Cooperative Interaction of Hypoxia-inducible Factor-2α (HIF-2α) and Ets-1 in the Transcriptional Activation of Vascular Endothelial Growth Factor Receptor-2 (Flk-1)*

Received for publication, November 5, 2002, and in revised form, November 26, 2002
Published, JBC Papers in Press, December 2, 2002, DOI 10.1074/jbc.M211298200

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Interactions between Ets family members and a variety of other transcription factors serve important functions during development and differentiation processed, e.g. in the hematopoietic system. Here we show that the endothelial basic helix-loop-helix PAS domain transcription factor, hypoxia-inducible factor-2α (HIF-2α) (but not its close relative HIF-1α), cooperates with Ets-1 in activating transcription of the vascular endothelial growth factor receptor-2 (VEGF-2) gene (Flk-1). The receptor tyrosine kinase Flk-1 is indispensable for angiogenesis, and its expression is closely regulated during development. Consistent with the hypothesis that HIF-2α controls the expression of Flk-1 in vitro, we show here that HIF-2α and Flk-1 are co-regulated in postnatal mouse brain capillaries. A tandem HIF-2α/Ets binding site was identified within the Flk-1 promoter that acted as a strong enhancer element. Based on the analysis of transgenic mouse embryos, these motifs are essential for endothelial cell-specific reporter gene expression. A single HIF-2α/Ets element conferred strong cooperative induction by HIF-2α and Ets-1 when fused to a heterologous promoter and was most active in endothelial cells. The physical interaction of HIF-2α with Ets-1 was demonstrated and localized to the HIF-2α carboxyl terminus and the autoinhibitory exon VII domain of Ets-1, respectively. The deletion of the DNA binding and carboxyl-terminal transactivation domains of HIF-2α, respectively, created dominant negative mutants that suppressed transactivation by the wild type protein and failed to synergize with Ets-1. These results suggest that the interaction between HIF-2α and endothelial Ets factors is required for the full transcriptional activation of Flk-1 in endothelial cells and may therefore represent a future target for the manipulation of angiogenesis.

The proliferation of vascular endothelial cells (which line the inner surface of all blood vessels) is a key mechanism of vascular growth involved in normal embryonic development and adolescence but is also a prerequisite of tumor growth and the progression of other destructive diseases (1, 2). Endothelial cell proliferation is mediated primarily by vascular endothelial growth factor (VEGF); also known as VPF, vascular permeability factor) signaling via its high affinity tyrosine kinase receptors. The VEGF receptors are expressed prominently on endothelial cells, thus establishing the cell specificity of VEGF. VEGF signaling is also important for behavioral changes of the capillary endothelium during blood vessel growth, such as invasive migration, matrix degradation, and sprouting, and also for the survival of endothelial cells in immature blood vessels. VEGF is induced in response to hypoxia and/or low intracellular glucose, thus stimulating neovascularization in accordance with the increasing metabolic demands of a growing tissue (3–5). The function of the high affinity VEGF receptors is not redundant. Although VEGF receptor-1 (Flt-1) is necessary for early embryonic vascular integrity (6), VEGF receptor-2 (KDR in humans/Flk-1 in mouse) is indispensable for the formation of the primordial vasculature during early embryogenesis; mouse embryos with targeted disruption of the Flk-1 gene fail to differentiate both blood and endothelial cells and die as a consequence of avascularity (7). Consistent with this phenotype, Flk-1 is strongly expressed by the mesodermal precursors of both endothelial and hematopoietic cells (hemangioblasts) (8, 9). Later on, during ontogeny, Flk-1 is expressed primarily on proliferating endothelial cells but becomes largely down-regulated in many vascular domains in the adult. However, Flk-1 expression is reactivated in newly forming blood vessels, e.g. in the corpus luteum, and in neoplastic lesions that express high amounts of VEGF, such as glioblastomas (10, 11). The level of VEGF receptor expression appears to be influenced by the availability of their ligand VEGF, which is secreted upon
tissue vascularization (12, 13). Signaling in the embryonic endoderm has been demonstrated convincingly only for Flk-1, whereas the role of Flt-1 in the developing vasculature is still puzzling (14, 15). Although other signaling systems, such as angioptietins/tie2 receptor and ephrins/ephrin receptors, as well are indispensable for vascular differentiation (16–18), the role of Flk-1 signaling is pivotal because inhibition of Flk-1 signaling, but not of any other receptor, prevents vascular development from its beginning and consistently inhibits tumor vascularization (2). Therefore, an understanding of the mechanisms that regulate the expression of Flk-1 in the endoderm would provide general insights into the mechanisms of vascular development in health and disease and might open up new approaches to the as yet frustrating therapy of ischemic and proliferative malignancies.

We recently characterized the essential regulatory elements of the Flk-1 gene (19). A 939-bp 5′-flanking region together with a 430-bp minimal enhancer from the first intron was essential and sufficient to convey endothelial cell-specific expression of the lacZ reporter gene in the transgenic mouse. By mutational analysis in transgenic animals, distinct SCL/TAL-1, GATA, and Ets consensus sites within the enhancer were shown to be essential for Flk-1 gene expression (20), indicating that members of the SCL, GATA, and Ets transcription factor families are not only important regulators of hematopoiesis (21) but also act upstream of Flk-1 expression during development. Consistently, members of all three families are expressed by endothelial cells and have been implicated in vascular development in the embryo and in tumor angiogenesis. SCL, a candidate master regulator of hematopoiesis, appears also to be involved in early endothelial cell differentiation and possibly acts upstream of Flk-1 at least in zebrafish (22–24). Ets factors stimulate the expression of a variety of endothelial cell-specific genes that are involved in vascular growth and sprouting, including urokinase, metalloproteinases, flt-1, tie-1, and tie-2 (25–30). Ets-1 is highly expressed in the lateral mesoderm when Flk-1 starts to be expressed in vascular endothelial cell precursors (31). In addition, the up-regulation of Ets-1 in capillary-invading tumors can be correlated with re-expression of VEGF receptors (32). Also the Ets family members NERF2 and TEL appear to serve important functions during angiogenesis (29, 30, 33). Others are important for cell lineage determination of lymphoid and mononuclear cells such as PU.1 (34) or may play a critical role in the differentiation of epithelia, such as ESE-1 and -2 (35).

In a variety of Ets-responsive promoters and enhancers analyzed thus far, DNA binding and transactivation by Ets family transcription factors is enhanced by the formation of a complex with distinct members of the bZIP and bHLH families of transcription factors (36). Recently, we and others cloned a novel member of the bHLH PAS family of transcription factors, which is expressed at high levels in vascular endothelium in mouse and quail embryos but also in tissues expressing high amounts of VEGF, and was denominated EPAS-1/HRF/HIF/MOP2 (37–41). This factor is highly homologous to hypoxia-inducible factor-1α (HIF-1α) and therefore was renamed HIF-2α (42). Like HIF-1α, HIF-2α protein is stabilized by hypoxia and, as a heterodimer with ARNT, binds to a six-base pair consensus sequence, the hypoxia-responsive element (HRE) in the regulatory sequences of hypoxia-responsive genes (40, 43). However, HIF-2α activates not only the transcription of hypoxia-inducible genes such as VEGF and erythropoietin (37, 40) but also of genes for endothelial receptor tyrosine kinases, such as Flk-1 and tie-2 (19, 40). Although in the erythropoietin enhancer and the VEGF promoter HIF-2α apparently utilizes the HRE, Flk-1 expression was not activated by HIF-1α, and no classical HRE was detected in the Flk-1 promoter.

The expression pattern and the biological activity of HIF-2α collectively suggested that this transcription factor is a dual candidate regulator of vascular development by activating both components of the VEGF/Flk-1 signaling system. Therefore, we aimed to analyze the mechanism by which HIF-2α activates transcription of Flk-1. In this study, we found that HIF-2α (but HIF-1α), although a relatively moderate activator of Flk-1 transcription, is synergistic with Ets-1 in stimulating the Flk-1 promoter. HIF-2α and Ets-1 (but not HIF-1α) physically interact via their carboxyl termini and exvon-VII domains, respectively. HIF-2 binds to two HRE-related sequences, each in close proximity to functional Ets binding sites in the Flk-1 promoter. These two pairs of transcription factor binding sites constitute enhancer elements that confer strong inducibility by HIF-2 and Ets-1 when fused to heterologous promoters. They are indispensable, positively acting elements for the Flk-1 5′-flanking region and are essential for endothelial cell-specific reporter gene expression in transgenic mice. As HIF-2α protein and Flk-1 are found to be co-regulated during development in brain capillaries, our data emphasize the importance of a cooperative interaction of the HIF-2α and Ets transcription factors for vascular growth and differentiation.

EXPERIMENTAL PROCEDURES

Antibody Preparation, Immunohistochemistry, Western Blot Analysis, and in Situ Hybridization—A 2315-bp SacI fragment comprising a large portion of the open reading frame of the murine HIF-2α cDNA was cloned in-frame into the SacI site of the prokaryotic His tag expression vector, pQE31 (Qiagen). By nickel affinity chromatography, an 87-kDa 6XHis-HIF-2α fusion protein was purified from Escherichia coli cell lysates after induction with isopropyl-1-thio-B-D-galactosanose according to the instructions of the manufacturer (Qiagen, Valencia, Calif). The protein was used for immunization of a rabbit according to standard protocols (Eurogentech), and immunoglobulin from sera was affinity-purified over protein G-Sepharose (Amersham Biosciences). The antisera readily detected HIF-2α in the nuclei and cell lysates of transfected cells and did not cross-react with HIF-1α upon Western blot or immunohistochemistry of cells transiently transfected with HIF-1α and HIF-2α, respectively. The antisera was applied at a dilution of 1:50–1:100 to cryostat sections of postnatal mouse brain, and bound antibody was detected using the Vectastain ABC™ Elite Kit (Vector Laboratories). Detection of Flk-1 protein was as described previously (44). For Western blot analysis, cell lysates from reporter gene assays were normalized for Renilla luciferase units and separated on 8% sodium dodecyl sulfate polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell) using a semidy blotting apparatus, and after blocking with bovine serum albumin, the blot was incubated with the primary antibody. Bound antibody was visualized with species-specific peroxidase-conjugated secondary antibody (Dako) or protein G-peroxidase (Pierce) and ECL™ techniques (Amersham Biosciences). Monoclonal anti-HIF-1α and HIF-1β antibodies were from Novus Biologicals, and anti-Ets antibody (C275) was from Santa Cruz Biotechnology. In situ hybridization was performed on frozen sections using [35S]UTP-labeled antisense RNA probes generated from a PstI–HindIII fragment of HIF-2α cDNA as described in detail previously (38).

Cell Culture and Reagents—HEK-293 cells were cultured in DMEM–F12 (Invitrogen) supplemented with 10% fetal calf serum (PAA Laboratories), 1 mM sodium pyruvate (Abimed), nonessential amino acids (1%, Abimed), 0.4% (w/v) β-mercaptoethanol, and antibiotics. Hepa1-C4, COS-7, NIH3T3 fibroblasts, bovine aortic endothelial cells (BAE), and normal umbilical vein endothelial cells were cultured in RPMI 1640 supplemented with fetal calf serum (Roche Molecular Biochemicals) and antibiotics. Endothelial cell media were supplemented with 0.4% (w/v) endothelial cell growth supplement (PromoCell). Chicken embryonic fibroblasts and Q2bn cells were grown in the presence of 8% fetal calf serum (Roche Molecular Biochemicals) and 2% chicken serum (Sigma). HepG2 cells were grown in RPMI medium (Invitrogen). For induction of HIFs, cobalt chloride (Sigma) to a final concentration of 100 μM was added to HEK-293 and BAE cells 16 h before cells were harvested.

Transient Expression Assays—Plasmid DNAs were purified using Qiagen columns. HepG2 and BAE cells were transfected by electropo-
roration. All other cell lines were transfected using Superfect™ transfection reagent (Qiagen). In a typical transfection experiment 200 ng of reporter plasmid together with 200 ng of expression vector and 2 ng of pRL-TK vector (Promega) were mixed in 20 μl of DMEM and Superfect™ transfection reagent (Qiagen), and after 10 min at room temperature the mixture was added to freshly passage subconfluent HEK-293 cells in 24-well plates. 48 h after transfection, cells were harvested in lysis buffer (Dual-Luciferase™ Reporter Assay System, Promega) and subsequently assayed for firefly and Renilla luciferase in a MicroLumat-Plus™ (Berthold) luminometer after the addition of luciferase assay reagent and Stop & Glow substrates (Dual-Luciferase™ Reporter Assay System, Promega), respectively. To correct for variable transfection efficiencies, the ratio of both luciferase activities was determined. All constructs were transiently transfected in 293 cells. In a second round of PCR were used to create the fusion product. The expression vectors for Flk-1 promoter/enhancer construct drives the expression of the lacZ reporter gene in endothelial cells during embryogenesis (19, 20). The generation, genotyping, and whole mount lacZ staining of transgenic mouse embryos was performed as described (19).

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—In the HEK-293 nuclear extracts from plasmids were performed at 4 °C essentially as described (48). Briefly, cells were scraped from plates in phosphate-buffered saline, washed in 5 packed cells volume of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT), resuspended in 3 packed cells volume of hypotonic buffer, and incubated on ice for 10 min. Nuclei were resuspended, collected by centrifugation, and again resuspended in an 0.5 packed nuclear volume of low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 20 mM KCI, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). Under constant stirring, an 0.5 packed nuclear volume of high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl2, 1.2 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) was added and mixed for 30 min under constant shaking. After pelleting of cell debris, nuclear extract was dialyzed against dialysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 100 mM KCI, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). Aliquots of nuclear extracts were shock-frozen in liquid nitrogen and stored at –80 °C until used.

An gel mobility shift assay performed was described as described (48, 49) using 15 μg of nuclear extract and 10,000 cpm of labeled oligonucleotide in a 10-μl reaction volume at 4 °C. Oligonucleotides were annealed with their corresponding reverse complementary strands and radiolabeled using T4 polynucleotide kinase and [γ-32P]ATP. Sequences are listed in Fig. 4B. Unlabeled oligonucleotides were added to the reaction mixture in a ratio of 1:1–1:500 to the labeled DNA in a 125 μl reaction volume. 5 μl of purified rabbit IgG was included into the reaction. Protein-DNA complexes were separated on polyacrylamide gels and visualized by autoradiography.

RESULTS

Flik-1 and HIF-2α Are Co-regulated during Development—We recently demonstrated that the endothelial bHLH PAS domain transcription factor, HIF-2α, is an activator of the VEGF receptor-2/Flik-1 promoter (19). To assess a possible role of HIF-2α in the regulation of Flk-1 expression in vivo, we generated a polyclonal antisera against an HIF-2α-His fusion protein and performed immunohistochemistry and in situ hybridization on frozen sections of postnatal mouse brain from different stages, comparing its pattern and time course of expression with that of the VEGF receptor Flik-1 (Fig. 1). It was shown previously that in rats and mice expression of Flik-1 is down-regulated in postnatal life in brain capillaries and that this down-regulation correlates with the decrease of the mitosis index of endothelial cells (51). Although HIF-2α mRNA was expressed constitutively in postnatal brain capillaries, the protein was detected in endothelial cell nuclei only until postnatal day 20 (P20). At P8, when proliferation of brain endothelial cells is at its maximum (52), a strong co-expression of HIF-2α and Flk-1 was found.
and Flk-1 was observed in brain capillaries, and HIF-2α was found translocated to the nuclei. Later, HIF-2α protein expression was detected in astrocytes and their end feet residing on the brain capillaries but was largely reduced in capillaries and absent from nuclei. At P30, when proliferation of brain endothelial cells is at its minimum (52), expression of Flk-1 mRNA and protein is almost absent from capillaries. Thus, the pattern and time course of HIF-2α expression correlate with the growth of brain capillaries, suggesting that this transcription factor regulates Flk-1 expression in the developing brain.

**Differential Activation of Flk-1 Promoter by HIF-2α and HIF-1α**

In previous studies it was shown that a 947-bp upstream sequence of the Flk-1 gene (referred to as Flk-1 promoter in the following) contains strongly positively acting elements (19, 47). Together with a 430-bp minimal enhancer from the first intron, the Flk-1 promoter conveys complete orthotope expression of the LacZ reporter gene (referred to as Flk-1 promoter in the following) in the endothelium of transgenic mouse embryos (19). To analyze whether hypoxia-inducible factors could be involved in Flk-1 expression, in a first series of experiments we co-transfected HIF-1α and HIF-2α, respectively, with a construct in which the firefly luciferase reporter gene is driven by the Flk-1 promoter in HEK-293 cells in concentrations from 2 to 400 ng of plasmid DNA/24-well (1.8 cm² of subconfluent cell monolayer). HIF-2α transactivated the Flk-1 reporter gene construct more strongly than HIF-1α. The titration experiment revealed that the transactivation effect was already saturated at 20 ng of transfected DNA (Fig. 2A, upper panel). In contrast, protein expression as estimated from the Western blot analysis of cell lysates was not saturated (Fig. 2A, lower panel). We then transfected the plasmids into different cell lines to exclude the possibility that the observation could be cell line-specific. In all cell lines tested, the outcome was similar (Fig. 2B). The data indicate that Flk-1 activation by HIF-2α is executed via a cell lineage-independent mechanism. As expected, the absolute basal activity of the Flk-1 promoter was low in most cell lines tested. The strongest relative activation of the Flk-1 promoter was obtained in HEK-293 cells. Therefore, this cell line was used for all further experiments. To exclude the possibility that the difference between HIFs was due to the larger instability of HIF-1α under normoxic conditions, reporter assays were performed in the presence of cobalt ions in the medium (which mimics induction of HIF-1α by hypoxia) (67). Cobalt chloride at a concentration of 50 μM enhanced the activation of the Flk-1

**FIG. 1.** Expression of HIF-2α and Flk-1 in freshly fixed mouse brain sections from postnatal day 8 (P8) (adolescent) and P30 (adult). HIF-2α mRNA is constitutively expressed in brain capillaries as shown by in situ hybridization with radiolabeled murine HIF-2α probe. In contrast, HIF-2α protein is regulated during postnatal development in parallel with Flk-1, as shown by immunohistochemistry with anti-HIF-2α and anti-Flk-1 antibodies. Although HIF-2α protein is detectable in endothelial cell nuclei at P8, labeling is absent from nuclei at P30. In addition to a weak labeling of the endothelial cytosol in the mature brain, HIF-2α protein is detectable in processes of astrocytes. Magnification: 1000×.

**FIG. 2.** Differential activation of Flk-1 reporter gene by HIFs. A, HIF-2α and HIF-1α expression plasmids were co-transfected with Flk-1-luciferase reporter plasmid in HEK-293 cells, respectively. The amount of co-transfected expression plasmid is indicated. The total amount of transfected plasmid DNA was kept constant by adding empty vector up to 400 ng. All further experiments were performed at saturation conditions by transfection of 200 ng of expression plasmid. In all reporter experiments of this study pRL-TK vector containing the Renilla luciferase reporter gene under the control of the HSV tk promoter was co-transfected as an internal standard. Relative luciferase activity was plotted as the ratio of reporter to standard. Data are the means from three independent assays. Expression of HIF proteins was checked by Western blotting as shown in the lower panel. Equal amounts of protein, as assessed from Renilla luciferase units, were loaded. B, HIF-2α and HIF-1α expression plasmids, respectively, were co-transfected with Flk-1-luciferase reporter plasmid in different cell lines. The luciferase read-out of vector only transfected cells was arbitrarily set to 1.
Deletion of the DNA-binding domain (HIF-2α dbn) abrogated the transcriptional activation as well (Fig. 3B).

We then tested whether HIF-1α and the HIF-2α mutants were able to compete with the wild type HIF-2α in a dominant negative manner. The results of this competition assay are shown in Fig. 3B. Only the HIF-2α dbn and HIF-2α ΔTAD N constructs significantly reduced activation of the Flk-1 promoter by wild type HIF-2α. These results indicate that binding of HIF-2α to both DNA and the TAD N is required for activation of the Flk-1 promoter. Because also HIF-1α reduced transactivation, albeit weakly, and all mutants and HIF-1α were readily translocated to the nucleus (as assessed by immunofluorescence; data not shown), it was possible that the dominant negative effects were due to a competition for the heterodimerization partner, ARNT/HIF-1β, which is necessary for DNA binding. However, forced over-expression of ARNT, which was substantiated by Western blot analysis, was not sufficient to overcome the dominant negative effects (data not shown).

HIF-2α Binds to HRE-related Sequences in the Flk-1 Promoter That Are Located in Close Proximity to Functional Ets Sites—To identify potential binding sites for HIF-2α in the Flk-1 promoter, we analyzed the DNA sequence using the MatInspector™ algorithm (Transfac data base, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). Two HRE-like GGCGTG motifs, which contained the ARNT/HIF-1β-binding consensus sequence CGTG (42), were located at −140 and −78, respectively (in the following referred to as HBS1 and HBS2, respectively). Both motifs were found in close vicinity to each of two Ets binding sites, which were proven to be functional in vitro in a recent study (20) (referred to as EBS3 and EBS6, respectively). The location of EBS and HBS motifs in the Flk-1 promoter is depicted in Fig. 4A. To determine whether the putative HBS were able to bind the HIF-2 complex, we prepared nuclear extracts from BAEs and performed electrophoretic mobility shift assays using radiolabeled double-stranded oligonucleotides containing the putative binding sites and flanking sequences. Protein-DNA complexes were separated on polyacrylamide gels and analyzed after autoradiography. The mutated control oligonucleotide as well as the HBS1 oligonucleotide and an oligonucleotide in which the HBS1 motif of the Flk-1 promoter was replaced by a classical HRE produced a strong nonspecific shift (Fig. 4B, indicated by “nsp” in the upper part of the gel). This was competed by unlabeled mutated as well as wild type oligonucleotide. The wild type and the HRE oligonucleotide formed an additional, smaller complex. The formation of this complex could be inhibited by an excess of unlabeled wild type oligonucleotide only but not by an excess of mutated control oligonucleotide (Fig. 4B). The intensity of the specific shift was enhanced about 2-fold after the treatment of the BAE cells with cobalt chloride before the nuclear extract was prepared (data not shown). This indicated that a hypoxia-inducible factor was responsible for the shifting. To examine whether HIF-2α from the BAE nuclear extract forms the complex with the HBS1 oligonucleotide, affinity-purified anti-HIF-2α antibody was added to the reaction mix, and an almost complete supershift of the complex resulted (Fig. 4C). Because the antibody did not cross-react with HIF-1α (not shown) and this supershift was not obtained with preimmune serum, the data clearly indicate that HIF-2α from endothelial cell nuclei can bind to the HBS1 motif of the Flk-1 promoter. Similar results were obtained with an HBS2 oligonucleotide.

We then performed DNase I footprint analysis of a 226 bp fragment from the Flk-1 promoter (−194 to +32), which contains the HBS1 and -2 sites, using nuclear extracts from BAE cells. Both the HBS1 and the HBS2 together with their adjacent EBS3 and -6, respectively, were found to be protected by
proteins from the nuclear extract from digestion by DNase. Protection of the pairs of binding sites was accompanied by the occurrence of new DNase I-hypersensitive sites flanking the footprints. Details are indicated in Fig. 4.

**HIF-2α, but Not HIF-1α, and Ets-1 Cooperate to Activate the Flk-1 Promoter**—In view of the close proximity of their binding sites, which are occupied by nuclear proteins in the footprint analysis, we next asked whether HIF-2α and Ets factors can cooperatively activate the Flk-1 promoter. Because in previous studies Ets-1 has been found to be a strong activator of endothelial cell-specific promoters, the co-transfection experiments were performed only with this family member. The results of representative co-transfection reporter experiments in HEK-293 cells are shown in Fig. 5. The effect of co-transfection of HIF-2α and Ets-1 was clearly more than additive (Figs. 5 and 8A). In contrast, HIF-1α did not influence transactivation by Ets-1 (Fig. 5). This failure could be attributed to the carboxy-terminal half of HIF-1α, because the HIF-2α/1α chimera, which contains the carboxyl terminus of HIF-1α, also failed to synergize with Ets-1 (Fig. 5). We addressed further the question of whether the dominant negative mutants of HIF-2α could interfere with activation by Ets-1. As shown in Fig. 5, none of the mutants reduced transactivation of the Flk-1 reporter construct by Ets-1. Interestingly, HIF-2α ΔTAD C, like the wild type, acted synergistically with Ets-1 to stimulate the Flk-1 promoter. In contrast, HIF-2α ΔTAD N did not co-activate. These data show that DNA binding of HIF-2α and the integrity of TAD N but not of TAD C domains are necessary for the interaction with Ets-1. The synergistic effect and the co-localization of their binding sites were suggestive of a physical interaction of HIF-2α and Ets-1 transcription factors on the Flk-1 promoter.

**HIF-2α and Ets-1 Interact Physically in Vitro**—To assess whether the transcription factors HIF-2α and Ets-1 can interact physically, in vitro translated HIF-2α, HIF-1α, and two HIF-2α mutants including the HIF-2α/1α chimera were subjected to pull-down assays with a series of purified glutathione S-transferase (GST)-Ets-1 fusion proteins. A schematic draw-

![Fig. 4. Analysis of the Flk-1 5′-flanking region for putative HIF-2α binding sites.](image-url)

**A**. Analysis of the Flk-1 5′-flanking region for putative HIF-2α binding sites. Two potential HIF-2α binding sites, HBS1 and HBS2, with the consensus binding motif GCCGTG were found by sequence analysis in the Flk-1 promoter as indicated. The HBS are in proximity to the previously detected functional Ets consensus sites, EBS3 and EBS6. **B**. Electrophoretic mobility shift assay for protein from BAE nuclear extracts binding to the HBS1 consensus sequence. The specific shift (sp) is supershifted (ss) after the addition of anti-HIF-2α antibody (HIF2) to the reaction mixture but not after the addition of preimmune serum (PIS). **C**. DNase I footprint analysis of a Flk-1 promoter fragment from bp −194 to +32 as referred to the transcriptional start site. NE, nuclear extract from BAE (2 to 20 μg) was included in the reaction mixture. Equal amounts of bovine serum albumin (BSA) were added to control reactions.
Ets-1 in combination with control vector, which was set to 100 (Promoter activity is expressed relative to that after stimulation with constructs only (Fig. 7A, left panel)). The expression of these constructs is shown in Fig. 6A, as compared with the wild type tk-luc reporter gene.

Cooperative activation of Flk-1 by HIF-2α and Ets-1

The EBS3/HBS1 element from the Flk-1 promoter is necessary for interaction with Ets-1 exon VII domain. As shown in Fig. 6B, the ability of HIF-2α to interact with the Ets-1 exon VII domain resides in the carboxyl-terminal part of the protein, just downstream of the PAS domains, because a mutant deleted from amino acid 325 can still weakly interact, whereas the substitution of the entire HIF-2α protein is necessary for interaction with Ets-1 exon VII (aa 238–328) or the more amino-terminal part of the protein, just downstream of the PAS domains, because a mutant deleted from amino acid 325 can still weakly interact, whereas the substitution of the entire HIF-2α protein abolishes the interaction completely. These data support the finding that HIF-2α but not HIF-1α cooperates with Ets-1 to activate the Flk-1 promoter.

The EBS3/HBS1 Element of the Flk-1 Promoter Confers Strong Inducibility by HIF-2α/Ets-1 to Heterologous Promoters and Is Highly Active in Endothelial Cells—The EBS3/HBS motifs in the Flk-1 promoter were identified as potential cis-acting elements, which could be important for endothelial cell-specific expression of Flk-1 during ontogeny. To assess the role of these elements, double-stranded oligonucleotides representing Flk-1 promoter sequences containing the EBS3/HBS elements were synthesized and fused upstream to the HSV tk promoter driving a luciferase reporter gene. Clones containing tandem repeats of the elements were electrophoretically transferred (referred to as [EBS3/HBS1]1tk-luc and [HBS2/EBS6]1tk-luc) and used for luciferase reporter assays in HEK-293 cells. A schematic drawing of these constructs is shown in Fig. 7A, left panel.

In this way, inducibility by HIF-2α and Ets-1 was conferred to a heterologous promoter. As compared with the wild type tk-luc construct, which was only slightly active and not inducible, relative induction by HIF-2α/Ets-1 was about 100-fold when the EBS3/HBS1 element was oriented as in the Flk-1 promoter and about 40-fold when oriented in the opposite direction (Fig. 7A). Mutation of the corresponding sites largely abolished inducibility by Ets-1 and HIF-2α. Interestingly, cooperative activation was lost when the Ets site was mutated. After mutation of the HBS1, however, cooperativity was retained but inducibility by Ets-1 was also reduced (Fig. 7A). This observation suggests that binding to DNA may not be absolutely necessary for HIF-2α to interact cooperatively with Ets-1. Experiments with the [HBS2/EBS6]1tk-luc constructs essentially revealed similar activation profiles, albeit to a much lower extent (6–20-fold; Fig. 7A). Essentially the same results were obtained when the SV40 minimal promoter was used instead of the HSV tk promoter. The SV40 promoter was less suitable, because moderate activation of the empty construct itself by Ets-1 was observed (not shown).

To test whether the EBS3/HBS1 element from the Flk-1 promoter is able to confer endothelial cell-specific expression to heterologous promoters, a variety of cell lines was transfected with the [EBS3/HBS1]1tk-luc constructs and subjected to analysis of reporter gene expression (Fig. 7B). The highest activity of this construct was obtained in endothelial cells, followed by COS and 293 cells. In CEFs, chicken embryonic fibroblasts) 3T3 fibroblasts, and HepG2 cells the construct was less active, and it was inactive in Hepa1-C4 cells, which lack functional ARNT/HIF-1β. The latter result suggests that HIF-2α, which needs heterodimerization with ARNT/HIF-1γ for activity, is required for transactivation of the [EBS3/HBS1]1tk-luc constructs. The data indicate that the EBS3/HBS1 element is a positively acting element of the Flk-1 promoter, which may contribute to restriction of gene expression to endothelial cells.

EBS3/HBS Elements of the Flk-1 Promoter Are Required for Endothelial Cell-specific Gene Expression in Vivo—To assess whether the EBS3/HBS elements in the native Flk-1 promoter are necessary for transcriptional activation in vivo, we sequentially mutated the individual Ets and HIF-2α binding sites within the Flk-1 promoter-luciferase reporter construct and tested their inducibility by Ets and HIF-2α in luciferase reporter assays before testing them in transgenic embryos. Mutation of individual Ets or HIF-2α binding sites resulted in a moderate but significant reduction of inducibility (data not shown). Even the complete mutation of the EBS3/HBS1 element, which was found to be a strong enhancer element, did not reduce the inducibility of the promoter by more than 60%. This finding suggested that the HBS2/EBS6 element also contributed considerably to the transcriptional activation of the Flk-1 promoter. Consistently, after mutation of both HBS/EBS motifs, the inducibility of the promoter by Ets-1 and HIF-2α was almost completely abolished (Fig. 8A). We then introduced the wild type and the mutated promoters into a lacZ reporter plasmid containing the enhancer element from the first intron of the Flk-1 gene (Fig. 8B). We have previously shown that this construct strongly recapitulates endothelial cell-specific expression of the Flk-1 gene during embryogenesis by lacZ reporter gene expression (19, 20). Mutation of the EBS3/HBS1 element did not alter the vascular expression pattern of the reporter gene ([mut EBS3/HBS1]), Fig. 8, C and D, Table I). Mutation of the HBS2/EBS6 element was needed in addition to abolish expression of the reporter gene completely (mut HBS1 + 2/EBS3 + 6; Fig. 8, E and F, and Table I). In only one double mutant embryo, faint endothelial and ectopic reporter gene expression was seen. These data indicate that the Ets/HIF-2 binding motifs are strong positively acting elements of the Flk-1 promoter, which are necessary for endothelial cell-specific expression of Flk-1 in vivo.
This promoter specificity, together with the endothelial cell-specific expression of HIF-2α, which is developmentally regulated concordantly with Flk-1 gene expression, constitutes a novel candidate regulatory system of angiogenesis.

DISCUSSION

Signaling via the VEGF receptor, Flk-1, is thought to be limiting for endothelial cell proliferation and survival and indispensable for the initial development of the vascular system. Because expression of Flk-1 is highly restricted to endothelial cells and tightly regulated during development, characterization of the gene regulatory elements of Flk-1 would provide insights into the mechanisms of both endothelial cell-specific restriction and temporal regulation of gene expression. In previous studies we identified a 430-bp minimal enhancer from the first intron of the Flk-1 gene, which in conjunction with the Flk-1 promoter is sufficient and necessary to drive endothelial cell-specific gene expression in transgenic mice (19). Although the enhancer partially conferred endothelial cell-specific expression of the lacZ reporter gene to a heterologous promoter, full recapitulation of endothelial expression was obtained only in conjunction with the Flk-1 promoter (19). These data together with the endothelial cell-specificity of the Flk-1 promoter (47) indicate that the binding of endothelial cell-specific transcription factors to the promoter is necessary to enhance the expression of Flk-1.
In this study, we have identified two functional binding sites for HIF-2 within the Flk-1 promoter, located in close proximity to functional Ets consensus sites. These binding sites constitute positively acting elements that confer inducibility by HIF-2α and Ets-1 and their cooperative effects to a heterologous promoter in an insertion-independent manner. Inducibility of the Flk-1 promoter was almost completely lost when these elements were mutated. Therefore, these elements were likely to represent the sought positively acting elements that are necessary for complete endothelial cell-specific expression of Flk-1. Finally, by mutational analysis in transgenic mouse embryos these elements were proved necessary for endothelial cell-specific reporter gene expression in vivo. Interestingly, mutation of both the HBS2/EBS6 and EBS1/HBS2 elements was necessary to obtain the loss of endothelial cell-specific transgene expression. These results suggest that the HBS/EBS elements may be used alternatively in vivo, which is consistent with our finding that both HBS readily bind HIF-2 from endothelial nuclear extracts and are occupied by nuclear protein. The in vivo findings do not explain the large difference in inducibility conferred by the HBS/EBS elements when fused to heterologous promoters. It can be speculated that the different distances between the neighboring HIF-2 and Ets binding sites and/or the distance to the transcriptional start site are critical parameters that influence the degree of inducibility conferred by the enhancer elements.

Cooperative activation of the Flk-1 promoter is reflected by the physical interaction between Ets-1 and HIF-2α. The physical interaction of Ets-1 and other Ets family members with a variety of other transcription factors has been demonstrated and implicated in differentiation processes mainly in hematopoiesis (36). Although Ets-1 interacts with the bHLH protein USF-1 at the DNA-binding domains (46), our GST pull-down experiments indicate that the carboxyl-terminal domain of HIF-2α strongly interacts with the exon VII domain of Ets-1. This domain is known as a negative regulatory domain, which needs to be conformationally changed to allow Ets-1 to bind DNA (55, 56). Thus, interaction of this domain with HIF-2α probably enhances DNA binding of Ets-1 and thereby establishes the mechanism underlying hyperadditive transactivation. The RUNT domain factor, AML1, interacts with Ets-1 in a similar manner (55).

Unlike its close relative HIF-2α, HIF-1α is not able to interact with the exon VII domain of Ets-1 functionally. By swapping the parts of the HIF molecules, the failure of interaction could be attributed to structural differences in the carboxy-terminal halves. The fact that deletion of the TAD N domain completely abrogated cooperativity suggests that TAD N is necessary for the recruitment of Ets-1 to the DNA via binding to the exon VII domain. In contrast, the TAD C domain does not appear to be required for this interaction. All mutants (HIF-2αΔN, HIF-2αΔN, HIF-2αΔTAD N) that fail to interact with Ets-1 are dominant negative over the wild type HIF-2α but do not reduce the basal level of Flk-1 promoter stimulation by Ets-1. Therefore, it can be concluded that endogenous HIF-2α is not necessary for the basal activation of Flk-1 promoter by Ets-1. This is further supported by the fact that high levels of basal activity were obtained in HEK-293 cells, which lack de-
Cooperative Activation of Flk-1 by HIF-2α and Ets-1

It cannot be ruled out that HIF-2α can also co-activate Ets-1 without specific DNA binding, because deletion of the HBS1 from our tk enhancer constructs did abrogate the induction by HIF-2α but not the cooperativity with Ets-1 completely. In contrast, intact EBS3 is absolutely required for induction by Ets-1 and synergism with HIF-2. If this speculation holds true, HIF-2α would represent another example of a DNA binding transcription factor that can transactivate via cofactors without specific DNA binding (57). However, as shown by deletion experiments, the DNA-binding domain of HIF-2α is indispensable for synergism with Ets-1. The seemingly conflicting observation, that a DNA-binding domain but not the specific recognition sequence is needed for activity, may be resolved by the hypothesis that HIF-2α requires binding to DNA for cooperative activation but, when associated with Ets-1, not necessarily binding to its specific recognition sequence.

It may be speculated that, depending on the genetic background HIF-1α can substitute for HIF-2α in endothelial cells in vivo. This could account for the varying outcomes of the HIF-2α knock-out in mice, such as the lack of any vascular phenotype and severe defects in angiogenesis (58, 59). Whether the weak interaction of HIF-1α with the amino terminus of Ets-1 (as observed in our GST-pull-down experiments) is of functional relevance remains to be elaborated. In addition, in endothelial nuclear extracts only little residual binding activity to the Flk-1 HBS was detected after supershifting with specific antisera to HIF-2α protein. This indicates that HIF-2α is the predominant endothelial HIF.

In the present study we show that HIF-2α protein is highly expressed in brain capillary endothelial cells of mice during angiogenesis and is localized to endothelial cell nuclei. The kinetics of endothelial cell proliferation were best studied in the brain, and down-regulation of Flk-1 was shown to precede the decrease in mitoses of capillary endothelial cells in the brain of postnatal mice and rats (51, 52). In parallel to Flk-1, expression of HIF-2α protein is largely down-regulated in the cytosol and disappears from the nuclei of endothelial cells, although strong expression of mRNA is still retained. This finding indicates that HIF-2α is regulated post-translationally in endothelial cells and raises the question of the underlying mechanism. Induction of HIF-2α by hypoxia has been proven and, similar to HIF-1α, is likely to depend on stabilization of the protein under hypoxia. This is achieved via an “oxygen-dependent degradation domain,” which under normoxia is tar-

**TABLE I**

LacZ reporter gene expression in transgenic mouse embryos

Embryos transgenic for the constructs listed above were generated, and LacZ staining and genotyping were performed at E10.5. TG, number of transgenic embryos; ES, number of transgenic embryos showing endothelial cell specific staining; ET, number of transgenic embryos showing ectopic staining; NO, number of transgenic embryos showing no staining.

| Construct | TG | ES | ET | NO |
|-----------|----|----|----|----|
| Wild type | 13 | 6  | 1  | 6  |
| [mut EBS3/HBS1] | 16 | 10 | 1  | 5  |
| [mut HBS1 + 2/EBS3 + 6] | 11 | (1) | (1) | 10 |

**Fig. 8.** Reporter gene analysis of HIF-2α and Ets-binding sites in the Flk-1 promoter in transient transfection assays and transgenic mouse embryos. A, the EBS3/HBS1 and HBS2/EBS6 elements were mutated in the Flk-1 promoter luciferase reporter plasmid, and inducibility of mutant promoters by HIF-2α and Ets-1 was tested as compared with the wild type promoter (wt) in transient co-transfection assays. mut EBS3/HBS1, EBS3/HBS1 sites mutated; mut EBS3/HBS1 + 2/EBS3 + 6, EBS3/HBS1 and HBS2/EBS6 sites mutated. B, structure of the reporter construct used for creating transgenic embryos. LacZ coding sequence is fused to the promoter and the enhancer sequence from the first intron of the Flk-1 gene (−646 to +299 bp and +3947 to +1677 to +3947 bp of the Flk-1 gene, respectively) C and D, LacZ staining of an E10.5 embryo expressing β-galactosidase under the control of the wild type [mut EBS3/HBS1] Flk-1 promoter/enhancer construct. D—F, E10.5 embryos transgenic for the [mut HBS1 + 2/EBS3 + 6] Flk-1 promoter/enhancer construct. Scale bar, 2 mm.
geted by hydroxylation of distinct proline residues to the degradation via the ubiquitin-proteasome pathway (54, 60, 61). Relative hypoxia in the growing brain may be an important factor that contributes to stabilization of endothelial HIF-2α protein and consequently influences transcription of its target gene, Flk-1. However, the data on a transcriptional induction of Flk-1 by hypoxia are controversial (62–65) (51). In contrast to Flk-1, the transcription of VEGF receptor Flt-1 is induced by hypoxia via a hypoxia-responsive element within the promoter (64). Whether Flt-1, like Flk-1 and tie-2, is another HIF-2α target gene, remains to be examined. Interestingly, gene regulatory elements of both tie-2 and Flt-1 contain functional Ets sites and, like many other endothelial genes, these can be transcriptionally activated by Ets-1 (26, 27, 29). The nature of the Ets factors that regulate expression of these genes in vitro is still elusive. Ets-1, Ets-2, TEL, NERF2, and other family members may be involved. The high functional redundancy of Ets family members expressed in the endothelium could account for the lack of a vascular phenotype after disruption of the Ets-1 gene (66). Whether HIF-2α can interact with other Ets family members and whether cooperative interaction of HIF-2α with Ets factors is also involved in the transcriptional regulation of other endothelial genes, and therefore represents a general mechanism for specifying endothelial gene expression, remains to be established.

Acknowledgments—We thank Dr. Hugo Marti (Bad Nauheim, Germany) for providing the ARNT/HIF-1α cDNA and the staff of the Max-Planck-Institut für physiologische und klinische Forschung transgenic facility (Bad Nauheim, Germany) for generating transgenic mice.

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