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Brain-specific angiogenesis inhibitor 2 regulates VEGF through GABP that acts as a transcriptional repressor

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Abstract Previously, we reported that decreased brain-specific angiogenesis inhibitor 2 (BAI2) induced increased VEGF expression. The regulatory mechanisms for this process are not understood. Here we show that GA-binding protein gamma (GABPγ) associates with the cytoplasmic domain of BAI2, and GABPα/γ or GABPα/β works as a transcriptional repressor of VEGF in SHSY5Y cells. Transcriptional activity of wild-type VEGF promoter was significantly increased in anti-sense BAI2-transfected cells, but not that of VEGF promoter harboring mutated GABP sites. In in vivo focal cerebral ischemia model, the decrease in BAI2 accompanied by decreased GABPα and GABPγ elicited increased VEGF expression before the onset of HIF-1α. Our results point out that BAI2 controls VEGF transcription through GABP under normal conditions and cerebral ischemia.

Keywords: Brain-specific angiogenesis inhibitor 2; Vascular endothelial growth factor; GA-binding protein; Cerebral ischemia; Transcriptional repressor

1. Introduction

Angiogenesis, a process by which new blood vessels are formed from preexisting ones, plays an important role in a wide range of physiological and pathological processes including wound healing and tumor growth, and is controlled by the balance between angiogenic factors and inhibitors. When angiogenic factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor prevail over angiogenesis inhibitors, angiogenesis occurs [1].

GA-binding protein (GABP) is an ETS transcription factor that controls gene expression in cell cycle control, protein synthesis, and cellular metabolism [2]. GABP is unique among ETS factors, since the active complex is an obligate heterotetramer that is composed of two distinct proteins. GABPα includes the ETS DNA binding domain, while two alternate subunits, GABPβ or GABPγ, contain ankyrin repeats and the transcriptional activation domain. The DNA-binding subunit GABPα cannot affect transcription by itself, but can modify GABP-dependent transcription in the presence of its associated subunits, GABPβ and GABPγ, which regulate GABP-mediated transcription by competing with each other [3,4].

Previously, we isolated and characterized the murine homologue brain-specific angiogenesis inhibitor 2 (mBAI2) of human BAI2, which is involved in the early stages of neovascularization of cerebral cortex after ischemia and the predicted structure of the mBAI2 protein suggests that mBAI2 may be a G-protein coupled receptor in the brain [5,6]. To date, however, there are no known ligands to bind and modulate BAI2. The increased VEGF expressions observed in anti-sense BAI2 cDNA transfected cells and during focal ischemia following the decreased BAI2 suggest a potential negative influence of angiostatic BAI2 on the angiogenic VEGF [5]. Thus, we hypothesized that cerebral ischemia could trigger hypoxia-adaptive molecular responses in neurons by suppression of brain-specific angiostatic genes, such as BAI2, and thereby eventually lead to stimulation of angiogenic proteins, such as VEGF.

In this study, we investigated the potential link underlying the inverse correlation between BAI2 and VEGF. A yeast-based two-hybrid assay was used to screen for BAI2-interacting cytoplasmic proteins in a human adult brain cDNA library, and GABPγ was identified as a binding protein to the cytoplasmic domain of BAI2. We found that GABP subunit, GABPα/β or GABPα/γ, acts as a transcriptional repressor in the regulation of VEGF expression at the promoter level. The decreased BAI2 during cerebral ischemia causes suppression of the available GABP subunit, thereby leading to increased VEGF in concert with HIF-1α, a transcriptional enhancer. Our study indicates that the regulation of the GABP by BAI2 is a novel mechanism involved in the control of VEGF expression for ischemia-induced neovascularization of brain as well as under normal conditions.

2. Materials and methods

2.1. Yeast two-hybrid assay

A yeast two-hybrid screening system was used to isolate cDNAs encoding proteins that interact with the cytoplasmic region of BAI2. To generate pLexA fusion constructs (pLexA202-mBAI2 S1/AS1,

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Abbreviations: VEGF, vascular endothelial growth factor; BAI2, brain-specific angiogenesis inhibitor 2; GABP, GA-binding protein; CV-1 cells, Green monkey kidney epithelial cells; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IP, immunoprecipitation; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; MCA, middle cerebral artery; TTC, triphenyl tetrazolium chloride

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S1/AS2, S2/AS1, S3/AS1, S4/AS1, S5/AS1), a cDNA sequence corresponding to each partial cytoplasmic region of mBAI2 was subcloned into pLexA202, and was used to screen a fetal brain cDNA library. The PCR primers: S1, 5′-TCCTGTCTCGTGGGT-3′; S2, 5′-TTCCAACCACC-ACCA-3′; S3, 5′-ACGGTAGCCCACACT-3′; S4, 5′-ACTT-TGAGCCTGCAG-3′; S5, 5′-AACCCTGTGTA-CATG-3′; AS1, 5′-AGCCCAGTGTCATAG-3′; AS2, 5′-GGTCATT-CCATACG-G-3′. The yeast reporter strain EGY48 (p80p-lacz; MAT, his, trp1, ura3, LexAop(x6)-LEU2) was sequentially transformed with pLexA-cytoBAI2 and the pB42-human adult brain cDNA library, and cotransformants were screened as described [7]. Plasmid DNA from positive clones was isolated and sequenced.

2.2. Quantitative β-galactosidase assay

For relative quantification of protein–protein interactions, β-galactosidase assays were performed. pLexA-BD-BAI2 was made by subcloning a partial cytoplasmic region of BAI2 (4247–4572 bp) into pLexA202. Full-length or three specific regions of the GABPγ were fused to pB42-AD. Yeast strains co-transformed by pLexA- and pB42-constructs were grown in supplemented minimal galactose medium lacking uracil, tryptophan and histidine at 30 °C for 24 h. They were then analyzed as described [8]. The activity of β-galactosidase was calculated in Miller units with the formula units = $A_{420} \times 1000/A_{600} \times \text{volume} \times \text{reaction time}$. Each sample was performed in triplicate.

2.3. Cell culture and transfection

SHSY5Y neuroblastoma and Green monkey kidney epithelial (CV-1) cells or human conjunctival (clone 1-5c-4) cells were cultured in DMEM or RPMI1640 containing 10% fetal bovine serum and antibiotic/antimycotic solution. Cells were then transfected with pFLAG-tagged GABPγ, control pFLAG vector or pREP4-anti-BAI2 plasmid [5] using FuGENE 6 (Roche) following the manufacturer's protocol. Colonies surviving in the G418- or hygromycin-containing medium were isolated and analyzed.

2.4. Production of anti-GABPγ antibody and Western blot analysis

GST-GABPγ fusion protein was prepared by subcloning of C-terminal portion containing the GABPγ-specific region (279–360 amino acid residues) into the pGEX-4T as previously described [7], and rabbit antiserum recognizing GABPγ was prepared using this protein. The serum recognizing GABPγ was filtered through a column of GST-GABPγ fusion protein, and the column was eluted with a low-pH buffer. It was then filtered through a column of GST protein to remove the anti-GST antibody component. Cell lysates were prepared from rat cerebral cortex tissues, resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred, and blotted with anti-BAI2 serum [5], anti-GABPγ serum, anti-GABPα, anti-GABPβ (Santa Cruz), anti-VEGF (NeoMarkers), or anti-HIF-1α (a gift form Dr. J.W. Park), and anti-rabbit, anti-goat, or anti-mouse Ig-HRP (Amersham) as described [5].

2.5. GST pull-down and immunoprecipitation (IP) assay

Escherichia coli BL21 cells transformed with each of pGEX-4T-BAI2-cytoplasm fusions were grown at 37 °C, and synthesis was induced by addition of 0.2 mM isopropyl-β-D-galactopyranoside as a final concentration. The GST-fusion proteins were isolated with glutathione-Sepharose-4B beads (Pharmacia Biotech), washed and incubated with [^{35}S] methionine-labeled GABPγ produced by in vitro translation using the TNT-coupled transcription–translation system (Promega). Bound proteins were eluted, analyzed by SDS–PAGE and autoradiographed.

IP was performed as previously described [7]. Briefly, SHSY5Y cells were resuspended in RIPA buffer, and solubilized supernatant was incubated with protein A/G beads and preimmune or anti-BAI2 serum for 2 h. Immunoprecipitates were resolved by SDS–PAGE, transferred and blotted with anti-GABPγ or anti-GABPα (Santa Cruz) and anti-rabbit or anti-goat Ig-HRP (Amersham).

2.6. VEGF promoter-reporter assay

A 2951-bp fragment containing the 5′ flanking region and transcriptional start site of the VEGF gene (−2362 ∼ +589 bp) was prepared by genomic PCR [9], and cloned into the pGL3-Basic vector (Promega) to make the VEGF-Luc promoter construct. Also, GABP binding site-directed mutagenesis of the VEGF-Luc promoter construct was performed as follows. GABP m1 and GABP m2 were generated by replacing the GABP binding element TTCCCT in −952/−947 and CGGATG in −236/−231 with GAATTC and CTCGAG, respectively. GABP m1 and m2 was prepared by replacing both sites.

CV-1 cells or stably anti-BAI2 transfected SHSY5Y cells were transfected with the indicated amount of expression plasmids (pGL3-VEGF-Luc with pcDNA3-GABPα, β, γ) and the pCMVβ as an internal control by using FuGENE 6. Total amounts of DNAs were kept constant by adding appropriate amounts of pcDNA3. At 48 h after transfection, cells were washed, harvested and assayed with the Dual Luciferase Reporter Assay System (Promega) as described [10]. The transfection experiments were performed at least three times. Data are presented as means ± S.E.M. of 3–4 independent experiments with triplicate dishes. The relative fold induction in each luciferase activity was determined by calculating the GABP/vector ratio when the vector (VEGF-Luc) activity was set at 1.

2.7. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with the following DNA probes and their complementary strands. The synthetic oligonucleotides were labeled and incubated for 30 min at room temperature with 10 μg of nuclear extract from CV-1 cells in a 20 μl reaction containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 1% Ficoll and 2.0 μg poly (dI–dC). For supershift experiments, reactions were preincubated for 20 min on ice with anti-GABPα or preimmune serum. Products were analyzed by SDS–PAGE, dried and autoradiographed.

2.8. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed on SHSY5Y and human conjunctival cells with ChIP assay kit (Upstate) largely following the supplier's recommendations. Cells grown on 100-mm dishes were collected and cross linked using 1% formalin for 10 min at room temperature, and the extracts were sonicated until the DNA fragments were 500–800 bp in size. Cell extracts were subsequently incubated with no antibody, 2 μg IgG or antibody against GABP alpha overnight at 4 °C. The extracts were incubated with salmon sperm DNA/protein A-sepharose beads (Upstate) for 1 h. After extensive washing of the beads, proteins were eluted and reversed by heating for 4 h at 65 °C. After DNA purification, PCR was performed at 35–40 cycles. Primers were: GABP site 1 (211 bp); 5′-TCCCCCTTTGGGTTTTGCCAGA-3′ (sense), 5′-TCAAAGTGAGCGGCAGCCAA-3′ (antisense), GABP site 2 (200 bp); 5′-AGAGGGAACGGCTCTCAGGC-3′ (sense), 5′-CTCTGCGGACGCCCAGTGAA-3′ (antisense).

2.9. RT-PCR

Total RNAs were prepared from cultured cells and reverse transcription was performed as described [5,7]. The annealing temperature was 58 °C for GABPα, GABPβ and GABPγ, and 60 °C for BAI2, VEGF, HIF-1α, β-actin and GAPDH. The amplification products were analyzed on agarose gels and visualized by ultraviolet epifluorescence. PCR primers: S1, 5′-GTGTCCAGCCTTCCATGAGATG-3′ and AS1, 5′-TTTCCGCATCCACCATGAAGC-3′ for hBAI2; S1, 5′-GCAGACCAAAG-AAAGATAGAGCAAG-3′ and AS1, 5′-CGCC-TCGGCTTGTCACAT-3′ for hVEGF; S1, 5′-AACATGATGGTT-CACTTTTTCAAGC-3′ and AS1, 5′-GTCAGCTGTGG-TAATCC-ACTTTCAT-3′ for hHIF-1α; S1, 5′-ACTACAGTGTTCTTTGG-ATGCTCATG-3′ and AS1, 5′-TGTGGACCACTGTATGGGATC-ATAGG-3′ for hGABPα; S1, 5′-GAATTCAGCTTGGAAATTTGC-ACT-3′ and AS1, 5′-TTAAACAGCTTCTTT-ATTAGTCT-3′ for hGABPβ; S1, 5′-CATCCAAGGC-AACAGATGAAACGG-3′ and AS1, 5′-TTGGATGACTGCGGCAAAGCACAC-3′ for hGABPγ, respectively.

2.10. Focal ischemia model in rat

Sprague-Dawley rats (200 g) were anesthetized with 4% halothane in an anesthetic chamber and maintained with 1% halothane in 100% O_2 using a rodent mask. Operation for focal ischemia was performed as described [5]. The rat was sacrificed after the time course of ischemia (0, 1, 2, 4, 8, 24 h) without reperfusion. The brain slices were stained with 2% triphenyl tetrazolium chloride (TTC) to visualize and measure

the infarct volumes in each group. The part of core infarct area due to the occlusion of middle cerebral artery (MCA) and the corresponding portion of sham-operated cerebral cortex were selected for production of protein.

2.11. Statistics

Experimental differences were tested for statistical significance using ANOVA and Students' *t*-test. A P value of less than 0.05 was considered significant.

3. Results and discussion

3.1. Interaction of GABPγ with the cytoplasmic region of mBAI2

The yeast two-hybrid system was used to identify proteins that associate with the cytoplasmic region of mBAI2. Initially, we found that the C-terminal residues (3830–4572 bp) of mBAI2 had auto-transcriptional activity. Six mBAI2 cytoplasmic protein fragments were examined to find the source of the auto-transcriptional activity (Fig. 1A) and nucleotide residues 4247–4572 of mBAI2 showed the least β-gal activity and were used as bait (Fig. 1B). From 6×10^5 transformants, several positive clone were isolated, one of which was hGABPγ.

Next, we examined the binding of mBAI2 to GABPγ in cultured cells. The in vitro translated GABPγ protein (~38 kDa) was found to interact with GST–BAI2 fusion proteins by pull-down assay (Fig. 1C). For IP analysis, we obtained stably GABPγ-overexpressing SHSY5Y neuroblastoma cells, which express endogenous BAI2 (Fig. 1D). After immunoprecipitating this cell lysate with anti-BAI2 serum, proteins were blotted with anti-GABPγ antibody and GABPγ was detected, indicating that GABPγ interacted with the cytoplasmic region of BAI2 (Fig. 1E). Interestingly, GABPα, which makes a heterodimeric complex with GABPγ, was found when the same blot was reprobed with goat anti-GABPα antibody (Fig. 1F). It indicated that GABPα also bound to the cytoplasmic region of BAI2 through interaction with GABPγ.

3.2. Full-length of GABPγ has high affinity to BAI2

To determine which part of GABPγ is essential for interaction with the BAI2, full-length and three specific regions of GABPγ (N-terminal portion containing ankyrin repeats, middle portion common to GABPγ and GABPβ, and C-terminal portion containing the GABPγ-specific region) were fused to pB42-AD (Fig. 2A). Co-transformation with pLexA-BD-BAI2 demonstrated that the whole region of GABPγ was necessary for strong interaction with the cytoplasmic region of BAI2 (Fig. 2B).

3.3. Expression of VEGF in GABPγ-transfected SHSY5Y cells

Our previous study demonstrated increased VEGF expression in SHSY5Y cells transfected with anti-sense BAI2 [5].

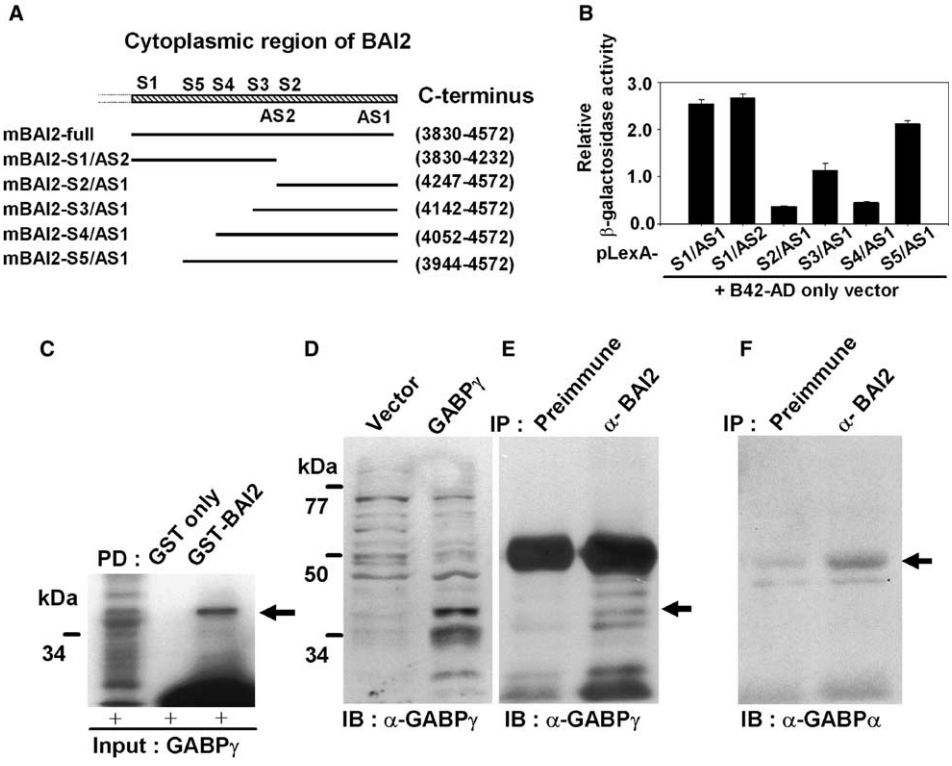

Fig. 1. Direct interaction of GABPγ with the cytoplasm region of BAI2. (A) Schematic representation of cytoplasmic fragment of BAI2 used as bait in yeast two-hybrid system. Full-length or deletion variants of the BAI2 cytoplasmic region are shown as lines. Numbers in parentheses are nucleotide residues. (B) To examine autotranscriptional activity, full-length or deletion variants of the BAI2 cytoplasmic region were cotransformed with pB42-AD empty vector, and β-galactosidase activities were measured. (C) GST–BAI2 fusion protein bound to glutathione-agarose beads was incubated with equivalent amounts of the ^{35}S-labeled GABPγ produced by in vitro translation, resolved by SDS–PAGE, and autoradiographed. Ten percent of the labeled proteins used in binding reaction were loaded as input. An arrow indicates GABPγ protein (~38 kDa) that interacted with GST–BAI2. (D) SHSY5Y cells were transfected with pFLAG-GABPγ or the control vector, and immunoblotted with anti-GABPγ antibody. (E) Each cell lysate in (D) was immunoprecipitated with anti-BAI2 serum, and blotted with anti-GABPγ antibody. An arrow indicates GABPγ. (F) The blot in (E) was reprobed with anti-GABPα antibody. An arrow indicates the ~52 kDa GABPα, which overlapped in size with rabbit IgG.

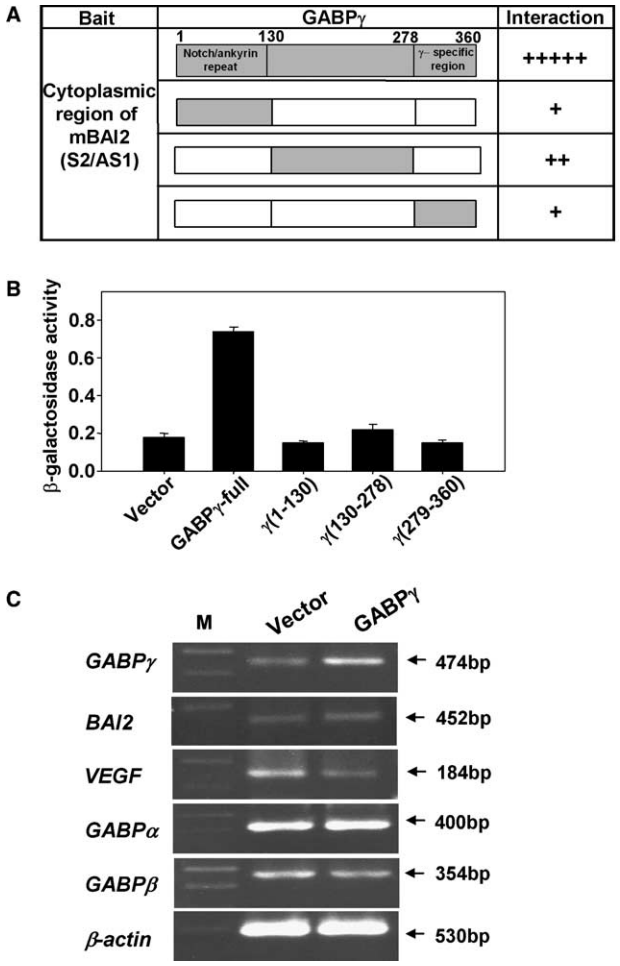

Fig. 2. Characteristic binding of GABPγ with BAI2 and effects of overexpressed GABPγ. (A) Specific regions of the GABPγ are shown. (B) The regions of GABPγ required for the interaction with cytoplasmic domain of BAI2 were determined by assessing β-galactosidase activity. Numbers in parentheses are the number of amino acids. (C) RT-PCR analysis of BAI2 and VEGF mRNA in SHSY5Y cells stably transfected with GABPγ. Expression of β-actin confirms the relative amounts and fidelity of the RNA. M, molecular size marker.

To test the causal link between GABPγ and VEGF, RT-PCR analyses were performed in SHSY5Y cells, which were stably transfected with a GABPγ construct (Fig. 2C). In GABPγ-overexpressed cells, *GABPγ* expression increased, whereas *BAI2* did not change. However, *VEGF* expression decreased, indicating that GABPγ might suppress the VEGF expression. Expression of *GABPα* was unchanged, whereas *GABPβ* decreased slightly (Fig. 2C).

3.4. Suppression of VEGF expression by GABP and binding of GABPα to VEGF promoter

Because overexpressed GABPγ, which makes a heterodimer with GABPα, suppressed VEGF expression (Fig. 2C), CV-1 cells were transiently co-transfected with VEGF-luciferase construct and increasing doses of GABP subunits, either GABPα/β or GABPα/γ, which enable GABPα to enter the nucleus and stimulate target gene transcription [3,4]. Transfection of GABP subunits significantly decreased the transcriptional activity of VEGF (Fig. 3A).

To elucidate whether the GABPα transcription factor actually binds to VEGF promoter, gel mobility-shift assays were performed. Inspection of *VEGF* 5′-flanking sequences by bioinformatics analysis revealed two potential GABP binding sites. Potential GABP binding site 1 (−960/−941, AGGTCCTC-TTCCCTCCCAGT) was in the reverse orientation, whereas potential GABP binding site 2 (−243/−224, CCGGGGG-CGGATGGGTAATT) was in the same orientation as the transcriptional unit. Two double-stranded DNA probes that corresponded to these two sites were used in EMSA with CV-1 cell nuclear extracts, which express high levels of GABPα. Two species from CV-1 cells were found to bind with the GABP binding site 1 (Fig. 3B, left column) and 2 (Fig. 3B, right column). Similar two complexes were reported to be formed between VEGF promoter and GABPα monomer/dimer [11]. Thus, the upper/lower bands corresponded to the dimeric/monomeric complex, respectively. In addition, two bands were abrogated by an anti-GABPα antiserum, indicating that GABP subunits participated as transcription factors in the regulation of VEGF expression at the promoter level. In addition, bindings of endogenous GABPα to the each GABP binding site (sites 1 and 2) on the VEGF promoter sequences were confirmed by chromatin immunoprecipitation (Fig. 3C).

To observe whether the mutation of putative GABP binding sites in the VEGF promoter sequence results in a loss of transcriptional repression in response to GABP subunits, CV-1 cells were transiently cotransfected with wild-type VEGF-Luc promoter construct or mutated VEGF-Luc promoter construct that has changed GABP binding site and increasing amounts of either GABPα/β or GABPα/γ expression plasmids. Transfection of GABPα/γ or GABPα/β, significantly decreased the transcriptional activity of wild-type, GABP m1 or GABP m2 VEGF promoter, but not that of GABP m1 and m2 VEGF promoter (Fig. 3D), indicating that GABPα acts as a transcriptional repressor of VEGF expression.

3.5. Increased VEGF expression in anti-sense GABPα- or anti-sense GABPγ-transfected SHSY5Y cells

To test that decreased GABP binding on the VEGF promoter sequence results in activated VEGF promoter activity and thereby increased *VEGF* expression, RT-PCR analysis of *VEGF* was performed in SHSY5Y cells stably transfected with anti-sense GABPα or GABPγ construct. The expression of *GABPα* or *GABPγ* decreased, whereas *VEGF* increased in anti-sense GABPα or GABPγ transfected SHSY5Y cells compared with vector transfected cells, though the magnitude of increment was larger in anti-sense GABPα transfected cells (Fig. 4A). However, expression of *BAI2*, *GABPβ* or *HIF-1α* did not change by decreased GABP binding on the VEGF promoter (Fig. 4A). It confirms that GABPα/γ acts as repressive transcriptional regulator on VEGF promoter activity.

3.6. Loss of BAI2 function leads to increased transcriptional activity of VEGF promoter in SHSY5Y Cells

To observe the causal link between decreased BAI2 expression and increased VEGF promoter activity, stably anti-sense BAI2 transfected SHSY5Y cells were transiently cotransfected with wild-type VEGF-Luc promoter or GABP binding site-mutated VEGF-Luc promoter. In the stably anti-sense BAI2-transfected SHSY5Y cells, expression of endogenous *VEGF* increased (Fig. 4B) as reported [5]. After cotransfection, the transcriptional activity of wild-type VEGF promoter was

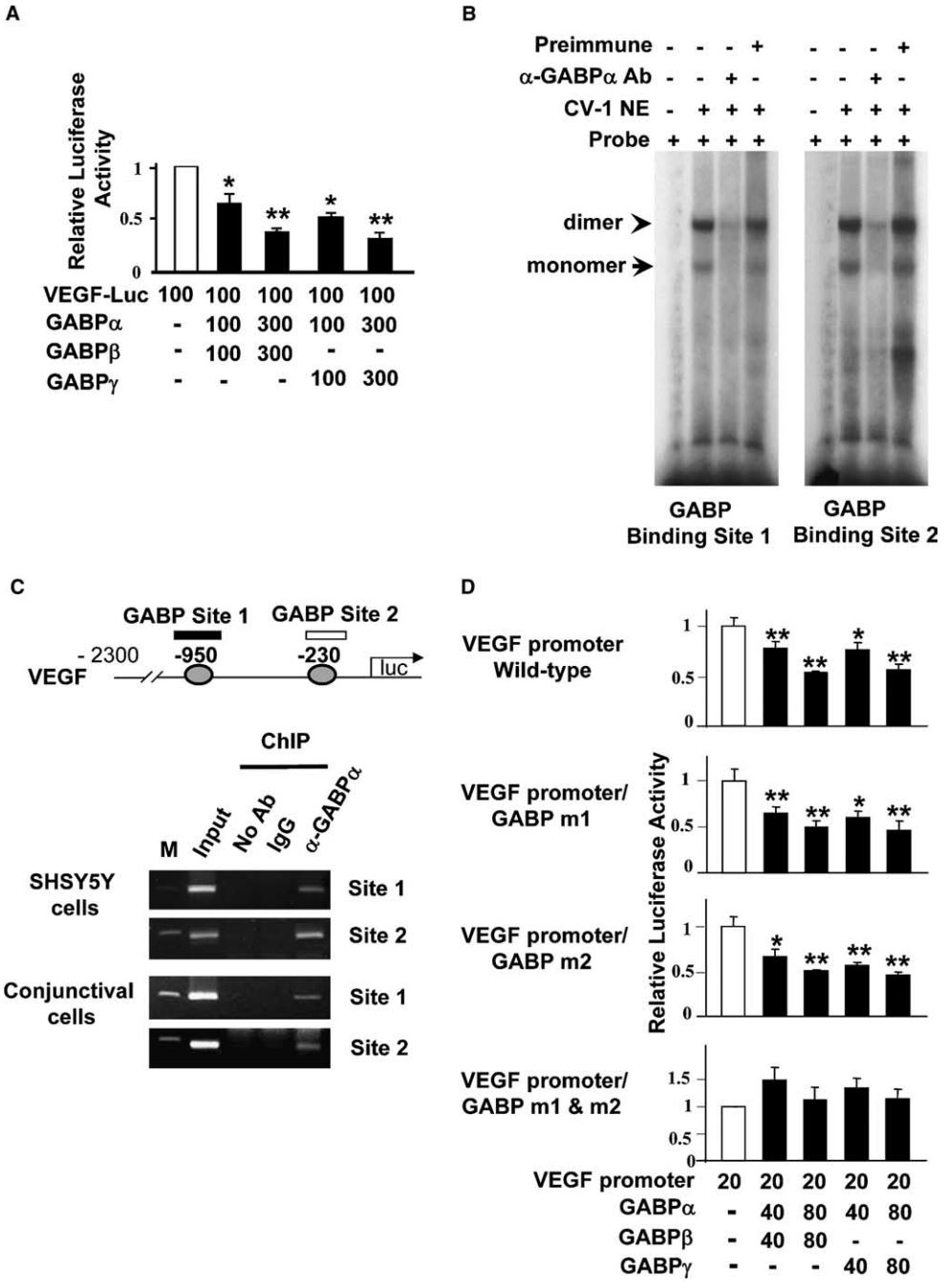

Fig. 3. GABP subunits act as transcriptional repressor for VEGF expression. (A) CV-1 cells were transiently cotransfected with VEGF-Luc promoter and increasing doses of either GABPα/β or GABPα/γ with CMV-β-gal as an internal control. After 48 h, luciferase activity was measured. All values are means ± S.E.M. ($n = 3$). Asterisks indicate significant differences between vector and GABP subunits group ($^{*}P < 0.05$, $^{**}P < 0.01$). (B) EMSA was performed with ^{32}P-labeled double-stranded oligonucleotide probe for the GABP binding site 1 (lanes 1–4) or the site 2 (lanes 5–8) of VEGF promoter using nuclear extract of CV-1 cells. Two complexes, upper (arrow head) and lower (arrow) bands, were formed with GABP binding sites 1 and 2. (C) ChIP assays showed that GABPα binds to the GABP binding sites on human VEGF promoter sequences in the SHSY5Y and human conjunctival cells. GABP site 1 or 2 represents promoter region containing of 5′ (−950)- or 3′ (−230)-GABP binding site on promoter sequence (upper column). (D) CV-1 cells were transiently cotransfected with wild-type VEGF-Luc promoter or mutated VEGF-Luc promoters having changed GABP binding site (GABP m1: TTCCCT in −952/−947 was replaced; GABP m2: CGGATG in −236/−231 was replaced; GABP m1 and m2: both sites were replaced) and increasing doses of either GABPα/β or GABPα/γ with CMV-β-gal. All values are means ± S.E.M. ($n = 4$).

significantly more increased, but not that of GABP m1 and m2 VEGF promoter (Fig. 4C) in the BAI2-suppressed SHSY5Y cells than that of in the control SHSY5Y cells. It indicates that angiostatic BAI2 suppresses the VEGF promoter activity through GABP transcription factors in cultured neuronal cells under normal condition.

3.7. Changes of BAI2 and GABP resulted in increased VEGF in focal cerebral ischemia model in rat

Because there are no known ligands to bind with BAI2 and modulate BAI2 activity, we examined the changes of GABPs and VEGF in an in vivo rat focal cerebral ischemia model, in which we observed the decreased BAI2 expression following

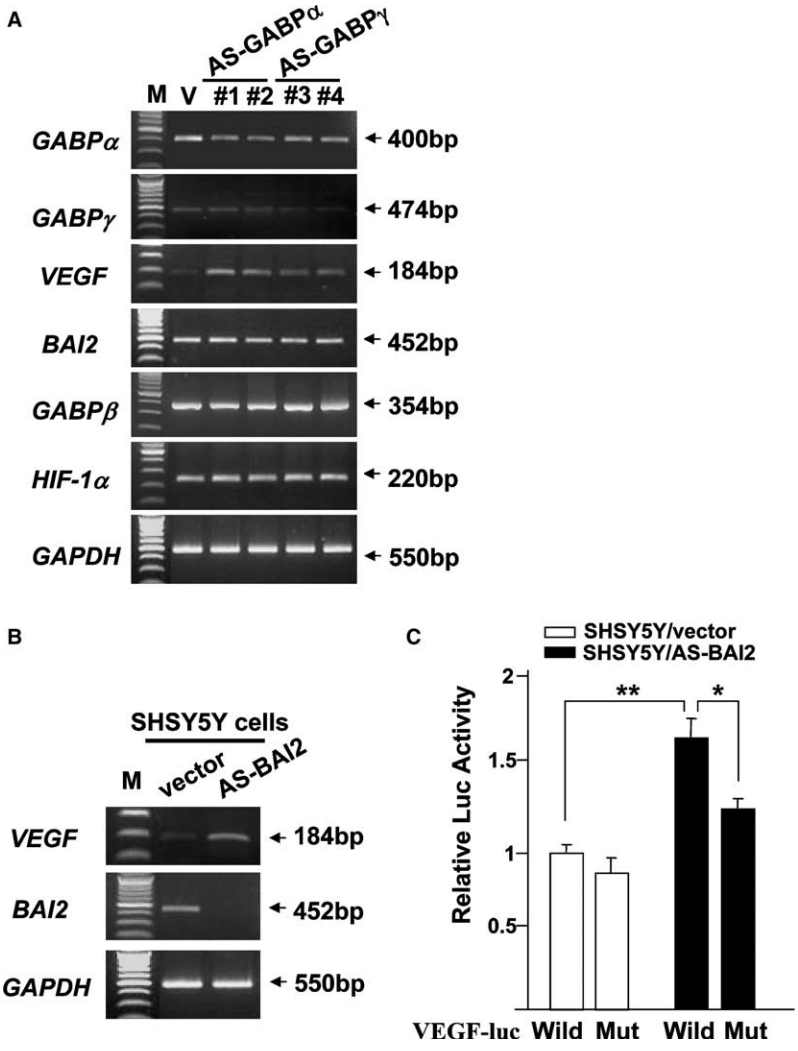

Fig. 4. Loss of GABPα/γ or BAI2 function leads to increased transcription of the endogenous VEGF gene or the exogenous wild-type VEGF promoter but not in the GABP site-mutated one. (A) RT-PCR analyses of *VEGF*, *BAI2* and *HIF-1α* were performed in SHSY5Y cells stably transfected with anti-sense GABPα (#1, #2) or GABPγ (#3, #4) construct. Expression of *GAPDH* confirms the relative amounts and fidelity of the RNA. (B) The endogenous *VEGF* expression was increased in the stably anti-sense BAI2-transfected SHSY5Y cells. (C) The transcriptional activity of wild-type VEGF promoter (Wild) or mutant VEGF promoter (Mut, GABP m1 and m2) was measured in anti-sense BAI2-transfected SHSY5Y cells. The results are means ± S.E.M. ($n = 3$). Asterisks indicate significant differences in relative luciferase activity between groups.

time window of MCA occlusion [5]. At first, we examined the ischemic region of whole brain by TTC staining at 8 and 24 h of the right MCA occlusion (Fig. 5A). The ischemic area increased with time-dependent manner in the right ipsilateral hemisphere.

Western blot analyses were done to observe the changes of BAI2, GABPs, VEGF and HIF-1α expressions in ischemic cerebral tissues. BAI2 decreased in the ischemic area after 1 h compared with in the control cerebral cortex and the decreased level was maintained until 4 h, but recovered at 8 h (Fig. 5B). BAI2 level was significantly decreased throughout all experimental periods compared with that of control (Fig. 5C). GABPα initially dropped at 1 h, but recovered at 2 h. But, after 4 h, it decreased again until 24 h. GABPγ showed the same expression pattern throughout the time window of ischemia as GABPα. However, GABPβ increased in the ischemic tissues throughout the time window. VEGF increased in the ischemic cortex after 1 h, peaked at 8 h, and it returned to basal level at 24 h after ischemia (Fig. 5B). Actually, there seems to recover to basal level in GABPα and GABPγ but a little decreased level of VEGF at 2 h compared with that of 1 or 4 h (Fig. 5C). HIF-1α was induced from 2 h and peaked at 8 h, but recovered to control level at 24 h (Fig. 5B). Taken together, suppression of the available GABP transcription factors led to the slightly increased VEGF expression in the early phase of cerebral ischemia (around 1 h) before the onset of HIF-1α. However, in the late phase (around 8 h), nearly dropped GABPα seemed to work together with increased HIF-1α to create a peak in VEGF expression. Therefore, our results showed that both decreased GABP repressors and increased HIF-1α enhancer affect VEGF transcription in focal ischemia-induced angiogenesis of cerebral tissues, and that a functional significance of GABP repressors as an additional factor in the regulation of angiogenesis after cerebral ischemia.

Both GABPβ and GABPγ contain a nuclear localization signal (NLS) and are localized predominantly in the nucleus regardless of the presence of GABPα. GABPα lacks an NLS and its entry into the nucleus absolutely requires the concom-

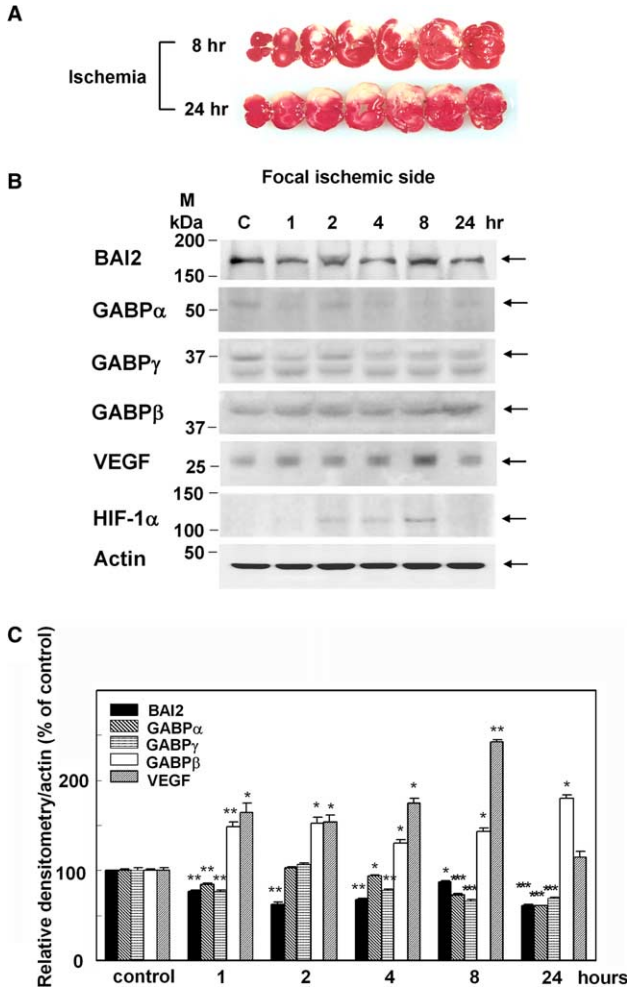

Fig. 5. Analyses of BAI2, GABP and VEGF in the in vivo focal ischemic cerebral tissues. (A) The representative staining of infarct area in focal ischemic rat cerebral tissues. MCA was occluded in given times (8 and 24 h) and the brain slices were stained with 2% TTC to visualize the infarct (white color) and non-infarct area (red color). (B) Western blot analyses were performed for time sequencial expressions of BAI2, GABPs, VEGF and HIF-1α in the focal ischemic rat cerebral tissues. C is sham-operated cerebral cortex. The same blot was reprobed with anti-actin antibody to control for loading. The data show one of three representative experiments. (C) Densitometric analyses of Western blot for BAI2, GABP and VEGF, which levels were normalized with respect to the corresponding actin level. The values are means ± S.E.M. ($n = 3$). Asterisks indicate significant differences in relative densitometric level (ratio of BAI2, GABP or VEGF versus actin value) of each period compared with control ($^{***}P < 0.001$).

itant expression of either GABPβ or GABPγ. So, GABPβ or GABPγ should bind GABPα in the cytoplasm to assist GABPα transport into the nucleus. Thus, GABPα acts as a transcription factor only after forming a complex with its partner protein GABPβ or GABPγ [3,4,12]. In this regard, our present results suggest that suppression of the available GABP by the deactivated BAI2 after ischemia leads to the depletion of the available GABPα for nuclear GABPα2/β2 heterotetramer or GABPα/γ heterodimer formation (Fig. 6), both of which act as transcriptional repressors for VEGF that contributes mainly to the neovascularization after ischemia [13]. Collectively, there exists a reciprocal relationship between BAI2 and VEGF in the brain angiogenesis in which angiostatic BAI2 regulates

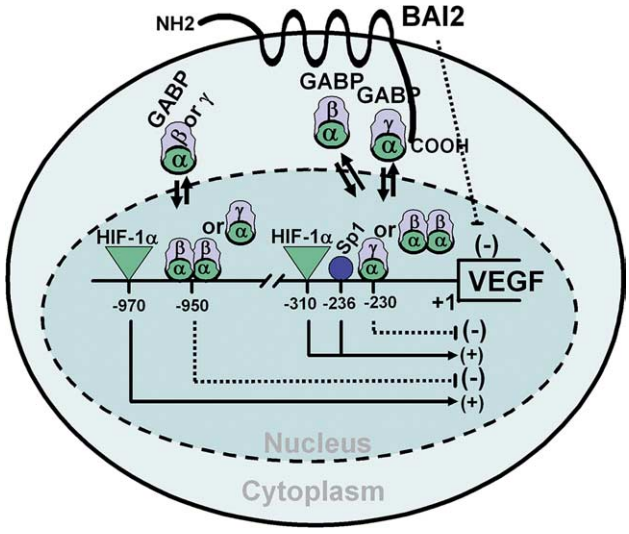

Fig. 6. A schematic diagram showing the transcriptional regulation of VEGF expression. It indicates that BAI2/GABP and HIF-1α act as transcriptional repressors and enhancer of VEGF, respectively.

VEGF expression through GABPs after cerebral ischemia as well as under normal conditions.

The hypoxia-induced upregulation of VEGF expression is mediated both by activation of VEGF transcription through HIF-1α and enhanced VEGF mRNA stability [14–16]. Also, there are many very well characterized angiogenic and angiostatic factors that can modulate VEGF expression, in which most of them are transcriptional activators [17]. In this study, it seems that the contribution of BAI2–VEGF transcription through GABP transcription factors after cerebral ischemia appears to be marginal (Fig. 5C) compared to that of HIF-1α from other studies [15,16]. However, our present study shows for the first time that GABP acts as a transcriptional repressor in the regulation of VEGF expression and this finding is potentially important for better understanding the regulation of VEGF, one of the most specific and critical regulators of angiogenesis.

It was reported that GABP regulates gene expression through its interaction with other transcription factors and co-activators bound to the cognate motifs in the vicinity of the GABP site, such as Sp1 [2,18]. The VEGF promoter was found to contain two HIF-1 binding sites (−975/−968, −313/−306) [15] and one Sp1 binding site (−240/−232) in the vicinity of the 5′ (−960/−941) and 3′ (−243/−224) GABP binding sites (Fig. 6). It is supposed that GABP bound at the 3′ binding site and Sp1 may cooperate to regulate the VEGF promoter [17]. Studies are undergoing to find the mechanisms for the regulation of VEGF promoter by cooperation of GABP with these transcription factors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.12.086.

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