Hypoxia-inducible Protein Binding to Vascular Endothelial Growth Factor mRNA and Its Modulation by the von Hippel-Lindau Protein

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Hypoxia induces an increase in the stability of the mRNA encoding vascular endothelial growth factor (VEGF). We have previously demonstrated that a 500-base region of the 3′-untranslated region of VEGF mRNA that is critical for stabilization of VEGF mRNA in an in vitro degradation assay forms a RNA-protein complex in a hypoxia-inducible fashion. We report here the identification of three adenylate-uridylate-rich RNA elements within this region that form an identical or closely related hypoxia-inducible RNA-protein complex. This complex is constitutively elevated in a tumor cell line lacking the wild type von Hippel-Lindau tumor suppressor gene and in which VEGF mRNA is constitutively stabilized. Furthermore, the glucose transporter-1 mRNA, which is also stabilized by hypoxia, forms a hypoxia-inducible RNA-protein complex with similar sequence and protein binding characteristics to that described for VEGF mRNA. Finally, RNA affinity purification and UV cross-linking were used to identify three proteins of 32, 28, and 17 kDa that are derived from this hypoxia-inducible RNA-protein complex.

Hypoxia has been shown to be an important stimulus for the new blood vessel formation seen in coronary artery disease (1), tumor angiogenesis (2), and diabetic neovascularization (3). VEGF, a also known as vascular permeability factor, is a potent angiogenic and endothelial cell-specific mitogen (4–6), whose growth factor B chain.

Porter-1; AUBP, AUUUA binding protein; PDGF-B, platelet-derived growth factor B chain.

The regulation of VEGF mRNA half-life appears to be mediated by both instability and stability elements (15). With a half-life of only 30–45 min under normal growth conditions (13–15, 18), VEGF mRNA falls within a class of labile mRNAs encoding for many transiently expressed proteins including cytokines, lymphokines, oncogenes, and transcriptional activators (19). A number of these mRNAs contain adenylate-uridylate-rich elements (AREs) in their 3′-UTR. Deletion of these sequences has been shown to enhance the stability of c-Fos and c-Myc mRNAs, and insertion of these sequences into the 3′-UTR of a normally stable globin mRNA significantly destabilizes it (19, 20). Analogously, in the VEGF 3′-UTR we have identified AREs that when deleted result in a significant stabilization of VEGF RNA in an in vitro degradation assay (15). Furthermore, insertion of these VEGF 3′-UTR sequences into a relatively stable mRNA can markedly destabilize it (21).

The mechanism whereby normally labile mRNAs are stabilized by stimuli such as phorbol ester, calcium ionophore, cAMP, tumor necrosis factor-α, and mitogenic antibodies is unclear (19, 22). However, a family of proteins that can bind with high affinity to AREs has been identified (19, 23, 24). On the basis of gel shift assays, the abundance of specific subsets of ARE binding proteins is altered when the mRNAs are stabilized in response to a specific agonist or stimulus (19, 22, 25). This has led to the hypothesis that stabilization of these labile mRNAs is mediated by the binding of specific ARE binding proteins that mask the ARE destabilizing motifs (25).

We have previously demonstrated in an in vitro degradation system that a 500-base pair region in the VEGF 3′-UTR is critical for the stabilization of VEGF RNA by hypoxic S-100 extracts and that this region binds a hypoxia-inducible protein complex (15). We report here the precise cis elements within this region of the VEGF 3′-UTR that bind the hypoxia-inducible protein complex. The trans-acting factors that bind to these cis elements were identified by RNA affinity purification and UV cross-linking. Similarities between the hypoxia-inducible protein binding to the VEGF mRNA 3′-UTR and to another hypoxia-regulated gene, Glut-1 (13), were also demonstrated.

Absence of the wild type von Hippel-Lindau tumor suppressor gene results in increased expression of VEGF.2 We have previously demonstrated that this is due, at least in large part, to increased VEGF mRNA stability.2 In this report we demonstrate increased constitutive formation of this VEGF mRNA 3′-UTR-protein complex in cells lacking wild type VHL protein.

1. Iliopoulos, O., Levy, A. P., Jiang, C., Kaelin, W. G., and Goldberg, M. A. (1996) Proc. Natl. Acad. Sci. U. S. A., in press.

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MATERIALS AND METHODS

Cell Lines and Culture Conditions—Rat H9C2 myoblasts, EBTR bovine trabecular epithelial cells, and the renal carcinoma cell line, 786-0, lacking p53 tumor suppressor gene, were obtained from the ATCC. The WT-8 cell line, derived by stably transfecting a wild type of the VHL cDNA under the control of a cytomegalovirus promoter in the 786-0 cells (27), was obtained from Dr. W. Kaelin (Dana-Farber Cancer Institute, Boston). Cells were routinely grown in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. The WT-8 cell line, derived by stably transfecting a wildtype VHL cDNA into the 786-0 cells, was kindly supplied by Dr. Beric R. Henderson.

Preparation of S-100 Extracts—The S-100 fraction of cytosolic proteins was obtained as described previously (28).

RNA Electromobility Shift Assay (EMSA)—The bacteriophage T7 RNA polymerase promoter sequence was appended to the 5' end of sense polynucleotide chain reaction (PCR) primers used to generate template DNA. The RNA electromobility shift assay (EMSA) was used to determine the position of the RNA-protein complex in the gel. The RNA-protein complex was resolved on 6% native polyacrylamide gel.

RESULTS

Mapping of the Cis Element(s) in the VEGF 3'UTR That Bind a Hypoxia-inducible Protein Complex—A hypoxia-inducible protein complex was previously demonstrated to bind to a riboprobe corresponding to the 3' 600 nucleotides of the VEGF 3'UTR (15). A battery of defined DNA fragments was generated by PCR using oligonucleotide primers from this region as described under "Materials and Methods." These PCR products in turn served as the template for the synthesis of riboprobes that allowed for the fine mapping of the cis elements in the VEGF 3'UTR that bind the hypoxia-inducible protein complex in the RNA electromobility shift assay (EMSA). Fig. 1A illustrates RNA EMSA experiments used to map the hypoxia-inducible complex, and Fig. 1B summarizes these data in a schematic fashion using the nomenclature defined under "Materials and Methods" for the different riboprobes. Riboprobes A1–A5 were previously shown to map to the hypoxia-inducible complex to a region between the Msel and Xbal site of the VEGF 3'UTR (nucleotides 1412–1754) (15). Further analysis of this region reveals that there are three separate elements in the VEGF 3'UTR that could each independently bind a hypoxia-inducible protein complex. Successively smaller riboprobes were generated that allowed fine mapping of these three elements with the minimal elements corresponding to nucleotides 1472–1510, 1508–1573, and 1631–1678 (VEGF GenBank accession no. U22372). Interestingly, all three hypoxia-inducible complexes migrated with identical electro-
phoretic mobility by RNA EMSA. While all three elements are adenylate-uridylate-rich, there is no consensus sequence between the three (Fig. 1C). Nonetheless they are capable of efficiently competing with one another for binding of the hypoxia-inducible complex. A representative example of this competition assay is shown in Fig. 1D which demonstrates that the hypoxia-inducible complex that binds to radiolabeled riboprobe C3 (VEGF nucleotides 1472–1573) is efficiently inhibited with 100-fold excess riboprobe Q3 (VEGF nucleotides 1631–1678). Utilization of 100-fold excess cold riboprobe Q7 that does not bind the hypoxia-inducible complex was 50-fold less efficient in competing with radiolabeled riboprobe C3 (data not shown).

**The Stability of VEGF mRNA and the Hypoxia-inducible Protein Complex Are Regulated by the von Hippel-Lindau (VHL) Tumor Suppressor Gene—Mutations in the VHL tumor**
The VHL gene regulates the RNA-protein complex in a hypoxia-specific fashion. S-100 extract was prepared from normoxic or hypoxic 786-0 (mutant VHL) or WT-8 (wild type VHL containing) cells and RNA EMSA performed with riboprobe Q3. In the 786-0 cells the protein complex was constitutively present (ratio 1% O2/21% O2 = 0.8 ± 0.2), whereas in the WT-8 cells the complex was hypoxia-inducible (ratio 1% O2/21% O2 = 2.4 ± 0.3). Similar results were obtained when using riboprobes from any of the three sites shown to independently bind a hypoxia-inducible complex in the H9c2 or EBTR cells. This experiment was repeated three times with the mean ± S.E. presented above for these independent experiments.

The 3′-UTR of Glut-1 mRNA shares sequence and protein binding characteristics with the ARE elements in the 3′-UTR of the VEGF gene. The 3′-UTR demonstrated to be capable of increasing Glut-1 mRNA stability (22). The increase in stability of Glut-1 mRNA in response to these agonists is correlated with increased binding of a specific ARE binding protein (AUBP) to an ARE element in the Glut-1 3′-UTR that normally directs rapid mRNA turnover (22). VEGF and Glut-1 appear to be stabilized by hypoxia in a similar manner based on the time course of the induction of the mRNA and the severity of hypoxia required for their induction (13). Furthermore, in the 786-0 and WT-8 cell lines described above, VEGF and Glut-1 are regulated in a similar fashion with respect to hypoxia in that mutation of the VHL gene results in a constitutive up-regulation of both mRNAs with restoration of the hypoxic inducibility of the genes dependent on the presence of wild type VHL protein.2

Fig. 3 demonstrates that a riboprobe from the 3′-UTR of Glut-1, containing the ARE that binds AUBP (22), binds a hypoxia-inducible protein complex that is similar to that formed with ARE elements in the 3′-UTR of the VEGF gene. First, the Glut-1 RNA-protein complex migrates with identical electrophoretic mobility to the VEGF RNA-protein complex. Second, the Glut-1 RNA-protein complex is hypoxia-inducible in cells containing wild type VHL protein. Third, the Glut-1 RNA-protein complex is efficiently competed by a 100-fold excess of riboprobe Q3 (VEGF nucleotides 1631–1678) but not by a riboprobe for the iron response element (IRE). Finally, Fig. 3C demonstrates the presence of significant sequence similarities between the region of the VEGF 3′-UTR demonstrated to bind the hypoxia-inducible protein complex and the region of the Glut-1 mRNA implicated in mediating its stabilization by multiple agonists (22). The Hypoxia-inducible VEGF and Glut-1 mRNA-Protein Complexes Are Approximately 65 kDa as Assayed by UV Cross-linking—To determine the size of the VEGF and Glut-1 mRNA-protein complexes that form in a hypoxia-inducible fashion, riboprobes corresponding to VEGF nucleotides 1472–1510, 1508–1573, or 1631–1678 or Glut-1 nucleotides 1967–2359 were cross-linked to the S-100 cytoplasmic extracts as described under “Materials and Methods.” As shown in Fig. 4 one major hypoxia-inducible RNA-protein complex of approximately 65 kDa was resolved with all three VEGF riboprobes and with the Glut-1 riboprobe. The amount of this complex was increased when protein extracts used for UV cross-linking were from cells that had been exposed to hypoxia thus corresponding to the increased binding observed in the RNA EMSA assays.

The 3′-UTR ARE Regions May Be Used to Affinity Purify Three Proteins of Molecular Mass 17, 28, and 32 kDa—Excess cold riboprobe corresponding to VEGF nucleotides 1631–1878

![Image](https://via.placeholder.com/150)
Hypoxia-inducible VEGF mRNA Binding Protein

FIG. 4. UV cross-linking of the hypoxia-inducible RNA-protein complex with radiolabeled VEGF riboprobe (C6, E2, Q3) and Glut-1 riboprobe. Cross-linking and gel electrophoresis were as described under “Materials and Methods.” The arrow points to the hypoxia-inducible complex of approximately 65 kDa. This experiment was repeated more than five times for the VEGF riboprobes and three times for the Glut-1 riboprobe.

was incubated with hypoxic or normoxic protein extracts, and the RNA-protein species was resolved by native polyacrylamide electrophoresis. The region of the gel at which the hypoxia-inducible complex migrates was cut out and subsequently soaked in SDS buffer with dithiothreitol. This gel slice was then loaded onto a SDS-polyacrylamide gel. Silver staining of this second gel revealed the presence of three major proteins with molecular mass of 17, 28, and 32 kDa (Fig. 5). Less abundant proteins of higher molecular mass were occasionally seen that did not appear to be hypoxia-inducible. Furthermore, the relative amounts of the 28- and 32-kDa species were variable.

DISCUSSION

We describe here a hypoxia-inducible protein complex that binds to three ARE elements in a region of the 3'-UTR of VEGF mRNA. We have previously shown that this region is critical for VEGF mRNA stabilization by hypoxic S-100 extracts in an in vitro degradation assay (15). We further demonstrate a significant correlation between the inducibility of this complex and the stability of VEGF mRNA and another hypoxia-inducible gene Glut-1. Finally, UV-cross-linking and affinity chromatography are used to provide an approximate size for this protein complex.

AREs are generally defined as 50–150-nucleotide regions that are involved in the rapid turnover of labile mRNAs (23). Generally, they contain multiple copies of the pentanucleotide AUUUA and have a high content of U and sometimes A residues. Not all AREs are destabilizing, and the critical sequence motifs within the ARE that direct rapid mRNA degradation are not known although a nonamer UUAUUUA(U/A)(U/A) may serve as the minimal instability sequence of some labile mRNAs (33, 34). At least nine proteins have been described that are capable of binding to AREs with high affinity (35–43). The strongest evidence for the role of these proteins in mediating the turnover of the labile mRNAs is the correlation of their binding activity negatively or positively with the stability of the mRNAs that have an ARE (19).

The AREs in the VEGF 3'-UTR that bind the hypoxia-inducible complex are distinct from the single canonical nanomeric instability sequence that is found between nucleotides 1686 and 1694 of the VEGF 3'-UTR. Mutation of this nonamer (riboprobe Q4 versus Q4m) does not affect the binding of the hypoxia-inducible complex (Fig. 1A). Furthermore, there are no AUUUA elements within any of the three AREs that bind the hypoxia-inducible complex, but there are 9 AUUUA elements in the VEGF 3'-UTR (16). The correlation between the increased binding of the hypoxia-inducible complex in S-100 extracts from normoxic cells lacking wild type VHL protein with increased VEGF mRNA stability in these same cells supports the role of the complex in mediating the hypoxic stabilization of VEGF mRNA. The precise mechanism whereby the VHL gene product mediates the binding of this hypoxia-inducible complex is the focus of ongoing studies, but no direct interaction between the VHL gene product and the hypoxia-inducible complex has been identified to date.

Concerted regulation of VEGF and Glut-1 has been reported in response to ischemia and hypoxia in differentially stressed microenvironments (13). Our studies demonstrate the existence of a hypoxia-inducible RNA-protein complex that binds to the Glut-1 mRNA 3'-UTR that is quite similar to the hypoxia-inducible complex that binds to the VEGF mRNA 3'-UTR. The 3'-UTRs of VEGF and Glut-1 show only a 42% overall homology, but both contain AREs that are highly homologous (Fig. 1C). These data strongly point toward a common role of the ARE and its cognate binding proteins in mediating the hypoxic stabilization of these two mRNAs. This contention is further supported by the similar regulation of PDGF-B chain mRNA by hypoxia2 since the PDGF-B chain mRNA, like VEGF and Glut-1 mRNA, contains multiple AREs in its 3'-UTR (26).

The size of the protein complex estimated by UV cross-linking differs from that of previously reported ARE binding proteins that range in mass from 32 to 46 kDa (23). Furthermore, the size of the proteins purified by RNA-affinity electrophoresis does not clearly correlate with previously reported ARE binding proteins including AUBP. Nonetheless, definitive comparison and further functional analysis of these different ARE binding proteins await their respective purification and cDNA cloning. Indeed, the RNA affinity purification described here may facilitate this goal.

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REFERENCES

1. Sabri, M. N., DiSciascio, G., Cowley, M. J., Alpert, D., and Vetrovec, G. W. (1991) Am. Heart. J. 121, 876–880
2. Siweki, D., Ilin, A., Soffer, D., and Keshet, E. (1992) Nature 359, 843–845
3. Aiello, L. P., Avery, R. L., Arrigg, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., Pasquale, L. B., Thieme, H., Iwamoto, M. A., Park, J. F., Nguyen, H. V., Aiello, L. M., Ferrara, N., and King, G. (1994) N. Engl. J. Med. 331, 1490–1497
4. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1306–1309
5. Levy, A. P., Tamargo, R., Brem, H., and Nathans, D. (1989) Growth Factors 2, 9–35
6. Senger, D., Ven De Water, L., Brown, L., Nagy, J., Yeo, K.-T., Yeo, T.-K., Berse, B., Jackman, R., Dvorak, A., and Dvorak, H. (1993) Cancer Metastasis Rev. 12, 303–324
7. Levy, A. P., Levy, N. S., Loscalzo, J., Calderone, A., Takahashi, N., Yeo, K.-T.,
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