Hypoxic Stabilization of Vascular Endothelial Growth Factor mRNA by the RNA-binding Protein HuR*

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Vascular endothelial growth factor (VEGF) is a potent angiogenic factor whose expression is dramatically induced by hypoxia due in large part to an increase in the stability of its mRNA. Here we show that HuR binds with high affinity and specificity to the element that regulates VEGF mRNA stability by hypoxia. Inhibition of HuR expression abrogates the hypoxia-mediated increase in VEGF mRNA stability. Overexpression of HuR increases the stability of VEGF mRNA. However, this only occurs efficiently in hypoxic cells. We further show that the stabilization of VEGF mRNA can be recapitulated in vitro. Using an S-100 extract, we show that the addition of recombinant HuR stabilizes VEGF mRNA markedly. These data support the critical role of HuR in mediating the hypoxic stabilization of VEGF mRNA by hypoxia.

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Vascular endothelial growth factor (VEGF) has been demonstrated both in vitro and in vivo to be a significant mediator of hypoxia-induced angiogenesis in such diverse disease processes as diabetic retinopathy (1), tumor angiogenesis (2), and coronary artery disease (3). Pharmacological manipulation of VEGF in these disorders either to augment or to inhibit neovascularization requires an understanding of the molecular mechanisms regulating VEGF induction by hypoxia.

We have demonstrated previously that the increase in VEGF protein and biological activity secreted by cells exposed to hypoxia is in large part the result of an increase in VEGF mRNA stability with the half-life of VEGF mRNA increasing 3–8-fold under hypoxic conditions (4–6). In vitro RNA degradation assays performed with VEGF mRNA and S-100 extracts from normoxic and hypoxic cells have permitted not only the identification of specific regions of VEGF mRNA which are responsible for the lability of VEGF mRNA under normoxic conditions but also regions that are critical for the stabilization of VEGF mRNA by hypoxia (5). In the case of the destabilizing elements in the VEGF 3′-untranslated region (UTR) the sequences correspond to canonical nonamer instability sequences (7–9) that have been shown to mediate the rapid turnover of many other cytokines and oncogenes.

With regard to the hypoxia-stabilizing element in the VEGF 3′-UTR we have described previously a novel hypoxia-inducible protein complex that binds to a adenylate-uridylate (AU)-rich element in this region (6). Affinity purification of the hypoxia-inducible protein complex using an RNA corresponding to this binding site in the VEGF mRNA 3′-UTR has allowed for the identification of three hypoxia-inducible proteins of approximately 34, 28, and 17 kDa.

The identity of these proteins has remained unknown. It is clear that at least one of the proteins in this complex binds specifically to an AU-rich element in the VEGF 3′-UTR. The cellular proteins that bind to such AU-rich elements have been the focus of much investigation (10–15). Recently, a 36-kDa RNA binding protein, HuR, which binds to AU-rich elements with high affinity and selectivity, has been identified and cloned (14, 16, 17). In view of its similarities in binding specificity and size to the 34-kDa component of the hypoxia-inducible protein complex, we sought to determine whether HuR might play a role in the hypoxic stabilization of VEGF mRNA.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—293T cells were obtained from the ATCC. WT-8 (18) and 786-0 cells were obtained from Dr. William Kaelin (Dana Farber Cancer Institute, Boston, MA). All cell lines were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Cells were cultured under either normoxic conditions (5% CO2, 21% O2, and balance N2) or hypoxic conditions (5% CO2, 1% O2, balance N2) in a Forma 3130 incubator (Forma Scientific, Marietta, OH).

Preparation of S-100 Extracts and in Vitro RNA Degradation Assays with HuR and HuR Antiserum—The S-100 fraction of cytosolic proteins was prepared and in vitro RNA degradation assays performed as described previously (5).

Preparation of Labeled RNA Transcripts—pSP65Hg (γ-globulin) was linearized with Sau3AI. This template was incubated with Sp6 RNA polymerase and [32P]UTP yielding a transcript of 165 nucleotides. pSP65Hg was provided by Dr. Gary Brewer. The DNA templates for the VEGF transcripts (fragment VRS and its subfragments A–F) were synthesized by polymerase chain reaction using the following oligonucleotides. For VEGF template VRS corresponding to VEGF 3′-UTR nucleotides 1252–1878 the oligonucleotides were T,1252 (CCATAACGGGACCTCATATAGGAGATTTCCAATATTTTATGAGGA) and 1878a (TTTGGAGATCAAGCTTTTGAGATTTTTAATACAAATG). For subfragment A corresponding to VEGF 3′-UTR nucleotides 1252–1470 the oligonucleotides were T,1252 and 1470a (TTCAAGGGATCTTTTGGTGTTGAGG). For subfragment B corresponding to VEGF 3′-UTR nucleotides 1472–1510 the oligonucleotides were T,1472 (CCATAACGGCAAATATACCTTCATTTTATGAGGA) and 1510a (TTTGGAGATCAAGCTTTTGAGATTTTTAATACAAATG). For subfragment C corresponding to VEGF 3′-UTR nucleotides 1508–1573 the oligonucleotides were T,1508 (CCATAACGGCAAATATACCTTCATTTTATGAGGA) and 1573a (CCATAACGGCAAATATACCTTCATTTTATGAGGA). For subfragment D corresponding to VEGF 3′-UTR nucleotides 1631–1678 the oligonucleotides were T,1631 (CCATAACGGCAAATATACCTTCATTTTATGAGGA) and 1678a (TTTGGAGATCAAGCTTTTGAGATTTTTAATACAAATG). For subfrag-
fig. 1. Structure of VEGF mRNA showing the sequence and location of the VRS and its subfragments A–F. The VRS (5), corresponding to VEGF 3'-UTR nucleotides 1695–1878, is shown as the boxed area adjacent to the poly(A) tail. The shaded element within the VRS box indicates the HuR binding site. The complete sequence of the VRS is shown below, and the element bound by HuR is shown as the boxed sequence. The locations of subfragments A–F of the VRS described in Fig. 4 are also shown. ORF, open reading frame.

![Diagram of VEGF mRNA structure](Image)

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**RESULTS**

**HuR Specifically Binds to the Regulatory Element in the 3′-UTR of VEGF mRNA**—Fig. 1 shows the structure of VEGF mRNA and of the RNAs (VRS, A, B, C, E, and F) used in these studies. VRS (the VEGF regulatory segment) contains the signals necessary for the post-transcriptional regulation of VEGF mRNA (5). Previous studies have shown that HuR binds specifically to similar regulatory elements in other mRNAs (14, 17). Thus we investigated whether HuR can bind to the regulatory elements of VEGF mRNA. Purified recombinant GST-HuR fusion protein was incubated with labeled transcripts and HuR-RNA complex formation assayed by gel retardation analysis (17, 20). HuR bound avidly to the VRS, and complex formation was easily detectable with 1 nM HuR (Fig. 2, lanes 5–8). No complex formation was detectable in the absence of HuR or with 200 nM GST. Complex formation is specific since no reaction was observed with a control RNA (the 3′-end of the human γ-globin mRNA) of similar composition which does not contain an AU-rich regulatory element (Fig. 2, lane 2).

**Interaction between HuR and the VRS** was investigated further using a quantitative RNA binding assay. We employed the same method as used originally for the R17 coat protein (21). A low concentration of labeled RNA was incubated with increasing concentrations of HuR protein as indicated. The reactions were filtered through nitrocellulose and the bound radioactivity determined. Fig. 3A shows that the formation of the VEGF mRNA-HuR complex is detectable at 0.1 nM, has a midpoint at about 9 nM, and reaches a plateau above 50 nM with about 80% of the input RNA bound. Complex formation with globin RNA was not detectable under these conditions (Fig. 3A). A plot of the log of complex/free RNA versus log HuR concentration shows a straight line with an intersect on the x axis at 9 nM (Fig. 3B). Thus the binding of HuR to VEGF mRNA is a simple molecular reaction with an apparent $K_d$ of 9 nM.

Next, we localized the HuR binding site within the VRS of VEGF mRNA. To do this we utilized the RNase T1 selection analysis that we have used previously in the analysis of other mRNAs (17). In this technique the HuR-RNA complex is formed and then digested with RNase T1. The specific RNA fragments bound to HuR are isolated by absorption of the complex to nitrocellulose and elution of the RNA fragments with phenol-chloroform (20). Fig. 4, lane 18, shows the RNase T1 digestion pattern of VRS RNA. HuR bound specifically to a 45-nucleotide fragment (Fig. 4, lane 17). The negative control (GST protein) did not select any fragments (Fig. 4, lane 16). This suggested that HuR bound specifically to the AU-rich element shown in Fig. 1. To confirm this we performed the same analysis on subfragments of the VRS. If our assignment of the binding site was correct we would expect that subfragment E would contain the binding site since it encodes the 45-nucleotide RNA-HuR complex. Indeed, this was the case. Only subfragment E yielded a selected fragment that was the same size as that selected from the VRS (Fig. 4, lanes 2, 5, 8, 11, and 14). It is interesting to note that the 45-nucleotide binding site is 100% conserved between VEGF mRNAs from rat and man (22), the only species for which sequence data are currently available from this region.

**Antisense HuR mRNA Blocks the Hypoxic Stabilization of**
VEGF mRNA—The in vitro binding data described above clearly demonstrate that HuR binds selectively to an AU-rich element in the regulatory element of VEGF mRNA. To determine if HuR plays an important role in the hypoxic regulation of VEGF mRNA stability in vivo, we next developed stable transformants of human 293T cells which expressed antisense HuR mRNA constitutively. 293T cells were chosen because the transformants of human 293T cells which expressed antisense HuR mRNA. 10 µg of total cell extract from hypoxic or normoxic R.11 or mvr 29 cells was subjected to Western blot analysis using an HuR affinity-purified antisera. The immunoreactive protein of 34 kDa present in mvr 29 cells is consistent with the previously reported size of HuR. This 34-kDa HuR immunoreactive protein is decreased in R.11 under both hypoxic and normoxic conditions. Panel A, antisense HuR mRNA blocks the hypoxic stabilization of VEGF mRNA. The half-life of VEGF mRNA in clone mvr 29 and R.11 was determined using actinomycin D as described previously (4, 43). The half-life of VEGF mRNA in clone mvr 29 and R.11 was identical under normoxic conditions (4.8 and 4.0 h in two independent experiments). Under hypoxic conditions only clone mvr 29 significantly increased the stability of VEGF mRNA (8.0 h in two independent experiments compared with 4.8 h for R.11).

**Table I**

| Cell line | VEGF mRNA half-life under hypoxic (1% O₂) or normoxic (21% O₂) conditions |
|-----------|--------------------------------------------------------------------------------|
|           | 1% O₂ | 21% O₂ |
| mvr 29    | 8.0   | 4.8    |
| H.26      | >20   | 3.8    |
| R.11      | 4.8   | 4.0    |

Although the antisense clone R.11 demonstrated a 2.2-fold increase in VEGF mRNA stability, actinomycin D chase experiments were performed with wild type clone mvr 29 or antisense clone R.11 (Fig. 5B and Table I). Whereas the half-life of VEGF mRNA in wild type and antisense HuR clones was virtually identical under normoxic conditions (4, 8, and 4, 0 h, respectively), only in the wild type cells did the half-life of VEGF mRNA increase with hypoxia (mean 8.0 h for mvr 29 and 4.8 h for R.11) (Fig. 5B and Table I). These stability data are consistent with the observation that, although wild type mvr 29 cells demonstrated a 2.2 ± 0.3-fold (n = 4) hypoxic induction of steady-state VEGF mRNA, the antisense clone R.11 demonstrated only a 1.2 ± 0.3-fold (n = 4) increase in steady-state VEGF mRNA with hypoxia.

**Overexpression of HuR mRNA Increases the Stability of VEGF mRNA under Hypoxic Conditions**—Although the antisense studies described above demonstrate that HuR is critical for the hypoxic stabilization of VEGF mRNA, they do not address whether HuR is sufficient to increase VEGF mRNA stability. We therefore sought to determine if overexpression of HuR could increase VEGF mRNA in vivo by developing stable transformants of human 293T cells which constitutively overexpressed HuR.

HuR under the control of the cytomegalovirus promoter was cloned into the pZeoSV2(−) expression plasmid (Invitrogen), and stable transfectants of the 293T cells expressing high constitutive levels of antisense HuR under the control of a cytomegalovirus promoter were obtained (e.g. 293T clone R.11). As a control, wild type 293T cells stably transformed with the pZeoSV2(−) vector backbone were also characterized (293T clone mvr 29). Panel A, HuR protein is decreased in cells expressing antisense HuR mRNA. 10 µg of total cell extract from hypoxic or normoxic R.11 or mvr 29 cells was subjected to Western blot analysis using the HuR affinity-purified antisera (Fig. 5A). The immunoreactive protein of 34 kDa present in mvr 29 cells is consistent with the previously reported size of HuR (14, 17). There was no significant difference in the amount of this protein between hypoxic and normoxic mvr 29 cells. We found that the 34-kDa HuR immunoreactive protein was decreased markedly in antisense clone R.11 compared with wild type clone mvr 29 (Fig. 5A).

To demonstrate that HuR is critical for the hypoxia-inducible increase in VEGF mRNA stability, actinomycin D chase experiments were performed with wild type clone mvr 29 or antisense clone R.11 (Fig. 5B and Table I). Whereas the half-life of VEGF mRNA in wild type and antisense HuR clones was virtually identical under normoxic conditions (mean 4.9 and 4.0 h, respectively), only in the wild type cells did the half-life of VEGF mRNA increase with hypoxia (mean 8.0 h for mvr 29 and 4.8 h for R.11) (Fig. 5B and Table I). These stability data are consistent with the observation that, although wild type mvr 29 cells demonstrated a 2.2 ± 0.3-fold (n = 4) hypoxic induction of steady-state VEGF mRNA, the antisense clone R.11 demonstrated only a 1.2 ± 0.3-fold (n = 4) increase in steady-state VEGF mRNA with hypoxia.
To determine whether overexpression of HuR increased VEGF mRNA stability, we performed actinomycin D chase experiments with the HuR-overexpressing clone H.26 and the wild type clone mvr 29. We found that the half-life of VEGF mRNA was similar in normoxic H.26 and mvr 29 cells (mean of 8.5 h in two independent experiments) (Table I). However, we found that in hypoxic HuR-overexpressing cells the half-life for VEGF mRNA was much longer than in hypoxic wild type cells (mean of >20 h in H.26 compared with a mean of 8 h in mvr 29 in two independent experiments) (Fig. 6B and Table I). These stability data are consistent with the observed steady-state mRNA levels in clones H.26 and mvr 29 in that there was no significant difference in steady-state VEGF mRNA between H.26 and mvr 29 under normoxic conditions, but under hypoxic conditions the half-life of VEGF mRNA in H.26 (mean of >20 h in two independent experiments) was greater than that of mvr 29 (8 h in two independent experiments).

Extract from hypoxic or normoxic H.26 or mvr 29 cells was subjected to Western blot analysis using the HuR affinity-purified antiserum. The immunoreactive protein of approximately 32 kDa was increased in HuR overexpressing clone H.26. Significantly, we found that in hypoxic HuR-overexpressing cells HuR protein was stabilizing VEGF mRNA in an in vitro RNA degradation assay. S-100 extracts were prepared and in vitro RNA degradation assays performed as described previously (5). All time points were performed in triplicate, and the experiment was repeated with different extracts with similar results. Panel A, representative autoradiograph of an in vitro degradation assay with and without HuR using S-100 extract from normoxic WT-8 cells. 30 µg of extract with or without 400 ng of the GST-HuR fusion protein was incubated with full-length rat VEGF 3'-UTR transcripts. The incubation was terminated after 5, 10, 20, or 40 min and the reaction products subjected to electrophoresis on a formaldehyde-agarose gel. After transfer to a nylon membrane the amount of primary undegraded transcript was quantitated by PhosphorImaging analysis. Panel B, linear regression analysis of the in vitro degradation assay with (○) or without HuR (■) presented in panel A. The half-life of VEGF mRNA in this study is 8.5 min in the absence and 30 min in the presence of recombinant HuR.
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The increase in the stability of VEGF mRNA in response to a reduction of the oxygen tension of the cellular milieu has been demonstrated to be mediated by specific sequences in its 3′-UTR. A clearer understanding of the mechanism of this regulation requires the identification and characterization of the transacting factors that interact with these elements. This work identifies HuR as one of the important transacting factors that bind selectively to these sequences and thereby regulate VEGF expression. HuR is a member of the Elav-like protein family. Elav, the founder member of this family, is a *Drosophila* RNA-binding protein required for neuronal differentiation (27). Interest in this family of proteins was originally fueled by the observation that their human homologs are tumor antigens (19). There are four members of the human Elav-like family, namely, HuD, HuC, Hel-N1, and HuR (17, 19, 28, 29). HuD, HuC, and Hel-N1 are expressed exclusively in postmitotic neurons and in specific neuroendocrine tumors (29–32). HuR, the fourth and newest member, is transcribed in all cells (17). The unique structural feature of the Elav-like proteins is the organization of their three ribonucleoprotein-2/ribonucleoprotein-1 type RNA recognition motifs (33). The first and second of these RNA recognition motifs are in tandem and are separated from the third by a segment rich in basic amino acids. The observation that these motifs bind selectively to the AU-rich elements that regulate mRNA turnover suggested that the Elav-like proteins are involved in the post-transcriptional control of gene expression (17, 20, 29, 34, 35). The results presented here on HuR and those recently obtained with HuD2 and Hel-N1 (36) confirm and extend this hypothesis.

Our data indicate that HuR is necessary for the post-transcriptional induction of VEGF expression. Inhibition of HuR expression by antisense constructs inhibits stabilization of VEGF mRNA by hypoxia. On the other hand our data do not permit us to conclude whether or not overexpression of HuR is sufficient for the stabilization of VEGF mRNA. Our data demonstrate that HuR protein was increased to a greater extent under hypoxic conditions than under normoxic conditions. We cannot rule out the possibility that the failure to stabilize VEGF mRNA in the overexpressing clones under normoxic conditions is due to an inadequate amount of HuR protein to mediate this effect. Thus we cannot conclude from these studies whether or not the stabilization of VEGF mRNA by hypoxia requires factors in addition to HuR which are also induced by hypoxia.

Why is HuR critical for the hypoxic stabilization of VEGF mRNA? Our data indicate that total cellular steady-state HuR protein is unchanged by hypoxia. This may suggest that HuR is a critical component of a hypoxia-inducible complex (6) whose other components are regulated by hypoxia. Such a precedent has been set recently for the hypoxia-inducible transcription factor HIF-1 (37–39). HIF-1 is a dimer of HIF-1α, which is regulated by hypoxia (39), and HIF-1β, which is not regulated by hypoxia. Nonetheless, elimination of cellular HIF-1β activity blocks the ability to transactivate hypoxia-inducible genes that are dependent on HIF-1 for this induction (38).

On the other hand, we have not measured directly the amount of active HuR in hypoxic and normoxic cells. Conceivably, HuR protein could be localized differentially in the cell under hypoxic or normoxic conditions, an intriguing possibility given the known ability of many such RNA-binding proteins to shuttle between the nucleus and the cytoplasm (40). Alternatively, HuR may be regulated by phosphorylation.

The observation that the Elav-like proteins stabilize mRNA that contains AU-rich elements has important implications for the understanding of the mechanism of mRNA turnover. The accepted model is that the AU-rich elements are recognized by specific factors. In some models the AU-rich element is recognized by a specific endonuclease. Cleavage of the transcript then occurs followed by rapid degradation catalyzed by a 3′–5′ exonuclease (9, 41). In other models it is proposed that the AU-rich element promotes the deadenylation of the mRNA. Deadenylation of the transcript is again followed by rapid 3′–5′ exonuclease degradation. In either case, it has been proposed that stability factors bind to the AU-rich element and inhibit either the endonuclease activity (42) or the deadenylase activity. In this paper we have mapped the binding site of HuR to be identical to the hypoxia-inducible complex RNA binding site. HuR binds within four nucleotides of a canonical nonamer instability element (7, 8) in the VEGF AU-rich element. Thus it is likely that HuR binding alters the structure of the AU-rich element and renders the site either inaccessible to the endonuclease or unable to promote deadenylation. Our ability to derive an *in vitro* system that responds to the addition of HuR will facilitate the purification of the putative factors and will permit a detailed examination of the mechanism of this reaction.

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