Opposing Functions of the Ets Factors NERF and ELF-1 During Chicken Blood Vessel Development

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Objective—The purpose of this study was to evaluate the role of the Ets factor NERF in the regulation of the Tie1 and Tie2 genes during chicken blood vessel development.

Methods and Results—We have isolated the full-length cDNA for the chicken homologue of the human Ets factor NERF2 (cNERF2). Northern blot analysis and in situ hybridization demonstrate that cNERF2 is enriched in the developing blood vessels of the chicken chorioallantoic membrane. Interestingly, cNERF2 functions as a competitive inhibitor of a highly related Ets factor cELF-1, which we have previously shown to be enriched in chicken blood vessel development. Although in vitro–translated cELF-1 and cNERF2 can bind equally well to conserved Ets binding sites in the promoters of the Tie1 and Tie2 genes, cELF-1 preferentially binds to the Ets sites in these promoters during early stages of chicken blood vessel development, suggesting that cNERF may bind during later stages of blood vessel development and vascular remodeling.

Conclusions—cNERF2 is enriched during embryonic and extraembryonic blood vessel development in the chicken and facilitates tight control of Tie1 and Tie2 gene regulation. (Arterioscler Thromb Vasc Biol. 2002;22:1106-1112.)

Key Words: angiogenesis ■ vasculogenesis ■ transcription ■ vascular biology ■ endothelium

Vasculogenesis is the development of a primary vascular network during embryogenesis. The Tie1 and Tie2 genes constitute a family of endothelium-specific receptor tyrosine kinases that are critical for vascular development.1 They are expressed predominantly on endothelial cells of the developing vasculature. Targeted disruption of the Tie genes leads to abnormalities in vascular development, including the formation of leaky dilated blood vessels, with associated edema and hemorrhage that result in early embryonic death.2 Mutations in the Tie2 gene have been identified in humans, resulting in venous malformations.3 Tie1 and Tie2 are also upregulated during tumor angiogenesis.4,5

Transcription factors have been shown to serve as master switches of several developmental processes, including hematopoiesis and myogenesis, in which several cell- or tissue-specific transcription factors have been identified. One approach toward identifying the types of transcription factors required for a developmental process is to examine the regulatory regions of genes known to be critical for this process and identify the classes of transcription factors that may be necessary to bind to these regions. Examination of the main regulatory elements of the Flt-1, Tie1, and Tie2 genes reveals several conserved putative binding sites for the Ets family of transcription factors that are critical for the activity of the promoters and enhancers of these genes.6–8 Mutations in the Ets binding sites of the promoter of the Flk-1 gene lead to a marked reduction in endothelium-specific LacZ-directed gene expression.9 Likewise, in transgenic animals in which LacZ expression is directed throughout the vasculature by the Tie2 promoter and enhancer, a mutation in an Ets binding site in the core enhancer leads to a marked reduction in vascularly directed LacZ gene expression. We have recently identified conserved Ets binding sites in the Tie1 and Tie2 promoters, which are necessary for the vascular-specific expression of these genes.10,11

The Ets genes are a family of transcription factors that play a central role in regulating the genes involved in development, cellular differentiation, and proliferation.12 All Ets factors share a highly conserved DNA binding domain, the ETS domain, which has a winged helix-turn-helix structure. Ets proteins recognize DNA with an internal conserved DNA binding core motif, GGAA/T. We have examined the ability of several different Ets factors to regulate the Tie1 and Tie2 genes. Our previous studies have demonstrated that NERF2 and ELF-1 (both are human Ets factors) are potent transactivators of the Tie1 and Tie2 genes and can bind to specific Ets sites within the promoters of these genes.10,11,13 To extend these studies and to demonstrate the potential in vivo relevance of Ets factors during vascular development, we have identified Ets factors that are expressed during chicken blood

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vessel development. Compared with mammals, as a model system, the chicken offers the advantage of easy access to developing blood vessels, particularly in the rich extraembryonic vessels within the chorioallantoic membrane (CAM). Using this model, we identified the chicken homologue of ELF-1 (cELF-1) and demonstrated that in addition to being a strong transactivator of the Tie1 and Tie2 genes, ELF-1 is enriched in developing blood vessels of the chicken.13 In the present report, we have extended these studies by identifying the chicken homologue of the Ets factor NERF2 (cNERF2) and have demonstrated that it functions as a competitive inhibitor of CELF-1. The expression of positive and negative transcriptional regulators of the Tie1 and Tie2 genes may facilitate tight control of their expression during different stages of blood vessel development.

Methods

Cell Culture and Northern Blot Analysis

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were grown in 10% FCS and DMEM. Total RNA was extracted from the CAM and from blood derived from the CAM at different developmental stages, as previously described.14 Ten micrograms of total RNA was electrophoresed and transferred onto a Nytran membrane (Schleicher & Schuell). The filter was blocked in prehybridization solution (50% deionized formamide, 6× SSPE, 5× Denhardt’s solution, 1% SDS, 0.1 mg/mL yeast tRNA, and 0.1 mg/mL salmon sperm DNA) and then hybridized with a cNERF-specific probe at 42°C overnight. To normalize for loading and transfer efficiency, the membranes were rehybridized with a probe for 36B4.

RT-PCR and Chicken λ-Phage Library Screen

To identify Ets factors that are expressed in the developing blood vessels of the chicken CAM, reverse transcription (RT)-polymerase chain reaction (PCR) was performed by using RNA extracted from CAM blood vessels. cDNA was generated from 2 μg total RNA by using random hexamer priming (GIBCO-BRL). Degenerate oligonucleotides corresponding to conserved regions within the Ets domain, allowing the identification of cERFs, were used as previously described.15 PCR fragments were subcloned into the TA cloning vector (Invitrogen), and clones containing fragments of the expected sizes were sequenced. An embryonic day (E)5 chicken yolk sac library (Stratagene) was plated and screened with a partial cDNA fragment for cNERF. Eight partial and 1 full-length cDNA clones were isolated and sequenced at the Beth Israel Deaconess Medical Center core sequencing facility by using an Applied Biosystems automatic DNA sequencer. Verification of the full-length cNERF sequence was also confirmed by RT-PCR with CDN generated from RNA that was extracted from the chicken CAM at E7 with the use of cNERF-specific primers. PCR reactions were performed as previously described.16

In Situ Hybridization

Whole-mount in situ hybridization on E5 chick embryos and E10 to 11E CAMs were carried out as described by Wilkinson et al.17 In brief, embryos were fixed, dehydrated, and rehydrated through a methanol series and washed in 1× PBT (containing PBS plus 0.1% Tween 20). Embryos were then permeabilized with RIPA buffer (containing 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mmol/L EDTA, and 30 mmol/L Tris-HCl, pH 8.0) for 30 minutes with agitation at room temperature. After color development to the appropriate intensity, specimens were washed several times in NTMT (containing 100 mmol/L NaCl, 100 mmol/L Tris-HCl, pH 9.5, 50 mmol/L MgCl2, and 1% Tween 20) and PBT and then rehydrated through the graded methanol baths. Images of the embryos were obtained and suspended in 80% glycerol with the use of a CCD Toshiba camera on a Nikon SMZ-U dissecting microscope. Digoxigenin-labeled RNA probes were prepared per the manufacturer’s recommendations (Roche). The level of digoxigenin incorporation was assessed by using a dot-blot comparison with a standard (Roche).

DNA Transfection Assays

Cotransfections of 1.5 to 2×10⁵ mouse endothelial cells (MS-1) or 293 HEK cells were performed by using 300 ng of the reporter gene constructs, pGL3 Tie1 (KH1) or pGL3 Tie2 (Pit-HindIII), as previously described,11,16 and a total of 300 ng of the expression vector DNA with 3 μL pre reagent and 2 μL Lipofectamine (GIBCO-BRL). Cells were washed with serum-free DMEM. A total of 400 μL serum-free DMEM was added per well. Liposomes were incubated with the DNA in 100 μL serum-free DMEM for 15 minutes at room temperature and then with the cells for 3 hours at 37°C. DMEM (500 μL) containing 20% FCS was added, and the cells were harvested 16 hours after transfection and assayed for luciferase activity. Transfections were performed in triplicate and were repeated independently in duplicate with the use of 2 independently cloned cNERF expression plasmids with similar results. Cotransfection of an additional plasmid for determination of transfection efficiency was omitted because potential artifacts with this technique have been reported18 and because many commonly used viral promoters contain potential binding sites for Ets factors.

Electrophoretic Mobility Shift Assay

DNA binding reactions were performed as previously described.15,19 In brief, 2-μL samples of in vitro–translated proteins or cellular extracts were incubated in the absence or presence of polyclonal antibodies specific to NERF (Santa Cruz) or ELF-1 at room temperature for 15 minutes.32 P-labeled double-stranded probes (30 000 cpm) were then added, along with 50 ng cold mutant oligonucleotides to reduce the background. Samples were incubated for 15 to 20 minutes at room temperature and run on a 4% polyacrylamide gel (acrylamide-bisacrylamide, 37:5.1) containing a buffer of 0.5× TBE (containing 45 mmol/L Tris borate and 1 mmol/L EDTA). Oligonucleotides used as probes and for competition studies were as follows: mouse Tie2 promoter wild-type oligonucleotide, 5′-TGCAAAGGAAAAGGAAAGGAACCTAAC-3′ and 3′-ACGTTCTTCCTTCCCTTCTGATTAATG-5′; mouse Tie1 promoter wild-type oligonucleotide, 5′-CCATCATTTCCTCTCCTCCCCAG-3′ and 3′-GGTAGTAAGGAGAAGGGGTC-5′; mouse Tie2 promoter mutant (bold) oligonucleotide, 5′-CTGAAAGAAAACGCAAAGGAAAGGATCAAACCTAAC-3′ and 3′-ACGTTCTTCCTTCCCTTCTGATTAATG-5′; and mouse Tie1 promoter wild-type oligonucleotide, 5′-CCATCATTTCCTCTCCTCCCCAG-3′ and 3′-GGTAGTAAGGAGAAGGGGTC-5′.

Results

Isolation of cNERF2

In an effort to identify transcription factors belonging to the Ets transcription factor family that are expressed during vasculogenesis, total RNA was extracted from developing blood vessels of the chicken CAM at E10. RT-PCR was performed with degenerate PCR primers corresponding to conserved regions of the Ets domain, allowing the identification of a partial DNA sequence with highest homology to the Ets family NERF. This fragment was used to screen a chicken yolk sac cDNA library and to isolate several cDNA clones encoding the full-length cNERF2. Several clones were sequenced to verify the nucleotide and amino acid sequence of the chicken NERF2 homologue. cNERF2 encodes a 595–amino acid protein, with an expected molecular mass of 68.5 kDa, compared with the 594–amino acid human...
NERF2, with a predicted molecular mass of 68.5 kDa (please see online Figure 1, available at http://atvb.ahajournals.org). The highest degree of homology to NERF2 exists in the DNA binding domain (100%), with overall protein sequence homology of 84%. We have previously identified additional regions of homology between NERF2 and a closely related Ets factor, ELF-1, in the transactivation domain. These 4 domains (A through D) are also highly conserved among human, mouse, and chicken NERF2.20

Expression Pattern of cNERF2 in the Developing Chicken
To determine the expression pattern of cNERF2 in the CAM, Northern blot analysis was performed by using total RNA derived from CAM at different developmental stages. As shown in Figure 1, cNERF2 is highly expressed in the CAM. Expression in this highly vascularized membrane appeared to be greatest early, and it gradually diminished during later stages of development. We have previously demonstrated that human NERF2 is highly expressed in a number of fetal tissues, including the heart, liver, and the brain.20 To determine cNERF2 expression, Northern blot analysis was performed with chicken fetal organs at progressive developmental stages (data not shown). cNERF2 is expressed in fetal liver and weakly expressed in the brain, with strong but transient expression in the developing heart and limb.

In Situ Hybridization of cNERF2
Having demonstrated strong expression of cNERF2 in the CAM blood vessels at different stages by Northern blot analysis, we wanted to define further the cellular expression pattern of cNERF2 by in situ hybridization. Examination of E11 and E14 CAMs (Figure 2B and 2D) demonstrates hybridization mostly in the large vessels, with weaker levels of expression in the smaller branching vessels and capillaries. This is in contrast to cELF-1, which is also highly expressed in the smaller developing blood vessels and capillaries.13 Minimal staining is observed in the sense controls (Figure 2A and 2C). Whole mount of the chicken embryos also demonstrates expression of cNERF2 in the developing limb buds (Figure 2F). To examine the expression of cNERF2 in the developing large vessels of the embryo, in situ hybridization of paraffin-embedded embryos (E3) sections was performed. cNERF2 was strongly expressed in the developing aorta (Figure 2H) and was also expressed in the developing neural tube. The fact that cNERF2 is expressed in tissues other than blood vessels suggests that it may also regulate genes required for other developmental processes.

cNERF2 Can Bind to Ets Sites in Tie1 and Tie2 Promoters
Although there is overall high protein sequence homology between chicken and human NERF2, we tested whether cNERF2 has the same DNA binding affinity to the conserved Tie2 Ets sites that we have previously shown to be required for transactivation by ELF-1 and NERF2.11 Gel mobility shift assays were performed with the use of oligonucleotide probes encoding either Tie2 or Tie1 Ets sites (Figure 3). These studies demonstrate that in vitro–translated cNERF2 can bind strongly to the Tie2 (lanes 1 and 2) and Tie1 (lanes 3 and 4) Ets sites, as is demonstrated by the presence of a clearly higher molecular mass protein-DNA complex not seen in the control extracts. To verify the specificity of complexes formed by cNERF2 and cELF-1, the ability of antibodies directed against NERF and ELF-1 to alter these complexes in gel mobility shift assays was tested. As shown in lanes 5 and 6 of Figure 3, only the antibody directed against NERF inhibited the complex formation when in vitro–translated cNERF2 was used. Similarly, when in vitro–translated chicken ELF-1 was used to bind to the Tie1 probe (lanes 8 and 9), only the polyclonal antibody to ELF-1 and not NERF was able to alter the complex formation and resulted in the formation of a supershifted complex (arrow; Figure 3, lane 9). These results also demonstrate the specificity of the NERF and ELF-1 antibodies because neither antibody recognizes or interferes with binding of the other highly related Ets protein.

cNERF2 Can Competitively Inhibit cELF-1 Transactivation of Tie1 and Tie2 Promoters
Having demonstrated that cNERF2 can bind to the Ets sites in the Tie1 and Tie2 promoters, we examined the ability of cNERF2 to transactivate the Tie2 promoter compared with human NERF2. As shown in Figure 4A and 4B, cNERF2 did not transactivate the Tie1 and Tie2 promoters significantly, despite its ability to bind equally well to conserved Ets binding sites in the promoters of these genes and the high protein homology to the human counterpart. We next tested the ability of cNERF2 to block the transactivation of the Tie1 and Tie2 promoters by cELF-1 by performing cotransfection experiments with cELF-1 and increasing the concentrations of cNERF2. As shown in Figure 4C and 4D, cNERF2 was able to inhibit the ability of cELF-1 to transactivate the Tie1 and Tie2 promoters in a dose-dependent manner. cNERF2 was similarly able to block cELF-1 transactivation of the Tie2 gene promoter in transfected endothelial cells (data not shown).

Assessment of In Vivo Binding of cELF-1 and cNERF2 During Vascular Development
To extend these studies, we sought to determine whether cNERF2 binds to the conserved Ets sites of the Tie1 and Tie2 promoters in vivo during development in the chicken CAM. As is shown in Figure 5, in vitro–translated chicken and human NERF and chicken ELF-1 were able to bind strongly to the Tie1 Ets site probe (lanes 2 to 4) compared with control.
extract (lane 1). When cell extracts derived from the chicken CAM blood vessels (E7) were used instead of the in vitro–translated products, a similar sized complex was formed (lane 5). However, the antibody to NERF had no effect on the formation of a similar sized complex (lane 6). In contrast, an antibody directed against ELF-1 was able to alter the formation of this complex completely, resulting in a supershift (arrow, lane 7), suggesting that cELF-1 and not cNERF2 is the Ets factor binding to the Tie1 Ets site in the chicken CAM. These results suggest that although in vitro–translated cELF-1 and cNERF2 bind equally well to conserved Ets sites in the Tie1 and Tie2 promoters, cELF-1 preferentially binds during the development of blood vessels in the chicken CAM. We similarly performed gel shifts with oligonucleotides encoding the Ets binding sites in the Tie2 promoter (data not shown). Chicken ELF-1 also bound preferentially to the Tie2 Ets sites.

**Discussion**

The goal of the present study was to further define the role of specific transcription factors in the regulation of vascular-specific gene expression during vascular development. Our particular focus has been on characterizing the role of members of the Ets transcription factor family in this process. In the present study, we demonstrate that cNERF2 is highly enriched in the developing blood vessels of the chicken CAM and chicken embryo.10,11 Whereas NERF2 acts as a positive regulator of transcription in humans, the chicken homologue of NERF2 acts as a competitive inhibitor of ELF-1, suggesting that the highly related Ets factors ELF-1 and NERF may act as positive and negative regulators of the same gene targets in the chicken. However, over evolution, the change in NERF2 function from a negative to a positive regulator may

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**Figure 2.** Whole-mount in situ hybridization of cNERF2 in E3 and E5 chicken embryos and in E11 and E14 chicken CAMs. A and B, cNERF2 is evident in the larger blood vessels of perfused E11 CAMs but absent in the capillaries. C and D, cNERF2 is also prominent along the perfused blood vessels of the E14 CAMs (red arrow). Note the punctate expression in the yolk sac (YS). E and F, cNERF2 expression is present in the limbs and mesenchyme (yellow lines) of E5 chick embryos. G and H, cNERF2 expression in the developing aortic arch (AA) and neural tube (NT) of E3 chicken embryos is shown. LL indicates lower limb; UL; upper limb. E3, E5, E11, and E14 correspond to Hamilton and Hamburger stages 21, 28, 37, and 40, respectively.27
have provided important redundancy in the regulation of critical developmental processes, such as hematopoiesis and vascular development.

Positive and negative regulators of developmental processes by Ets factors have similarly been shown in Drosophila during eye development. The pointed gene is an Ets factor that acts as a positive regulator of photoreceptor determination and is activated by the Ras/mitogen-activated protein kinase pathway. In contrast, the related Drosophila Ets factor, yan, is a negative regulator of transcription of the same gene targets as pointed. However, on phosphorylation by activation of the Ras/mitogen-activated protein kinase pathway, yan loses the ability to act as a transcriptional repressor and allows pointed to bind to DNA and activate the same gene targets. A similar process may occur with respect to NERF2 and ELF-1 during chicken blood vessel development. In contrast to cNERF2, which appears to be expressed predominantly in the larger vessels of the developing chicken CAM, cELF-1 is expressed not only in the larger vessels but also in the smaller vessels and capillaries of the CAM.

The Tie1 and Tie2 genes are both expressed in the early embryonic vascular system. Tie2 is also upregulated in the extraembryonic blood vessels. The results of the present study demonstrate that cNERF2 is expressed in the developing embryonic as well as extraembryonic vasculature. We have previously demonstrated that cELF-1 is similarly expressed in embryonic and extraembryonic blood vessels. Some differences in the expression of NERF and ELF-1 are as follows: ELF-1 was expressed in very small as well as larger extraembryonic vessels, whereas NERF was predominantly expressed in the larger extraembryonic vessels. NERF and ELF-1 are both expressed in the CAM several days after primary vasculogenesis has occurred, suggesting they may also be involved in regulating blood vessel remodeling and maturation during later stages of development. The ability of cELF-1 and cNERF2 to act as positive and negative transcriptional regulators of the Tie1 and Tie2 genes may provide an important mechanism for regulating the expression levels of the Tie receptors during different stages of blood vessel development and vascular remodeling. Data from the gel mobility shift assays demonstrating preference...
terial binding of cELF-1, a positive transactivator in the CAM at E7, most likely reflects a proliferative phase in vascular development, whereas at later stages of relative quiescence, the balance could shift either in favor of preferential binding of cNERF2 to the Tie Ets sites or simply through a reduction in the amount of cELF-1. The coexpression of these factors at various stages of vascular development suggests that additional mechanisms of control, such as phosphorylation of the factors, may also be determinants of the relative activity of these transcription factors.

Further support for the role of the Ets factor in vascular development and angiogenesis comes from knockout experiments of selected Ets factors. Another Ets factor, Fli-1, was also similarly shown to be critical for blood vessel development. Targeted disruption of this gene resulted in defects in the development and angiogenesis of the extraembryonic blood vessels. Targeted disruption of the gene leads to abnormalities in vitelline vein development. The vascular defects appear to be mainly extraembryonic and restricted to larger vessels with normal-appearing capillaries. One of the recently identified targets of Tel is stromelysin, a matrix metalloproteinase (MMP). Tel acts as a transcriptional repressor of the stromelysin gene. The Ets factor Ets-1 is upregulated in endothelial cells during angiogenesis. The gene targets for Ets-1 involve several MMPs, including MMP-1 and MMP-9. Finally, the expression of the extracellular matrix glycoprotein SPARC and thrombospondin in endothelial cells is dependent on the expression of the Ets factor Erg.}

In conclusion, the present study provides substantial support for the role of Ets factors in vascular development and vascular-specific gene expression. Furthermore, the present study provides evidence of the opposing roles of the chicken Ets factors NERF2 and ELF-1 in the regulation of Tie genes during various stages of chicken blood vessel development and maturation.

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