGG*TACGTC*GGGCCAGCCT*CGTCTCGGTTT*-3′
EM2	5′-ATGG*TACGTCTT*GCCAGCCTAT*TCTCGGTTT*-3′
EM3	5′-ATGG*TACGTCTTTAA*AGCCTATGAG*CGGTTT*-3′
EM4	5′-ATGGGCATGAGGGCC*CTAAGCGTCTCGGTTT*-3′
EM5	5′-ATGG*TACGTCTTTAACTAAGCT*GAGATTGGG-3′
BTE	5′-GATCGAGAAGGAGGCGTGGC-3′
Sp1	5′-ATTCGATCGGGGCGGGGCGAG-3′
CACC box	5′-TAGAGCCACACCCTGGTAA-3′
CAT box	5′-ACTTTTAACCAATCAGAAAAAT-3′
AP2	5′-GATCGAACTGACCGCCCGCGGCCCGT-3′

Sequences of oligonucleotides SE1 and EM0 through EM5 are listed with the mutated bases appearing in italics. The consensus binding sites for BTE, Sp1, CACC box, CAT box, and AP2 are underlined.

DNA Binding Activity of GST-BTEB2 Protein

Further confirmation that BTEB2 is an SE1 sequence-binding protein was obtained by incubating the GST-BTEB2 protein with the labeled oligonucleotide containing SE1 sequence (see Table for sequence). The oligonucleotide EM0 was retarded by GST-BTEB2 and not by GST alone in a gel-shift assay (Figure 3A). Binding to EM0 was completely competed by a 500-fold molar excess of unlabeled EM0. To define the sequence to which BTEB2 binds, duplex oligonucleotides, EM1 to EM5, were used as competitors. The complex was efficiently competed with a 500-fold molar excess of unlabeled EM1, which keeps the sequence from −105 to −96 unchanged. Unlabeled EM2, which keeps the sequence from −103 to −94 unchanged, competed for binding but less efficiently. EM3, EM4, and EM5 with unchanged sequences from −100 to −91, −115 to −101, and −94 to −85, respectively, did not compete. These results indicate that a sequence from −105 to −100 is required for GST-BTEB2 to bind to the EM0. This was further confirmed by testing the binding of GST-BTEB2 to radiolabeled EM0 to EM5 (Figure 3B). A DNA:GST-BTEB2 complex was formed with either EM0 or EM1, but not with EM2, EM3, EM4, or EM5.

Given that BTEB2 is expected to bind GC-rich sequences,[32] we tested whether the GST-BTEB2 protein specifically binds to consensus sequences of the Sp1-binding site,[36] CACC-box,[37] or BTEB site,[38] each of which is known to be a binding site for Sp1 or Sp1-related zinc finger transcription factors. Indeed, the shifted complex formed with EM0 and GST-BTEB2 protein was completely abolished by addition of unlabeled oligonucleotide carrying an Sp1 binding site, CACC box, or BTE binding site, but not by the unlabeled oligonucleotide containing the binding sites for CAT (Figure 3C).

Transactivation of the SMemb/NMHC-B Promoter by BTEB2

Given the capability of BTEB2 to bind to the SE1, an element important for the expression of the SMemb/NMHC-B gene, we determined whether BTEB2 activates transcription from the SMemb/NMHC-B promoter. The full-length BTEB2 cDNA under the control of the cytomegalovirus promoter was cotransfected into COS-7 cells with a series of 5′-deletion constructs of rabbit SMemb/NMHC-B promoter (Figure 4). Overexpression of BTEB2 resulted in a significant induction of Del-105 reporter gene expression (7.5±0.4–fold). Deletion of a sequence to −99 bp resulted in a marked decrease in transactivation by BTEB2 (2.1±0.2–fold). To address further the effects of BTEB2 on the SMemb/NMHC-B promoter activity, mutations were introduced into SE1 sequence within the context of Del-138, which contains SMemb/NMHC-B promoter sequence up to −138 bp. Luciferase activity of Del-138 reporter gene was significantly increased by BTEB2 (7.4±0.4-fold) (Figure 5). Mutations of sequence either between −105 and −101 (pGVm1) or between −100 and −96 (pGVm2) resulted in a noticeable reduction of fold activation by BTEB2. In contrast, mutations of sequence either between −95 and −91 (pGVm3) or between −90 and −86 (pGVm4) failed to block the BTEB2-induced expression of the reporter gene. Overall, these results indicate that BTEB2 can activate SMemb/NMHC-B promoter activity and the sequence between −105 and −96 mediates at least in part the induction by BTEB2.

To verify that SE1 sequence mediates the activation of the SMemb promoter by BTEB2, we inserted 5 copies of SE1 sequence upstream of the simian virus 40 promoter-luciferase vector pGVC to generate the construct 5×SE-pGVC. Cotransfection of BTEB2 expression vector stimulated transcription of 5×SE-pGVC by 6.2-fold, whereas it activated pGVC by 2.8-fold (data not shown). These results indicate that SE1 sequence confers the BTEB2-induced activation on the heterologous promoter.

Regulation of SMemb Promoter Activity by Phorbol 12-Myristate 13-Acetate (PMA)

Having demonstrated that overexpression of BTEB2 leads to the SE1 sequence-dependent activation of the SMemb promoter, we examined whether this promoter is also responsive to stimuli that induce endogenous BTEB2 expression. Stimulation of C2/2 cells with PMA leads to the sustained increase in BTEB2 mRNA levels (K.-K. Kowase, M. Kurabayashi, R. Nagai, unpublished results, 1999). Figure 5C shows that PMA increases luciferase activity derived form wild-type SMemb promoter, and this effect of PMA was attenuated when the 10-bp sequence located between −105 and −96 was mutated (see pGVm1, pGVm2 in Figure 5C).

Expression of the BTEB2 in Adult Tissues and Developing Aorta

To determine the expression profile of BTEB2 mRNA, we performed Northern blot analysis of RNAs from various tissues of adult rabbit. The mRNA is expressed most abundantly in the small and large intestines, followed by lung, bladder, and uterus, but not in cerebrum, ventricle, liver, skeletal muscle, aorta, spleen, and kidney (Figure 6A). A more refined analysis of BTEB2 mRNA distribution was achieved by RNase protection assay (Figure 6B). A fully protected band was clearly detected only in the samples from

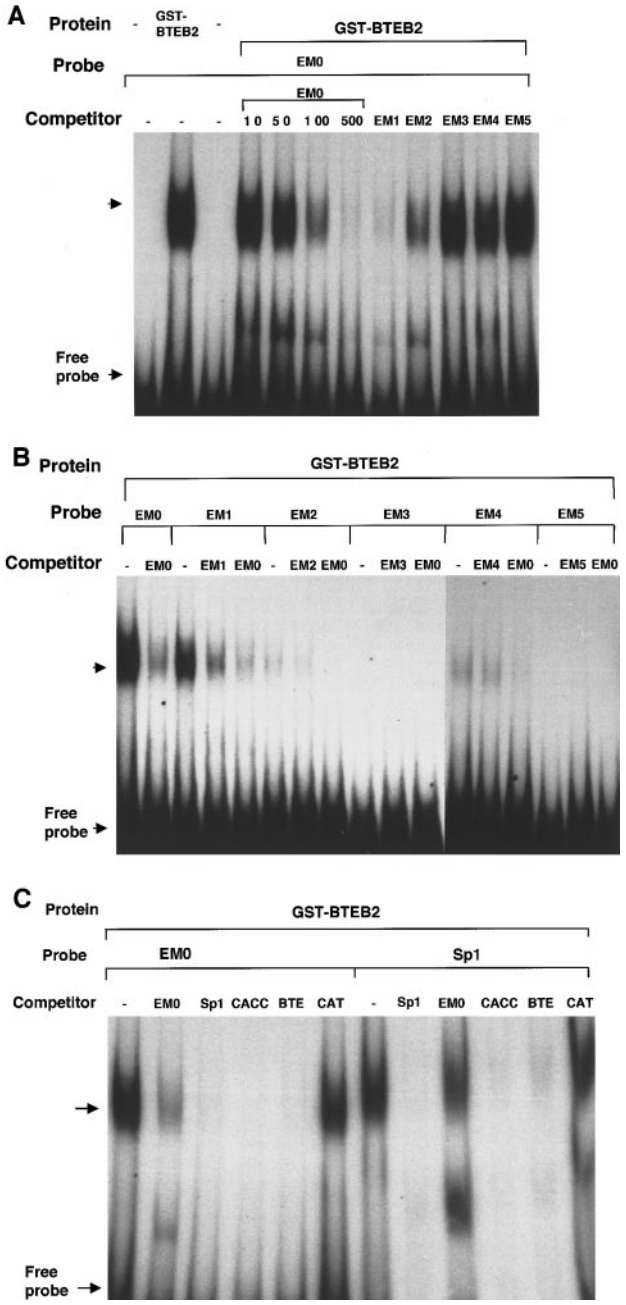

Figure 3. EMSAs showing DNA binding of GST-BTEB2 fusion protein. A, Approximately 1 μg of GST-BTEB2 fusion protein or GST alone was incubated with the ^{32}P-labeled double-stranded oligonucleotide EM0, which contains SMemb/NMHC-B promoter sequence between −111 and −85 (see the Table for sequence). Competition was carried out by using either unlabeled self-competitor at an indicated molar excess, or the mutant competitors EM1 through EM5. Unlabeled mutant competitors were used at a 500-fold molar excess. B, GST-BTEB2 fusion protein was incubated with ^{32}P-labeled double-stranded oligonucleotides EM0 through EM5, which contain either wild-type (EM0) or mutated sequence (EM1–EM5) of the SMemb/NMHC-B promoter sequence between −111 and −85. Competition was carried out by using either unlabeled self-competitor or EM0. Unlabeled competitors were used at a 500-fold molar excess. C, GST-BTEB2 fusion protein was incubated with either the ^{32}P-labeled double-stranded oligonucleotide EM0 or the consensus binding site for Sp1. Unlabeled EM0, Sp1-binding sequence, CACC box, BTE and CAT box were used as competitors and were added at a 500-fold molar excess. Nucleotide sequence for each oligonucleotide is shown in Table 1. A through C, Arrows indicate sequence-specific protein:DNA complexes, except where noted.

small intestine and lung, whereas a faint band was present in the liver, and no signal was detected in the cerebrum, atrium, ventricle, spleen, skeletal muscle, and kidney. We next examined the expression of BTEB2 mRNA in various cell lines, such as C2/2, NIH3T3, COS-7, and A10 cells. BTEB2 was abundantly expressed in C2/2 cells and scarcely expressed in other cells (Figure 6C). Furthermore, the RNase protection assay revealed that BTEB2 mRNA levels are downregulated in developing rabbit aorta; BTEB2 mRNA is abundantly expressed in fetal aorta but barely detectable in adult aortas (Figure 6D).

BTEB2 Protein Expression in a Developing Aorta and an Injured Aorta

Presence of BTEB2 protein was determined by immunoblot analysis (Figure 7). The specificity of the BTEB2 antibody was confirmed by its ability to recognize GST-BTEB2 fusion protein but not GST (Figure 7A). The BTEB2 antibody detected a major band, the molecular mass of which was ≈35 kDa in C2/2 cells (Figure 7B), although the calculated molecular mass of BTEB2 protein deduced from its cDNA sequence is ≈23 kDa. BTEB2 could be easily detected in neonatal aorta, but not in adult aorta. From these results, we conclude that BTEB2 is expressed in a tissue-restricted and developmentally regulated manner in the aorta. To examine the induction of BTEB2 in injured vessels, balloon injury of rat aortas was performed as described (see Materials and Methods). At 2 weeks after balloon injury, SMCs in the neointima were positive for BTEB2 (Figure 7C) as well as for SM1 and SMemb (data not shown). In the medial SMCs, however, only a few cells adjacent to the internal elastic lamina were positive for BTEB2.

Discussion

We undertook this study as a first approach toward identifying transcription factors that contribute to phenotypic modulation of vascular SMCs. We have recently shown that the 15-bp sequence located between −105 and −91, designated SE1, is important for directing transcription of the SMemb/NMHC-B gene in vascular SMCs but not in other cell types, including NIH3T3 and COS-7 cells.[30] In the present study, using the concatenated SE1 sequence as a probe, we have screened an expression cDNA library prepared from an SMC line. We have isolated the cDNA clones encoding BTEB2, a C_2H_2 zinc finger transcription factor that has previously been cloned from human placenta, and explored the role for BTEB2 in regulating SMemb/NMHC-B gene expression.[32] Our studies have defined BTEB2 as a transcription factor that regulates the SMemb/NMHC-B gene on the basis of the following 4 different criteria. (1) We verified that BTEB2 is a component of nuclear factor:DNA complexes formed with SE1 probe by the supershift assays using the BTEB2 antibody as well as by the gel-shift assays of bacterially expressed GST-BTEB2 fusion proteins. Consistent with the previous report in which BTEB2-binding sequence is similar or identical to Sp1-binding sequence,[38] binding of BTEB2 to SE1 sequence was efficiently competed by excess amounts of unlabeled Sp1 or Sp1-related factor-binding sequences, such as CACC box and BTE. (2) Cotransfection analysis showed

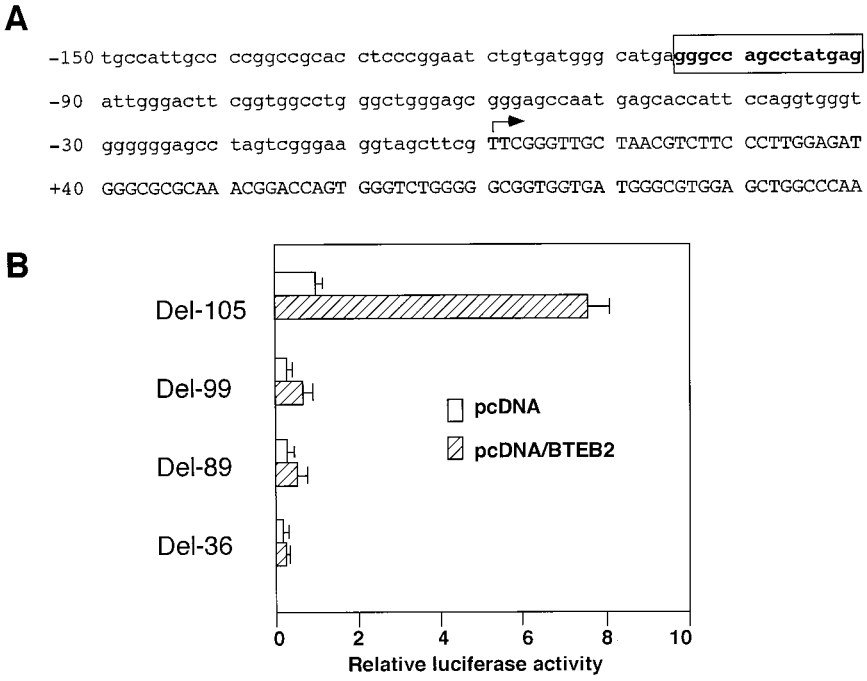

Figure 4. Transactivation of the SMemb promoter by BTEB2. A, Nucleotide sequence of the 5′-flanking region of the rabbit SMemb/NMHC-B gene. Numbers indicate position relative to transcription start site (arrow). Sequence corresponding to SE1 is bold-faced and boxed. B, Transactivation of the SMemb/NMHC-B promoter by BTEB2 was assessed by luciferase assays. Luciferase/reporter plasmids used in these assays were Del-105, Del-99, Del-89, and Del-36, which contain promoter region between −105 and +24, −99 and +24, −89 and +24, and −36 and +24, respectively. Each of the plasmids was transfected into C2/2 cells with either vector alone (pcDNA3) or BTEB2 expression vector (pcDNA/BTEB2). Bars represent relative luciferase activities normalized to protein concentration of the cell lysate and are shown by the relative value to the activity of the Del-105, which is arbitrarily set at 1.0. Transfections were performed in at least 3 independent experiments with duplicate samples. Data are mean±SE.

that BTEB2 significantly activates the SMemb/NMHC-B promoter/luciferase reporter gene. (3) BTEB2 expression is tissue restricted in adult rabbit and developmentally down-regulated in the aorta as assessed by Northern blot and RNase protection analyses. (4) BTEB2 protein is reinduced in the neointimal layer after balloon injury in adult aorta. This pattern of expression correlates remarkably with that of SMemb/NMHC-B.[9] These lines of evidence suggest that BTEB2 participates in the regulation of SMemb/NMHC-B gene expression and may play an important role in modulating the phenotypes of vascular SMCs.

It should be noted that we originally characterized the binding protein to SE1 to be a member of CCAAT binding.[30] However, by modifying the protocol of nuclear extract preparation, we found that besides CCAAT binding protein, Sp1-like proteins could also bind to this element, which turned out to be BTEB2 in the present study.

BTEB2 belongs to the Krüppel family of transcription factors, which contain 3 C_2H_2 zinc finger domains. The family of C_2H_2 zinc finger genes represent a class of DNA binding proteins, many of which have been demonstrated to have roles in regulating transcription in diverse genes.[39,40] BTEB2 has been implicated in the regulation of transcription of eukaryotic genes on the basis of cotransfection analysis using BTEB2 expression vector and SV40 early promoter construct which contains GC-rich sequence.[32] However, cellular genes specifically regulated by BTEB2 and the function of BTEB2 in physiologically relevant context have remained

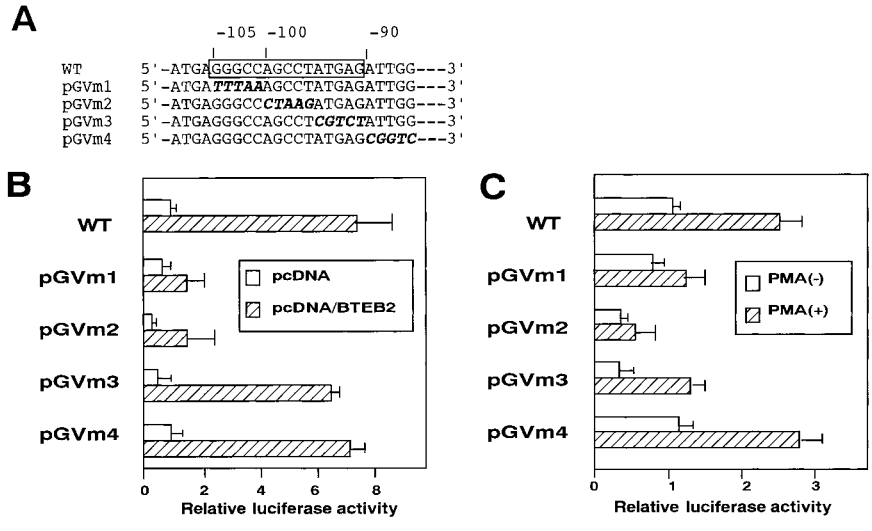

Figure 5. Effects of site-specific mutations on the SMemb/NMHC-B promoter activity. A, Nucleotide sequence between −109 and −86. The wild-type (WT) construct contains SMemb/NMHC-B promoter spanning from −138 to +24 in front of the luciferase gene. Box indicates SE1 sequence; boldface italics, 5 successive mutations that were introduced into −105 to −85 region of the wild-type construct. B, C2/2 cells were transfected with luciferase reporter constructs (WT and pGVm1 through pGVm4) along with either vector alone (pcDNA3) or BTEB2 expression vector (pcDNA/BTEB2). Results are shown as relative luciferase activities normalized to protein concentration of the cell lysate and are shown by the relative value to the activity of the wild-type construct without BTEB2 expression vector. C, C2/2 cells transiently transfected with luciferase reporter constructs (WT, and pGVm1 through pGVm4) were treated with 100 ng/mL of PMA for 12 hours and assayed for luciferase activity. Results are shown as relative luciferase activities normalized to protein concentration of the cell lysates and are shown by the relative value to the activity of the wild-type construct in vehicle-treated control cells. Transfections were performed in at least 3 independent experiments with duplicate samples. Data are mean±SE.

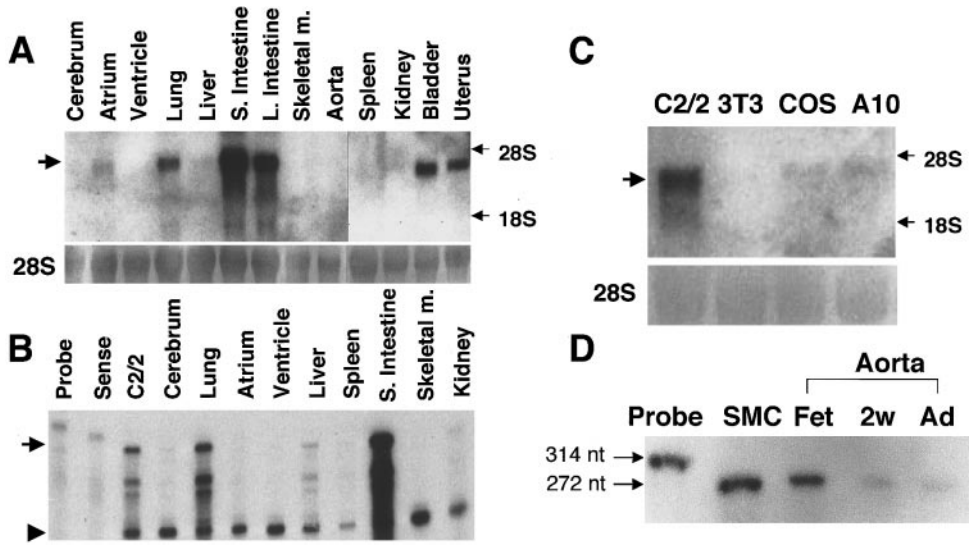

Figure 6. BTEB2 mRNA distribution in adult rabbit tissues and cultured cells. A, Northern blots of total cellular RNA were probed with a ^{32}P-labeled rabbit BTEB2 cDNA and then autoradiographed. S. Intestine, L. Intestine, and Skeletal m. indicate small intestine, large intestine, and skeletal muscle, respectively. Before hybridization with BTEB2 cDNA probe, membranes were stained with methylene blue, and the band corresponding to 28S rRNA was photographed to assess RNA quality, loading, and transfer efficiency. B, RNase protection assay for BTEB2 mRNA in various tissues. GAPDH mRNA was used as an internal control. Arrow and arrowhead indicate BTEB2 and GAPDH, respectively. C, BTEB2 mRNA expression in various cell lines. Northern blot analysis was performed with 20 μg of the total RNA extracted from confluent cultures of indicated cells. Only the C2/2 cell line showed high-level expression of BTEB2. D, Developmental changes in BTEB2 expression in aorta. RNase protection assays were performed with 5 μg of the total RNA extracted from rabbit cultured aortic SMCs or rabbit aortas at fetal stage (Fet), 2 weeks of age (2w), and adult stages (Ad). The band at 314 nucleotides (nt) indicates full protection of the probe, and the band at 272 nt indicates partially protected probe.

to be determined. In the present study, we found that tissue distribution of BTEB2 is clearly different from that of Sp1 in that BTEB2 is abundantly expressed in smooth muscle tissues, whereas Sp-1 is almost ubiquitously expressed.[41] Because there exist many transcription factors that recognize the GC-rich sequence as a binding site, it is difficult to ascribe a specific function to particular members of GC-box binding proteins. In the case of BTEB2, its unique features in tissue distribution and developmental regulation allow us to speculate that BTEB2 plays a role in regulating the SMC genes the expression of which is developmentally regulated. Conclusively establishing a specific role of BTEB2 will need to wait for studies on the consequences of the targeted disruption of this gene.

Recently, several C_2H_2 zinc finger genes that are implicated in the regulation of tissue-specific gene expression have

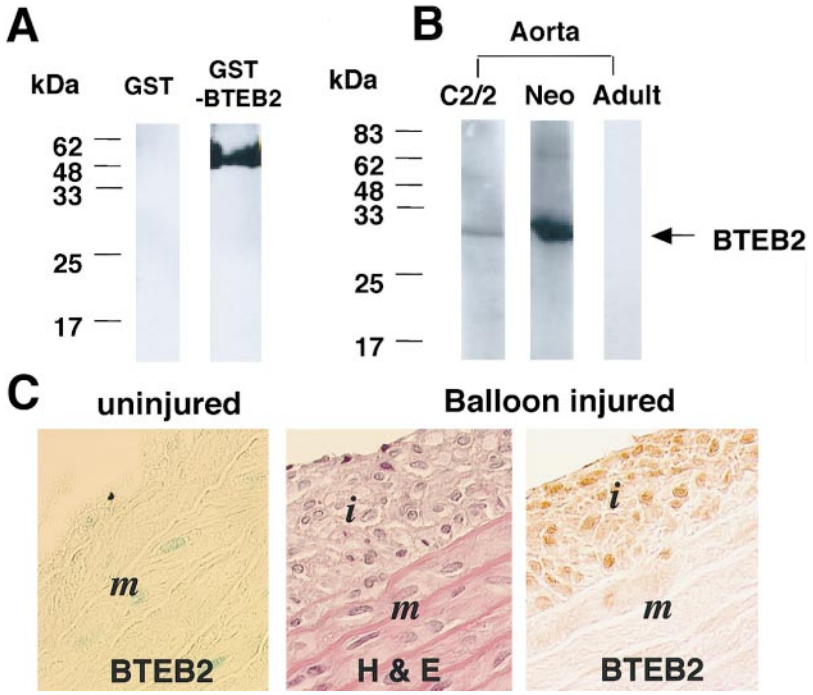

Figure 7. Expression of BTEB2 protein. A, Immunoblots of either GST-BTEB2 fusion protein or GST with anti-BTEB2 antibody. GST-BTEB2 protein was expressed in *E. coli* and purified as described in Materials and Methods. Anti-BTEB2 antibody reacted with GST-BTEB2 fusion protein but not with GST. B, Cell lysates or tissue lysates from either neonatal rat aorta (Neo) or adult rat aorta (Adult) were immunoblotted with anti-BTEB2 antibody. The anti-BTEB2 antibody detected the prominent band with approximate molecular mass of 33 kDa in C2/2 cells and 2-day-old rat aorta but only a faint band, if any, in the adult rat aorta. C, Neointimal cells upregulate BTEB2 expression. Shown are photomicrographs of cross sections of rat aorta stained with hematoxylin and eosin (H & E) or polyclonal antibody against BTEB2 (BTEB2). Cells within the intima (*i*) exhibit a marked increase in BTEB2 immunoreactivity as compared with cells within the media (*m*) 2 weeks after injury. In contrast, BTEB2 immunoreactivity is not evident in uninjured control aorta.

been cloned. The cDNA encoding a Krüppel-related polypeptide, designated EKLF, was shown by both a biochemical and a genetic approach to represent a CACC binding protein that controls the β-globin gene.[42,43] The BKLF/TEF-2, another member of Krüppel family of transcription factors, which binds strongly to CACC box, contains a unique basic region and its expression is less tissue restricted.[44] More recently, 2 other genes that contain structural homology to BTEB2 have been reported, LKLF and GKLF, which are preferentially expressed in lung and gut, respectively.[42,45] The amino acid sequence of the zinc finger domain is remarkably conserved among the Krüppel family of transcription factors. Although any specific function of each of these factors remains to be determined, we observed that both LKLF and GKLF are expressed in the aorta and are able to activate either SMemb/NMHC-B or SM1/2 promoters in transient transfection assays (data not shown). These observations support the notion that certain members of Krüppel-like zinc finger proteins, including BTEB2, LKLF, and GLKF, can potentially play a physiologically significant role in the control of SMC genes.

We also found that BTEB2 expression in the balloon-injured aorta is confined to the neointima, where SMCs exhibit the activated or synthetic phenotype. Recently, the transcription factors that display SMC phenotype-restricted expression have been cloned. HoxB7 and HoxC9 show preferential mRNA expression in fetal SMCs.[46] MEF2A, MEF2B, and MEF2D expression in smooth muscle is associated with phenotypically activated SMCs.[47] Interestingly, like BTEB2, MEF2 expression is upregulated in neointima in a pattern similar to that of BTEB2 after balloon injury. It is tempting to speculate that induced expression of BTEB2, in conjunction with that of MEF2s and other injury-induced transcription factors, such as Ets-1[48] and Egr-1, may play a role in regulating the activated SMC phenotype-specific pattern of gene expression in neointima. Indeed, we have recently found that BTEB2 is capable of activating transcription of the c-jun and Id2 genes, which are implicated in cellular proliferation (M. Kurabayashi et al, unpublished data, 1999). Thus, it is possible that BTEB2 mediates the proliferative response after balloon injury.

In conclusion, we have identified BTEB2 as a transcription factor regulating SMemb/NMHC-B gene expression. Because BTEB2 expression is associated with the activated phenotype of vascular SMCs, our present data should provide the insight into the molecular mechanisms of phenotypic modulation underlying the development of vascular diseases.

Acknowledgments

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BTEB2, a Krüppel-Like Transcription Factor, Regulates Expression of the SMemb/Nonmuscle Myosin Heavy Chain B (SMemb/NMHC-B) Gene

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