Regulation of Human Vascular Endothelial Growth Factor mRNA Stability in Hypoxia by Heterogeneous Nuclear Ribonucleoprotein L

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A 126-base region of human vascular endothelial growth factor (VEGF) 3′-untranslated region, which we identified as the hypoxia stability region, forms seven hypoxia-inducible RNA-protein complexes with apparent molecular masses ranging from 40 to 90 kDa in RNA-UV-cross-linking assays. In this study, we show that proteins that form the 60-kDa RNA-protein complex with the hypoxia stability region were present in both cytoplasmic and nuclear compartments. We purified the protein associated in the 60-kDa complex and identified it as heterogeneous nuclear ribonucleoprotein L (hnRNP L) by protein sequencing. Removal of hnRNP L by immunoprecipitation specifically abolished formation of the 60-kDa complex. Synthetic deoxyribonucleotide competition studies defined the RNA-binding site of hnRNP L as a 21-base-long sequence, 5′-CACCCACCCCAUAUCAUCAU-3′. Immunoprecipitation of hnRNP L followed by reverse transcription-polymerase chain reaction showed that hnRNP L specifically interacts with VEGF mRNA in hypoxic cells in vivo. Furthermore, when M21 cells transfected with antisense oligodeoxyribonucleotide to the hnRNP L RNA-binding site, the VEGF mRNA half-life was significantly reduced under hypoxic conditions. Thus, we propose that specific association of hnRNP L with VEGF mRNA under hypoxia may play an important role in hypoxia-induced post-transcriptional regulation of VEGF mRNA expression.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a potent angiogenic and endothelial cell-specific mitogen (1, 2). VEGF is expressed and secreted at low levels by most normal cells but constitutively expressed at high levels by many human tumors and tumor cell lines (1–4). Hypoxia up-regulates VEGF expression and several studies have demonstrated that increase in transcription alone does not account for all of the increase in VEGF mRNA (3–8). The post-transcriptional regulation of VEGF mRNA stability also plays a critical role in the observed hypoxic induction (6–8).

Post-transcriptional regulatory mechanisms, especially modulation of mRNA stability, has been shown to play a major role in gene expression (9). The turnover rate of a given mRNA can be determined by interactions of trans-acting factors with specific cis-element located within 3′-untranslated regions (3′-UTR) (9, 10). Many labile mRNAs, including those that encode lymphokines, cytokines, transcription factors, and proto-oncogenes, contain AU-rich elements (AREs) in their 3′-UTR (9). Identification of the interaction of AREs with trans-acting proteins has been the first step in understanding the molecular regulation of mRNA stability (9, 10). The presence of a reiterated pentamer (AUUUA), in many AREs has been shown to be associated with rapid mRNA turnover and translation attenuation (10–12). In the case of granulocyte-macrophage colony-stimulating factor, c-Fos, and c-Myc mRNAs, deletion of the ARE region enhances their stability, and insertion of the region into the 3′-UTR of a normally stable globin mRNA significantly destabilizes it (10–15). A variety of AUUUA-binding proteins have been identified, and examples of these include (i) a 32-kDa nuclear protein from HeLa cells (15); (ii) AU-A, AU-B, and AU-C, 30–43-kDa nuclear and/or cytoplasmic proteins from human T lymphocytes (16, 17); (iii) AUBF, a heterotrimeric protein formed by 15-, 17-, and 19-kDa subunits and present in both nucleas and cytoplasm (18, 19); and (iv) AUBP, a 36-kDa cytoplasmic protein from human spleen identified as glyceraldehyde-3-phosphate dehydrogenase (20). How these mechanisms of these proteins affect mRNA turnover remains unclear.

Our previous work identified a 126-base hypoxia stability region (HSR) in human VEGF 3′-UTR that is critical for the stabilization of VEGF mRNA under hypoxia (21). This region is able to form seven hypoxia-inducible mRNA-protein complexes (21). Here we report hnRNP L as a protein that interacts with the VEGF HSR and forms a hypoxia-inducible 60-kDa RNA-hnRNP L complex. The cytoplasmic hnRNP L specifically interacts with VEGF mRNA in hypoxic cells in vivo and regulated VEGF mRNA stability. Thus, we propose that a specific interaction of hnRNP L with VEGF mRNA may play an important role in hypoxia-induced post-transcriptional regulation of human VEGF mRNA stability.

MATERIALS AND METHODS

Antibodies and Oligodeoxyribonucleotides—Monoclonal antibodies 4D11 (anti-hnRNP L) and 4F4 (anti-hnRNP C) were generously provided by Dr. Gideon Dreyfuss (University Pennsylvania, Philadelphia) (22, 23). All of the oligodeoxyribonucleotides used in the study were synthesized from Genemed Synthesis (San Francisco, CA).

Cell Lines and Culture Conditions—Human melanoma cell line M21 was obtained from Dr. Romaine Saxton (UCLA, Los Angeles, CA). Cells were grown and processed for RNA isolation as described previously (24). Human melanoma cell line M21 was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). All other materials were purchased from Sigma. Reagents were added to culture media at the following final concentrations: sodium pyruvate, 100 μM; streptomycin, 100 μg/ml; penicillin, 100 units/ml; FBS, 10%.

This paper is available on line at http://www.jbc.org

(Received for publication, June 29, 1998, and in revised form, September 25, 1998)
were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM t-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin. Cells were cultured under either normoxic conditions (5% CO₂, 21% O₂, 74% N₂) in a humidified Queue incubator (Asheville, NC) at 37 °C or hypoxic conditions (5% CO₂, 3% O₂, 92% N₂) in a humidified triple gas Heraeus incubator (model 6060, Hanau, Germany) at 37 °C.

Preparation of Cytoplasmic and Nuclear Extracts—Cytoplasmic and nuclear extracts were obtained as described by Claffey et al. (21). Following exposure to normoxia or hypoxia (3% O₂), M21 cells were washed three times in ice-cold PBS followed by lysis in 1% Triton X-100 lysis buffer containing 50 mM Hepes, pH 7.5, 10 mM sodium pyrophosphate, 150 mM NaCl, 100 mM NaF, 0.2 mM NaOAc, 1 mM EGTA, 15 mM MgCl₂, 10% glycerol, and 5 mM 4-(2-aminoethyl)benzene-sulfonyl fluoride (Sigma). The cytoplasmic extract was collected and centrifuged at 14,000 × g for 15 min, and the nuclei pellet were further extracted with the 1% Triton X-100 lysis buffer containing 400 mM NaCl for 15 min on ice. The nuclear extract was recovered after centrifuged at 14,000 × g for 15 min.

In Vitro Transcription—The sense and antisense HSR of human VEGF were transcribed in vitro as described previously (21), using T7 RNA polymerase transcription of NolI-linearized and T3 RNA polymerase transcription of EcoRI-linearized plasmid, respectively. Both biotin-UTP-labeled and [³²P]UTP-labeled RNA transcripts were generated in in vitro transcription (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol and then treated with RNase-free DNase (Promega, Madison, WI) for 15 min at 37 °C. The [³²P]UTP-labeled transcripts were extracted once with phenol:chloroform and loaded onto RNase-free G-50 spin columns (Boehringer Mannheim). The transcripts were then immobilized with 3 volumes of ethanol.

Affinity Purification of mRNA-binding Proteins—Cytoplasmic extracts were prepared after M21 cells were cultured in hypoxia for 24 h. The lysate was sequentially loaded onto poly(A)+, poly(U), antisense HSR, or A-Sepharose 4B affinity columns at room temperature. The sense HSR-Sepharose column was then extensively washed with phosphate-buffered saline, 0.2% Triton X-100. The mRNA-binding proteins were then eluted with 0.9 M NaCl and precipitated with 3 volumes of ethanol.

Identification of hnRNP L as 3′-UTR-binding Protein

RESULTS

Indentification of VEGF 3′-HSR mRNA-binding Proteins—The post-transcriptional regulation of VEGF mRNA stability in response to hypoxia may, in part, be due to the interaction of VEGF 3′-UTR with specific binding protein(s) (6–8, 27, 28). Our previous work identified a 126-base HSR in human VEGF 3′-UTR (Fig. 1A), which is critical for hypoxia-induced human VEGF mRNA stability (21). The HSR formed seven RNA-protein complexes with M21 human melanoma cell cytoplasmic proteins with apparent molecular masses of 90, 88, 72, 60, 56, and 40 kDa in RNA-UVXL (21).

Studies have shown that RNA-binding proteins are also present in nuclear compartments (15–17). Thus, we prepared both cytoplasmic and nuclear extracts from hypoxic or normoxic cultured M21 cells and compared RNA-protein complex formation by RNA-UVXL with [³²P]labeled VEGF HSR. As shown in Fig. 1B, 24-h hypoxia (3% O₂) substantially up-regulated the formation of the 90-, 88-, 72-, and 60-kDa complexes when compared with cytoplasmic extracts made from normoxic (21% O₂) cells. The 90-, 88-, 72-, and 56-kDa complexes were selectively cytoplasmic with little or no nuclear localization (Fig. 1B). Significantly higher levels of the 60-kDa complex were formed with nuclear extracts than cytoplasmic extracts, and the complex was markedly increased by hypoxia treatment in both extracts (Fig. 1B). Other than the 60-kDa complex, the nuclear extract also formed three unique complexes with ap-
parent molecular masses of 45, 48, and 120 kDa (Fig. 1B). Taken together, these results suggest that proteins that form the 90-, 80-, 72-, and 56-kDa complexes with HSR mainly appear in cytoplasm. In contrast, the protein that forms the 60-kDa complex with HSR is present in both nuclear and cytoplasmic compartments with the majority in the nucleus. Thus, given the distribution and the hypoxia induction of the 60-kDa complex, we established a protocol to purify the RNA-binding protein in the 60-kDa complex.

**Purification of the RNA-binding Protein in the 60-kDa RNA-Protein Complex**—The VEGF HSR is a highly AU-rich element containing 54 A bases and 52 U bases out of 126 bases, resulting in 43% A and 41% U distribution (Fig. 1A). To purify the binding protein that forms the 60-kDa complex, hypoxia-treated M21 cell lysates were loaded sequentially onto poly(A), poly(U), and antisense HSR-coupled columns to remove non-specific RNA-protein interactions prior to loading to the sense HSR-coupled column. To ensure that the protein that formed the 60-kDa complex was not removed by poly(A), poly(U), and antisense HSR-coupled columns, the flow-through from each column was collected, and RNA-UVXL was performed with 32P-labeled HSR. As shown in Fig. 2A, a poly(A) column effectively interacted with proteins forming the 90- and 72-kDa complexes and removed them from the cell lysates, whereas the poly(U) column removed the proteins that formed the 88-, 56-, 46-, and 40-kDa complexes. Further incubation of the lysate with the antisense HSR column completely removed proteins forming the 72-kDa complex, whereas the 60-kDa complex selectively bound to the sense HSR column (Fig. 2A). These results suggest that the proteins forming the 90- and 72-kDa complexes can interact with poly(A) sequences, whereas proteins forming the 88-, 56-, 46-, and 40-kDa complexes can interact with poly(U) sequences. In contrast, the proteins present in the 60-kDa complex only specifically interact with a unique sequence in the HSR.

To analyze proteins that bound to the four mRNA columns, small aliquots of the column material were directly analyzed in reducing 6% SDS-PAGE. As shown in Fig. 2B, all four mRNA columns interacted with numerous proteins. However, the sense HSR column contained one unique protein band with an apparent molecular mass near 60 kDa (indicated by an arrow). When eluted with various concentrations of NaCl, the 60-kDa protein was successfully released from the sense HSR column with 0.9 M NaCl (Fig. 2B, indicated by an arrow). The purified 60-kDa protein was then separated in a preparative 6% SDS-PAGE, stained with Coomasie Blue, and sequenced after tryptic digest and HPLC purification (Harvard Microchemistry Protein Sequencing Facility). A 16-amino acid sequence of an HPLC-purified peptide was determined as SDALETLGFNLHYQMK, which is identical to hnRNPL, amino acid residues 522–537 (22).

**The RNA-binding Protein in the 60-kDa Complex Is hnRNPL**—To further confirm the RNA-binding protein in the 60-kDa complex as hnRNPL, we used anti-hnRNPL monoclonal antibody to immunoprecipitate hnRNPL from M21 cell lysates followed by detection of the 60-kDa complex formation by RNA-UVXL. For hnRNPL control, anti-hnRNPC monoclonal antibody was used. Direct Western blot analysis of M21 cytoplasmic extracts showed that the anti-hnRNPL monoclonal antibody identified three proteins with apparent molecular masses of 66, 60, and 56 kDa, with the 60-kDa band being the most abundant of the three (Fig. 3A), indicating that hnRNPL is expressed as three different isoforms or differentially modified proteins in M21 cells. Anti-hnRNPL immunoprecipitation removed all of the 56- and 66-kDa bands and most of the 60-kDa hnRNPL band (Fig. 3A). Control antibodies (anti-hnRNPC and normal mouse serum) did not interact with any of the three hnRNPL proteins (Fig. 3A). Control antibodies (anti-hnRNPC and normal mouse serum) did not interact with any of the three hnRNPL proteins. Control antibodies (anti-hnRNPC and normal mouse serum) did not interact with any of the three hnRNPL proteins.

Taken together, these results further prove that hnRNPL is indeed the RNA-binding protein responsible for the 60-kDa RNA-protein complex. Removal of hnRNPL from cell lysates did not affect the formation of the other six RNA-protein complexes, suggesting that hnRNPL does not form specific com-

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**Note:** The text contains references to figures (Fig. 1, Fig. 2, Fig. 3) and tables, which are not visible in the provided image. The description of these figures and tables is crucial for understanding the context and conclusions drawn in the text. The full text context is necessary for a complete understanding of the research.
complexes with other RNA-binding proteins interacting with VEGF HSR.

**Hypoxia Regulation of hnRNP Expression**—Hypoxia substantially increases the steady state level of VEGF mRNA (4–8). The formation of hnRNP L–VEGF 3′-HSR complex markedly increased when cytoplasmic protein extracts were obtained from M21 cells exposed to hypoxia (3% O2) as compared with those exposed to normoxia (21% O2) (Fig. 1B). Thus, it was of interest to compare the effect of hypoxia on synthesis and distribution of hnRNP L in nuclear versus cytoplasmic compartments. As shown in Fig. 4, M21 cells grown in normoxic conditions contained high levels of hnRNP L, especially in the nuclear compartment. A minor induction of hnRNP L can be seen under hypoxia in both cytoplasm and nucleus, with the 56-kDa immunoreactive band in cytoplasm being the most significantly increased (Fig. 4). Conversely, the hnRNP C level in cytoplasm decreased to an undetectable range after a 24-h hypoxia incubation, although it remained high in the nucleus (Fig. 4). These results suggest that hypoxia differentially regulates the distribution of hnRNPs in different cellular compartments.

**Identification of hnRNP L mRNA Binding Site**—To define the hnRNP L mRNA binding site, six antisense oligodeoxyribonucleotides were synthesized from different regions of the HSR (Fig. 5A, AS1–AS6). To ensure the stability of oligonucleotides in the RNA-UVXL, we synthesized oligodeoxyribonucleotides instead of oligoribonucleotides. As shown in Fig. 5B, AS1 (1 μM) efficiently blocked the interaction of hnRNP L with 32P-labeled HSR and abolished formation of the 60-kDa RNA-hnRNP L complex. The blocking of HSR-hnRNP L complex formation can be seen as low as 0.05 μM AS1 (data not shown). AS6 abolished the formation of the 90- and 88-kDa complexes and partially inhibited the HSR-hnRNP L complex, whereas AS2, AS3, AS4, and AS5 did not show any effect (Fig. 5B). These results suggest that the complementary region of AS1 on HSR contains the hnRNP L binding site and that hnRNP L interacts with the single-stranded mRNA region. AS1 and AS6 lack sequence homology; thus, the partial inhibition of hnRNP L interaction with HSR observed with AS6 may be functioning by blocking the 90- and 88-kDa complexes that may promote hnRNP L binding in some manner.

To further explore the hnRNP L mRNA binding site, we chemically synthesized the sense oligodeoxyribonucleotide (S1), which was complementary to AS1 (Fig. 6A) and attempted to compete for hnRNP L binding to 32P-labeled HSR. As shown in Fig. 6A, S1 (10 μM) successfully competed with 32P-labeled HSR for hnRNP L binding and substantially reduced HSR-hnRNP L complex formation (85% inhibition of hnRNP L binding). The competition of S1 for hnRNP L binding to HSR can be seen as low as 0.1 μM AS1 (data not shown). To define the hnRNP L binding site, eight different oligodeoxyribonucleotides were synthesized after modification of the internal base sequences of S1 (Fig. 6). As shown in Fig. 6A, substitution of base 333 G to A in S1 or further substitution of base 335 C to A in S1.2 showed strong competition for 32P-RNA-HSR. As shown in Fig. 6B, S1.1 and S1.2 showed 74 and 82% inhibition, respectively, thus demonstrating that neither 333 G or 335 C were required for hnRNP L binding. However, substitu-
hnRNP L and VEGF mRNA Stability—To study whether hnRNP L plays an important role in the regulation of VEGF mRNA expression, M21 cells were transfected with antisense oligodeoxyribonucleotide AS1 to block interaction of hnRNP L with VEGF mRNA in hypoxic cells in vivo. The AS3 oligodeoxyribonucleotide, which did not block the formation of any HSR-protein complex formation (Fig. 5), was used as control. As shown in Fig. 8, M21 cells transfected with AS1 had substantially lower levels of VEGF mRNA when compared with mock-transfected control cells (54.3% by PhosphorImager analysis), whereas the GLUT-1 mRNA level was not affected by the transfection (97.3%), indicating that AS1 is specifically affecting VEGF mRNA expression. The control oligodeoxyribonucleotide AS3-transfected cells did not show changes for either VEGF mRNA (109.5%) or GLUT-1 mRNA (94.5%). These results suggest that blocking of hnRNP L and VEGF mRNA interaction in vivo will specifically affect VEGF mRNA accumulation in hypoxia.

The hnRNP Ls are a family of abundant nuclear proteins that are involved in pre-mRNA processing and splicing (22–24). To address whether the effect of AS1 repression of VEGF mRNA accumulation is through VEGF mRNA stability, we transfected M21 cells with AS1, AS3, or mock reagent and examined VEGF mRNA decay under hypoxic conditions. The rate of decay of the mature VEGF mRNA was determined by Northern blot hybridization after treatment of cells with the transcriptional inhibitor actinomycin D. As shown in Fig. 9A, M21 cells transfected with AS1 showed a substantial decrease in VEGF mRNA level after 30 min of actinomycin D treatment, whereas mock- and AS3-transfected cells maintained higher VEGF levels for up to 1 h of actinomycin D treatment. Conversely, GLUT-1 mRNA did not show any changes by AS1 when compared with mock- and AS3-transfected cells (Fig. 9A). When the VEGF mRNA decay curve of a triplicate assay was plotted over a 2-h actinomycin D treatment, M21 cells transfected with AS1, AS3, or mock reagent showed similar decay half-lives of 53 ± 4.1 and 57 ± 5.6 min, respectively (Fig. 9B). AS1-transfected cells showed half-lives of 53 ± 4.1 and 57 ± 5.6 min, respectively (Fig. 9B). AS1-transfected cells showed statistically significant differences (p ≤ 0.0005) in VEGF mRNA levels when compared with mock-transfected cells at both 30 and 60 min. These results suggest that blocking of the hnRNP L and VEGF mRNA interaction decreases VEGF mRNA stability under hypoxia. These also suggest that although hnRNP L is important for mRNA processing, interaction of hnRNP L with VEGF mRNA 3′-UTR plays a critical role in post-transcriptional regulation of VEGF mRNA stability.
Identification of hnRNP L as 3'-UTR-binding Protein

With a half-life of only 30–45 min under normal growth conditions (6–8), VEGF mRNA falls within a class of labile mRNAs encoding for many transiently expressed proteins including cytokines, lymphokines, oncogenes, and transcriptional activators (9). The mechanisms whereby normally labile mRNAs are stabilized by stimuli such as hypoxia, growth stimulation, and viral infection are unclear. On the basis of RNA-binding and UV cross-linking, the interaction of specific cellular proteins with mRNAs have been shown to be altered in response to stimuli, which correlates with changes in mRNA stability (9–21). This has led to the hypothesis that mRNA turnover is mediated principally through mRNA-binding proteins that specifically recognize AREs and other sequence motifs.

Our previous work demonstrated a 126-base HSR in human VEGF 3'-UTR, which is responsible for hypoxia-induced VEGF mRNA stability and forms seven hypoxia-inducible RNA-protein complexes with M21 human melanoma cell extracts (21). In the present study, we showed that the protein that forms the 60-kDa RNA-protein complex is present in both nuclear and cytoplasmic compartments (Fig. 1). Protein sequencing and immunodepletion studies identified that protein in the 60-kDa RNA-protein complex as heterogeneous nuclear ribonucleoprotein L (hnRNP L) (Figs. 3 and 4). The specific mRNA binding site of hnRNP L was identified as 3'-CAACCCACCCACAAUACAUACAU-5', a 21-base single-stranded element, which is unique to human and bovine VEGF 3'-UTR sequences (Figs. 5 and 6). Immunoprecipitation of hnRNP L followed by RT-PCR showed that hnRNP L specifically interacted with VEGF mRNA in hypoxic cells in vivo (Fig. 7). Furthermore, when M21 cells were transfected with antisense oligodeoxyribonucleotide to the human VEGF L RNA-binding site, the hypoxia-induced VEGF mRNA half-life decreased from 53 ± 4.1 min to 32 ± 5.7 min (Fig. 8). This study identifies for the first time that hnRNP L as a protein present in human cells that is capable of interacting with VEGF mRNA 3'-UTR and is functionally involved in the post-transcriptional regulation of VEGF mRNA under hypoxic conditions.

It is well documented that unstable mRNAs containing AREs generally consists of either scattered AUUUUA pentanucleotides, contiguous AUAUUA repeats, nonamer motif UUAUUAUAUAUA (U/A), or a stretch of AU residues lacking either motif (10–15). These AREs interact with cytoplasmic and/or nuclear proteins and usually form ARE-protein complex with average molecular mass less than 40-kDa (10, 15–20). The VEGF HSR is also a highly AU-rich element (43% A and 41% U) and contains one AUUAUAU pentanucleotide as well as two stretches of AU residues. However, interaction of the HSR with cellular proteins formed seven RNA-protein complexes with higher molecular masses (ranging from 40 to 90 kDa). The HSR also formed same protein complexes with five other cell lines we tested (data not shown), indicating that those RNA-binding proteins are common proteins to different cell lines. The 3'-UTRs of two other hypoxia up-regulated genes, erythropoietin (25) and tyrosine hydroxylase (26), interacted with mRNA-binding proteins with apparent molecular masses ranging from 66 to 140 kDa. However, the VEGF HSR and the 3'-UTRs of both erythropoietin and tyrosine hydroxylase lack significant homology. Moreover, the interaction site for a 66-kDa protein to tyrosine hydroxylase 3'-UTR is different from the hnRNP L mRNA binding site. Thus, VEGF HSR-binding proteins may be distinct from those that recognize other hypoxia-inducible genes.

Studies of rat VEGF mRNA demonstrated a 600-base region covering nucleotides 1251–1877 in the 3'-UTR required for rat VEGF mRNA stability (27, 28). However, little homology exists between the human VEGF HSR sequence and the rat 600-base region. In addition, the protein complexes observed by EMSA in the rat ARE-binding proteins are 17, 28, and 34 kDa (27, 28),

**FIG. 8.** Blocking of hnRNP L and VEGF mRNA interaction reduces steady state VEGF mRNA level. M21 cells were transiently transfected in the absence (−) or presence of 1 μM AS1 or AS3 oligodeoxyribonucleotides, and VEGF, GLUT-1, and 36B4 mRNA levels were detected by Northern blot analysis. VEGF and GLUT-1 mRNA signals were normalized with the ribosome-associated mRNA, 36B4, from each lane and quantified by PhosphorImager. The mRNA from AS1- and AS3-transfected cells were calculated as percentage of VEGF or GLUT-1 of mock-transfected mRNA (−) signals designated as 100%.

**FIG. 9.** Blocking of hnRNP L and VEGF mRNA interaction by AS1 oligodeoxyribonucleotide decreases VEGF mRNA stability. M21 cells were transiently transfected in the absence (−) or presence of 1 μM AS1 or AS3 oligodeoxyribonucleotide, and VEGF mRNA stability was analyzed in an 8-h hypoxia assay. Actinomycin D (5 μg/ml) was periodically added beginning at 6 h hypoxia and incubated for 2, 1, or 0.5 h, respectively, to triplicate plates. Total mRNA was extracted, and VEGF, GLUT-1, and 36B4 mRNA levels were detected by Northern blot. A, representative Northern blot analysis of VEGF mRNA level after actinomycin D treatment. B, VEGF mRNA decay curves and mRNA half-life (t½[mean±SEM]), 4.1 min to 32±5.7 min. VEGF mRNA signal was normalized with the ribosome-associated mRNA, 36B4, from each lane after quantification by PhosphorImager and calculated mean ± S.D. from a triplicate experiment. VEGF mRNA signal without actinomycin D treatment was defined as 100%, and actinomycin D treated VEGF mRNA levels was calculated as percentage of decay. The asterisk indicates p ≤ 0.0005 when AS1- and mock (−)-transfected cells were compared at 30- and 60-min actinomycin D treatment using Student's t test.

**DISCUSSION**

With a half-life of only 30–45 min under normal growth conditions (6–8), VEGF mRNA falls within a class of labile mRNAs encoding for many transiently expressed proteins including cytokines, lymphokines, oncogenes, and transcriptional activators (9). The mechanisms whereby normally labile mRNAs are stabilized by stimuli such as hypoxia, growth stimulation, and viral infection are unclear. On the basis of RNA-binding and UV cross-linking, the interaction of specific cellular proteins with mRNAs have been shown to be altered in response to stimuli, which correlates with changes in mRNA stability (9–21). This has led to the hypothesis that mRNA turnover is mediated principally through mRNA-binding proteins that specifically recognize AREs and other sequence motifs.

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none of which correlate to the hypoxia-induced complexes described here. Recent studies identified that the 34-kDa rat VEGF ARE-binding protein as HuR, which interacted with a 45-base element of rat VEGF 3′-UTR and stabilized the mRNA under hypoxia (28). This element is also present in the human 3′-UTR covering nucleotides 1682–1726 3′ to the translation stop codon, a region close to the poly(A) tail. Taken together, these studies suggest that there are a variety of mRNA-binding proteins present in cells, and the regulation of mRNA turnover probably depends on the mRNA sequences, the metabolic and activation state of the cells, and the regulation of the RNA-binding protein expression and distribution.

The hnRNPs are a family of abundant nuclear proteins that are involved in pre-mRNA processing and splicing (22–24). The hnRNPL differs from other ARE-binding hnRNPs in that it binds at the AUUUA motif and may participate in mRNA destabilization (15, 29–32), whereas hnRNLP interacts with a unique sequence in the human and bovine VEGF 3′-UTR (3′-CACCCACCCACAUAUCAU-5′) and probably has a predominant stabilization effect. In support of this hypothesis, hypoxia substantially increased hnRNPL in both nucleus and cytoplasmic compartments, whereas hnRNPA was dramatically decreased to undetectable levels in the cytoplasm after a 24-h hypoxic treatment. This differential regulation of hnRNPs under hypoxia, a decrease in the hnRNPA and an increase in the hnRNPL in cytoplasm, may define a key balance regulating mRNA metabolism during metabolic stress. The 21-base-long mRNA binding site of hnRNPL is juxtaposed to the only AUUUA pentanucleotide and a long stretch of AU residues. Therefore, it is possible that the interaction of hnRNPL may block other proteins such as hnRNPA from interacting with and destabilizing the mRNA. Furthermore, under hypoxia we observe increased expression of hnRNPL and the specific interaction of hnRNPL with VEGF mRNA in vivo, along with reduced VEGF mRNA stability observed with transfection of antisense oligodeoxyribonucleotide to the hnRNPL binding site. Thus, hnRNPL appears to play a significant role in the post-transcriptional regulation of VEGF mRNA during hypoxic stress.

Using Western blot analysis, anti-hnRNPL monoclonal antibody identified three proteins with apparent molecular masses of 66, 60, and 56 kDa, with the cytoplasmic 56-kDa protein being the most strongly up-regulated by hypoxia. Since there was only one RNA-hnRNPL complex formed in RNA-UVXL, it is unlikely that all three hnRNPL isoforms interact with HSR. Interaction of hnRNPL with the entire 21-base binding site would account for approximately 6 kDa of the 60-kDa HSR-hnRNPL complex after UV-cross-linking and RNase treatment. Thus, it is reasonable to predict that the 56-kDa hnRNPL isoform is the molecule that is specifically interacting with HSR. Comparing the minor induction of hnRNPL levels in hypoxia by Western blot to the marked increase of hnRNPL and HSR interaction in RNA-UVXL, assays lead us to speculate that post-translational modification of hnRNPL isoforms may promote a higher hnRNPL binding affinity to HSR under hypoxia, an interesting possibility we are currently investigating.

To effectively define the complex cellular mechanisms that control mRNA stability such as VEGF, identification of the mRNA-binding proteins is required. Purification and identification of hnRNPL protein as one of the VEGF mRNA-binding proteins is a significant step in defining the molecular regulation of VEGF mRNA expression under hypoxic conditions. Here we have detailed the characterization of hnRNPL as one of the hypoxia-inducible RNA-binding proteins that recognizes a specific region of the human VEGF HSR. The potential signaling mechanisms that regulate hnRNPL isoform expression and association with VEGF mRNA can now be investigated in detail. Since a complex of at least seven proteins bind to VEGF HSR, interaction of hnRNPL with other proteins in the same complex may be required to protect the VEGF mRNA from nuclelease digestion. Interference of hnRNPL binding to VEGF HSR significantly affected VEGF mRNA expression in hypoxia, indicating that it plays an important and novel role in VEGF expression under hypoxic stress. Identification of the other mRNA-binding proteins that recognize VEGF HSR, how they are regulated, and their potential interaction with hnRNPL will be necessary to get a complete understanding of the molecular mechanisms resulting in hypoxia-mediated VEGF mRNA stability.

Acknowledgments—We thank Dr. Gideon Dreyfuss for providing antibodies against hnRNPL and hnRNPC. We are grateful to Andrew Mullen and Kristin Abrams for helpful technical assistance.

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