Poly(A)-binding Protein-interacting Protein 2, a Strong Regulator of Vascular Endothelial Growth Factor mRNA*

Expression of vascular endothelial growth factor (VEGF) is tightly regulated, particularly at the level of its mRNA stability, which is essentially mediated through the 3′-untranslated region (3′-UTR) of VEGF mRNA. To identify new protein partners regulating VEGF mRNA stability, we screened a cDNA expression library with an RNA probe corresponding to the entire VEGF mRNA 3′-UTR. We identified the “poly(A)-binding protein-interacting protein 2” (PAIP2) as a new VEGF mRNA 3′-UTR interacting protein. By RNA electromobility shift assays, we showed that PAIP2 binds to two distinct regions of a domain encompassing base 1 to 1280 of the VEGF 3′-UTR. Such in vitro interaction was confirmed using cell extracts in which PAIP2 expression is induced by tetracycline (Tet-on cells). Moreover, we demonstrated that PAIP2 can modulate VEGF mRNA 3′-UTR stabilization in Tet-on cells, leading to increased VEGF secretion. Moreover, RNAi-mediated knock-down of PAIP2 significantly reduces the steady-state levels of endogenous VEGF mRNA. We also showed, by in vitro protein-protein interactions and co-immunoprecipitation experiments, that PAIP2 interacts with HuR, an already known VEGF mRNA-binding protein, suggesting cooperation of both proteins for VEGF mRNA stabilization. Hence, PAIP2 appears to be a crucial regulator of VEGF mRNA and as a consequence, any variation in its expression could modulate angiogenesis.

In the absence of sufficient oxygen delivery, organs induce production of angiogenic factors that function to recruit new blood vessels to hypoxic tissues (1, 2). Vascular endothelial growth factor is one of the most important regulators of angiogenesis as inactivation of only one allele of its gene leads to embryonic lethality in mice (3). Expression of VEGF is tightly regulated. At the transcriptional level, hypoxia-inducible factor-1 is recruited under hypoxia to the VEGF promoter (4). Oncogenic transformation also induces activation of VEGF transcription by recruiting AP-2 and Sp1 transcription factors to the proximal region of the promoter (5). VEGF expression is also regulated post-transcriptionally by stabilization of its mRNA (6–9). Previous experiments have shown that the VEGF mRNA 3′-UTR contains nine copies of the nonamer consensus for AU-rich element (ARE) present in the 3′-UTR of many labile mRNAs (9). Consistent with their presence, the VEGF 3′-UTR can confer instability to a reporter mRNA in a cellular model (9–11). Precedent reports have demonstrated the presence of binding sites for hypoxia or stress inducible proteins (7, 9, 11). Even if several proteins were shown to interact with the VEGF mRNA 3′-UTR, only two proteins have been identified: HuR, a member of the ELAV family (8) and the ribonucleoprotein hnRNPL (12). Moreover, the molecular mechanisms underlying VEGF mRNA stability, which is modified under physiological and pathological situations, remain poorly understood. The aim of our study was to identify VEGF mRNA protein partners that are essential for the modulation of VEGF mRNA stability. Our screening experiments identified 15 independent clones that interact with the VEGF mRNA 3′-UTR. The most strongly interacting clone was the poly(A)-binding protein-interacting protein 2 (PAIP2). At the same time, PAIP2 was identified as a translational repressor by using bicistronic vectors (13, 14). Our study consisted in further analyzing the role of PAIP2 as a modulator of VEGF mRNA expression.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA-modifying enzymes were obtained from New England Biolabs or Eurogentec (Liège, Belgium). Synthetic oligonucleotides were from Eurogentec. [α-32P]UTP, [α-32P]ATP, and [α-32P]dCTP were from Amersham Biosciences. The monoclonal anti-myc antibody (9E10) was from Roche and the polyclonal anti-myc antibody was from MBL.

Cell Lines and Culture Conditions—The A431, C6, HeLa, LoVo, PRoh, and Rau3Vol-12 cells (15) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 7.5% heat-inactivated fetal calf serum and containing penicillin (50 units/ml) and streptomycin sulfate (50 μg/ml). S19-R443 cells are a derivative of CCL39 Chinese hamster lung fibroblasts, which stably express a tetracycline repressor. They were stably transfected with an expression vector encoding a tetracycline inducible myc-tagged PAIP2 (cloned into the pCDNA4/TO/myc-His A vector, Invitrogen) and cultured as described (16). Induction of myc-tagged PAIP2 was obtained by stimulating cells with tetracycline (1 μg/ml).

cDNA Library Screening—We screened a mouse testis cDNA library constructed in the λ ZAP Express vector (Stratagene). Nitrocellulose filters soaked in 10 mM isopropyl-1-thio-β-D-galactopyranoside were

*S-transferase; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; RNP, ribonucleoprotein; ELISA, enzyme-linked immunosorbent assay; hnRNP, heterogeneous nuclear ribonucleoprotein; DRB, 5,6-dichlorobenzimidazole riboside.

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applied to the surface of plates previously inoculated with bacteria. Phage wells were then incubated overnight at 42 °C. Filters were then peeled off the plates and immersed in a large volume of RNA binding buffer containing a final concentration of 10 mM HEPES (pH 7.5), 5 mM MgCl₂, 50 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 100 μg/ml RNA. After two washes in this binding buffer, radiolabeled RNA transcript (10° cpm) corresponding to the entire human VEGF cDNA 3'-UTR (NCBI accession number AF027410) and heparin (5 μg/ml) were added to the filters. After incubation for 1 h with the probe at 30 °C, RNase T1 was added to a final concentration of 1000 units/ml. The filters were then washed five times in RNA binding buffer and autoradiographed. The process of screening and plating was repeated until a homogenous population of positive recombinant bacteriophage was obtained. By using the ExAssist helper phage, a pBK-CMV phagemid vector containing the cDNA of interest was excised from the ZAP Express vector according to the manufacturer's protocol.

Cloning of Full-length PAIP2 cDNA—First-strand cDNA was synthesized from 1 μg of human colon carcinoma cell HT29 poly(A)⁺ RNA, using avian myeloblastosis virus reverse transcriptase with an oligo(dT) primer. This material was used as a template for polymerase chain reaction amplification. The following oligonucleotides derived from the human PAIP2 (NCBI accession number NM_016480) were synthesized and used as primers for the polymerase chain reaction: oligonucleotide 1, 5’-GTC-GATCAGATTGAGGGCGCTGCTGCT-3’; oligonucleotide 2, 5’-TAGTCGACTAATCTCTTCGATCTCACCCAGG-3’. An aliquot of cDNA was amplified in a 50-μl reaction volume with 200 μM dNTPs, and 2.5 units of Ampli-Taq (Roche Applied Science) in buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin. Polymerase chain reaction amplification was performed in a DNA thermal cycler (Biometra) using the following parameters: 30 s at 95 °C, 1 min at 55 °C, and 1 min at 72 °C for 30 cycles, followed by an extra cycle with a 10-min extension step at 72 °C. An expected fragment of 381 bp was analyzed by restriction enzymes and sequenced.

Plasmid Constructs—The 381-bp DNA fragment corresponding to the full-length PAIP2 cDNA was cloned into the pGEX-6P1 and pCMV-Tag3B vectors (Amersham Biosciences and Stratagene, respectively) with Sall restriction sites. Both restriction sites were artificially added to the cDNA extremities during the PCR (BamHI site 5’ and Sall site 3’). A DNA fragment of 454 bp was excised from the pCMV-Tag3B vector with NotI and ApaI and subcloned into the corresponding sites of the pCDNA3/myc-His A vector (Invitrogen). The full-length human HuR cDNA (NCBI accession number BC003376) was introduced into the pCMV-Tag3B vector within EcoRI/XhoI sites. The full-length human HuR cDNA (NCBI accession number BC003376) was introduced into the pCMV-Tag3B vector within EcoRI/XhoI sites.

Preparation of Cytosolic Extracts, REMSA, and in Vitro RNA Degradation Assay—S19-R443/PAIP2 cells were stimulated or not with tetracycline, in a buffer containing 100 μg of S100 extract from cells stimulated or not with tetracycline. At each time point, an aliquot of the reaction was taken and treated as previously described (11).

RNA Affinity Purification—2 μg of in vitro transcribed polyadenylated RNA, corresponding to the 3′ UTR of the cDNAs encoding or the full-length human cDNAs encoding ERK1 mRNA 3′-UTR (18) were incubated with polyethylene-latex beads with dC₃T₉ oligonucleotides covalently linked to the surface (OligoGen, Qiagen), in a binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM NaCl, 2 mM EDTA, and 0.2% SDS). After 2 washes in a wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA), the RNA-competitors were incubated with 100 μg of S100 extracts from cells stimulated or not with tetracycline, in the RNA binding buffer used for REMSA experiments, supplemented with 1% bovine serum albumin. After incubation for 10 min at 30 °C, the beads were extensively washed with RNA binding buffer. Laemmli sample buffer was added to the beads. Proteins bound to the RNA probe were resolved on SDS-PAGE and subjected to Western blotting using the monoclonal anti-myc antibody described below.

Anti-PAIP2 and Anti-HuR Antibodies—Antibodies were generated by Eurogentec (Liege), by injecting four times two rabbits with 100 μg of a GST-PAIP2 fusion protein or a GST-HuR fusion protein. Sera were affinity purified using an EAH-Sepharose 4B column (Amersham Biosciences) to which GST-PAIP2 or GST-HuR was coupled. The GST-PAIP2 or GST-HuR IgG were then eluted with 10 μg glycine, pH 2.8, and neutralized with 20 μM Tris, pH 11.

Immunofluorescence—S19-R443/PAIP2 cells expressing inducible myc-tagged PAIP2 were plated on glass coverslips. After tetracycline induction for 12 h, cells were fixed with 3% paraformaldehyde at room temperature for 30 min and permeabilized with 0.2% Triton X-100. Coverslips were washed with PBS containing 10% fetal calf serum, incubated for 2 h with the first antibody diluted in PBS, 10% fetal calf serum (monoclonal anti-myc, 1/1000), then washed with PBS and incubated with the second antibody (Alexa 594-coupled anti-mouse antibody, Molecular Probes, 1/250). 4',6-Diamidine dihydrochloride (Roche) was added at the same time to a final concentration of 0.2 μg/ml. Coverslips were washed with PBS and distilled water and analyzed with a DMR Leica microscope.

Western Blotting—For cell extracts, cells were washed twice with ice-cold PBS and immediately lysed in Laemmli sample buffer. For the extracts derived from mice, tissues were frozen and homogenized with a Polytron in lysis buffer containing 1% Triton X-100. Protein extracts were resolved on a 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Membranes were incubated with a polyclonal anti-PAIP2 antibody (1/2000) or a monoclonal or polyclonal anti-myc antibody (both 1/1000) and then with an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody. Bound antibody was revealed using an ECL system (Amersham Biosciences).

Immunoprecipitation of Ribonucleoprotein (RNP) Complexes and Analysis by Reverse Transcriptase-PCR—RNP complexes were immunoprecipitated from whole HeLa cells extracts as described below in “Co-immunoprecipitation Experiments.” Briefly, pre-cleared lysates were incubated 2 h at 4 °C with the anti-PAIP2, an irrelevant antibody, or their respective preimmune sera, prior to incubation with protein A-Sepharose saturated with antibodies. The reaction mixtures were resolved on a 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). Membranes were incubated with a polyclonal anti-PAIP2 antibody (1/2000) or a monoclonal or polyclonal anti-myc antibody (both 1/1000) and then with an anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody. Bound antibody was revealed using an ECL system (Amersham Biosciences).

Measurement of VEGF mRNA Half-life—S19-R443/PAIP2 cells expressing inducible myc-tagged PAIP2 were stimulated or not with tetra-
FIG. 1. The GST-PAIP2 fusion protein interacts with the VEGF mRNA 3′-UTR in vitro. A, schematic map of the VEGF 3′-UTR illustrating the templates used for generation of riboprobes for REMSA. □ refers to the AREs (pentanucleotide AUUUA) present on the VEGF 3′-UTR. □ corresponds to the HuR binding site. Probes interacting (+) or not (−) with GST-PAIP2 are mentioned. B, REMSA were performed by incubating the radiolabeled VEGF 3′-UTR transcripts with purified GST-PAIP2, GST, or GST-HuR. For the full-length and NsiI transcripts, 750 nM of each GST fusion protein was used (left panels). For the StuI and ΔStuI transcripts, increasing concentrations of GST-PAIP2 (75 and 325 nM) and a fixed concentration of GST (325 nM) were used (right panels). Competition with specific competitor (×100 molar excess) corresponding to unlabeled StuI and ΔStuI transcripts, respectively, was also performed in the presence of 325 nM GST-PAIP2. The arrows point to RNA-protein complexes. The brackets encompass free and degraded probe.
complexes were subjected to SDS-PAGE and Western blotting. With lysis buffer, Laemmli buffer was added to the beads, and protein rabbit reticulocyte lysates using the TNT-coupled transcription/translation-prior to incubation with the different antibodies. Protein A-Sepharose lysed and analyzed either by Western blotting for PAIP2 expression or phosphate method. 48 h following the second transfection, cells were –

corresponds to the coding region 310–328 of the human PAIP2 complete cDNA. The siRNA sequence targeting the coding region 477–495 relative to the first nucleotide of the EGFP complete cDNA and used as an irrelevant siRNA is the following: 5'-GAACGGCAUC-AAGGUGAACTT-3'. The two RNA strands were mixed in equimolar ratios and annealed by heating to 95 °C for 1 min and then to 37 °C for 1 h. A431, HeLa, and S19-R443 cells were transiently transfected twice

corresponds to the coding region 295–313 relative to the first nucleotide of the human PAIP2 complete cDNA (NCBI accession number NM_016480). The second one is 5'-GAUCUGUGGUCAAGCAGCAT-T-3' and corresponds to the coding region 305–313 relative to the first nucleotide of the human PAIP2 complete cDNA. The siRNA sequence targeting the coding region

corresponds to the first nucleotide of the EGFP complete cDNA and used as an irrelevant siRNA is the following: 5'-GAACGGCAUC-AAGGUGAACTT-3'. The two RNA strands were mixed in equimolar ratios and annealed by heating to 95 °C for 1 min and then to 37 °C for 1 h. A431, HeLa, and S19-R443 cells were transiently transfected twice

RESULTS

A GST-PAIP2 Fusion Protein Interacts with the VEGF mRNA 3'-UTR in Vitro—To identify proteins interacting with the VEGF 3'-UTR, we screened an expression mouse testis cDNA library with a [32P]UTP-radiolabeled RNA probe corresponding to the full-length VEGF mRNA 3'-UTR. We obtained several clones, the hybridization of which resisted RNase T1 treatment. The relative affinity of the different clones was evaluated by the intensity of hybridization of the VEGF probe to the λ phage plaques. Moreover, total protein extracts from bacteria obtained with the rescued phagemid vectors (see “Experimental Procedures”), stimulated or not with isopropyl-1-thio-β-D-galactopyranoside, were used in REMSA. The intensity of complexes obtained with identical amounts of protein extracted from isopropyl-1-thio-β-D-galactopyranoside-stimulated bacteria, also allowed us to measure the relative affinity for the VEGF RNA probe of the different proteins obtained

Fig. 2. Expression of PAIP2 in different tissues and tumor cell lines. A, 20 μg of total RNA isolated from various human and mouse tissues (upper and middle panels, respectively) or tumor cell lines (lower panel) were analyzed by Northern blotting for expression of PAIP2. 28 S or 18 S ribosomal RNA is shown as a loading control. B, 100 μg of mouse tissue extracts (upper panel) or tumor cell extracts (lower panel) were subjected to Western blotting using a polyclonal anti-PAIP2 antibody. The amounts of ERK2 are shown as a loading control.
FIG. 3. PAIP2 interacts with VEGF mRNA in vivo. A, S19-R443 cells were stably transfected with an expression vector encoding a tetracycline inducible myc-tagged PAIP2. Cells were stimulated for various times with tetracycline (1 μg/ml). Inducible overexpression of myc-PAIP2 was then visualized either by Western blotting (upper panel), or by immunostaining (lower panel), using a monoclonal anti-myc antibody as described under “Experimental Procedures.” B, S100 cytosolic extracts (5 μg) derived from S19-R443/PAIP2 cells treated (+) or not (−) for 4 h with tetracycline (1 μg/ml) were incubated with the radiolabeled NsiI transcript in the absence or presence of ×100 or 1000 molar excess of cold competitor. The specific competitor corresponds to the unlabeled NsiI transcript, whereas the nonspecific competitor corresponds to the unlabeled full-length Chinese hamster ERK1 3′-UTR transcript (642 bp). The arrow points to the RNA-protein complex. The bracket encompasses free and degraded probe. This experiment is representative of three independent experiments. C, 2 μg of unlabeled polyadenylated transcripts (NsiI for the specific probe and ERK1 3′-UTR for the nonspecific probe) were bound to oligo(dT) beads. S100 extracts (100 μg) derived from S19-R443/PAIP2 cells treated (+) or not (−) for 4 h with tetracycline (1 μg/ml) were incubated with the RNA-coated beads. Proteins interacting specifically with the unlabeled transcripts were then subjected to Western blotting, using a monoclonal anti-myc antibody (upper panel). The Amido Black staining for total protein is shown as a loading control (lower panel). D, whole HeLa cell extracts were immunoprecipitated with no antibody (lane beads), with the preimmune serum, or with a polyclonal anti-PAIP2 antibody. Equal aliquots of purified total RNA isolated from the immunoprecipitates (P) (lanes 1, 3, and 5), and from the supernatants (S) (lanes 2, 4, and 6), were assayed by reverse transcriptase-PCR to detect the VEGF (upper panel) and β-actin (lower panel) transcripts. The reactions in lanes 7 and 8 serve as negative controls.
during the screening. Thus, we selected the clone interacting with the strongest affinity with the VEGF mRNA 3′-UTR. This clone corresponded to the full-length VEGF 3′-UTR and the PAIP2 transcript expression was examined by Northern blotting of mouse and human tissues as well as of different tumor cell lines (Fig. 2A). For these cells, there is also a good correlation between mRNA and protein levels, with the highest amounts in HeLa cells. We note a correlation between the mRNA and proteins levels, except for liver where the PAIP2 protein is more abundant than the mRNA, compared with others tissues. For the tumor cell lines, there is also a good correlation between mRNA and protein levels, with the highest amounts in HeLa cells.

**PAIP2 Interacts with VEGF mRNA in Vivo**—To analyze the relevance of the PAIP2/VEGF mRNA interaction, we stably transfected S19-R443 cells (CCL39 derivatives) with an expression vector encoding a tetracycline inducible myc-tagged PAIP2 (16). S19-R443 cells were chosen because of their very low basal level of PAIP2 (data not shown). Fig. 3A shows that exogenous PAIP2 is detected after only 4 h of tetracycline treatment and reach a maximum after 12 h (4–6-fold induction at 4 h to 45-fold induction at 24 h compared with the endogenous level of PAIP2). Moreover, overexpression does not affect its cytoplasmatic localization (13) (lower panel). The effects of PAIP2 overexpression on RNA binding were tested after 4 h of tetracycline induction. REMSA experiments with the NsiI part of the VEGF mRNA 3′-UTR were performed with S100 extracts derived from cells treated or not with tetracycline. Fig. 3B shows a constitutive protein(s)-RNA complex, the intensity of which is further increased with S100 extracts from tetracycline-treated cells (compare lanes 1 and 5). This complex is completely inhibited by competition with an excess of unlabeled specific transcript corresponding to the NsiI region (lanes 2, 3, 6, and 7), but not competed out with an excess of unlabeled nonspecific ERK1 3′-UTR transcript (lanes 4 and 8). These results suggest that PAIP2 itself and/or protein(s) dependent on PAIP2 recruitment is(are) associated in this complex. We performed the
same REMSA experiments on the ΔNsiI probe (bases 1276 to 2211). The RNA-protein complexes visualized with this probe are not modified upon tetracycline treatment (data not shown), which confirms our previous results using GST-PAIP2.

To further demonstrate the presence of PAIP2 in the RNA-protein(s) complex, we performed RNA affinity purification assays. The unlabeled polyadenylated transcript corresponding to the NsiI probe was bound to oligo(dT) beads. S100 extracts from cells treated or not with tetracycline were incubated with the RNA-coated beads. After extensive washing, proteins associated with the bound probe were resolved by Western blotting. Fig. 3C shows that PAIP2 induced by tetracycline strongly interacts with the NsiI transcript. This interaction appears to be specific because, in the same conditions, PAIP2 is not able to bind the polyadenylated ERK1 3′-UTR transcript, used as a negative control. Even if proteins are unspecifically trapped on the beads (see Amido Black), the intensity of an irrelevant protein (i.e. ERK2) is not modified by tetracycline treatment (data not shown). This result clearly demonstrates the presence of PAIP2 in the protein(s)-RNA complex described above and then, the ability of PAIP2 to bind VEGF 3′-UTR.

To confirm that PAIP2 not only interacts with an in vitro transcript corresponding to a region of the VEGF 3′-UTR, but also with the endogenous VEGF mRNA, we performed ribonucleaseprotein complexed immunoprecipitation experiments with whole cell extracts from HeLa cells (a cell line expressing strongly PAIP2). Fig. 3D shows that immunoprecipitation of PAIP2 results in the co-precipitation of VEGF mRNA (lane 5, upper panel). Such co-precipitation is highly specific because immunoprecipitation with either preimmune serum (lane 3) or an irrelevant antibody (data not shown) does not co-precipitate VEGF mRNA. As expected, VEGF mRNA is also detected in total RNA prepared from the HeLa cell extracts used for immunoprecipitations (input, lane 7). Moreover, it is noteworthy that immunoprecipitation of PAIP2 does not lead to co-precipitation of a nonspecific transcript such as β-actin mRNA (lane 5, lower panel), which is used here as a negative control. The same results were obtained in A431 cells, another cell line that highly expresses PAIP2. These results provide convincing evidence that VEGF mRNA is specifically associated with PAIP2.

**PAIP2 Overexpression Increases VEGF mRNA Half-life in an in Vitro RNA Degradation Assay**—We then used a specific in vitro RNA degradation assay to evaluate the role of PAIP2 on VEGF mRNA stability, a protocol already used to demonstrate the stabilization of VEGF mRNA induced by the HuR protein or by stress-activated protein kinases (6–8, 11, 19). S100 extracts were prepared from cells treated or not with tetracycline and incubated with the in vitro radiolabeled NsiI transcript. Fig. 4 shows that this transcript is 2.5 ± 0.3-fold more stable (n = 3) following incubation with extracts from tetracycline-stimulated cells, demonstrating that PAIP2 overexpression confers higher stability of the VEGF mRNA via its 3′-UTR, at least in vitro, when the protein is bound to the NsiI region.

**PAIP2 Overexpression Increases VEGF mRNA Half-life in Vivo**—To analyze the relevance of the PAIP2/VEGF mRNA interaction in vivo, we determined whether overexpression of PAIP2 increased endogenous VEGF mRNA stability. For this purpose, DRB chase experiments in stably transfected cells treated or not with tetracycline were performed. Four VEGF mRNA species ranging from 1.5 to over 4.0 kb are detected on the Northern blot (Fig. 5A). All the isoforms are stabilized upon tetracycline treatment, except form 2 with a long time exposure to DRB. The total amount of VEGF mRNA at each point corresponds to the sum of the global quantity of each isoform measured with a PhosphorImaging system. Hence, we show that the VEGF mRNA half-life is increased 4.4 ± 0.4-fold (n = 3) when PAIP2 is overexpressed (Fig. 5, A and B).

As PAIP2 was shown to act as a translational repressor (13, 14), we decided to test whether increased VEGF mRNA stability was because of a general inhibition of protein synthesis, which is responsible for accumulation of many mRNA including oncogenes, growth factors, and VEGF mRNA itself (20). As compared with untreated cells, Fig. 5C shows no major modification of protein synthesis after 4 h of incubation in the absence or presence of tetracycline in S19-R443/PAIP2 cells, which are the previous experimental conditions. This result demonstrates that the PAIP2 stabilizing effect on VEGF mRNA does not reflect a nonspecific inhibitory effect on protein synthesis. PAIP2 specifically stabilizes VEGF mRNA by interacting with the VEGF mRNA 3′-UTR.

**PAIP2-mediated VEGF mRNA Stabilization Contributes to Increased VEGF Secretion**—To correlate VEGF mRNA stability with VEGF production, we measured VEGF protein levels...
PAIP2 overexpression contributes to increased VEGF secretion. Secreted VEGF was measured by ELISA on cell culture supernatants from S19-R443 control cells or S19-R443/PAIP2 cells stimulated without (white box) or with (hatched box) tetracycline (Tet; 1 μg/ml) for 48 h. Fold induction of secreted VEGF is presented as a mean of three independent experiments performed in duplicate (upper panel). The data were corrected for the cell number. Protein extracts (20 μg) prepared in parallel were analyzed, as a control, by Western blotting using a polyclonal anti-myc antibody (lower panel). The amounts of ERK2 are shown as a loading control.

Silencing of PAIP2 by RNA Interference Reduces Steady-state Levels of Endogenous VEGF mRNA—To obtain independent verification of the conclusions raised from the overexpression of PAIP2, we turned to paip2 gene silencing by using siRNA duplexes. We performed these experiments in S19-R443 cells, the same cells used for PAIP2 overexpression assays, as well as in two tumor cell lines: A431 and HeLa. Cells were transfected twice with 50 nM of a 21-nucleotide duplex homologous to the same cells used for PAIP2 overexpression assays, as well as duplexes. We performed these experiments in S19-R443 cells, (Fig. 7A, lower panel). The values are normalized to 36B4 RNA. Control values obtained with the siRNA against green fluorescent protein (GFP) are taken as 100%. The data shown represent the mean ± S.E. of three independent experiments.

VEGF mRNA levels are affected by the knock-down of PAIP2, no change was detected in VEGF mRNA stability measured by a DRB chase experiment (data not shown; see “Discussion”). The same experiments were performed using an independent siRNA sequence targeting PAIP2 (see “Experimental Procedures”). An equivalent decrease of steady-state VEGF mRNA levels was obtained (data not shown), confirming the specificity of the PAIP2 silencing effect on VEGF mRNA expression.

PAIP2 and HuR Physically Interact—To explain the molecular mechanism leading to PAIP2-mediated VEGF mRNA stabilization, we analyzed potential interactions of PAIP2 with proteins that were previously described as VEGF mRNA stabilizing proteins. HuR interacts with the VEGF mRNA 3′-UTR and participates in VEGF mRNA stabilization under hypoxia. It is part of a protein complex recruited under hypoxia that is composed of HuR itself and two unknown proteins of 29 and 17 kDa (7, 8). As the size of the 29-kDa protein was compatible with that of PAIP2 (13), we have first determined whether PAIP2 and HuR could interact in vitro. As shown in Fig. 8A, in vitro translated HuR interacts with immobilized GST-PAIP2 fusion protein but not with GST protein alone, demonstrating that PAIP2 and HuR interact directly, at least in vitro, without need of any partners. Moreover, this interaction is highly specific because an in vitro translated irrelevant protein does not interact with immobilized GST-PAIP2 (data not shown).
To demonstrate an in vivo association, HeLa cells were transiently transfected with an expression vector encoding myc-tagged HuR or myc-tagged PAIP2. We showed that the overexpressed forms of both proteins interact, because they co-immunoprecipitate (data not shown). We then performed co-immunoprecipitation experiments directly on the endogenous proteins to confirm what we have observed in vitro and with overexpressed HuR and PAIP2. Fig. 8B shows that endogenous HuR can be co-immunoprecipitated with an anti-PAIP2 antibody (lane 4). Endogenous PAIP2 can also be co-immunoprecipitated (to a lower proportion than HuR) with an anti-HuR antibody (Fig. 8B, lane 2). Moreover, interaction between both endogenous proteins is specific because neither anti-HuR nor anti-PAIP2 preimmune sera (lanes 1 and 3), nor an irrelevant antibody (lane 5), are able to co-immunoprecipitate endogenous HuR and PAIP2. These results clearly demonstrate an interaction between both proteins in vivo. To evaluate whether this association could be RNA dependent, because PAIP2 and HuR bind to the same target mRNA, we performed co-immunoprecipitations with RNase A-treated cell extracts. Fig. 8C demonstrates that treatment with RNase A does not abolish the interaction between PAIP2 and HuR. Hence, PAIP2 and HuR physically interact without any partner RNA. Nevertheless, RNase A treatment seems to increase the pool of immunoprecipitated HuR, whereas it has no effect on the amount of co-immunoprecipitated PAIP2 (Fig. 8C, lane 4).

**DISCUSSION**

To better understand processes controlling VEGF mRNA stability, we cloned PAIP2 as a new protein partner of the VEGF mRNA 3′-UTR. At the same time, PAIP2 was shown to interact with the poly(A)-binding protein, which prevents poly(A)-binding protein binding to the poly(A) tail of mRNAs and inhibits cap-dependent and cap-independent translation (13). Here, we show that PAIP2 strongly interacts with the VEGF mRNA 3′-UTR both in vitro and in vivo, leading to VEGF mRNA stabilization. Furthermore, PAIP2-mediated VEGF mRNA stabilization correlates with an increase in VEGF production.

The double effect of PAIP2 on mRNA stability (this report and Ref. 21) and mRNA translation (13) has already been described for the iron regulatory proteins IRP1 and IRP2 as well as for CUGBP2, which binds to the cyclooxygenase-2 mRNA. IRP1 and IRP2 proteins tightly regulate ferritin and transferrin receptor mRNA stability and translation, to maintain steady-state levels of iron in the blood (22). In the case of CUGBP2, it positively regulates cyclooxygenase-2 mRNA stability but negatively regulates cyclooxygenase-2 mRNA translation (23). Hence, PAIP2 seems to be a multifunctional protein that controls two important aspects of post-transcriptional regulation of gene expression, such as mRNA stability and translation. VEGF mRNA could represent a particular class of mRNA where the main role of PAIP2 is devoted to mRNA stability.

This increase in mRNA stability is consistent with that mediated by other stabilizing proteins like HuR or hnRNPL (8, 12). We also observed that PAIP2 is implicated in the stability of sugar transporters GLUT5 mRNA (21) and GLUT1 mRNA (data not shown), the later being also stabilized by Hu proteins (24). Our results suggest that RNA structures or sequences targeted by PAIP2 are present on several labile mRNAs (25).
The role of PAIP2 in mRNA stability correlates with its tissue distribution. We observed high levels of PAIP2 in testis, a tissue were mRNA stabilization is a crucial mechanism for gamete maturation (26, 27). PAIP2 is also highly expressed in liver where the mRNA stability is crucial for the regulation of the expression of enzymes such as the cholesterol-7a-hydroxylase (28, 29).

From the results of PAIP2 overexpression experiments, it was argued that PAIP2 silencing in the same cells would lead to a decrease in VEGF mRNA stability. Surprisingly, PAIP2 knock-down by RNA interference does not induce such an effect on VEGF mRNA stability, not only in S19-R443 cells but also in two other cell lines: A431 and HeLa. However, following PAIP2 knock-down, we observe a strong decrease in steady-state levels of VEGF mRNA in all the cell lines we have tested. Similar results have been described in the case of HuR and two other labile mRNAs are involved in the rapid turnover of these mRNAs (34). Four AREs corresponding to the pentamer -UTR (36), a region that we show to interact between PAIP2 and the StuI region. Therefore, these conserved sequences could represent potential targets for PAIP2. PAIP2 could also target this motif, as we show an interaction with HuR, a member of a protein complex recruited to the 3′-UTR of many mRNA 3′-UTR of many labile mRNAs are involved in the rapid turnover of these mRNAs (35). Four AREs associated in mediating basal VEGF mRNA stability. HuR was also observed in HuR-depleted cells (31, 32). One hypothesis to explain our results is that PAIP2 also affects the processing or export of VEGF pre-mRNA. In this context, it is noteworthy that PAIP2, although mainly cytoplasmic, is able to shuttle between the nucleus and the cytoplasm (data not shown). Further experiments are needed to definitely resolve this issue.

PAIP2 does not contain any RNA-binding motifs (33) that are common features of proteins that bind pre-mRNA, mRNA, pre-ribosomal RNA, and small nuclear RNA. However, some proteins that were shown to interact with and to stabilize labile mRNA, such as the Wilms’ transcription factor, do not contain such sequences (34). The AREs present in the 3′-UTR of many labile mRNAs are involved in the rapid turnover of these mRNAs (35). Four AREs corresponding to the pentamer AUUUA were previously described in the ΔStuI region of the VEGF mRNA 3′-UTR (36), a region that we show to interact with PAIP2. A polyuridylate sequence motif, which has also been demonstrated to mediate changes in mRNA stability (37, 38), was described in the StuI region of the VEGF 3′-UTR (36). PAIP2 could also target this motif, as we show an interaction between PAIP2 and the StuI domain. Therefore, these consensus sequences could represent potential targets for PAIP2. We demonstrate in this study that PAIP2 directly interacts with HuR, a member of a protein complex recruited to the VEGF mRNA 3′-UTR following hypoxia (6). The molecular weight of PAIP2 matches that of one of the three proteins described in this complex. However, RNA as well as protein levels of PAIP2 and HuR are not modified under hypoxia (8) (data not shown). PAIP2 and HuR could be constitutively associated in mediating basal VEGF mRNA stability. HuR was shown to interact with a region downstream of the PAIP2 interacting domain (8, 19). Interaction between both proteins could influence the overall RNA structure by bringing together disparate RNA sequences. Such stable structures would make RNA inaccessible to endonucleases.

All our experiments lead us to conclude on a crucial role of PAIP2 in regulating VEGF mRNA expression. Henceforth, we are investigating others target mRNAs for PAIP2 by differential screening using the RNAi-mediated knock-down of PAIP2. Moreover, because PAIP2 may modulate angiogenesis, the regulation of PAIP2 expression upon growth factor or cytokine stimulation and stress situations, where angiogenesis is affected, is also under investigation.

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