NF1 Regulatory Element Functions as a Repressor of Tissue Plasminogen Activator Expression

Nhat-Long Pham, Amy Franzen, Eugene G. Levin

Objective—Analysis of the distribution of endothelial cell tissue plasminogen activator (tPA) in the vasculature of rodents and primates demonstrated that tPA is constitutively expressed predominately in small artery endothelial cells of brain and lung. The regulatory elements responsible for the highly selective expression of arterial endothelial cell tissue plasminogen activator were sought.

Methods and Results—Transcription factor binding sites were defined by electrophoretic mobility-shift assay (EMSA) analysis using rat lung and brain nuclear extracts and the tPA promoter sequence from −609 to +37 bp. Protein binding to the promoter was found to be mediated by an NF1 site between −158 and −145 bp upstream from the transcriptional start site. Specific binding was confirmed through mutational analysis and competition binding studies. Infection of endothelial cells with a tPA promoter-green fluorescent protein (GFP) (−609 to +37 bp) reporter construct resulted in expression of the GFP, whereas no expression was found in smooth muscle cells. Mutation of the NF1 site increased the GFP expression indicating that the element acts as a repressor.

Conclusions—These results suggest that the 600 bp of the tPA promoter upstream of the transcription start site conveys cell specificity to tPA expression and that an NF1 site within this region acts as a repressor. (Arterioscler Thromb Vasc Biol. 2004;24:982-987.)

Key Words: endothelial cells ■ tissue plasminogen activator ■ gene expression ■ promoter ■ NF1

The endothelium is a functionally heterogeneous group of cells that respond to the diverse needs of specific tissue and organ environments, both constitutively and in response to changing physiological and pathologic conditions.1,2 Little is known about the origins of phenotypic diversity of endothelial cells, although inheritance from distinct sublineages (clonality) and microenvironmental effects have been proposed.3,4 The role of environmental factors promoting specific endothelial phenotypes involves the interaction of endothelial cells with other cell types such as astrocytes, smooth muscle cells, and myocytes, as well as the hemodynamic forces within the vessel lumen. These factors promote gene transcription of specific endothelial cell genes leading to unique endothelial cell phenotypes.5–8

One protein whose expression is highly regulated in endothelial cells is tissue plasminogen activator (tPA). tPA expression occurs in a selective subset of endothelial cells that are defined by both vessel type and organ.9–11 The production of tPA and its secretion into the vasculature has been long assumed to be the responsibility of all endothelial cells. However, studies have demonstrated that in vivo expression of tPA is restricted to a distinct minority of endothelial cells. Immunohistochemical and in situ hybridization analysis of several large vessels in the nonhuman primate showed tPA antigen and mRNA exclusively localized to the endothelium of the vasa vasora, with none found in the large vessel endothelium.11 Physiological studies have indicated that tPA also is found in the brachial and coronary arteries.12–14 When a more thorough analysis of tPA distribution was performed in rodents, endothelial cell tPA expression was found to be predominately localized to the pulmonary arterial endothelium and the pia mater of the brain.14,15 This suggests that constitutive tPA expression is a characteristic of specific endothelial cells.

There have been numerous studies on the molecular regulation of tPA.15,16–18 Some of the experiments involved promoter mapping studies using various types of cultured cells, whereas others have used transgenic animals in which various lengths of promoter were inserted and site specific reporter-gene expression documented. Studies on endothelial cell regulation have been performed primarily with cultured cells from sources that do not express the protein in vivo. Because endothelial cells from most sources begin to express tPA when placed in culture it is not clear whether the results from the studies performed in vitro represent the mechanisms used in the intact animal. In the present study, we have characterized the tPA 5′ flanking region for elements that promote constitutive tPA expression within the endothelial cells of the brain and lung arterioles using freshly prepared tissues. We have examined the first 600 bp upstream from the
transcriptional start site, a sequence that contains a region of homology between the rat, mouse, and human promoter, for protein binding activity and have identified the participation of an NF-1 consensus sequence.

**Methods**

**Nuclear Extract Preparation and EMSA**

Nuclear extracts were prepared from the brains and lungs of 15-day-old rats (Harlan Breeders, Indianapolis, IN). Animals were anesthetized, decapitated, and the brains, lungs, kidneys, and livers removed and placed in buffer A (10 mmol/L HEPES, pH 7.9, 25 mmol/L KC1, 1 mM EGTA, 1 mmol/L EDTA, 0.32 mol/L sucrose, 0.15 mmol/L Spermine, 0.5 mmol/L spermidine, 1 mmol/L DTT, 0.5 mmol/L PMSF, 0.1 mmol/L Benzamid, 1X complete protease inhibitor cocktail [Roche Molecular Biochemicals]), finely minced, and the volume of buffer A increased to a final volume equaling 5× the tissue weight. The samples were homogenized and the homogenate diluted 2 times in buffer A containing 2 mol/L sucrose. This mixture was layered onto undiluted buffer A containing 2 mol/L sucrose and the nuclei isolated by centrifugation for 45 minutes at 70 000	t t at 4°C. The nuclear pellet was washed twice in 600 µL of buffer A containing 20% glycerol and homogenized. To extract the nuclear proteins, 400 µL of buffer A containing 20% glycerol and 1 mol/L KC1 was added to the nuclear homogenate and the mixture incubated for 1 hour at 4°C with rocking. The samples were centrifuged at 244 587g for 50 minutes at 4°C. Nuclear extracts were partially purified by ion exchange chromatography using DEAE-Sephael. Fractions containing eluted protein were dialyzed against 10 mmol/L Tris-HCl, pH 7.5, 50 mmol/L NaCl, 1 mmol/L MgCl2, 4% glycerol, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 0.5 mmol/L DTT, 0.15 mmol/L Spermine, 0.5 mmol/L Spermidine, 0.5 mmol/L PMSF, 1 mM Benzamid, concentrated, and stored at –80°C.

To identify potential binding sites within the promoter –609 to +37, oligonucleotide probes representing 4 overlapping regions of ~300 base pairs each were generated by polymerase chain reaction (PCR)-labeled on the 5 ‘ end with biotin. The oligonucleotides were added at final concentration of 20 fmol to the reactions mixture containing 10 mmol/L Tris, pH 7.5, 50 mmol/L KC1, 1 mmol/L MgCl2, 4% glycerol, 50% glycerol, 100 mmol/L MgCl2, 1 µg/µL Poly(dIdC), and 1% NF-40, and, if required, 2 pmol of specific or nonspecific competitor. The mixture was incubated for 90 minutes at room temperature and fractionated on 6% polyacrylamide gels. The contents of the gel were transferred to nitrocellulose and detection of protein binding activity and have identified the participation of an NF-1 consensus sequence.

**Assessment of GFP Expression in Cultured Cells**

HeLa cells and smooth muscle cells were cultured in DMEM with 10% fetal calf serum and dorsal endothelial cell and umbilical vein endothelial cells were grown in EGM-2 medium (Clonetics) until confluence was achieved. Before infection, the adenovirus was diluted to 5.0×10⁵ pfu/mL in the appropriate growth medium and the cells infected with either 20 pfu/cell (fluorescent microscopy) or 50 pfu/cell (fluorescence-activated cell sorter [FACS]). The cells were incubated for 24 hours to 48 hours at 37°C. For fluorescence microscopy, the cells were washed with PBS and images captured with a Leica fluorescence microscope equipped with a Hamamatsu CCD camera and images analyzed using the OpenLab Software (Improvision). For FACS analysis, the cells were washed with phosphate-buffered saline twice and removed from the dish with trypsin. The cells were resuspended in 300 µL of cold 2% FBS and FACS analysis was performed with a Becton-Dickinson FACScan calibrated before use with CaliBRITE beads (Becton-Dickinson). Data were acquired using CellQuest Software.

**Results**

**Protein Binding Activity**

Comparison of the rat, mouse, and human tPA promoter sequence shows a high degree of homology within the first 300 bp proximal to the transcriptional start sites (Figure 1). The protein binding activity of a 600 bp fragment (~609 to +37) of the rat tPA promoter was examined by EMSA analysis using nuclear extracts derived from rat brain or lung. In the brain, both endothelial cells and neurons express tPA, whereas in the lung, endothelial cells are the only source of this protein. Therefore, to maximize the likelihood that endothelial cell-specific elements were being identified, only results that were identical with both lung and brain extracts were pursued. The initial EMSA analysis was performed with oligonucleotides representing 4 overlapping promoter fragments of ~300 bp each. Specific binding was observed with fragment 1 (~287 to +37) and fragment 2...
Two bands appeared in each and lung and brain had identical patterns with each probe (Figure 2B). To determine the sequence to which the proteins bound, fragment 1 was divided further into increasingly smaller overlapping oligonucleotides. The upper band observed in the 300-bp fragment disappeared as the fragments became smaller while the lower band remained, regardless of probe size. EMSA analysis showed complex formation with a 33-bp oligonucleotide probe representing the sequence between −165 and −132 (Figure 2C). Within this oligonucleotide exists an NF1 transcription factor consensus binding sequence.

Evidence that the NF1 site was specifically responsible for the protein binding was sought by performing EMSA analysis with 3 25-bp overlapping fragments containing either the complete or a partial NF1 binding sequence (Figure 3A). Only the fragment containing the complete NF-1 consensus sequence showed binding activity in both lung and brain. Neither kidney (Figure 3A) nor liver (data not shown) displayed specific binding of a protein to the probe indicating that the DNA–protein interaction was not a common occurrence among the various organs. To determine whether the protein binding region represented the NF1 consensus sequence, competition binding studies and mutational analysis were performed. The NF1 consensus sequence was mutated at positions critical for the recognition by NF1 (Figure 3B) and the mutated oligonucleotide analyzed by EMSA. No complexes were formed between the mutated NF1 sequence in either the lung or brain extracts. Binding could also be eliminated by competition with the complete NF1 sequence. Competition for binding with fragments a or c (sequence shown in Figure 3A) that contained partial NF1 consensus sequences had no effect on the binding activity of fragment b indicating that the intact sequence was necessary for protein binding.

**Functional Role of the NF1 Consensus Sequence**

To determine whether the promoter fragment studied can support transcriptional activity in cells that express tPA endogenously, adenoviral constructs containing the tPA promoter sequence −609 to +37 placed upstream from the coding sequence of GFP were added to cultures of HeLa cells, dermal microvascular cells, umbilical vein endothelial cells, and GFP expression evaluated (Figure 4). Photomicrographic analysis shows that GFP is expressed at detectable levels in each of these cells within 48 hours after infection. Infection of B109 cells, a neuroendocrine cell line, showed no GFP expression in the presence of the tPA promoter but were positive when the CMV promoter was substituted (data not shown). Mock infection controls were blank. A more detailed analysis of tPA promoter-driven GFP expression was performed by FACS using the 600 bp promoter-GFP (−609 to +37) construct, a shorter promoter sequence containing sequence −289 to +37, and each of these with a mutation residing in the NF-1 site (Figure 5). In endothelial cells, the lowest level of GFP expression was observed with the 600-bp fragment. A significant increase in expression (2.8-fold) occurred when the NF1 site was mutated (as in Figure 3C). Reduction in the length of the promoter to include the sequence from −287 to +37 also resulted in an increase in GFP expression even though the NF1 site was intact. A further increase in GFP expression was observed when the NF1 site was altered in this shortened fragment substantiating the results with the 600 bp promoter. When these studies were repeated with smooth muscle cells, no GFP expression was observed with any of the constructs despite the fact that cells infected with virus containing the CMV in place of the tPA promoter expressed as efficiently as the endothelial cells. In HeLa cells, GFP expression followed the same pattern as with the endothelial cells, with an increase when the NF1 site was mutated and when the fragment was shortened to 300 bp.

**Discussion**

In this study, we examined the possibility that regulatory elements within the tPA promoter region are involved with the control of limited endothelial cell tPA expression. Using a fragment consisting of the promoter sequence spanning −609 to +37, we performed EMSA analysis with nuclear fractions derived from brain and lung. The rationale for choosing these 2 organs is the presence of tPA producing endothelium, a condition that is absent from other organs analyzed. Fifteen-day-old animals were used because of the continued development of the lung and brain vasculature with the attending proliferation of arterial endothelial cells. Such development would require continued transcriptional activity of the tPA gene. Within this fragment there appeared a protein binding activity that resided in an NF1 consensus sequence within 200-bp of the transcriptional start site. Commensurate with its protein binding activity is the repression of GFP expression as demonstrated by an increase in GFP expression in both endothelial and HeLa cells when the NF1 was altered. Cell specificity was suggested because smooth muscle cells and neuroendocrine cells infected with the virus containing the wild-type promoters had no detectable GFP expression. These findings are consistent with our hypothesis that the tPA promoter is responsible for regulating tPA expression in a cell type-dependent manner. This is also
supported by the absence of specific binding in kidney and liver, neither of which have detectable levels of endothelial cell tPA expression.10 Parallel results between endothelial cells and HeLa cells also were reported in other studies using plasmids containing promoter-reporter gene constructs containing 410 bp of the proximal promoter region.24 These constructs were sufficient to generate detectable levels of basal reporter activity in transfected endothelial cells and...
HeLa cells and in both cases were responsive to phorbol esters. HeLa cells produce endogenous tPA in culture and studies of the regulation of tPA antigen expression show a pattern consistent with endothelial cell regulation.24–26 An unexpected result in these studies was the increase in repressor activity when the portion of the promoter containing sequence /H11002 609 to /H11002 288 was removed from the construct. With this shorter construct, GFP expression increased considerably whether the NF1 was mutated. This region has no known regulatory elements and binding assays of fragment 2 and 3 (Figure 2) that contain all or part of this region show no bands that are specific to both lung and brain. These data suggest that the presence of this promoter fragment enhances the repressor activity of the NF1 through secondary effects, eg, by altering the structure of the DNA to allow for increased function at the NF1 site or that a factor exists within the cultured cells that is not present/detectable in tissues by EMSA.

The NF1 family of nuclear regulatory factors can both activate and repress gene transcription.27,28 In addition, NF1 can repress a promoter in 1 type of cell whereas activating the same promoter in another.29 Transcriptional repressors regulate gene expression by at least 2 distinct mechanisms. Nuclear proteins may compete with positive transcriptional activators for common DNA-binding sites, preventing binding of such factors, or destabilizing bound factors.30 They may also contain intrinsic repressor activity and the capacity to downregulate transcription through internal domains. It is not clear in the case of the tPA promoter whether the NF1 binding protein is acting passively or actively. However, the proximity of the NF1 site to a CRE (Figure 1) creates the opportunity for interaction between the 2 factors, an event that has been observed in other promoters.31–33 In addition to the NF1 site, other regulatory elements within the first 200 bp of the proximal promoter region have been associated with regulation of constitutive t-PA expression as well as induction of expression in response to various agonists.34–35 In HeLa cells, a cAMP response-like element and an SP-1 site act cooperatively to mediate phorbol-ester–mediated induction of tPA gene expression, and in endothelial cells cyclic...
strain induction was shown to be dependent on the CRE and an AP-2 site.26,36
In summary, this study identifies a binding sequence in the tPA promoter that binds to an as yet unidentified protein found in the nuclear extract of lung and brain. This protein acts as a repressor of tPA expression in endothelial cells and other cells that produce tPA in vitro. In addition, promoter activity is absent in smooth muscle cells that are incapable of producing tPA in vivo.

References
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