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The Tie2 5′ untranslated region is inhibitory to 5′ end-mediated translation initiation

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Abstract **Tie2 is an endothelium-specific receptor tyrosine kinase required for normal blood vessel maturation, remodeling, and stability. Tie2 expression is also upregulated in various cancers implicating a role in tumor angiogenesis. Its mRNA transcript contains an unusually long (372 nucleotides) 5′ untranslated region (UTR) with five upstream open reading frames (uORFs) and an internal ribosome entry site (IRES) that allows this mRNA to be translated under hypoxic conditions. This sets up an alternative initiation pathway with the potential to clash with 5′ end-mediated initiation from the same template. Herein, we define experimental conditions under which the Tie2 IRES is not active, allowing us to assess the contribution of the 5′ UTR to cap-dependent translation on the Tie2 transcript. We find that the Tie2 5′ UTR is inhibitory to translation initiation with ribosome flow decreasing following encounters with each uORF. No single uORF was found to harbor significant *cis*-acting inhibitory activity. Our results suggest that the uORFs within the Tie2 5′ UTR serve to decrease the percent of ribosomes competent for reinitiation as these traverse the mRNA 5′ UTR, thus minimizing interference with the IRES.**

Keywords: Internal initiation; Tie2; uORF; 5′ UTR

1. Introduction

Angiogenesis is a process of new blood vessel formation that is the culmination of mitogenic and tissue remodeling events resulting in neo-vascularization. It is a physiological process that is required for normal embryonic development, female reproductive function, and wound healing. During this process, angiogenesis is tightly regulated by a balance of positive and negative factors. However, in various disease states, including diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, and several cancers, deregulation of angiogenesis contributes to disease progression [1]. It is also well documented that tumor angiogenesis is essential for solid tumor growth and metastasis [2]. Previously published reports have indicated that angiogenesis involves the coordinated activities of at least two families of receptor tyrosine kinases (RTKs), the vascular endothelial growth factor receptor (VEGFR) and the Tie receptor families [3].

The Tie receptors, Tie1 and Tie2, are endothelial-specific receptor tyrosine kinases that share a number of common structural features. Although the functional significance and signaling partners of Tie1 are not well defined, the angiopoietins Ang1 and Ang2, have been identified and characterized as ligands for the Tie2 receptor [4,5]. Disruption of Tie2 function in mice results in embryonic lethality due to defects in vascular development, characterized by a reduction in endothelial cell number and a defect in the morphogenesis of microvessels [6,7]. Disrupting the function of Ang1, an agonist of the Tie2 receptor, or overproduction of the antagonist Ang2, yields a phenotype similar to disruption of the Tie2 gene, confirming the importance of the Ang/Tie2 pathway during embryonic vascular development [4,5]. Tie2 is also expressed and phosphorylated in quiescent adult endothelial cells suggesting that it plays an active role in the maintenance of blood vessels [8]. Moreover, this receptor is upregulated in capillaries during the process of neovascularization, including skin wounds and tumors [8–11]. Consistent with an essential role for Tie2 in angiogenesis, a missense mutation in the Tie2 gene has been shown to be associated with venous malformations – the most common error of vascular morphogenesis in humans, typically resulting from an imbalance of endothelial cells and smooth muscle cells [12].

While much research effort has focused on the function and biochemistry of the Tie2 gene product, very little is known about the translational regulation of Tie2 expression. The Tie2 gene encodes an mRNA with an unusually long (372 nucleotides) 5′ UTR with 5 uORFs [13]. Upstream open reading frames (uORFs) in mRNAs are known to regulate translation in eukaryotes and are particularly common in mRNAs coding for proto-oncogenes, transcription factors, and genes involved in the control of cellular growth and differentiation (reviewed in [14]). A variety of translational control mechanisms mediated by uORFs have been documented, ranging from *cis*-acting peptide-induced ribosome stalling, position- or length-dependent influences on downstream initiation events, regulation of IRES activity, and effects on mRNA stability [14,15]. When uORFs induce reinitiation of translation, this is generally an inefficient mechanism that is possible only after translation of short uORFs [16,17]. It is thought that the distance between the termination codon of the upstream ORF and the initiation codon at the downstream ORF affects the rate of reloading of the eIF2/GTP/Met-tRNA$_i$ ternary complex onto scanning ribosomes, with increasing distance (time) allowing for higher reinitiation

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efficiencies. In some cases, the frequency of reinitiation at a downstream ORF depends on the coding content of the uORF (e.g., the *Neurospora crassa arg-2* gene), the sequence context of the termination codon, or the ability of the uORF to induce shunting (reviewed in [14,17]).

Cellular IRESes have been identified in many genes involved in cell growth, proliferation, apoptosis and angiogenesis (reviewed in [18,19]). We have previously demonstrated that translation of the Tie2 mRNA is maintained during hypoxic conditions due to the presence of an IRES [13]. The presence of an IRES on a capped mRNA raises an interesting problem to the cell – how to regulate the two initiation mechanisms (cap-dependent and IRESmediated) in a manner that avoids non-productive interference. In this regard, it has been proposed that for the mRNA encoding the arginine/lysine transporter *cat-1*, cap-dependent initiation mechanism inhibits IRES activity. For *cat-1*, ribosomes that have initiated in a cap-dependent fashion disrupt RNA–RNA interactions between the upstream and downstream ends of an IRES and prevent inducible internal initiation [15]. Physiological situations that decrease cap-dependent protein synthesis induce cat-1 IRES activity. Interestingly, Tie2 expression does not significantly change between normoxic and hypoxic states [13]. In this report, we investigate the contribution of 5′ end-mediated initiation to Tie2 expression and find that ribosomes competent for reinitiation decrease as they traverse the 5′ UTR and encounter downstream uORFs.

2. Materials and methods

2.1. Generation of monocistronic and bicistronic constructs

The CAT reporter plasmid, pSKII/CAT, has been previously described [20]. A fragment (441 bp) corresponding to the hTie2 5′ UTR sequence (nucleotides −372 to +69) was fused to the CAT-reporter gene in pSKII/CAT. To generate this construct, RT-PCR was performed by using total RNA isolated from primary human umbilical vein endothelial cells (Clonetics; Walkersville, MD). Primer sequences based on the Tie2 −5′ UTR sequence are as follows: sense ($^{5'}$TTTGATATCAGATCTAAGCTTAAATTCCTCTGCCCCTACAGCAGC$^{3'}$) and antisense ($^{5'}$TTTTAGATCTGGCACCTTCCACAGTTCCAG$^{3'}$). The obtained PCR product was cloned into pSKII/CAT. Mutants of the Tie2 5′ UTR were constructed using PCR and subcloned into pSKII/CAT. For generating mammalian expression vectors, fragments containing the Tie2 5′ UTR and CAT reporter gene were subcloned into pcDNA3. All recombinant clones were sequenced to ensure that no additional changes had occurred. A monocistronic luciferase reporter construct was generated based on FLUC/pcDNA3. A fragment containing the Tie2 5′ UTR was excised from Tie2/CAT and subcloned into FLUC/pcDNA3.

To generate expression constructs with individual Tie2 uORFs (Fig. 5), complementary oligonucleotide pairs encompassing each uORF were annealed and cloned into the pGL-Basic plasmid (Promega; Madison, WI). DNA fragments containing individual uORFs and firefly luciferase were then transferred into pcDNA3.

Bicistronic constructs generated were based on pGEMCAT/FLUC [20]. The hTie2 5′ UTR sequence was amplified by PCR and cloned into the intercistronic region of pGEMCAT/FLUC. CAT/EMCV/FLUC was generated by inserting the EMCV 5′ UTR into the intercistronic region of plasmid pGEMCAT/FLUC. The accuracy of all constructs was confirmed by sequencing.

2.2. In vitro transcription/translation reactions

For in vitro translation studies, plasmids were linearized and mRNA transcripts synthesized in the presence of m^7GpppG or ApppG, as previously described [21,22]. Capped RNA transcripts were quantified by monitoring the incorporation of ^{3}H CTP and the quality of each RNA preparation assessed by SYBR gold (Molecular Probes; Eugene, OR) staining following fractionation on formaldehyde/1% agarose gels. In vitro translations in rabbit reticulocyte lysates and wheat germ extracts were carried out using ^{35}*S*-methionine as instructed by the manufacturer (Promega). Krebs translation extracts were prepared and used for in vitro translation reactions, as previously described [23].

2.3. RNA stability

^{32}P-labeled mRNAs were incubated under standard in vitro translation conditions in wheat germ extracts at 25 °C for 0, 15, 30, 60, and 120 min, respectively. Each translation reaction (10 μl) was then treated with 50 μg of proteinase K at 37 °C for 15 min, followed by phenol/chloroform extraction. After ethanol precipitation, RNA samples were resolved on formaldehyde/1% agarose gels. The gels were dried and exposed to X-ray films at −70 °C.

2.4. Cell culture and transient DNA/RNA transfections

Primary human umbilical vein endothelial cells (HUVECs) were maintained in endothelial growth media-2 (EGM-2) supplemented with growth factors, 2% fetal bovine serum (FBS), 50 μg/ml gentamicin, and 50 μg/ml amphotericin according to the manufacturer's instructions. DNA transfections into primary HUVECs were performed using Lipofectin (Invitrogen; Carlsbad, CA), as specified by the manufacturer. Briefly, $1.5–1.8 \times 10^5$ cells were seeded per 10 cm^2 plate and grown in EGM-2 medium supplemented with 2% FBS. Cells were harvested 24 h post-transfection and CAT and Renilla luciferase activities were measured using the CAT ELISA (Roche Applied Science; Penzberg, Germany) and the Renilla Luciferase reporter assay systems (Promega; Madison, WI). Transient RNA transfections were performed using the cationic lipid reagent, DMRIE-C (Invitrogen). Approximately 1.8×10^6 HUVECs were seeded per 10 cm^2 plate 24 h prior to transfection. Cells were then transfected with 20 μg of capped and polyadenylated mRNA and harvested 8 h post-transfection. Luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega). Transfections were performed in duplicate and repeated three times. All experiments with HUVECs were performed between passages two through five.

3. Results

The human Tie2 5′ UTR contains five uORFs, the last of which overlaps with the putative Tie2 initiation codon (Fig. 1A). Comparison of the Tie2 5′ UTR sequence from the human and mouse mRNAs revealed that although the absolute number of uORFs is not conserved, the relative positions of four of the uORFs are (Fig. 1A). Further analysis reveals that if uORF5 is translated, it should shunt ribosomes past the Tie2 initiation codon (which lies embedded within the uORF5 coding region) to a downstream AUG codon (Fig. 1A). The presence of five uORFs, as in the Tie2 mRNA 5′ UTR, is unusually rare.

3.1. The Tie2 5′ UTR is significantly inhibitory to translation in vitro

In order to obtain insight into the putative inhibitory effects of the Tie2 5′ UTR on translation initiation, we generated a series of CAT reporter constructs containing the full-length Tie2 5′ UTR (Tie2/CAT) or the 5′ UTR lacking all uAUGs except for the predicted initiation codon (AUG_{373}; Tie2/CAT-ΔuORF; Fig. 1B). Two reporter constructs were generated in which the CAT initiator AUG was placed in-frame with the Tie2 initiation codon at AUG_{373}. Tie2/CAT contains all 5 uORFs, whereas in Tie2/CATΔuORF, these have been removed by site-directed mutagenesis of the uAUGs (Fig. 1B). Translations of Krebs-2 extracts programmed with mRNAs derived from these reporter constructs demonstrated that very little CAT protein is produced from Tie2/CAT, whereas a