Stability of the vascular endothelial growth factor (VEGF) mRNA is tightly regulated through its 3'-untranslated region (3'-UTR). Here, we demonstrate that VEGF mRNA levels are increased by anisomycin, a strong activator of stress-activated protein kinases. Hence, VEGF mRNA induction is inhibited by SB202190, an inhibitor of JNK and p38/HOG kinase. Furthermore, VEGF mRNA expression is increased in cells that over-express JNK and p38/HOG by an increase in its stability. We show by two different approaches that anisomycin exerts its effect on the VEGF mRNA 3'-UTR. First, by using an in vitro mRNA degradation assay, the half-life of the VEGF mRNA 3'-UTR region transcript was found to be increased when incubated with extracts from anisomycin-treated cells; and second, the 3'-UTR was also sufficient to confer mRNA instability to the Nhe3 (Na+/H+ exchanger 3) heterologous reporter gene, and anisomycin treatment stabilized the chimeric mRNA (Nhe3 fused to the VEGF mRNA 3'-UTR). This chimeric mRNA is also more stable in cells overexpressing p38/HOG and JNK that have been stimulated by anisomycin. We show that such regulation is mediated through an AU-rich region of the 3'-UTR contained within a stable hairpin structure. By RNA electrophoretic mobility shift assays, we show that this region binds proteins specifically induced by anisomycin treatment. These findings clearly demonstrate a major role of stress-activated protein kinases in the post-transcriptional regulation of VEGF.

Vascular endothelial growth factor (VEGF) is implicated in both normal and pathological angiogenic processes (1–4). Its expression is mainly regulated by hypoxia. Hypoxia increases the transcription of the VEGF gene (5–10), the stability of its mRNA (11–13), and the translation of its mRNA through a mechanism implicating protein interaction with intraribosomal entry sites within its 5'-untranslated region (UTR) (14, 15). A recent report described a cooperation of different elements in the 5'- and 3'-UTRs for the hypoxic regulation of VEGF (16). Growth factors such as epidermal growth factor and transforming growth factor-α (17–19); cytokines such as transforming growth factor-β (20), interleukin-1, -6, and -8, and tumor necrosis factor (21–24); and transforming oncoproteins such as ras and raf (25) have been shown to induce the expression of VEGF. We have previously demonstrated that growth factors and transforming oncoproteins of the mitogen-activated protein kinase pathway increase VEGF transcription by activation of AP-2 and Sp1 transcription factors, which bind to a proximal region of the promoter (26). This action was shown to be elicited strictly by the activation of p42/p44 mitogen-activated protein kinase (26).

In this study, we have analyzed the transduction pathways implicated in the increased expression of VEGF mRNA by anisomycin, a strong activator of stress-activated protein kinases (SAPKs). We demonstrate that anisomycin increases VEGF mRNA stabilization through the activation of p38/HOG and JNK. This action is presumably mediated by specific recruitment of proteins, which remain to be identified, on various parts of the 3'-untranslated region of VEGF mRNA.

**EXPERIMENTAL PROCEDURES**

Cell Culture—The established Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection), its derivatives PS120 and PS200 (which lack Nhe1 antipporter activity) (27), and human embryonic kidney 293 and corresponding transfected cells were cultivated in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 7.5% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin sulfate. For the p38/HOG and JNK stable transfectants, we have used two CCL39 derivatives, PS200 and PS120, in which we cotransfected hemagglutinin-tagged JNK and p38/HOG, respectively (28, 29). The Na+/H+ exchanger expression vectors (Nhe1 isoform) were cotransfected as a selection gene (30) with a 20:1 ratio. Nhe1-expressing clones were selected by the acid load test (27), and p38/HOG or JNK expression was detected by using anti-hemagglutinin antibody in Western blot experiments. For the selection of cells stably transfected with Nhe3 chimeras, we used the acid load test in the presence of amiloride derivatives, which specifically inhibit Nhe1, but not Nhe3 (31).

Materials—Restriction and DNA-modifying enzymes were obtained from New England Biolabs Inc. or from Eurogentec (Liège, Belgium). [α-32P]dCTP and [α-32P]UTP were from ICN or Amersham Pharmacia Biotech. Synthetic oligonucleotides were from Eurogentec. SB202190 (32, 33) was from Calbiochem.

Cloning of the Human VEGF 3'-UTR—First-strand cDNA was synthesized from 1 μg of human embryonic kidney 293 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 VEGF 3'-UTR (7, 34) were synthesized and used as primers for the polymerase chain reaction: oligonucleotide 1, 5'-CGATTGACACGC-CAAGCCGCTG-3'; and oligonucleotide 2, 5'-GAGGACTTTGAAT-CAGAAG-3'. Intermediate oligonucleotides were also chosen to amplify smaller fragments. These oligonucleotides overlapped the StuI restriction site situated in the middle of the 3'-UTR sequence. The sequences of these oligonucleotides are as follows: oligonucleotide 3, 5'-GCAGAT-
were treated with RNase-free DNase (Stratagene) for 15 min at 37 °C, (Promega) (12) using the SP6 promoter, and the RNA transcripts were synthesized from linearized (200 ng/ml) CCL39 cells for the times indicated was analyzed by Northern blotting for expression of VEGF. 28 S ribosomal RNA is shown as a loading control. The specific signal for VEGF was quantified with the use of a phosphoimaging system (lower panel). This experiment is representative of three independent experiments.

Preparation of RNA—Cells were washed in ice-cold PBS and lysed in RNA Insta-Pure buffer (Eurogentec). The supernatant was cleared by centrifugation, isopropanol alcohol-precipitated, and resuspended in sterile water. In mRNA stability experiments, cells were treated with 5 μg/ml actinomycin D for the times indicated on the figures. For determination of mRNA half-life, 18 S ribosomal RNA was used as an internal standard. The entire cDNA for mouse VEGF (kindly provided by Dr. Werner Risau) or Nhe3 was used as a probe in Northern blot experiments.

Preparation of Cytosolic Extracts, RNA Electromobility Shift Assays, and in Vitro mRNA Stability Assay—CCL39 cells and PS200 hemagglutinin-tagged JNK- and p38/HOG-containing clones were grown to confluence and stimulated with 500 ng/ml anisomycin for different periods of time. Cells were then rinsed with ice-cold PBS and lysed, as described previously (34). RNA transcripts were synthesized from linearized poly(A) 3′-UTR template (Promega) (12) using the SP6 bacteriophage RNA polymerase with the RNA transcription kit (Promega) according to the manufacturer’s protocol. The coding region corresponding to Nhe3 (35) was subcloned in the pECE vector (36) at the Nhe3 3′-UTR TOT vector with SpeI and SmalI, blunting with Klenow, and religating. The 3′-UTR SpeI vector was obtained by cutting the Nhe3 3′-UTR TOT vector with SpeI and SmalI, blunting with Klenow, and ligating.

Preparation of GST Fusion Protein, in Vitro Kinase Assays, and Immunoblotting—Cell cultures were washed with ice-cold PBS and lysed in Triton lysis buffer (37). For GST fusion protein precipitation, 5 μg of GST-Jun was added per sample, followed by 20 μl of glutathione-Sepharose. For kinase assays, 4 μl of total antiserum directed against murine p38/HOG (38) was preincubated with 20 μl of protein A-Sepharose before the addition of the extract. Precipitates were washed four times with lysis buffer plus an additional wash with kinase buffer (37) and then resuspended in 40 μl of kinase reaction buffer for 15 min. Samples were boiled in Laemmli buffer and resolved on SDS-polyacrylamide gels. The radioactivity incorporated into GST-Jun or GST-ATF2 (the p38/HOG substrate) was analyzed with the use of the Fuji phosphoimaging system. For immunoblotting, 60 μg of proteins was run on a 10% SDS-polyacrylamide gel and transferred to an Immobilon membrane (Millipore Corp.), and the membrane was incubated overnight with the appropriate primary antibody at 4 °C. The protein was decorated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody and developed using the ECL system (Amersham Pharmacia Biotech). The 3′-UTR TotI/SmalI vector was obtained by cutting the 3′-UTR SspI construct with PmlI and SmalI and ligating. The plasmids containing the VEGF 3′-UTR used in EMSA and RNA degradation assays contained the rat VEGF 3′-UTR and have been previously described (12). For generating the StyI construct, the vector was digested with HindIII (polylinker of the pSP64 vector) and StyI, blunted with Klenow, and ligated.

FIG. 1. Anisomycin induces VEGF mRNA expression. 30 μg of total RNA isolated from exponentially growing or anisomycin-stimulated (200 ng/ml) CCL39 cells for the times indicated was analyzed by Northern blotting for expression of VEGF. 28 S ribosomal RNA is shown as a loading control. The specific signal for VEGF was quantified with the use of a phosphoimaging system (lower panel). This experiment is representative of three independent experiments.

RESULTS

Anisomycin Induces an Increase in VEGF mRNA Expression—To identify if SAPKs play a role in the signaling pathway leading to VEGF mRNA expression in CCL39 cells, we studied the effects of a strong activator of these kinases, anisomycin. As shown in Fig. 1, VEGF mRNA was rapidly induced by anisomycin and reached a maximum after 7 h of stimulation. Such an induction could be obtained with a low concentration of anisomycin (5 ng/ml) for the CCL39 cells (Fig. 2) and PS200 cells (data not shown). However, the maximal induction was obtained at higher concentrations in all cell lines tested (CCL39 and its derivatives PS120 and PS200, HeLa cells, 9L cells, and pro-B cells) (Fig. 2 and data not shown). As anisomycin

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Anisomycin is a strong inhibitor of protein synthesis (Fig. 2b), we have compared the effects of anisomycin and cycloheximide, which was previously shown to induce VEGF mRNA (39, 40). Fig. 2a shows that in our cell system, only very high concentrations of cycloheximide (total protein synthesis inhibition was obtained at 10 μg/ml) could slightly induce VEGF mRNA. This result shows that the anisomycin-dependent induction of VEGF cannot be completely attributable to its effect on protein synthesis inhibition.

Anisomycin Increases VEGF mRNA Stability—As anisomycin has no effect on VEGF gene transcription (26), we suspected that it could act at the post-transcriptional level by increasing VEGF mRNA stability. To demonstrate this effect, we performed a decay assay in the presence of actinomycin D. Consistent with our hypothesis, removal of anisomycin resulted in a rapid decay of VEGF transcripts (Fig. 3a, lanes 1–3), whereas the maintenance of anisomycin induced a persistence in VEGF mRNA (lanes 5–7). Under this last condition, anisomycin increased the VEGF mRNA half-life from 30 min to >4 h. Thus, anisomycin increases VEGF mRNA stability by blocking its degradation.

SAPKs p38/HOG and JNK Play a Key Role in the Stabilization of VEGF mRNA by Anisomycin—To analyze the implication of SAPKs in the induction of VEGF mRNA by anisomycin, we used a specific inhibitor of p38/HOG and JNK, SB202190 (32, 33). At a concentration of 20 μM, this drug inhibited the phosphorylation of c-Jun and ATF2, which are the direct targets of JNK and p38/HOG, respectively (Fig. 4a). We then evaluated the ability of SB202190 to block VEGF mRNA stabilization by anisomycin. Indeed, pretreatment of the cells with SB202190 totally abolished the effect of anisomycin (Fig. 4b). This result demonstrates that anisomycin stabilizes VEGF mRNA through the activation of SAPKs. To confirm the implication of the SAPKs in VEGF mRNA stabilization, we produced cells overexpressing p38/HOG or JNK. Fig. 5a shows that basal and anisomycin-stimulated JNK and p38/HOG activities were significantly increased in these cells. We next analyzed the level of VEGF mRNA in these cells. Fig. 5b shows that not only the basal, but also the anisomycin-induced VEGF mRNA levels were increased in stably transfected cells compared with control cells. VEGF mRNA levels were 5- and 10-fold higher in these cells than in their transfected controls, respectively, after anisomycin stimulation (200 ng/ml). This induction was particularly elevated in the JNK-expressing clone tested, which expressed 5–10-fold more JNK than the control cells. Such induction is directly dependent on the JNK implication of the SAPKs in VEGF mRNA stabilization, we substituted anisomycin with actinomycin D for the indicated periods of time. As expected, overexpression of SAPK was sufficient to slow down the VEGF mRNA degradation (Fig. 6), a result reflecting the increased basal levels of p38/HOG and JNK. Quantification of the transcript levels showed that the VEGF mRNA half-life was 30 min in control cells, 1 h and 30 min in p38/HOG-transfected cells, and 2 h and 30 min in JNK-transfected cells (Fig. 6). These results are in agreement with those of Li et al. (41), who observed a similar increase in the VEGF mRNA half-life in rat aortic smooth muscle cells after interleukin-1β stimulation, another p38/HOG and JNK activator.

Fig. 2. Anisomycin induces VEGF mRNA expression by a mechanism independent of its effect on protein synthesis. a, exponentially growing CCL39 cells were stimulated with anisomycin (ANISO) or cycloheximide (CYCLOHEX) at different concentrations for 5 h. 30 μg of total RNA was analyzed by Northern blotting. 18 S ribosomal RNA is shown as a loading control. This experiment is representative of three independent experiments. b, cells were incubated with SB202190 totally abolished the effect of anisomycin stabilization by anisomycin. Indeed, pretreatment of the cells with SB202190 totally abolished the effect of anisomycin (Fig. 4b). This result demonstrates that anisomycin stabilizes...
Anisomycin Exerts Its Stabilization Effect through the VEGF 3′-UTR—Most of the elements implicated in VEGF mRNA turnover have been shown to be located in the 3′-UTR (12, 16, 34). Thus, we wondered whether anisomycin is able to induce VEGF 3′-UTR stabilization in an in vitro degradation assay. Fig. 7 shows that [32P]UTP-labeled, capped, and polyadenylylated in vitro transcribed RNA containing the full-length VEGF 3′-UTR had a significantly longer half-life when incubated with anisomycin-treated cell extracts versus unstimulated cell extracts (ratio of 1.7 ± 0.2, n = 12). This protection is even more important with cellular extracts issued from p38/HOG- and JNK-expressing cells either treated or not with anisomycin (data not shown). These results highlight the importance of these protein kinases in anisomycin-mediated VEGF mRNA stabilization. These results also suggest that the effects observed in vivo are mediated through the 3′-UTR.

VEGF mRNA 3′-UTR Confers Instability to a Heterologous Transcript—We next wanted to study whether the VEGF 3′-UTR will confer anisomycin sensitivity to a stable heterologous transcript. The entire VEGF 3′-UTR or an appropriate deletion of this 3′-UTR was fused to the Na+/H+ exchanger reporter gene (Nhe3 isoform) (Fig. 8a). Since transcription of all the chimeric constructs was driven by the vector containing the SV40 promoter, differences at the chimeric mRNA levels could be attributed to fused VEGF 3′-UTR sequences. Fig. 8a recapitulates the different constructs obtained by deletion on the 3′-UTR. The chimeric constructs WT, 3′-UTR TOT, 3′-UTR SpeI, 3′-UTR SspI, and 3′-UTR Pml/IISspI were stably transfected into CCL39 cells. Expression of transfected constructs was assayed by the ability of Nhe3 to confer acid load resistance in the presence of amiloride derivatives (31). This method of selection is particularly interesting in our study since low amounts of Nhe3 are sufficient to confer resistance to the acid load test. Therefore, it allows selection of cells expressing very low levels of chimeric mRNA, a result anticipated by grafting the VEGF 3′-UTR. As shown in Fig. 8b, in the absence of any stimulus, Nhe3/VEGF 3′-UTR TOT chimeric mRNA amounts were strongly reduced compared with the Nhe3 mRNA. This result is in favor of a constitutive degradation mechanism through the instability elements contained within the 3′-UTR (12). This result is best illustrated after treatment with actinomycin D. As was the case for the endogenous VEGF mRNA, the chimeric Nhe3/VEGF 3′-UTR TOT mRNA was rapidly degraded. However, control Nhe3 mRNA was very stable as judged by the absence of degradation in the presence of actinomycin D (Fig. 8b). It is important to note that we could also detect different sizes of chimeric RNA after hybridization with the Nhe3 probe. This is probably due to the utilization of different polyadenylation signals present in the VEGF 3′-UTR. We have also measured the half-lives of the chimeric RNAs containing different regions of the VEGF mRNA 3′-UTR. SspI and SpeI constructs have half-lives that are similar to chimeric RNA containing the entire 3′-UTR (Fig. 8c). These results are in agreement with recent reports that have shown that sequences present in both constructs contain instability elements similar to those previously found in c-fos mRNA (12, 13, 34).

Anisomycin Increases Chimeric mRNA Stability by Stimulating SAPK—To evaluate whether the activation of SAPK by anisomycin can protect chimeric mRNA from degradation, we
transfected the 3′-UTR TOT construct in control, p38/HOG, and JNK stable transfectants. Fig. 9 shows that anisomycin increased chimeric mRNA stability in control cells. This effect was more pronounced in the p38/HOG- and JNK-transfected cells. As described above for endogenous VEGF mRNA, these cells conferred mRNA stabilization in the absence of any stimulation, which reflects the basal p38/HOG or JNK activity. Interestingly, the stability of chimeric mRNA was more intense in cells overexpressing JNK, as was the case for endogenous VEGF mRNA.

**Target Region for SAPK Can Form a Hairpin Structure**—It has been shown that JNK exerts its regulatory effect on interleukin-3 mRNA stabilization through an AUUU-rich region (33). We therefore investigated the effect of anisomycin on a region of the VEGF 3′-UTR containing such sequences. We found five AUUU regions in the VEGF 3′-UTR, but the one comprised between the PmlI and SspI sites (Fig. 8c) seems to be particularly interesting. By using an RNA-folding algorithm, MFOLD (42, 43), we showed that this region can form a very stable hairpin structure (Fig. 10a). The folding energy of the stem-loop formed was −12.8 kcal/mol. Within this region, there exists a “bubble” containing the UUAUUUA/U(A)/U(A) consensus sequence that corresponds to an instability domain equivalent to those described in c-fos, c-myc, and granulocyte/macrophage colony-stimulating factor mRNAs (44). This region also contains another AUUU sequence, but it does not fit the exact consensus described above. Furthermore, Ming et al. (33) and Winzen et al. (45) have shown that such sequences are the target of JNK- and p38/HOG-mediated stabilization of interleukin-3, -6, and -8 mRNAs. Since the presence of secondary structure was previously shown to be required for protein recognition (34, 46), we tested whether this domain can confer anisomycin sensitivity to the Nhe3 heterologous transcript. Fig. 10b shows that the chimeric mRNA containing this short domain was almost undetectable under basal conditions. This result suggests that the PmlI/SspI region is sufficient to confer mRNA instability. However, following anisomycin stimulation, it was highly induced and even more stable than a chimeric mRNA containing the total VEGF 3′-UTR (half-life of 2 h and 30 min instead of 1 h). These results allow us to identify at least a minimal target region for anisomycin-induced VEGF mRNA stabilization.

**Anisomycin Stimulation Induces Protein Binding to the Target Region**—The total in vitro transcribed VEGF 3′-UTR and a subdomain containing the stem-loop described above were incubated with S100 extracts to allow identification by EMSA of anisomycin-inducible VEGF mRNA-binding proteins. Fig. 11 shows that anisomycin-inducible protein complexes bound to the full-length (FL) and Sty1 probes. These complexes cannot
be visualized in the presence of an excess of unlabeled probe (data not shown). The region delimited by the Styl site in the rat VEGF mRNA 3'-UTR corresponds to the same region delimited by the PmlI and SspI sites in the human VEGF mRNA 3'-UTR. This domain presents 93% homology between the two species and can form a hairpin structure in the human as well as rat VEGF 3'-UTRs. This result points out the importance of the stem-loop structure in the anisomycin-mediated mRNA stabilization.

**DISCUSSION**

In this work, we have identified the signal transduction pathways implicated in the stabilization of VEGF mRNA in response to anisomycin. Anisomycin has been shown to be an inhibitor of protein synthesis as well as a strong activator of the SAPKs JNK and p38/HOG (28, 38, 47, 48). In this study, we show that anisomycin induces VEGF mRNA independently of an effect on protein synthesis. Therefore, the increase in VEGF mRNA must be mediated through p38/HOG and JNK activation. Indeed, VEGF mRNA amounts are higher in cells overexpressing p38/HOG and JNK, and this effect is enhanced following anisomycin stimulation. The fact that SB202190, a strong inhibitor of p38/HOG and JNK (32, 33), inhibits anisomycin-dependent VEGF mRNA induction confirms the essential role played by both kinases in the regulation of VEGF mRNA stabilization. We also provide evidence that anisomycin exerts its effects by increasing the stability of VEGF mRNA. Our data are
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We then focused our attention on the 3′-UTR of VEGF mRNA, which contains cis-active sequences previously shown to be important for mRNA stabilization in response to hypoxia (12, 13, 34), and asked whether the 3′-UTR is the target for regulation by SAPKs. By in vitro degradation assays, we demonstrated that anisomycin also exerts its effect via sequences present in the VEGF 3′-UTR region. We also demonstrated that the anisomycin- as well as SAPK-mediated VEGF mRNA regulation through the 3′-UTR can be conferred to a heterologous gene. In this context, we have shown that a chimeric Nhe3 mRNA containing the entire VEGF mRNA 3′-UTR is rapidly degraded, as was the case for the VEGF mRNA. We also confirmed the presence of sequences implicated in rapid degradation of VEGF mRNA by using overlapping chimeric constructs. Such data are compatible with the findings of Levy et al. (12, 13), who have shown by an independent approach the presence of such cis-active sequences within the 3′-UTR of VEGF mRNA. As was the case for the endogenous VEGF mRNA, p38/HOG and JNK participated in chimeric mRNA stabilization upon anisomycin stimulation. Our results are compatible with those of Chen et al. (32), Ming et al. (33), and Winzen et al. (45), who have demonstrated that JNK and p38/HOG signaling pathways are implicated in the stabilization of inter leukin-2, -3, -6, and -8 mRNAs. According to the results of Ming et al. (33), JNK exerts its effects through the AU-rich element (ARE) domains of interleukin-3 mRNA. Claffey et al. (34) identified five consensus domains (UUAAUU(UA)(UA)) in the VEGF mRNA 3′-UTR, and one of them is situated in a region of the 3′-UTR that can form a very stable hairpin structure.

Using the chimeric constructs, we show that this domain alone is sufficient to confer remarkable instability to the heterologous Nhe3 transcript as well as anisomycin-induced stabilization. Finally, we have shown by EMSA experiments that this region recruits protein(s) upon anisomycin stimulation. This domain is highly conserved between the rat and human sequences, whereas a lot of divergence usually exists in the 3′-UTR of mRNAs. The fact that this sequence is conserved in both species points out its importance in the post-transcriptional regulation of VEGF. However, it is not excluded that other regions participated in the regulation of VEGF mRNA stabilization by anisomycin. The AUUUUA sequence is the target of the already described AUF1 proteins, which are responsible for rapid mRNA degradation. Nakamaki et al. (50) have shown that their binding is independent of any stimulation of the granulocyte/macrophage colony-stimulating factor mRNA 3′-UTR. Recently, Sirenko et al. (51) showed that their binding to the GROα mRNA is a phosphorylation-dependent mechanism sensitive to protein kinase inhibitors. It will be interesting to test whether AUF1 proteins can interact with the hairpin structure and if they are direct targets for phosphorylation by p38/HOG or JNK. Identification of the in vivo VEGF 3′-UTR-interacting proteins will add to the knowledge of the regulation of VEGF expression during stress situations.

Acknowledgments—We particularly thank Dr. Darren Richard for critical reading of the manuscript, Emmanuel Gothie for RNA structure analysis, and Dominique Grall for technical assistance.

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J. Biol. Chem. 2000, 275:26484-26491.
doi: 10.1074/jbc.M002104200 originally published online June 9, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M002104200

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