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p42/p44 MAP Kinase Module Plays a Key Role in the Transcriptional Regulation of the Vascular Endothelial Growth Factor Gene in Fibroblasts*

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Vascular Endothelial Growth Factor (VEGF) is a potent mitogen for vascular endothelial cells that has been implicated in tumor neovascularization. We show that, in hamster fibroblasts (CCL39 cells), VEGF mRNAs are expressed at low levels in serum-deprived or exponentially growing cells, whereas it is rapidly induced after stimulation of quiescent cells with serum. CCL39 derivatives, transformed with Polyoma virus or with active members of the p42/p44 mitogen-activated protein (MAP) kinase pathway, Gly/Val point mutant of Ras at position 12 (Ras-Val^{12}), MKK1 in which Ser^{218} and Ser^{222} were mutated to Asp (MKK1-SS/DD)), express very high levels of VEGF mRNA. To analyze the contribution of the p42/p44MAP kinase in this induction, we used the CCL39-derived cell line (Raf-1:ER) expressing an estradiol-activable Raf-1. We show a time and an estradiol dose-dependent up-regulation of VEGF mRNA clearly detectable after 2 h of stimulation. The induction of VEGF mRNA in response to conditioned activation of Raf-1 is reverted by an inhibitor of MKK1, PD 098059, highlighting a specific role for the p42/p44 MAP kinase pathway in VEGF expression. Interestingly, hypoxia has an additive effect on VEGF induction in CCL39 cells stimulated by serum or in Raf-1:ER cells stimulated by estradiol. In contrast to VEGF, the isoforms VEGF-B and VEGF-C are poorly regulated by growth and oncogenic factors. We have identified a GC-rich region of the VEGF promoter between −88 and −66 base pairs which contains all the elements responsible of its up-regulation by constitutive active Ras or MKK1-SS/DD. By mutation of the putative binding sites and electrophoretic mobility supershift experiments, we showed that the GC-rich region constitutively binds Sp1 and AP-2 transcription factors. Furthermore, following activation of the p42/p44 MAP kinase module, the binding of Sp1 and AP-2 is increased in the complexes formed in this region of the promoter. Altogether, these data suggest that hypoxia and p42/p44 MAP kinase independently play a key role in the regulation of the VEGF expression.

Angiogenesis is a fundamental physiological process by which new blood vessels are formed (1). One of the most widely described mechanisms controlling neovascularization associated with pathological processes (2) is the increased secretion by the "stressed cells" (inflammation, psoriasis) or nutrient-deprived tumor cells of multiple growth factors (3–7) and cytokines (8, 9). Among growth factors, two major classes have been characterized: acid and basic FGF of the FGF family (3, 4) and Vascular Endothelial Growth Factor, VEGF,[1] a new family of secreted growth factors structurally related to PDGF (40% homology at the amino acid level) (5–7). VEGF, also described as a permeability factor, stimulates endothelial cell migration and proliferation *in vitro* and has angiogenic activity *in vivo* (10, 11). Different isoforms of 121, 165, 189, and 206 amino acids resulted from alternative splicing of the same gene (12). Many tissues and cell types express VEGF mRNA, especially tissues which are highly vascularized in addition to tumor-derived cell lines (13). Stimulation of serum-deprived NIH 3T3 cells by PDGF also results in VEGF induction in a Ras- and Raf-dependent manner (14). Deprivation of oxygen during cell culture, which mimics the necrotic hypoxic regions in solid tumors, induces VEGF mRNA expression by both an increase in the rate of transcription but also by stabilization of its mRNA (15–17). Considering the key role played by VEGF in the control of neovascularization (6, 7), it is of primary importance to decipher the growth factor-activated signaling pathways involved in controlling its expression.

In the present report, we have compared the expression of VEGF in resting, serum-stimulated, or oncogenically transformed CCL39 fibroblasts (18, 19). Exploiting a CCL39-derived cell line in which Raf-1 can be rapidly activated by estradiol (Raf-1:ER) (20–22), we demonstrated that the p42/p44 MAP kinase cascade is critical in the control of VEGF expression. To further characterize the effect of constitutively active Ras or MKK1 on the VEGF expression, we have assayed different constructs of the VEGF promoter in order to define cis-active regions sufficient to promote regulation of VEGF transcription by members of the p42/p44 MAP kinase module. By electrophoretic mobility assays (EMSAs) and supershift assays, we also defined transcription factors whose binding on the VEGF promoter is regulated through p42/p44 MAP kinase cascade.

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; Ras-Val^{12}, Gly/Val point mutant of Ras at position 12; p38/HOG, protein kinase of M_r 38 activated by osmotic shock (mammalian homolog of the yeast kinase HOG); p42/p44 MAPK, mitogen-activated protein kinases of 42 and 44 kDa, respectively; JNK, c-Jun N-terminal kinase; MKK1 or MEK1, MAP kinase kinase 1; MKK1-SS/DD, MKK1 in which Ser^{218} and Ser^{222} were mutated to Asp; Raf-1:ER cells, cells stably expressing an estradiol-inducible Raf-1; AP-1, activator protein 1; AP-2, activator protein 2; EMSA, electrophoretic mobility shift assay; PDGF, platelet-derived growth factor; PCR, polymerase chain reaction; bp, base pair(s); PBS, phosphate-buffered saline; DTT, dithiothreitol; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; FCS, fetal calf serum; TGF, transforming growth factor.

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EXPERIMENTAL PROCEDURES

Cell Culture—The established Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection), their derivatives PS120 and PS200, which lack NHE1 antiporter activity (23), and corresponding transfected cells were cultivated in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 7.5% fetal calf serum, penicillin (50 units/ml), and streptomycin sulfate (50 μg/ml). Growth-arrested cells were obtained by total deprivation of serum for 16–24 h. Raf-1:ER cells (clone 18 or 19) are a derivative of CCL39, and they stably expressed a fusion protein between the catalytic domain of Raf-1 and the hormone binding domain of the estrogen receptor (20–22). These cells were cultivated in the same medium described above without phenol red to reduce the basal activity of the Raf-1:ER construct. Hypoxia was generated by placing the cells in hermetic jars together with the Gas Pak Plus system from Becton Dickinson. In this system, hydrogen generated from sodium borohydride following the addition of water combines with the oxygen present in the jar in the presence of palladium catalyst to form water. Oxygen deprivation is almost complete after 1 h of incubation. Approximately 4–10% carbon dioxide is generated during this oxygen removal. Thus, cells are cultivated in the same bicarbonate-buffered medium.

Materials—Restriction and DNA modifying enzymes were obtained from New England Biolabs or from Eurogentec, Liège, Belgium. [α-^{32}P]dCTP, [α-^{33}P]dATP were from ICN. Synthetic oligonucleotides were from Eurogentec, Liège, Belgium.

Production of VEGF, VEGF-B, and VEGF-C Probes—First strand cDNA was synthesized from 1 μg of CCL39 poly(A)$^+$ RNA using avian myeloblastosis virus reverse transcriptase with oligo(dT) primer. This material was used as template for polymerase chain reaction (PCR) amplification. The following oligonucleotides derived, respectively, from human VEGF, mouse VEGF-B (24), human VEGF-C (25) sequences, were synthesized and used as primers for the PCR reaction: 5′-ATGAACTTTCTGCTGTCTTGGG-3′ and 5′-CCGCCTCGGCTTGTCACATCTGC-3′; 5′-ATGAGCCCCCTGCTCCGTCGCCTG-3′ and 5′-CTTTCGCGGCTTCCGGCACC-3′; and 5′-ATGACTGTACTCTACCCAGAATATTG-3′ and 5′-GCTCATTTGTGGTCTTTTC-3′. An aliquot of cDNA was amplified in a 50-μl reaction volume with 200 ng of each primer, 200 μM dNTPs, and 2.5 units of GoldstarTaq DNA polymerase (Eurogentec) or ampli-*Taq* from Boehringer Mannheim in a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin. The PCR amplification was performed in a DNA thermal cycler (Biotechnia) 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. Expected fragments of approximately 600 and 550 bp for VEGF, 600 bp for VEGF-B, and 1200 bp for VEGF-C were obtained. These fragments were purified on agarose gels and used as probes for Northern analysis. The fragments were also cloned in the pTAG vector using the manufacturer protocol (R & D Systems Europe Ltd.). The different inserts were sequenced using a Universal or T7 primer or specific oligonucleotides for each sequence. No specific problems related to low abundance of mRNA were encountered during the cloning of VEGF-B and VEGF-C from Chinese hamster lung fibroblasts even though lung is a tissue where they represent poorly abundant mRNA species (24, 25). The high percentage of homology (98%) with Chinese hamster, mouse, or human homologs has allowed us to use both mouse and hamster VEGF probes for Northern experiments.

Transient Transfection and Luciferase Assay—CCL39 cells in 12-well dishes (10^5 cells/well) were transiently transfected by $CaPO_4$ precipitation with the indicated plasmids (250 ng/well of the reporter plasmid, 200 ng of expression vector, and 100 ng of CMV β-galactosidase as a control of transfection efficiency). Sixteen hours after addition of DNA, the cells were washed twice with PBS and incubated with Dulbecco's modified Eagle's medium with or without 7.5% fetal calf serum. Two days later, the cells were washed with cold PBS, and luciferase assays were performed as follows (Promega protocols and applications guide). Cells were lysed in lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1, 2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid, 10% glycerol, and 1% Triton X-100) for 15 min at room temperature, and the lysate was cleared by centrifugation. The assay of luciferase activity was performed in a chemioluminometer in a buffer containing 20 mM Tricine, 1.07 mM $(MgCO_3)Mg(OH)_2$, $5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferine, and 530 μM ATP. Protein concentration was measured using the bicinchonic acid (BCA) protein assay kit (Pierce) with bovine serum albumin as standard.

Preparation of RNA—Cells were washed in ice-cold PBS and lysed in the "RNA Insta-Pure" buffer from Eurogentec. The supernatant was cleared by centrifugation, ethanol precipitated, and resuspended in sterile water.

Promoter Construction and Mutagenesis Experiments—Dr. Werner Risau kindly provided the human VEGF promoter gene construct (−1176/+54) (16) cloned in the pGL2 basic vector from Promega. Construct −88/+54 was obtained by cutting the above vector by *Sma*I (one site in the vector and one site at position −88 of the promoter) and re-ligating. Construct −27/+54 was obtained by subcloning a *Dra*I/*Nhe*I fragment within the *Sma*I/*Nhe*I sites of pGL2 basic. The −66/+54 and −52/+54 constructs were generated by PCR by using oligo 1, 5′-GCGGGTACC(**T**)CCCGGCGGGGCGG-3′; and oligo 2, 5′-GCGGGTACC(**A**)GCCATGCGCCCCC-3′, respectively. Bases shown in bold correspond to positions −66 and −52, respectively. At their 5′ ends, both oligos contain the *Kpn*I restriction site. They were used in a PCR reaction with oligo 3, 5′-CTTTATGTTTTTGGCGTCTTCCA-3′, which corresponds to a sequence within the vector at the 3′ end of the promoter. The amplified fragments were digested with *Kpn*I and *Nhe*I and inserted into the pGL2 basic vector (Promega). We mutated the AP-2 site, both Sp1 sites or all three binding sites by the PCR method (26, 27). The following oligonucleotides were chosen: oligo 4, 5′-TGTATCTTATGGTACTGTAACTG-3′; oligo 5, 5′-GGGGCGGGCC(**TA**)GGGCGGGG-3′; oligo 6, 5′-CCCCGCCC(**TA**)GGCCCGCCCC-3′; oligo 7, 5′-GCCCCCCGGG(**AA**)GGGCCGGGG(**AA**)GGGGTCCG-3′; oligo 8, 5′-CGGACCCC(**TT**)CCCCGGCCC(**TT**)CCCGGGGGGC-3′; oligo 9, 5′-GGG(**AA**)GGCC(**TA**)GG(**AA**)GGGGTC-3′; and oligo 10, 5′-GAACCCC(**TT**)CC(**TA**)GGCCC(**TT**)CCC-3′. Oligo 4 corresponds to a sequence within the vector at the 5′ end of the promoter. For oligos 5, 6, 7, 8, 9, and 10, bases shown in bold indicate those modified in relation to the wild type sequence (oligos 5 and 6 for AP-2 mutation; oligos 7 and 8 for mutation of both Sp1 sites; oligos 9 and 10 for mutation of the AP-2 site and both Sp1 sites; see also Fig. 6*a*). After obtention of the –1176/+54 mutated constructs, we digested them with *Sma*I to obtain the corresponding −88/+54 constructs. For the triple mutant, we used the construct −1176/+54 that was mutated for both Sp1 binding sites and oligos 9 and 10 for mutation of the remaining AP-2 binding site before digestion with *Sma*I to obtain the −88/+54 construct. The presence of the mutations were verified by gel sequencing.

Preparation of Nuclear Extracts and Gel Mobility Shift Assays—Confluent Raf-1:ER cells cultures were serum-starved overnight followed by stimulation with or without estradiol (1 μM) for 3 h. Nuclei were isolated by the isotonic/Nonidet P-40 procedure: cells were resuspended in HNB (0.5 M sucrose, 15 mM Tris, pH 7.5, 60 mM KCl, 0.25 mM EDTA, pH 8, 0.125 mM EGTA, 0.5 mM spermidine, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 50 mM NaF, 40 mM β-glycerophosphate, 200 mM paranitrophenylphosphate, 0.2 mM orthovanadate) and homogenized in HNB containing 0.2% Nonidet P-40. Nuclei were recovered by centrifugation at 3000 rpm and rinsed in HNB alone. Nuclear extracts were then prepared by the method described by Dignam *et al.* (28). The probe was synthesized to span the region of the human VEGF promoter comprised between the −88 and −66 bp: 5′-*TTT*CCGGGGCGGGCCGGGGGCGGGG*TAT*-3′ (random sequences added to the wild type sequence are shown in italic letters). Protruding 5′ ends were filled in with (exo-) Klenow fragment (from Stratagene) and [α-^{32}P]-dCTP and dATP. The DNA binding reaction was performed for 15 min at room temperature in a final volume of 15 μl. A first volume of 7.5 μl was prepared containing 5 μg of nuclear extracts, 0.75 mg/ml poly(dI:dC) (Sigma), dialysis buffer (20 mM Hepes, pH 7.9, 60 mM KCl, 20% glycerol, 0.25 mM EDTA, 0.125 mM EGTA, 1 mM DTT), protease and phosphatase inhibitors) (see HNB). The residual 7.5 μl was comprised of 90 fmol of labeled probe (1–2.10^5 cpm), with or without excess (60–600-fold) of unlabeled probe, and with or without excess (100-fold) of Sp1 or AP-2 consensus oligonucleotides (Promega): Sp1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′ and 3′-TAAGCTAGCCCCGCCCCGCTCG-5′; AP-2, 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′ and 3′-CTAGCTTGACTGGCGGGCGCCGGGCA-5′.

The competitors were added 15 min before addition of the probe. Supershift assays were performed with 2.5–3 μg of nuclear extracts. The Sp1 (PEP-2) and AP-2α (C-18) antibodies were purchased from Santa Cruz Biotechnology. 0.2 μg of Sp1 antibody was incubated 30 min at 4 °C after addition of the probe, whereas 1 μg of AP-2α antibody was added 30 min before the addition of the probe. Electrophoresis was performed on a 4% polyacrylamide (30 acrylamide, 0.8 bisacrylamide) gel, in 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA), for 4–5 h at 280–300 V/10–12 mA .

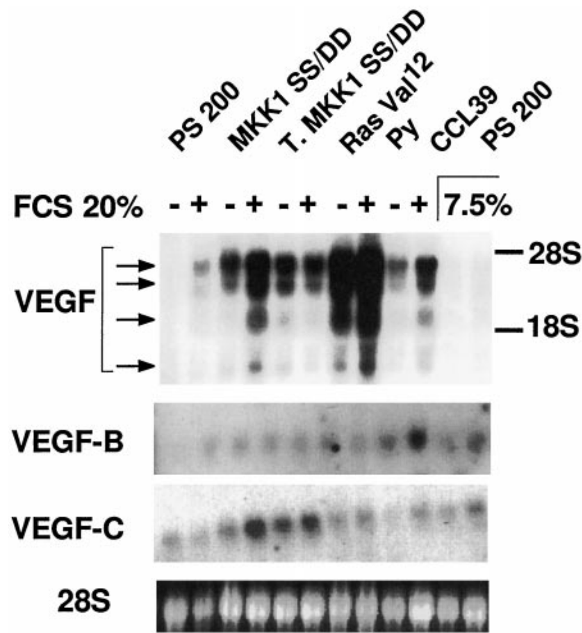

FIG. 1. **Expression of VEGF in resting, serum-stimulated, and transformed cells.** 20 μg of total RNA isolated from cells transformed by Ras-Val^{12}, Polyoma virus (*Py*), constitutively active MAP kinase kinase (MKK1-SS/DD) or from T. MKK1-SS/DD cells recovered from nude mice (19) were analyzed by Northern blot in comparison with control cells (*PS 200*). Cells were serum-deprived for 16 h (–) and then stimulated with 20% FCS for 3 h (+). RNA isolated from exponentially growing normal cells were loaded as controls (CCL39, PS200/7.5%). The blot was hybridized with probes corresponding to VEGF, VEGF-B, and VEGF-C. Ethidium bromide coloration of 28 S ribosomal RNA is shown as loading control. Probes used to specifically identify VEGF, VEGF-B, and VEGF-C were as described under "Experimental Procedures."

RESULTS

VEGF mRNA but Not VEGF-B and VEGF-C Are Regulated by Growth and Oncogenic Factors in CCL39 Cells—Under normoxic conditions, exponentially growing CCL39 or its derivative PS 200 Chinese hamster lung fibroblasts express barely detectable levels of VEGF mRNA (*right lanes* of Fig. 1). Serum stimulation of growth-arrested CCL39 (data not shown) or PS 200 cells (*left lanes* of Fig. 1) triggers the induction of VEGF mRNA. However, this expression is strongly elevated in cells transformed either with Polyoma virus, Ha-Ras (Ras-Val^{12}) (18) or a constitutive active form of MAP kinase kinase (MKK1-SS/DD) (19). At least four isoforms that correspond to the spliced variants described (12) detectably hybridize to a mouse VEGF probe. Fig. 1 shows that, in the Polyoma virus, Ha-Ras and MKK1-SS/DD transformed cells, the different VEGF mRNA isoforms are expressed at a level approximately 10-fold superior to that of control cells. This overexpression is particularly prominent for the clone 5c that overexpressed Ha-Ras (18). For each of the cell lines tested, FCS was able to increase the amount of VEGF mRNA, although in transformed cells the basal level was extremely elevated. However, this is not the case for cells expressing MKK1-SS/DD and isolated from a tumor produced in nude mice (T.MKK1-SS/DD). Interestingly, these cells were shown to be fully independent of serum growth factors (19). This could explain the inability of serum to further modify the elevated level of VEGF mRNA in these tumor cells. In the different cell lines tested, the other members of the VEGF family, VEGF-B and VEGF-C, are constitutively expressed showing that both genes are not tightly regulated via growth or oncogenic factors even if VEGF-C seems to be up-regulated in MKK1-SS/DD transformed cells.

p42/p44 MAP Kinase Cascade Specifically Induces VEGF mRNA Expression in Raf-1:ER-expressing Cells—To further

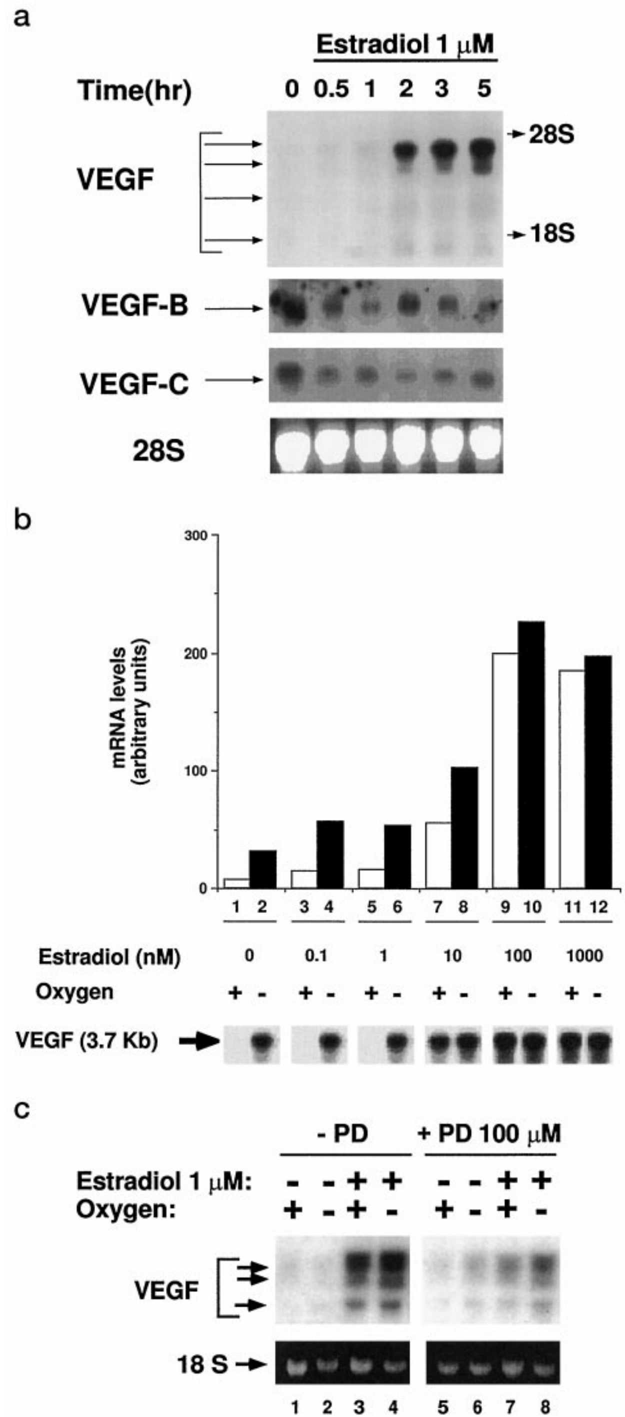

FIG. 2. **Regulation of expression of VEGF in Raf-1:ER expressing cells.** *a,* 20 μg of RNA isolated from quiescent or estradiol-stimulated Raf-1:ER cells (1 μM) for the times indicated were analyzed by Northern blot for expression of VEGF, VEGF-B, and VEGF-C. As described in Fig. 1, 28 S ribosomal RNA is shown as loading control. *b,* Raf-1:ER cells were serum-deprived for 16 h and then stimulated with estradiol for 4 h at different concentrations (0, 0.1, 1, 10, 100, 1000 nM) in the presence or absence of oxygen (see "Experimental Procedures"). 20 μg of RNA were analyzed by Northern blot. The specific signal for VEGF was quantified by using a phosphoimager. *c,* the MKK1 inhibitor PD 098059 inhibits the estradiol induction of VEGF mRNA. Raf-1:ER cells were serum-deprived for 16 h and then stimulated or not with estradiol (1 μM) for 4 h in the presence (+) or absence (–) of PD 098059, 100 μM, and in the presence or absence of oxygen. Levels of VEGF mRNA were quantitated using a phosphoimager. 18 S ribosomal RNA is shown as loading control.

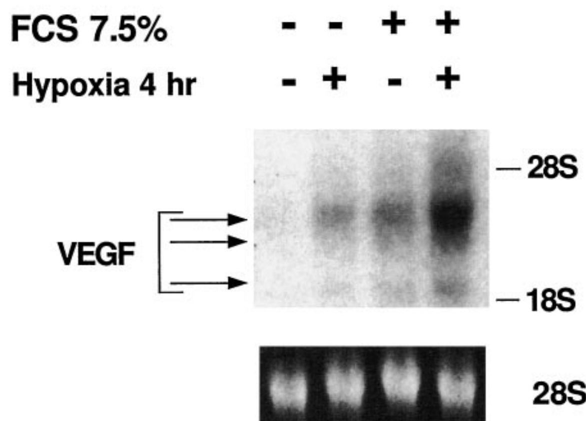

FIG. 3. **Hypoxia and growth factors have additive effects on VEGF mRNA induction.** Quiescent or exponentially growing CCL39 cells were submitted or not to hypoxia. 20 μg of RNA isolated from the cells submitted to the different situations were analyzed by Northern blot. 28 S ribosomal RNA is shown as loading control.

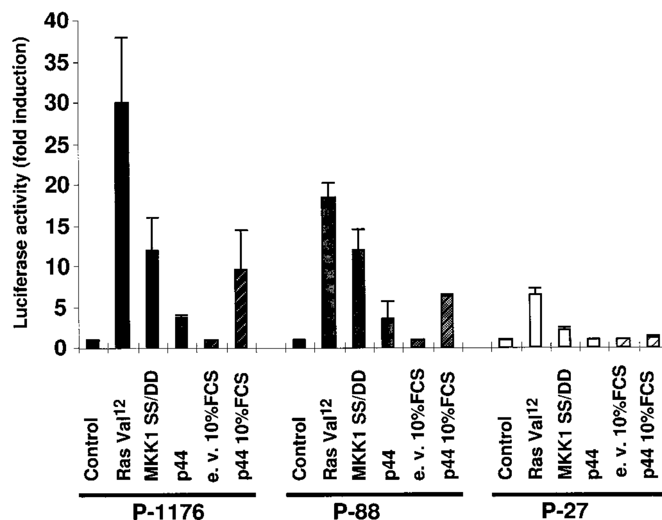

FIG. 4. **The p42/p44 MAP kinase pathway is required to up-regulate VEGF gene transcription.** 250 ng of different constructs of the VEGF promoter (–1176/+54; –88/+54, –27/+54) coupled to the luciferase reporter gene were transfected together with 200 ng of empty expression vector (*Control*) or 200 ng of expression vector coding for constitutively active human Ras (Ras-Val^{12}), constitutively active MEK1 (MKK1-SS/DD), or p44 MAP kinase. In all cases, 100 ng of an expression vector coding for β-galactosidase was co-transfected in order to normalize for transfection efficiency. 24 h after transfection, cells were serum-deprived for 20 h, and then luciferase activity was measured. When cells were transfected with p44 MAP kinase, they were also stimulated with 10% FCS. The results shown correspond to four independent experiments. Each set of data are the mean of triplicate determinations.

examine the contribution of the Ras/p42/p44 MAP kinase pathway in VEGF expression, we have chosen a cell line expressing an estradiol-inducible Raf-1 (Raf-1:ER cells) (20–22). In this case, the p42/p44 MAP kinase activity is rapidly activated by estradiol, eliminating the contribution of SAP kinase cascade (p38MAPK/JNK) (29, 30, 31) and phosphatidylinositol 3-kinase cascade (32, 33) that are generally activated by serum or constitutively active Ras. Raf-1:ER-expressing cells (see "Experimental Procedures") were serum-deprived for 16 h and then stimulated by the addition of estradiol for the times indicated. Fig. 2*a* shows that VEGF transcripts are expressed at a detectable level after 2 h and are maximally expressed after 3 h of estradiol stimulation, the expression being sustained for up to 5 h. A longer exposure of the blot shows detectable transcripts after only 30 min of stimulation (data not shown), and the expression of the three other spliced variants are revealed in Fig. 1. This rapid induction is compatible with the kinetics of activation of p42/p44 MAP kinases in these cells (22). The expression of VEGF-B and VEGF-C mRNA species are not modified by estradiol treatment, confirming that activation of the p42/p44 MAP kinase pathway does not play any role in controlling their expression in these cells. Induction of VEGF mRNA in response to estradiol was dose-dependent, with 70% of the maximal induction obtained after a stimulation with 10 nM estradiol (Fig. 2*b*, *lane 7*), and maximal induction obtained with 100 nM estradiol (Fig. 2*b*, *lane 9*). This dose-response activity reflects the magnitude of activation of p42/p44 MAP kinase in these cells (22). If the activity of MKK1, the kinase directly downstream of Raf, is blocked by the specific inhibitor PD 098059 (34) (Fig. 2*c*, *lane 7*), the p42/p44 MAP kinase pathway-dependent induction of VEGF is inhibited by 50% after 4 h of stimulation by estradiol (compare *lanes 3* and *7* of Fig. 2*c*). This result supports the hypothesis that the p42/p44 MAP kinase cascade plays a key role in VEGF gene induction. The partial inhibition of MKK1 by PD 098059 (60–70% inhibition at this concentration)[2] explains the residual VEGF mRNA amount in Fig. 2*c*.

Additive Effect of Hypoxia and Growth Factors on VEGF mRNA Induction—To test whether induction of VEGF mRNA by hypoxia is dependent or not on the action of growth factors, we submitted quiescent or exponentially growing CCL39 cells to hypoxia for four hours. Fig. 3 shows that oxygen deprivation on its own is able to induce VEGF mRNA induction in quiescent cells to a level that is comparable with the basal level present in exponentially growing cells. When serum is present, VEGF mRNA levels reached a level superior to that present in quiescent cells following hypoxia. There is no discrepancy between the level observed in this experiment and the results presented in Fig. 1. In Fig. 1, the blot was underexposed to compare the high levels of mRNA in transformed cells. We routinely observed a basal level of mRNA in exponentially growing cells. However, the amounts of mRNA obtained after serum stimulation of quiescent cells is at least five times more elevated. Again, to analyze more directly the contribution of p42/p44 MAP kinase, we used the Raf-1:ER cells stimulated by estradiol in the presence or absence of oxygen (see Fig. 2*b*). At suboptimal concentrations of estradiol (0.1 and 1 nM), which do not maximally activate p42/p44 MAP kinase (22), we observed the induction of VEGF by hypoxia. Estradiol and hypoxia exert additive effects on VEGF mRNA induction at a dose of estradiol (10 nM) that induces half of the p42/p44 MAP kinase activity (see *lane 8* of Fig. 2*b*). This situation is comparable with that observed in Fig. 3 where exponentially growing cells are submitted to hypoxia. When a maximal dose of estradiol is used (100 nM or 1 μM), a small additive effect with hypoxia persists, but it is less detectable than that observed with 10 nM estradiol. In the presence of PD 098059, the induction of VEGF mRNA by hypoxia still occurs even if the estradiol-mediated induction of VEGF mRNA is inhibited by 50% (see Fig. 2*c*). This result suggests that the hypoxia-mediated VEGF mRNA increase is independent of p42/p44 MAP kinase activity. This is further emphasized by the fact that hypoxia is not capable of activating p42/p44 MAP kinase activity in CCL39 cells (data not shown).

Expression of Constitutively Active Members of the p42/p44 MAP Kinase Pathway Increase VEGF Gene Transcription—To assess the mechanism by which p42/p44 MAP kinase cascade stimulates VEGF mRNA production, we directly analyzed the activation of the VEGF promoter coupled to the luciferase

[2] F. R. McKenzie, J. M. Brondello, and A. Brunet, unpublished results.

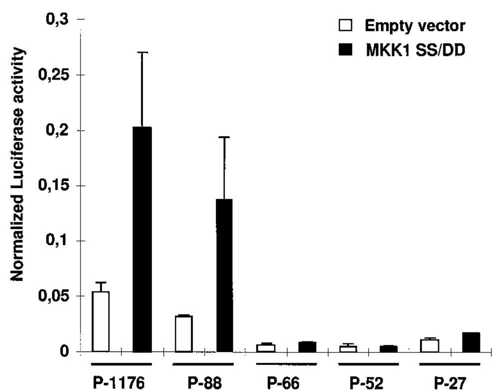

FIG. 5. **Identification of a p42/p44 MAP kinase pathway responsive region between −88 and −66 of the VEGF gene promoter by 5′ deletional analysis.** CCL39 cells were transfected with 250 ng of the −88/+54, −66/+54, –52/+54 and –27/+54 in the presence or absence of 200 ng of expression vector coding for constitutively active MEK1 (MKK1-SS/DD). In all cases, 100 ng of an expression vector coding for β-galactosidase was co-transfected in order to normalize for transfection efficiency. 16 h after the transfection, the cells were rinsed with PBS and grown in medium supplemented with 7.5% FCS for 48 h. The cells were then lysed, and luciferase activity was measured. The results correspond to three different experiments. Each set of data are the mean of triplicate determinations.

reporter gene as previously reported (15, 16, 35, 36). Fig. 4 shows that constitutively active Ras (Ras-Val[12]) or constitutively active MKK1 (MKK1-SS/DD) can strongly stimulate the VEGF promoter (–1176/+54) in the absence of FCS when compared with empty vector. Thus, both members of the p42/p44 MAP kinase pathway can stimulate VEGF gene transcription. VEGF gene transcription is not significantly increased when a p44 MAP kinase encoding construct is cotransfected with the reporter vector in the absence of serum. However, when cells are transfected with a p44 MAP kinase encoding construct in the presence of serum, the level of transcription is significantly increased when the –1176/+54 and the −88/+54 constructs are used. VEGF promoter constructs (−888/+54 (data not shown) and −88/+54) still conserved the capacity to be induced by constitutively active Ras or MKK1 even if the response to Ras-Val[12] is reduced by 40% for the −88/+54 construct and the response to MKK1-SS/DD is not modified. This is surprising since the –1176/– 88 region contains putative AP-1 consensus binding sites which are targets of transcription factors whose activity is up-regulated by the p42/p44 MAP kinase pathway (Jun/Fos). The difference observed between the stimulatory effects of Ras and MKK1 is not attributable to the stress-activated kinase pathways that are also activated by Ras because transfection of c-Jun N-terminal kinase (JNK) or p38/HOG in the presence of activating agents (anisomycin, IL-1β) or their constitutively active activating kinases (MKK3 or MKK4) (31) has no effect on VEGF promoter activity (data not shown). However, if AP-2 and Sp1 binding sites are deleted (–27/+54), the induction obtained with MKK1-SS/DD is totally abrogated and the activation obtained with Ras-Val[12] is inhibited by 80%.

Characterization of the p42/p44 MAP Kinase Pathway Responsive Region of the VEGF Promoter—We next constructed and analyzed the activities of the −66/+54 and –52/+54 constructs in comparison with the –1176, −88 and –27/+54 constructs in the presence or absence of MKK1-SS/DD to localize the p42/p44 MAP kinase pathway responsive element. Fig. 5 shows that while the –1176 and −88/+54 constructs displayed constitutive base-line activity, which was increased by cotransfection with MKK1-SS/DD by a factor of 3.3 and 4.6, respectively, analysis of the −66, –52, and –27/+54 constructs showed a loss of both basal and MKK 1 SS/DD transcriptional activation. These results suggest that sequences between −88 and −66 are absolutely required for basal and p42/p44 MAP kinase-dependent pathway-stimulated promoter activity. The loss of both basal and stimulated transcriptional activity could reflect a truncation of transcription factor binding sites in the −88/+54 construct that can regulate the overall activity of the promoter.

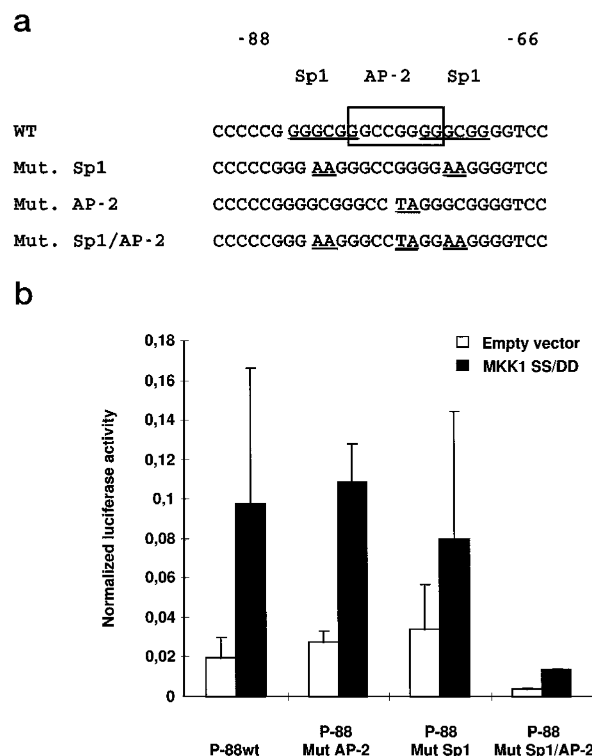

FIG. 6. **Cooperation of Sp1 and AP-2 for VEGF promoter activation.** *a,* sequence of −88/–66 human VEGF promoter region. AP-2 binding site is *boxed*, and Sp1 binding sites are *underlined*. The mutations for Sp1 or AP-2 binding sites are *underlined*. *b,* CCL39 cells were transfected with 250 ng of the −88/+54 promoter containing or not a mutation for AP-2, both Sp1, or the three binding sites in the presence or absence of 200 ng of expression vector coding for active MEK1 (MKK1-SS/DD). In all cases, 100 ng of an expression vector coding for β-galactosidase was co-transfected in order to normalize for transfection efficiency. 16 h after the transfection, the cells were rinsed with PBS and grown in medium supplemented with 7.5% FCS for 48 h. The cells were then lysed, and luciferase activity was measured. The results correspond to three different experiments. Each set of data are the mean of triplicate determinations.

Cooperative Effects of AP-2 and Sp1 in MKK1-SS/DD Stimulation of VEGF Promoter—As shown by Gille *et al.* (37), TGF-α-mediated induction of the VEGF promoter is a mechanism involving the AP-2 transcription factor but not Egr-1, a member of the same family. TGF-α acts via the EGF receptor whose signaling is in part mediated by the p42/p44 MAP kinase pathway (for reviews, see Refs. 38 and 39). Transfection of an expression vector encoding AP-2 can stimulate the VEGF promoter in CCL39 cells (data not shown), which is in agreement with the data of Gille *et al.* (37). Based on these results, we have investigated whether the p42/p44 MAP kinase pathway induced VEGF mRNA levels by directly activating AP-2 or Sp1, whose binding sites are present between −88 and −66. Fig. 6*a* shows that within this region there exists two putative binding sites for Sp1 and one for AP-2. These sites are conserved between human, mouse, and rat promoters (35, 40–42). Therefore, the intact −88/+54 construct or a construction with point mutations in the AP-2, both Sp1, or the three binding sites (Fig. 6*a*) were transfected in exponentially growing cells, and activation of the VEGF promoter was analyzed by luciferase assay.

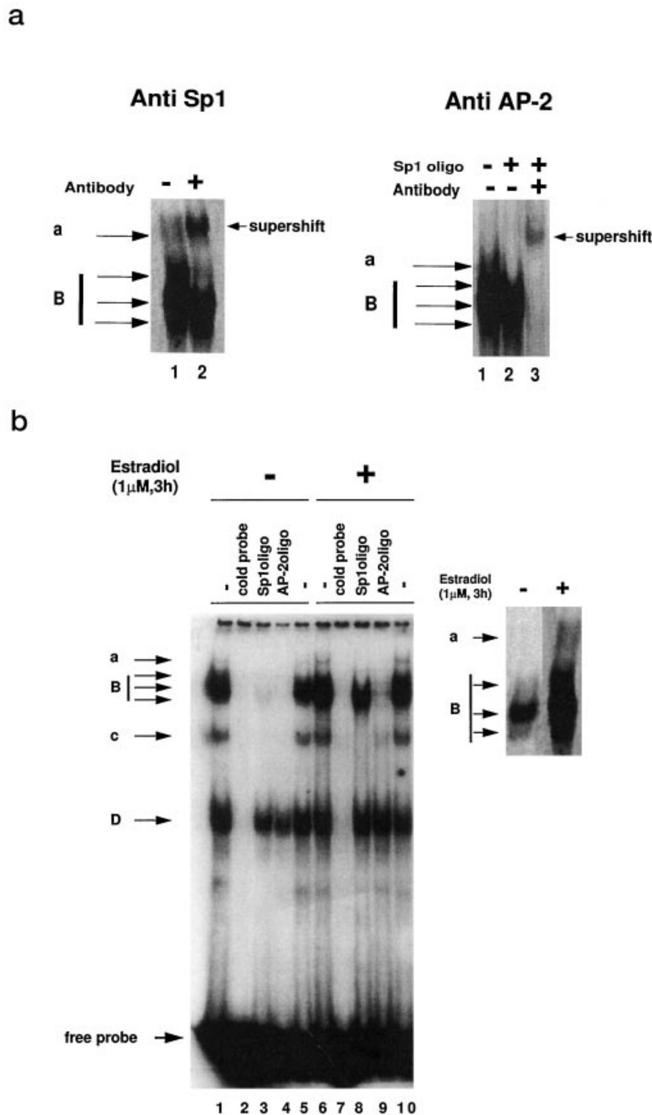

FIG. 7. **Identification of AP-2 and Sp1 transcription factors in the p42/p44 MAP kinase pathway-inducible DNA binding complex.** *Panel a, left*, EMSAs with nuclear extracts of estradiol-stimulated Raf-1:ER cells (*lanes 1* and *2*) in the absence (*lane 1*) or the presence (*lane 2*) of Sp1-specific antibodies (0.2 μg). *Panel a, right,* EMSAs with nuclear extracts of estradiol-stimulated Raf-1:ER cells in the absence (*lane 1*) or the presence (*lanes 2* and *3*) of unlabeled doubled-stranded Sp1 oligonucleotides and in the absence (*lanes 1* and *2*) or the presence (*lane 3*) of AP-2 specific antibodies (1 μg). The different complexes observed (*a*, *B*) are shown by *arrows*. *Panel b,* EMSAs with nuclear extracts of untreated (*lanes 1* to *5*) or estradiol-stimulated (*lanes 6* to *10*) Raf-1:ER cells in the absence (*lanes 1, 5, 6,* and *10*) or the presence of excess unlabeled probe (*lanes 2* and *7*), or excess of double-stranded Sp1 consensus oligonucleotides (*lanes 3* and *8*), or excess of doubled-stranded AP-2 consensus oligonucleotides (*lanes 4* and *9*). Formation of specific complexes is indicated on the *left* (*a*, *B*, *c,* and *D*). The DNA sequence of the probe used is indicated under "Experimental Procedures." Competitor was used at a concentration of 100 molar excess. An enlargement of complexes a and B upon resting or stimulated conditions is shown on the *right* of the figure.

The response to the p42/p44 MAP kinase module was assessed by co-expression of constitutively active MKK1. Fig. 6*b* shows that mutations of the AP-2 or both Sp1 putative binding sites done individually do not significantly modify basal and MKK1-SS/DD-stimulated promoter activity. However, a combined mutation of AP-2 and both Sp1 binding sites dramatically decreases basal and MKK1-SS/DD-dependent transcriptional activation. This result suggests a cooperative effect of AP-2 and Sp1 for maximal transcriptional activation of the VEGF promoter.

p42/p44 MAP Kinase Pathway Controls the Binding Activity of Nuclear Protein Extracts to the −88/−66 VEGF Promoter Region in Raf-1:ER Cells—In order to confirm the specific role of AP-2 and Sp1 transcription factors in the regulation of the VEGF promoter, we utilized a double-stranded probe encompassing the −88/−66-bp region in EMSA experiments. Fig. 7 shows four constitutive DNA binding complexes in resting or in estradiol-stimulated Raf-1:ER cells (*complexes a*, *B*, *c*, *D*; see *lanes 1* and *6* of Fig. 7*b*). To demonstrate that either Sp1 or AP-2 are present in at least the large complex B, we performed supershift experiments. Indeed, Sp1 antibodies supershifted part of complex B formed with extracts from resting cells (data not shown) or estradiol-stimulated cells (Fig. 7*a, left*, *compare lanes 1* and *2*). Similarly, AP-2 antibodies supershifted part of complex B with extracts from estradiol-stimulated cells (Fig. 7*a, right,* compare *lanes 2* and *3*). This supershift, however, is more evident in the presence of Sp1 neutralizing oligonucleotides. The binding specificity of the complexes formed were determined by exclusive competition with an excess of identical unlabeled DNA (Fig. 7*b*). Under resting conditions, DNA binding of complexes a, B, and c are clearly inhibited by either Sp1 or AP-2-specific oligonucleotides (Fig. 7*b*, compare *lane 1* or *5* with *lanes 3* and *4*). However, a remarkable change is observed when p42/p44 MAP kinase was specifically stimulated with estradiol. Nuclear extracts of cells stimulated for 3 h with estradiol show a strong increase in the binding of complex B. This is seen in Fig. 7*b*, *right panel*, where complex B is better resolved and enlarged. Another striking change occurs when binding is inhibited with a 100-fold excess of Sp1 oligonucleotides (Fig. 7*b*, compare *lanes 3* and *8*). Under stimulated conditions, complex B resists the competition with the Sp1 oligonucleotides, reflecting that more proteins are bound and/or have a higher affinity. The same result is observed when AP-2 oligonucleotides are used as a competitor, even if in this case the labeling of the resistant complex B is less intense. Altogether, these data clearly demonstrate that Sp1 and AP-2 transcription factors bind to the −88/−66 region of the VEGF promoter and that p42/p44 MAP kinase activity plays a key role in controlling the VEGF promoter activity via these sites.

DISCUSSION

The expression level of the VEGF mRNA is tightly regulated by both transcriptional and post-transcriptional mechanisms (15–17, 35, 43). A variety of cytokines and growth factors, including epidermal growth factor, transforming growth factor α, transforming growth factor β, interleukins 1 and 6 (44–47), as well as transforming agents such as v-Ha-Ras and v-Raf (14) were shown to induce VEGF expression in several cell lines, and Pedram *et al.* (48) have proposed a role for Erk in the endothelin activation of the VEGF promoter. However, the signaling cascades involved have not been fully deciphered. Here, we have analyzed VEGF, VEGF-B (24), and VEGF-C (25) expression in quiescent, serum-stimulated, or oncogenically transformed CCL39 cells. In all the conditions where p42/p44 MAP kinase activity is strongly enhanced, the VEGF mRNA levels are especially increased; this is particularly notable in Ras-Val^{12} or MKK1-SS/DD transformed cells. This first result suggests that regulation of VEGF gene expression occurs through a p42/p44 MAP kinase-dependent mechanism. To confirm more directly the role of the p42/p44 MAP kinase pathway in this mechanism, we have used a cell line where p42/p44 MAP kinase activity is tightly regulated by an estradiol-inducible Raf-1 (Raf-1:ER). The stimulation of p42/p44 MAP kinase cascade via the estradiol-dependent Raf activity eliminates the contribution of alternative signaling cascades such as stress-activated protein kinases (p38 HOG/JNK) or phosphatidyli-

nositol 3-kinase activated by serum or oncogenic Ras. Hence, production of VEGF mRNA is stimulated by estradiol in a time- and a dose-dependent manner. Furthermore, utilization of the MKK1 inhibitor PD 098059 (34) in Raf-1:ER cells after a maximal activation of p42/p44 MAP kinase confirmed the importance of the p42/p44 MAP kinase pathway in VEGF gene induction by growth factors. Also notable is that the two new VEGF-related mRNAs, VEGF-B and VEGF-C, show a different mode of regulation. Their mRNA levels do not vary or do not present the same spectacular induction in the transformed cells tested and are not regulated by estradiol stimulation in Raf-1:ER cells (compare Figs. 1 and 2*a*). A possible interpretation of this data is that the cellular system investigated is not appropriate for testing the fine regulation of both genes. Enholm *et al.* (49) have observed an induction of VEGF and VEGF-C in IMR-90 cells by serum, PDGF, EGF, and TGF-β but no regulation of VEGF-B after such stimulations, which is in favor of a specific cell context for such an induction.

We then analyzed the contribution of members of the p42/p44 MAP kinase module on VEGF transcription. The human, the mouse, and the rat VEGF promoter contains binding sites for AP-2, Sp1, or Sp1-related factors (35, 40–42, 50) in addition to binding sites for AP-1 (51) and HIF-1 (16, 35), which regulate the transcription of the gene during hypoxia (43). The mouse VEGF promoter also contains additional NFκB binding sites (42). A recent report by Gille *et al.* (37) described the AP-2 transcription factor as the major factor implicated in the TGF-α stimulation of VEGF gene transcription in A431 cells. They also showed that Sp1 is constitutively bound to the promoter (37). However, the promoter region responsible for such a regulation can bind AP-2 as well as Egr-1, but AP-2 only regulates the promoter activity. Furthermore, they showed that promoter activity still remains even after AP-2 binding site mutation but is reduced by 50%. In the present study, we have dissected the events responsible for the increase of VEGF transcription. The proximal promoter elements contained within construct −88/+54 were found to be sufficient to drive the stimulation of transcription induced by p42/p44 MAP kinase activation. Interestingly, the −88/+54 construct can still be activated by Ras-Val^{12} and MKK1-SS/DD. This is surprising since the region comprised between –1176 and −88 contains a consensus AP-1 binding site that is regulated via the p42/p44 MAP kinase module. However, mutations on the AP-2 or Sp1 binding sites, and in particular mutations in the three binding sites, strongly reduced basal and MKK1-SS/DD-stimulated VEGF promoter transcription. This is in accord with the results of Gille *et al.* (37) who showed that even if the AP-2 binding site is mutated, the promoter still displays high basal activity, possibly driven through Sp1 binding.

We have further analyzed the −88/–66 region by EMSA as well as supershift experiments. We observed four major DNA-proteins complexes either with extracts from stimulated or unstimulated Raf-1:ER cells. With the use of specific antibodies, we confirmed that AP-2 and Sp1 are components of the more intense complex B. This fundamental role of Sp1 is in agreement with the results of Yoshida *et al*. (52) who have shown that Sp1 is required for the stimulation of VEGF transcription by TNF-α and that antisense oligo-nucleotides can partially inhibit the TNF-α-dependent production of VEGF. We also show a spectacular increase in the labeling of part of complex B upon estradiol stimulation, which is resistant to competition with Sp1 oligonucleotides. Part of complex B is also enhanced upon estradiol stimulation when AP-2 competitor oligonucleotides are used. This result confirmed that activation of p42/p44 MAP kinase activation has a direct effect on AP-2 and Sp1. We are presently investigating whether this effect is mediated through an increase in binding affinity which could be regulated by phosphorylation or mediated by an increase in the amounts of both factors. However, we cannot exclude that p42/p44 MAP kinase may activate other transcription factors that could be components of the B complex. Egr-1 is a good candidate for such a regulation even if it has been shown that the transfection of an Egr-1 encoding construct has no effect on the VEGF promoter activity (37). We also observed two other complexes in EMSAs (a and c) that are inhibited by an excess of cold Sp1 or AP-2 oligonucleotides. While complex a is enhanced by estradiol treatment, complex c is not affected. Complex a could be the result of an association between Sp1 and AP-2. Complex c could be the result of Sp1 (41, 53) or AP-2-related factors binding (54, 55). This is in agreement with previous results which have shown that Egr-1 can bind to this region of the promoter (37).

Another interesting feature of VEGF regulation is its strong up-regulation upon oxygen deprivation by both transcriptional induction and stabilization of the mRNA by interaction of proteins with the 3′-untranslated region (15–17, 35, 43). Mukhopadhyay *et al*. have shown that hypoxic induction of VEGF is blocked by genistein and that c-Src is implicated in such an activation. They also showed that the dominant negative form of c-Src or Raf-1 can block hypoxic induction of VEGF (56). In our cell system, we show that such an induction is totally independent on growth factor action but that a combination of growth factor stimulation and oxygen deprivation have additive effects on VEGF mRNA induction. In Raf-1:ER cells, the inhibition of p42/p44 MAP kinase pathway by PD 098059 does not affect induction of VEGF by hypoxia (Fig. 2*c*), confirming that the induction of VEGF mRNA strictly attributable to p42/p44 MAP kinase activation and induction of VEGF by hypoxia are two independent mechanisms. There is no discrepancy between our results and those of Mukhopadhyay *et al*. (56) since Raf-1 can signal independently of Erk (22, 33), and dominant negative forms of Raf-1 can also titrate signals emerging from Ras which then activate independent pathways (32, 33). Our results have a strong physiological implication. The role of Ras oncogenes in the pathogenesis of human cancers is well established. Here we show that one of the target genes of the p42/p44 MAP kinase pathway which is activated by Ras is VEGF. As tumors are known to secrete growth factors activating the p42/p44 MAP kinase pathway such as FGF or relatives (3, 4, 57), VEGF expression initiated at least by constitutively active members of the p42/p44 MAP kinase pathway is amplified by a paracrine mechanism via the same transduction pathway. Such a regulation is strictly transcriptional and depends on activation of the VEGF promoter through at least two transcription factors, AP-2 and Sp1. We also show that growth factors and oxygen deprivation have additive effects which contribute to the increase of VEGF expression. We are now deciphering how the signal mediated by oxygen deprivation is sensed by the cells and which transduction pathways are implicated in VEGF induction.

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