Idenitication of Two Sp1 Phosphorylation Sites for p42/p44 Mitogen-activated Protein Kinases

THEIR IMPLICATION IN VASCULAR ENDO THELIAL GROWTH FACTOR GENE TRANSCRIPTION

Sp1 regulates activation of many genes implicated in tumor growth and cell cycle progression. We have previously demonstrated its implication in the up-regulation of vascular endothelial growth factor (VEGF) gene transcription following growth factor stimulation of quiescent cells, a situation where p42/p44 mitogen-activated protein kinase (MAPK) activity is dramatically increased. Here we show that p42/p44 MAPK directly phosphorylates Sp1 on threonines 453 and 739 both in vitro and in vivo. Mutation of these sites to alamines decreases by half the MAPK-dependent transcriptional activity of Sp1, in the context of the VEGF promoter, in SL2 Drosophila cells devoid of the endogenous Sp1 protein. Moreover, inducible overexpression of the (T453A,T739A) Sp1 double mutant compromises MAPK-driven VEGF mRNA transcription in fibroblasts. These results highlight Sp1 as a key molecular link between elevated activation of the Ras \( \Rightarrow \) p42/p44MAPK signaling pathway and increased VEGF expression, two major steps deregulated in tumor cells.

Normal and pathological angiogenesis depends on the secretion of growth factors needed for proliferation and survival of endothelial cells. Among these factors the vascular endothelial growth factor (VEGF), which is overexpressed by a wide variety of human tumors (1), has been shown to be crucial for tumor neovascularization. Regulation of VEGF expression is complex, because it is modulated by numerous stimuli at multiple levels, including gene transcription (2–9), mRNA stabilization (10–14), and mRNA translation (15, 16). Interestingly, the low tension of oxygen (hypoxia), which occurs in the core of solid tumors, induces VEGF expression by modulating all three levels of regulation cited above (2, 3, 11, 12, 15, 16). Oncogenes, including activated forms of Ras, Src, and Raf (17, 18), have also been implicated in increased VEGF expression. We have recently shown that the p42/p44 mitogen-activated protein kinase (MAPK) pathway plays a critical role in the transcription of VEGF gene following Ras transformation and growth factor stimulation. For this first study we used a cell line derived from CCL39 fibroblasts in which rapid and exclusive activation of MAPK can be achieved in response to estradiol (19–21). In these cells, activation of the MAPK pathway is sufficient to induce VEGF transcription (22). However, the phosphatidylinositol 3-kinase pathway via activation of protein kinase C \( \zeta \) is also highly important in human fibrosarcoma and renal cell carcinoma for driving VEGF transcription (23, 24). In both cases, the main transcription factor implicated in the regulation of VEGF transcription following Ras activation is Sp1 (8, 22, 25).

Sp1 is one of the first eukaryotic transcription factors to be identified and cloned (26). It plays an important role in the transcriptional regulation of genes, and its knock out in mice results in embryonic lethality (27). Originally described as a cellular transcription factor required for SV40 gene expression, Sp1 was shown to stimulate transcription through binding to GC-rich boxes present on a wide variety of promoters. Sp1 is a highly glycosylated and phosphorylated protein (28). Several kinases have been shown to phosphorylate Sp1, including DNA-dependent protein kinase (29), casein kinase II (30), protein kinase A (31), and protein kinase C \( \zeta \) (8), but the sites targeted by these kinases in vivo are still unknown. However, a recent report (32) described phosphorylation of human Sp1 by cyclin A-CDK 2 on serine 59. The level of Sp1 phosphorylation is also regulated during cell cycle progression (33, 34) and differentiation (35), two processes in which the MAPK cascade is switched on.

MAPK are activated by a wide variety of stimuli and particularly by growth and differentiation factors. These serine/threonine kinases are cytoplasmic in quiescent cells, but once activated they translocate to the nucleus (36). They phosphorylate multiple substrates, including membrane-associated (37), cytoplasmic (38–41), and nuclear proteins (42–45). An important role in the control of gene expression has been attributed to MAPK, because many of their nuclear targets are transcription factors, such as Elk-1 (46), which is directly activated, c-Fos, which is stabilized (47), and p53, which is degraded following phosphorylation by MAPK (48).

Constitutive activation of the MAPK pathway has been observed in many tumors (49–54). Concomitantly, VEGF is overexpressed in many tumors and contributes to their neovascularization (55). In a previous study we have identified a short region of the VEGF promoter, which is targeted by the p42/p44 MAPK. This region containing two Sp1 sites actually binds the transcription factor (22). Another study showed that recombinant active p42 MAPK enhances DNA binding capacity of Sp1...
in vitro (56). These observations prompted us to test the hypothesis that Sp1 is a link between the MAPK pathway and VEGF expression. First we observed that MAPK stimulation rapidly enhanced DNA binding of Sp1 and Sp3 to the VEGF promoter. By using in vitro kinase assays as well as antibodies directed against phosphopeptides we identified two major sites targeted by p42/p44 MAPK on Sp1. Both sites are indispensable for Sp1 activity following activation of the p42/p44 MAPK pathway. Thereafter, we demonstrated that phosphorylation of Sp1 by p42/p44 MAPK is a crucial event for the regulation of at least VEGF, one of the key genes implicated in neovascularization.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA modifying enzymes were obtained from New England BioLabs or from Eurogentec (Liège, Belgium). [α-32P]dCTP and [α-32P]dATP were from ICN. Synthetic oligonucleo-
tides were from Eurogentec. Recombinant human proteins Sp1 and AP-2 were purchased from Promega. Recombinant active p42 MAPK/ERK2 was purchased from New England BioLabs. Anti-Myc antibody (9E10) was from Roche Molecular Biochemicals. Anti-Sp1 (PEP-2) was from Santa Cruz Biotechnology.

Cell Culture—ΔRaf:ER cells are a derivative of CCL39 fibroblasts that stably expressed a fusion protein comprised of the catalytic domain of Raf-1 and the hormone binding domain of the CCL39 fibroblasts that stably expressed a fusion protein comprised of the catalytic domain of Raf-1 and the hormone binding domain of the estrogen receptor (19–21). These cells were cultivated in Dulbecco’s modified Eagle’s medium (Invitrogen) without phenol red containing 7.5% fetal calf serum, penicillin (50 units/ml), streptomycin sulfate (50 μg/ml), and L-glutamine supplemented with 10% heat-inactivated fetal calf serum. ΔRaf:ER cells (10 cells/10-cm diameter dish) were transfected by CaPO4 precipitation technique with 15 μg of the different pcDNA4/TO vectors. SL2 cells were transiently transfected as described above (see luciferase assays).

Plasmid Constructs—All the plasmids coding for the different subdomains of Sp1 fused to GST were a generous gift of Dr. J. Horowitz except the GST-D, which was obtained by PCR using the following oligonucleotides on the GST-Zn matrix: forward, 5′-CATGCTCGAGGATCCCGGAGC-3′; reverse, 5′-GGAATTCCTAGTTGGCCATGC-3′ (57). The full-length Sp1 cDNA excised from the pcDNA vector was introduced in the pCMVTag3B vector (Stratagene). The different point mutations of the MAPK consensus phosphorylation sites were obtained using a QuikChange site-directed mutagenesis kit supplied by Stratagene. The vector containing the VEGF promoter fused to the luciferase reporter gene has already been described (22). The MEK3(Shk)-MAPK construct corresponding to a fusion of the MEK3(Shk) cDNA with the ERK2 cDNA (kindly provided by Dr. Y. Miyata (58), was subcloned in the XhoI site of the pCMTag3B vector (Stratagene).

Preparation of RNA—Cells were washed in ice-cold phosphate-buffered saline (PBS) and lysed in the “RNA Insta-Pure” buffer from Eurogentec. The supernatant was cleared by centrifugation, ethanol-precipitated, and resuspended in sterile water. Ten micrograms of RNA was introduced in the pcDNA4/TO vector (Invitrogen). The supernatant was cleared by centrifugation, ethanol-precipitated, and resuspended in sterile water. Ten micrograms of RNA was introduced in the pcDNA4/TO vector (Invitrogen).

Luciferase Assays—SL2 Drosophila cells in 12-well dishes (105/well) were transiently transfected using the calcium phosphate technique.

Preparation of Nuclear Extracts and Gel Mobility Shift Assays—

Confluent ΔRaf:ER cell cultures were serum-deprived overnight prior to stimulation with 1 μM estradiol for 15 min. Nuclear extracts, electrophoretic mobility shift assays (EMSA), and supershift assays were performed, as previously described (22). The probe used in these experiments was synthesized to span the region of the human VEGF promoter designated –88 to –66 (56); TTTCCCAGGTCGGCCGGGCGGCGGGGGGTAT-3′ (random sequences added to the wild type sequence are shown in italic letters).

Antibodies—Anti-phospho-protein sera were generated by Neosystem (Strasbourg, France) by injecting two rabbits each with the following phosphopeptides. Phosphopeptide 1 (Phospho Thr1751); NH2-KRRSEG-STAPA(PO4)-H9004 (1/200), coupled to KLH. Phosphopeptide 2 (Phospho Thr583); NH2-KSGPIIR-(PO4)-H9262 (1/100). Anti-phospho-Thr453 was a generous gift of Dr. J. Horowitz except the GST-D, which was obtained by PCR using the following oligonucleotides on the GST-Zn matrix: forward, 5′-CATGCTCGAGGATCCCGGAGC-3′; reverse, 5′-GGAATTCCTAGTTGGCCATGC-3′ (57). The full-length Sp1 cDNA excised from the pcDNA vector was introduced in the pCMVTag3B vector (Stratagene).

Preparation of Nuclear Extracts and Gel Mobility Shift Assays—

RESULTS

Increase Sp1 Binding to the VEGF Promoter following a Short p42/p44 MAPK Stimulation—We have previously shown that long term activation of the p42/p44 MAPK pathway results in increased VEGF transcription. This effect was found to be directly dependent on the recruitment of Sp1 and AP-2 transcription factors to a GC-rich region located on the proximal region of the VEGF promoter (–88/-66) (22). To determine whether this effect of p42/p44 MAPK on the DNA binding activity of these transcription factors is direct, we performed EMSA experiments with nuclear extracts of cells expressing an estrogen-inducible Raf:ER (59) or Raf:ER mutant (59). In these cells, p42/p44 MAPK activity is rapidly and specifically activated by estradiol (21). ΔRaf:ER cells were serum-deprived for 48 h and then stimulated by the addition of estradiol. When incubated with nuclear extracts of untreated ΔRaf:ER-1 cells, a basal DNA binding activity on a double-stranded probe encompassing the –88/-66 bp region of the human VEGF promoter
was detected (Fig. 1A, lane 1). In nuclear extracts of cells stimulated with estradiol for 15 min (Fig. 1A, lane 3) or 3 h (Fig. 1A, lane 5), an increase DNA binding activity was observed. Both basal and the stimulated complexes, in extracts of estradiol-stimulated cells for 15 min (Fig. 1A, lane 4) or 3 h (Fig. 1A, lane 6), were almost entirely disrupted in the presence of an excess of unlabeled double-stranded Sp1 consensus oligonucleotide, demonstrating that these complexes contain Sp1 or Sp1-related proteins. However, when extracts of cells stimulated with estradiol for 3 h (Fig. 1A, lane 6) were used, one part of complex B resists competition with unlabeled Sp1 consensus oligonucleotide. One part of this complex can be attributed to the presence of the AP-2 transcription factor, which is recruited to the VEGF promoter after long term p42/p44 MAPK stimulation as previously described (22). To demonstrate the presence of Sp1 in the different complexes observed with extracts of estradiol-stimulated H9004Raf-1:ER cells for 15 min, we performed supershift experiments with anti-Sp1 antibody. Our results clearly demonstrate that Sp1 (present at least in complexes B1 and B2) is recruited to the VEGF promoter following a short term stimulation of p42/p44 MAPK by estradiol (Fig. 1B). Supershift experiments with anti-Sp3 antibody also show that

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FIG. 3. In vivo phosphorylation of Sp1 by MAPK occurs on threonine 453 and threonine 739. A, specificity of the immune serum affinity-purified using peptides containing phospho-threonine 453 or phospho-threonine 739. GST/BQ or GST/Zn-D were phosphorylated or not by recombinant active p42 MAPK. Equal amounts of proteins were submitted to immunoblot analysis with anti-GST purified antibody as a loading control, and with anti-phospho-Thr453 or anti-phospho-Thr739-purified sera (H9251-PT453 and H9251-PT739). B, phosphorylated Thr453 Sp1 was examined by immunofluorescence analysis on the WT2 cells. This CCL39-derived clone expresses a p42 MAPK: ER chimera (21), the tetracycline repressor, and tagged-Sp1 (WT) under control of the tetracycline operator. WT2 cells were serum-deprived 48 h. At the same time, Sp1 overexpression was induced by tetracycline (1 μg/ml). Cells were then stimulated or not with estradiol for 15 min. Immunostaining with purified anti-phospho-Thr453 shows...
Sp3 is recruited to the VEGF promoter following a short term stimulation with estradiol and is contained within complex B3 and c (data not shown). Band shift as well as supershift experiments were also performed with nuclear extracts of estradiol-stimulated cells in the presence of cycloheximide, an inhibitor of protein synthesis. The same results were also obtained in such conditions (data not shown). Because this effect occurred rapidly and in conditions where protein neo-synthesis is blocked, we postulated that it could be accounted for by direct phosphorylation of the transcription factors.

MAPK Phosphorylates Sp1 on Two Sites In Vitro and In Vivo—We have then investigated whether Sp1 could be directly phosphorylated by p42/p44 MAPK. Thus, we performed in vitro kinase assays using equivalent molar amounts of affinity-purified Sp1, AP-2, bovine serum albumin (BSA), GST-ATF2, GST-Jun, GST-Elk1, and myelin basic protein. GST-ATF2, a specific substrate for the SAPK/Jun NH2-terminal kinase and p38/HOG MAPK (60, 61), GST-Jun, a specific substrate for the SAPK/Jun NH2-terminal kinase (62) and BSA can be considered as negative controls. GST-Elk1 (63, 64) and myelin basic protein (65), commonly used substrates for p42/p44 MAPK, are considered as positive controls. We observed that recombiant active p42 MAPK strongly phosphorylates Sp1 as well as GST-Elk1 and myelin basic protein, whereas AP-2, GST-ATF2, GST-Jun remain poor substrates for MAPK under the same conditions. In these conditions, BSA is not phosphorylated at all (Fig. 2A). The regions of Sp1 targeted by MAPK were determined by performing in vitro kinase assays on the functional domains of Sp1, schematized in Fig. 2B, fused to glutathione S-transferase (GST) (57). Two of these domains, but not the others (data not shown), are phosphorylated by p42 MAPK in vitro. These include the glutamine-rich transactivating domain, B5, and the COOH-terminal domain, D, which is implicated in protein-protein interactions and required for synergistic transactivation via several Sp1 sites (66, 67). Phospho-amino acid analysis of these domains revealed that the phosphorylation occurs only on threonine residues (data not shown). To identify the putative MAPK phosphorylation sites within these domains, we mutated the three threonines contained in MAPK consensus phosphorylation sites to alanine; two in the B5 domain (GQT565P and IR745P) and one in the D domain (TAT60P).

As shown in Fig. 2C, point mutation of the potential B5 domain sites revealed that threonine 453 is phosphorylated by MAPK in vitro, whereas threonine 355 is not. Similarly, active p42 MAPK efficiently phosphorylates wild type GST/D protein but not the T739A mutant form, confirming that threonine 739 is also targeted by MAPK in vitro. Therefore, this first in vitro approach allowed us to identify two sites for direct MAPK action: 451IRTP454 and 737TATP740. We next developed anti-phospho-specific antibodies directed against the sequences targeted by p42/p44 MAPK to further analyze the in vivo relevance of these phosphorylations. The specificity of the immunopurified antibodies was analyzed by Western blotting to GST/B5 and GST/Zn-D proteins phosphorylated, or not, by p42 MAPK in vitro. Fig. 3A shows that each antibody recognizes only the phosphorylated form of the appropriate specific amino acid sequence. Using the affinity-purified anti-phospho-Thr453 antibodies, we were unable to detect in vivo Sp1 phosphorylation by immunoblotting, however, it could be visualized by immunofluorescence staining. Indeed, in control ΔRaf-1-ER quiescent cells, no staining was detectable (Fig. 3B, Control, −). But when p42/p44 MAPK was activated for 15 min with estradiol, we observed an increased nuclear immunoreactivity (Fig. 3B, Control, +). To verify that this reactivity toward anti-phospho-Thr453 was specific for Sp1, we attempted to amplify the signal by developing a cell line in which overexpression of epitope-tagged Sp1 (Myc-tagged at the NH2 terminus, and HA-tagged at COOH terminus) could be induced with tetracycline. Under induced conditions (right panels of Fig. 3B), MAPK activation with estradiol potently enhanced nuclear immunolabeling with anti-phospho-Thr453 antibodies. This result demonstrates that the protein detected by this antibody in vivo is indeed Sp1. The level of tagged Sp1 after tetracycline induction was monitored both by anti-myc immunofluorescence staining, and by Western blotting (see Fig. 3, B and C). Activation of endogenous MAPK was monitored with anti-phospho-MAPK antibodies (Fig. 3C). The same results were obtained in two independent clones, and in CCL39 cells stimulated by fetal bovine serum (data not shown). Concerning the D domain, phosphorylation of Thr739 in vivo could be readily detected by Western blotting of extracts of ΔRaf-ER cells stimulated with estradiol or serum. Anti-phospho-Thr739 antibodies detected a protein of 95 kDa corresponding to Sp1 and only under stimulated conditions. Both the intensity- and time-dependent phosphorylation of the 95-kDa protein correlated with the increase in MAPK activity (see Fig. 3D). Furthermore, the myc-tagged Sp1-inducible transgene product is also labeled by the anti-phospho-Thr739 in tetracycline-treated cells, as shown in Fig. 3E. These data clearly demonstrate that Sp1 is rapidly phosphorylated on Thr739 in vivo following p42/p44 MAPK activation.

Phosphorylated Forms of Sp1 Bind to the VEGF Promoter—To demonstrate that Thr453, and Thr739-phosphorylated Sp1 was present in the DNA complexes shown in Fig. 1, we performed supershift experiments with the Thr453 and Thr739 anti-phospho-Sp1 antibodies. Antibodies directed against total Sp1 were used as a positive control (Fig. 4). Indeed, anti-total Sp1 antibody supershifted part of complex B as shown on Fig. 1B (Fig. 4, lane 2). In the presence of Thr739 anti-phospho-Sp1 antibody, an evident supershifted complex was observed (Fig. 4, lane 3) whereas only a weak supershift was obtained in the presence of Thr453 anti-phospho-Sp1 (Fig. 4, lane 4). Simultaneous addition of both antibodies results in a reduction in the intensity of complexes a and B (data not shown). These results clearly demonstrated that Sp1, phosphorylated on both threonines 453 and 739, is recruited to the VEGF promoter following activation of the p42/p44 MAPK pathway. However, the fact that anti-phospho-antibodies are not able to induce supershifts equivalent to those obtained with anti-total Sp1 a nuclear staining after Raf-ER stimulation with estradiol (Texas red staining). The overexpression of tagged-Sp1 is detected by anti-myc staining, which gives an exclusive nuclear staining, only in tetracycline-treated cells (fluorescein isothiocyanate staining). Nuclei are visualized by a 4',6-diamidine dihydrochloride staining (top row of images). C, phosphorylation of p42/p44 MAPK in response to ΔRaf-ER activation is detected in the WT2 clone, treated or not with tetracycline, by anti-phospho-MAPK immunoblotting Sigma) (lower panel). The level of tagged-Sp1 expression is controlled by anti-HA immunoblotting (upper panel). D, phosphorylated Thr739 Sp1 was examined by immunoblotting with purified anti-phospho-Thr739 on quiescent estradiol (1 μM)- or fetal bovine serum (FBS, 20%)-stimulated ΔRaf-ER cells (upper panel). To verify the co-localization of the bands, anti-total Sp1 hybridization was performed with the PEP-2 antibody (Santa Cruz Biotechnology) on the same membrane. The antibodies directed against phosphorylated p42/p44 MAPK and total MAPK were used, respectively, to control for MAPK activation and as a loading control (lower panels). E, WT2 cells were serum-deprived 48 h. At the same time, Sp1 overexpression was induced by tetracycline (1 μg/ml). Cells were then stimulated by estradiol (1 μM) for the times indicated. As in D, phosphorylated Thr739 Sp1 was examined by immunoblotting (upper panel). The level of endogenous and epitope-tagged Sp1 expression is controlled by anti-Sp1 immunoblotting (PEP-2, Santa Cruz Biotechnology). The level of MAPK activation is also shown as a control.
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Phosphorylation of Sp1 by p42/p44 MAPK Increases Transcriptional Efficiency of the Minimal VEGF Promoter—We next examined the functional role of these MAPK-dependent phosphorylation sites by comparing wild type and mutated (T453A or/and T739A) Sp1 forms on transcriptional activation of the VEGF promoter. For that purpose, we analyzed the activity of the minimal VEGF promoter containing the Sp1 target sites (22) in SL2 Drosophila cells, which lack endogenous Sp1. Using an Sp1-negative cell line ensured that all the activity was attributable to the transfected constructs. Expression of wild type Sp1 in normally growing cells stimulated VEGF promoter activity by 6- to 8-fold. When a constitutive active form of p42 MAPK was co-expressed (MEKSDSE-p42MAPK fusion protein) (58), a condition where total MAPK activity is highly enhanced, VEGF promoter activity was further increased by ~4-fold. Interestingly, each single-mutated or double-mutated Sp1 form transactivated transcription from the VEGF promoter as well as wild type Sp1. However, in the presence of the MEKSDSE-p42MAPK fusion protein, the mutated Sp1 forms are less efficient in promoting transcriptional activation than the wild type protein and activation was reduced by more than 50% in the presence of (T453A,T739A) Sp1 (Fig. 5). This type of inhibition is in the same order of magnitude of that induced by mutation of serine 59, a site targeted by cyclin A/CDK 2 (32). These results indicate that both MAPK phosphorylation sites contribute to intrinsic Sp1 activity. However, direct phosphorylation of Sp1 by p42/p44 MAPK represents only one half of the p42/p44 MAPK-mediated VEGF transcription. The fact that p42/p44 MAPK could allow the recruitment of Sp1 partners to the VEGF promoter probably explains why we do not observe a total inhibition in the presence of the mutated forms of Sp1.

Overexpression of Sp1 Double Mutant Inhibits Estradiol-induced VEGF Transcription in ΔRaf:ER Cells—In a second biological assay, we directly analyzed the expression of specific MAPK-dependent VEGF gene expression under conditions in which the wild type or the double Sp1 mutant was induced with tetracycline. Fig. 6A shows the time course of p42/p44 MAPK-stimulated VEGF expression in cells in which wild type or (T453A,T739A) Sp1 was induced or not with tetracycline. Ecotropic expression of wild type Sp1 (Fig. 6A, +Tet, left) increased basal and estradiol-induced VEGF expression particularly after stimulation for 1 or 2 h. In contrast, expression of the double-mutated form of Sp1 (T453A,T739A) (Fig. 6A, +Tet, right) reduced by half the basal and p42/p44 MAPK-dependent VEGF expression (Fig. 6B). Therefore, the mutated Sp1 form behaves as a dominant negative construct on VEGF transcription in vivo. The less potent effect of estradiol in inducing VEGF expression in the double Sp1 mutant compared with wild type Sp1-expressing cells, in the absence of tetracycline, can be interpreted as the following: 1) this clone is intrinsically less sensitive to estradiol, 2) the tetracycline regulated promoter is leaky and allows for basal expression of the double Sp1 mutant, which exerts a partial dominant negative effect even in the absence of tetracycline. Another surprising result is the effect of overexpression of wild type Sp1 (positive effect) or mutant Sp1 (negative effect) on basal VEGF mRNA levels in growth factor-deprived cells. Such basal VEGF mRNA levels are attributed to residual promoter activity induced by the remaining p42/p44 MAPK activity that persists in all cell lines tested even after a long term serum deprivation. We suppose that basal expression of VEGF is enhanced because of the presence of increased “basal” p42/p44 MAPK-activated Sp1 when the wild type form of Sp1 is induced by tetracycline. Overexpression of the mutated form of Sp1 competed with endogenous basal p42/p44 MAPK-activated Sp1 for binding to the VEGF promoter resulting in a reduction of the basal VEGF mRNA level. Fig. 6C shows by Western blot analysis the level of induction of ectopic Sp1 after tetracycline induction and estradiol stimulation for 1 h to verify that no major differences of expression between wild type or (T453A,T739A) Sp1 exist (top panel). Overexpression of this blot shows basal expression of...
the Sp1 transgenes even in the absence of tetracycline (data not shown). This observation favors the second interpretation given above, which explains the differential effect observed with estradiol between wild type and (T453A,T739A) Sp1-expressing cells in the absence of tetracycline. Under these conditions, ectopic expression of wild type or (T453A,T739A) Sp1 does not modify p42/p44 MAPK activation by estradiol stimulation (Western blot anti-phosphorylated forms of MAPK, middle panel). All these results highlight the regulatory role of p42/p44 MAPK phosphorylation on Sp1 activity in the context of the VEGF promoter.

**DISCUSSION**

The work reported here was first designed to identify a molecular link between the Ras signaling pathway and the transcription of the VEGF gene. Our previous results identified the proximal GC-rich box (−58/−66) of the VEGF promoter, where Sp1 and AP-2 bind, as the main target for growth factors stimulation and oncogenic activation, an action mediated via the p42/p44 MAPK signaling cascade (22). The present study reinforces this conclusion and establishes Sp1 activation by MAPK as one of the molecular links bridging growth factor action/oncogenic transformation and VEGF expression. Phosphorylation of Sp1 by p42/p44 MAPK on threonines 453 and 739 is a key element in this regulatory action. Although Sp1 is a substrate of several protein kinases in vitro, this is the first time that two MAPK phosphorylation sites have been identified in vivo. Mutation of both threonines to alanines decreases but does not abolish Sp1 activity, which is still able to transactivate an artificial VEGF/Luciferase promoter in SL2 Drosophila cells. Furthermore, our supershift experiments suggest that the proportion of phosphorylated Sp1 on both sites is relatively low after activation of the p42/p44 MAPK pathway for 15 min.

However, our results strongly suggest that these phosphorylations enhance Sp1 binding and Sp1 transcriptional activity. Our experiments also corroborate the results of Merchant et al. (56) who have described that phosphorylation of Sp1 by ERK2 in vitro enhances binding of Sp1 to a target sequence. However, we do not know if the p42/p44 MAPK-dependent phosphorylation of Sp1 directly affects transcriptional activity or if phosphorylation of threonines 453 and 739 allows the recruitment of one or more Sp1 partners required for efficient transcription. We cannot also rule out the possibility that p42/p44MAPK phosphorylates one or more other factors that interact with Sp1 to activate transcription. It is probably the reason why the increment in transcriptional activation induced by the MEK/MAPK chimera is not entirely blocked when the mutated forms of Sp1 are used in Drosophila cells. Another result that corroborates this hypothesis is that Gal4/Sp1A fusion protein, which contains neither threonines 453 or 739, could activate a reporter gene following NGF stimulation, a strong activator of
Sp1 Phosphorylation by p42/p44 MAPK

p42/p44 MAPK, in PC12 cells (68). However, overexpression of the double-mutant forms of Sp1 reduced by half the estradiol-mediated induction of endogenous VEGF mRNA in ΔRaf-1/ER cells probably by preventing efficient phosphorylation of endogenous Sp1 by p42/p44 MAPK. This result confirms that phosphorylation of Sp1 by p42/p44 MAPK is necessary for full Sp1 activity.

The phosphorylation site Thr453 is interesting, because the domain B of Sp1 has been shown to interact directly with both the TATA-box protein accessory factor TAF\_II110 (69), and transcription factors (70). Phosphorylation of this site, conserved in all mammalian Sp1 sequences, could be essential for coupling TATA-less promoters to the polymerase machinery via Sp1 and TATA box-binding protein-associated factor interaction. The second site, Thr739, also conserved in all cloned mammalian Sp1 proteins, belongs to a region essential for synergistic activation of transcription by Sp1 (67) and is known to interact with different partners (71, 72). Furthermore, this site is proximal to putative docking sites for MAPK recognition of substrate proteins. The first sequence, 679-faced\_A, is closely related to the FFXX motif present in specific MAPK substrates whereas the second sequence, 614-KKQK/LICHHI-625, closely related to the (K/R)(K/R)(K/R)X\_1(X/L/X/X/L) mediates binding to MAPK and JNK. One potential serine, four threonines, and docking sites for MAPK recognition are also present on Sp3 (Ser\_5, Thr\_47, Thr\_151, Thr\_288, and Thr\_422, for putative phosphorylation sites and F\_613ECP\_616 and K\_549KKQHICHI\_557 for the docking sites). Indeed, Sp3 could also represent a p42/p44 MAPK target, and the presence of both sequences could explain the recruitment of Sp3 to the VEGF promoter following short p42/p44 MAPK stimulation. Our results prompted us to investigate if p42/p44 MAPK and Sp1 could co-immunoprecipitate. As expected, we have reproducibly obtained acceptable co-immunoprecipitation of p42/p44 MAPK with endogenous Sp1.

One key point of this study is the demonstration that the Raf \(\rightarrow\) MAPK-driven VEGF transcription requires the direct phosphorylation of Sp1 by p42/p44 MAPK. This finding raises two important questions: 1) Is Sp1 action mandatory for VEGF expression in response to various external stimuli such as growth factors and hypoxia? 2) Considering the wide list of Sp1-dependent promoters, how general is the p42/p44 MAPK control of transcription via Sp1 phosphorylation?

An answer to the first question was rapidly obtained by stimulating ΔRaf/ER cells by fetal bovine serum instead of estradiol. Under this condition, ectopic expression of the double Sp1 mutant (T453A,T739A) only moderately suppressed VEGF transcription. This result indicates, as anticipated, that phosphorylation of Sp1 on threonines 453 and 739 is not as important when a multiplicity of signaling pathways are elicited instead of a single one. It is important to recall that, in different cellular contexts, the VEGF transcriptional control is more sensitive to phosphatidylinositol 3-kinase than p42/p44 MAPK signaling (24). Further work will address the specific contribution of phosphorylated Sp1 in response to platelet-derived growth factor, fibroblast growth factor, and α-thrombin alone or in combination with hypoxia.

The second question was addressed by monitoring in parallel to VEGF, the expression of the cyclin-dependent kinase inhibitor, p21\_Waf1/Cip1. The promoter of this gene is notorious for its regulation by Sp1 (73, 74). Surprisingly, p21\_Waf1/Cip1 expression upon p42/p44 MAPK activation (estradiol stimulation) is magnified by the ectopic expression of (T453A,T739A) Sp1 (data not shown). One of the major differences between both promoters is the presence of a TATA box on the p21\_Waf1/Cip1 promoter (75). Overexpression of an unphosphorylatable form of Sp1 could favor the interaction of TATA box-binding protein with the TATA box and thus increase transcription. Indeed, phosphorylation of NF\_κB by p38 MAPK is essential to mediate a positive regulation by transcription factor IID in the context of cytokine promoters (76). As it was shown that phosphorylation of transcription factor IID could inhibit transcription (77), we could envisage that an unphosphorylatable form of Sp1 would bypass this phenomena and thus enhance transcription.

Two alternative mechanisms could be proposed to explain the positive effects of phosphorylation of Sp1 in the context of VEGF promoter. Phosphorylation could mediate the release of inhibitory proteins. Several candidate proteins exist, including p53 and its homologue p73, which have both been shown to be implicated in the down-regulation of VEGF transcription (78, 79). Another putative candidate is Sp1 Inhibitor (Sp1-I) a small protein that prevents binding of Sp1 to DNA (80). The von Hippel-Lindau tumor suppressor (pVHL) could also be considered because of its implication in tumoral angiogenesis and because it was shown to inhibit Sp1 activity through a direct interaction (9). Alternatively, phosphorylation of Sp1 could mediate the recruitment of co-activators. Two likely partners are AP-2 and Egr-1, which have been shown to be associated to Sp1 on the VEGF promoter (25). The transcriptional co-activator p300 could also be associated to phosphorylated Sp1. Indeed, a complex between Sp1 and p300 has been identified on the p21\_Waf1/Cip1 promoter following nerve growth factor stimulation of PC12 cells, a situation where p42/p44 MAPK is strongly activated (68).

Another interesting feature of Sp1 phosphorylation is its effect on stability. It has been previously shown that long term stimulation of GH4 cells with EGF, a strong stimulator of p42/p44 MAPK, induces Sp1 degradation (81). Thus phosphorylation of Sp1 by p42/p44 MAPK could be important for stimulating its transcription potential following a short term stimulation and for mediating its degradation at later times to avoid a general dysfunction of the system. Phosphorylation-dependent regulation of protein stability has already been described for the transcription factor c-Jun or the MAPK phosphatase 1 or the tumor suppressor p53, c-Jun and MAPK phosphatase 1 are less sensitive to degradation by the proteasome machinery following JNK (82) or p42/p44 MAPK (83) phosphorylation, respectively, whereas p53 becomes sensitive to degradation after phosphorylation by p42/p44 MAPK (48). We are currently investigating how phosphorylation of Thr\_453 or/and Thr\_739 modifies the stability of Sp1 and next how phosphorylation mediates the recruitment of specific Sp1 partners depending on the promoter context and specific physiological situations.

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