Identification of MAPK Phosphorylation Sites and Their Role in the Localization and Activity of Hypoxia-inducible Factor-1α*

Hypoxia-inducible factor 1 (HIF-1) controls the expression of most genes induced by hypoxic conditions. Regulation of expression and activity of its inducible subunit, HIF-1α, involves several post-translational modifications. To study HIF-1α phosphorylation, we have used human full-length recombinant HIF-1α as a substrate in kinase assays. We show that at least two different nuclear protein kinases, one of them identified as p42/p44 MAPK, can modify HIF-1α. Analysis of in vitro phosphorylated HIF-1α by mass spectroscopy revealed residues Ser-641 and Ser-643 as possible MAPK phosphorylation sites. Site-directed mutagenesis of these residues reduced significantly the phosphorylation of HIF-1α. When these mutant forms of HIF-1α were expressed in HeLa cells, they exhibited much lower transcriptional activity than the wild-type form. However, expression of the same mutants in yeast revealed that their capacity to stimulate transcription was not significantly compromised. Localization of the green fluorescent protein-tagged HIF-1α mutants in HeLa cells showed their exclusion from the nucleus in contrast to wild-type HIF-1α. Treatment of the cells with leptomycin B, an inhibitor of the importin α (CRM1), reversed this exclusion and led to nuclear accumulation and partial recovery of the activity of the HIF-1α mutants. Moreover, inhibition of the MAPK pathway by PD98059 impaired the phosphorylation, nuclear accumulation, and activity of wild-type GFP-HIF-1α. Overall, these data suggest that phosphorylation of Ser-641/643 by MAPK promotes the nuclear accumulation and transcriptional activity of HIF-1α by blocking its CRM1-dependent nuclear export.

The transcriptional activator hypoxia-inducible factor-1 (HIF-1) is the key mediator of the cellular response to hypoxia. HIF-1 controls the expression of many genes that are involved in angiogenesis, erythropoiesis, glucose uptake, cell metabolism, apoptosis, invasion, or metastasis and are, therefore, critical for adaptation to low oxygen and also tumor progression. HIF-1 consists of two subunits: HIF-1α and HIF-1β (or ARNT). Both contain basic helix loop helix and PER-ARNT-SIM (PAS) domains in their NH₂-terminal parts that mediate heterodimerization and binding to DNA regulatory sequences. These sequences, called hypoxia response elements (HRE), are present in the promoter or enhancer regions of HIF-1 target genes. HIF-1α contains, in addition, an oxygen-dependent degradation (ODD) domain and two transactivation domains, N-TAD and C-TAD. These two latter domains are separated by the inhibitory domain, a protein part that when removed increases the transcriptional activity of both TADs. Whereas ARNT is constitutively expressed, also serving as the partner of Aryl hydrocarbon receptor, HIF-1α is unique to HIF-1 and is regulated at multiple levels by hypoxia or other cell signaling pathways (1).

Under normoxic conditions, the protein levels of HIF-1α are kept very low by von Hippel-Lindau (VHL)-mediated polyubiquitination and subsequent targeting to the proteasome (2, 3). Interaction of HIF-1α with VHL, a tumor suppressor protein and part of an E3 ubiquitin ligase complex, is regulated by hydroxylation of two proline residues in the ODD domain of HIF-1α (4–6). The proline hydroxylases that modify HIF-1α (members of the EGLN family, termed PHDs or HPHs) are Fe²⁺ dependent and require molecular oxygen as a substrate (7). Thus normally HIF-1α is hydroxylated, bound by VHL, and rapidly degraded. When oxygen becomes scarce, inhibition of hydroxylation leads to HIF-1α protein stabilization and its rapid accumulation (8, 9). Stabilized HIF-1α can then enter the cell nucleus, heterodimerize with ARNT, bind to DNA, and induce expression of its target genes by recruiting transcriptional coactivators such as CBP/p300. Some of these steps may also be regulated by oxygen tension. It has been suggested that nuclear import of HIF-1α is enhanced under hypoxic conditions (10). Furthermore, the interaction of HIF-1α with CBP/p300 is also controlled by hydroxylation. An asparaginyl-hydroxylase, called factor inhibiting HIF-1, modifies the C-TAD domain of HIF-1α and HIF-1β (or ARNT).

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† To whom correspondence should be addressed: 22 Papakyriazi Str., Larissa 41222, Greece. Fax: 30-2410-565054; E-mail: simos@med.uth.gr.

‡ Biochemistry and Physiology, Department of Medicine, University of Thessaly, Larissa 41222, Greece and the Protein Chemistry Laboratory, Biomedical Sciences Research Center “Alexander Fleming,” Vari 16672, Greece.

§ The abbreviations used are: HIF-1, hypoxia-inducible factor-1; ARNT, aryl hydrocarbon receptor nuclear translocator; PAS, PER-ARNT-SIM domain; ODD domain, oxygen-dependent degradation domain; TAD, transactivation domain; HRE, hypoxia response element; VHL, von Hippel-Lindau protein; MAPK, mitogen-activated protein kinase; LMB, leptomycin B; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; ERK, extracellular signal-regulated kinase; DBD, DNA binding domain; PBS, phosphate-buffered saline; GFP, green fluorescent protein; GST, glutathione S-transferase; MS, mass spectrometry; NLS, nuclear localization signal.
In addition to oxygen-dependent control, expression and activity of HIF-1α under normoxic conditions can be regulated by major signal transduction pathways including those involving phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase (MAPK/ERK), both of which can be induced by growth factors, cytokines, oncogenes, or hypoxia-mimetic chemicals (1, 12, 13). However, the exact mechanisms underlying HIF-1α stabilization and subsequent activation by these pathways are not completely understood. A number of reports concerning HIF-1α activation by the phosphatidylinositol 3-kinase/AKT pathway imply that AKT activation causes increased translation of HIF-1α mRNA leading to an enhanced rate of HIF-1α protein synthesis. On the other hand, the MAPK pathway is implicated both in regulation of HIF-1α protein synthesis (14) as well as potentiation of its transcriptional activity (15–17). Furthermore, HIF-1α can be directly phosphorylated by p44/p42 MAPK (ERK1/2) both in vitro and in vivo (18–20). However, the exact position of the phosphorylation sites as well as their effect on HIF-1α activity have remained so far unknown (21). Phosphorylation of HIF-1α by other, as yet unidentified, kinases has also been suggested (22).

To clarify the issue of HIF-1α phosphorylation we have tried in this work to identify the responsible kinases and map their in vitro phosphorylation sites. Using purified bacterially expressed recombinant HIF-1α as a phosphorylation substrate, we provide evidence that at least two distinct nuclear protein kinases from HeLa cells target the amino- and carboxyl-terminal parts of HIF-1α, respectively. Furthermore, we show that one of these kinases, p42 MAPK, modifies two distinct serine residues at positions 641 and 643, inside the inhibitory domain of HIF-1α. Introduction of mutations in these two sites does not influence the transcriptional activity of HIF-1α in a yeast reconstitution system but strongly inhibits both its activity and nuclear accumulation in HeLa cells. However, this inhibition can be reversed when nuclear export is inhibited by leptomycin B (LMB). Based on these findings, we suggest that phosphorylation of Ser-641/643 by MAPK enhances the nuclear accumulation and activity of HIF-1α by blocking its CRM1-mediated nuclear export.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—Construction of NH₂- and COOH-terminal deletions of HIF-1α cloned into pBS-SK(+) was described previously (23). The corresponding cDNA inserts were subcloned as BamHI fragments into pGEX-4T-1 bacterial expression vector (Amersham Biosciences) yielding pGEX-HIF-1α-1–251, -1–530, -1–652, -348–826, and -652–826. Single point HIF-1α mutants HS641A, HS643A, and the double mutant HS634A were constructed using as template pGEX-HIF-1α (24), containing the full-length cDNA of HIF-1α, with the QuikChange® II site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the primers HS641A sense, 5’-GTGTTATTAGAGATGGAGCTGCAATCAATATTTTAATGC-3’; HS641A antisense, 5’-GACATATTAAATATTGAGTTTCGGCCTCACTTCCCTACCACCCAC-3’; HS643A sense, 5’-CTTTATATGTGGTTAGGAGCTGAGCTGCAATAT-3’; HS634A sense, 5’-GATTTCAGTCCAGCTCACTTACCACATTAAAG-3’; and HS634A antisense, 5’-CTTTATGTTGGGTTAGGAGCTGGAGCTGCAATAT-3’.

The DNA sequence of the point mutants was confirmed by sequencing performed by Lark Technologies Inc. (Hope End, Essex, UK). All forms of HIF-1α were then subcloned as BamHI fragments into the mammalian expression vector pEGFP-C1 (Clontech) and in the yeast expression vector pBEVY-GU (25). To construct GAL4-DBD fusions, the cDNA fragment corresponding to HIF-1α (348–826) (HAN) was obtained by PCR using as template pGEX-HIF-1α (wild-type and point mutant forms) and the primers H348F (5’-TTTTGATGATCGCTGATTCTTCTCCTCC-3’) and HIF-C-B (5’-TTTTGTGATCTTCAGTTAACTTGATACCAAGG-3’). The derived fragments were subcloned as BamHI inserts into the pBXGI mammalian expression vector, a gift by Dr. A. Kretsovali (IMBB, FORTH, Heraklion, Greece), in-frame with the DNA-binding domain (DBD) of the yeast transactivator GAL4 (amino acids 1–147) yielding GAL4-HΔN, -HΔN-S641A, -HΔN-S643A, and -HΔN-SDMA.

**Cell Lines, Transfection, and Luciferase Assays**—Human HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transient transfections were carried out in 10-cm dishes or 12-well plates by using the TransPass™ D2 Transfection Reagent (New England Biolabs Inc., Beverly, MA). In each experiment the total amount of DNA was adjusted to 1–2 μg/well or to 10 μg/dish. When required, 19 h after transfection cells were treated for 5 h with 50 μM PD98059, 10 μM MG132, or 20 ng/ml LMB, purchased from Sigma. Fractionation of HeLa cells was performed as previously described (26). Luciferase assays were performed in cells transiently co-transfected with plasmids expressing different forms of HIF-1α (or the empty parental vectors pEGFP-C1 and pBXGI as controls), the firefly luciferase reporter plasmids pGL3–5HRE–VEGF, kindly provided by Dr. A. J. Giacia (Stanford University) or gal4-tk-luc, a gift by Dr. G. U. Ryffel (Institut fur Zellbiologie Tumorforschung, Germany), and as controls the β-galactosidase expressing plasmid CMV–lacZ, a gift by Dr. A. Kretsovali (IMBB, FORTH, Heraklion, Greece) or the Renilla luciferase expressing plasmid pCInpRenilla, generously provided by Dr. M. U. Muckenthaler (University of Heidelberg, Germany). 24 h post-transfection, cells were washed with PBS, lysed, and luciferase activity was determined using the luciferase assay kit or Dual Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activity values from cells expressing GFP-HIF-1α were normalized to their β-galactosidase or Renilla luciferase activities and are expressed as fold-increase in relation to cells expressing GFP or GAL4 alone and as relative luciferase units.

**Protein Purification and Phosphorylation Assays**—GST–HIF-1α, GST–HS641A, GST–HS643A, GST–HS6MDA, and the GST–HIF-1α deletion mutants were expressed in Escherichia coli and purified as previously reported for GST–HIF-1α (24). Phosphorylation reactions were carried out at 30 °C for 30 or 60 min, in a total volume of 25 μl with phosphorylation buffer (25 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM NaCl, 50 μM ATP), 0.5 μCi of [γ-32P]ATP (6000 Ci/mmol) when required, 1–3 μg of the appropriate substrate, and an aliquot of cell extract or

CACATACATAAAAG-3’; and HS6MDA antisense, 5’-CTTTATGTTGGGTTAGGAGCTGGAGCTGCAATAT-3’.
recombinant active p42 MAPK (New England Biolabs, Beverly, MA) as indicated in the figure legends. The reaction was stopped by adding SDS sample buffer and heating at 95 °C for 3 min. Samples were analyzed by SDS-PAGE followed by Coomassie Blue staining and autoradiography. To quantify the relative phosphorylation levels of the different substrates, bands corresponding to the substrates were excised from the gels and their radioactivity was measured by scintillation counting. Phosphorylation assays in gels (in situ assays) were performed essentially as previously described (27) by adding 0.1 mg/ml GST-HIF-1α or GST (as control) into the separating gel prior to its polymerization and using 10 μCi of [γ-32P]ATP for the kinase assay.

**SDS-PAGE and Western Blot**—Proteins were resolved by 8% SDS-PAGE, and analyzed by Coomassie Blue or Western blotting using an anti-HIF-1α mouse monoclonal antibody (BD Transduction Laboratories), a rabbit polyclonal anti-GFP serum generously provided by Dr. H. Boleti (Hellenic Pasteur Institute, Athens, Greece), or antibodies against p44/42 MAP kinase and phospho-p44/42 MAP kinase (Cell Signaling, Beverly, MA). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) or goat anti-rabbit IgG (Cell Signaling) followed by ECL or by detection with color reaction using chloronaphthol and H2O2 as substrates.

**Mass Spectrometry**—Proteins were visualized by staining SDS-PAGE gels with silver nitrate, the gel bands were excised and cut into small pieces. The gel particles were reduced with dithiothreitol and alkylated with iodoacetamide before being subjected to in-gel digestion with mass spectroscopy grade gold trypsin (Promega) in 40 mM ammonium carbonate overnight. The resulting peptides were eluted from gel particles with 10 μl of [γ-32P]ATP and subjected to in-gel digestion with mass spectroscopy grade gold trypsin (Promega) in 40 mM ammonium carbonate overnight. The resulting peptides were eluted from gel particles with sucrose–phenylmethylsulfonyl fluoride, EDTA-free protease inhibitor mixture (Roche, Basel, Switzerland), 50 mM β-glycerolphosphate, and 10 mM Na3VO4. After centrifugation, the sample volume was adjusted to 800 μl with lysis buffer and samples were incubated for 3 h at 4 °C with 1 μl of anti-GFP serum. 20 μl of Protein A-Sepharose (Amersham Biosciences) bead slurry was added and incubation continued for 16 h at 4 °C under gentle shaking. Beads were collected by centrifugation, washed 3 times with lysis buffer, and bound proteins were eluted by SDS sample buffer.

**Yeast Transformation and β-Galactosidase Assay**—A yeast strain expressing human ARNT and carrying the reporter gene plasmid pHRE-lacZ (23) was transformed with pBEVY-GU-derived plasmids expressing the wild-type or point mutant forms of HIF-1α. Independent transformants were selected, cultured, and assayed for β-galactosidase assay as previously described (23).

**Fluorescence Microscopy**—HeLa cells grown on coverslips were transiently transfected with GFP-tagged versions of HIF-1α and 24 h post-transfection were washed once with PBS and fixed with 3% formaldehyde in PBS for 5 min at room temperature. After washing twice with PBS, cells were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride for 2 min and mounted on slides. Images were collected on a Optiphot-2 Nikon microscope.

**RESULTS**

**p42/44 MAPK and a Second Protein Kinase Target Distinct Domains of HIF-1α**—To study the phosphorylation of HIF-1α, we have used as kinase substrate recombinant full-length GST-HIF-1α, the production of which in bacteria was recently reported (24). As shown in Fig. 1A incubation of GST-HIF-1α with HeLa cell cytosolic or nuclear extracts in the presence of [γ-32P]ATP resulted in its phosphorylation (lanes 6 and 8). The phosphorylation level was higher in the case of nuclear extract (Fig. 1A, compare lanes 6 and 8) and was specific for the HIF-1α moiety of the fusion protein as GST alone was not detectably phosphorylated (lanes 5 and 7). When extracts from primary rabbit tracheal smooth muscle cells were used, a similar pattern of phosphorylation was observed but the overall level of phosphorylation of GST-HIF-1α was lower (data not shown). Therefore, HeLa cell nuclear extracts were chosen as the kinase source for subsequent experiments. Incubation of GST-HIF-1α with nuclear extract followed by isolation of the fusion protein on GSH-Sepharose beads and incubation with [γ-32P]ATP also resulted in its specific phosphorylation confirming the direct association of HIF-1α with the kinase(s) responsible for its modification (data not shown).

To characterize the kinase activity targeting HIF-1α, nuclear extracts were analyzed by SDS-PAGE using gels containing GST-HIF-1α (or GST as control), which was added into the gel mixture prior to its polymerization. After the end of the electrophoresis, the nuclear proteins separated in the gel were subjected to in situ kinase assay, i.e. they were renatured and the gel was incubated in kinase buffer with [γ-32P]ATP, washed, and subjected to autoradiography. Under these conditions, the appearance of a radioactive band in the gel would signify the position of a kinase that can phosphorylate the recombinant protein inside the gel. As shown in Fig. 1B, three bands were...
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**FIGURE 1.** Recombinant GST-HIF-1α is phosphorylated by at least two kinases from HeLa nuclear extracts. A, 1 μg of recombinant GST-HIF-1α (lanes 2, 4, 6, and 8) or GST (lanes 1, 3, 5, and 7) were incubated with 2 μg of HeLa cytoplasmic (lanes 1, 2, 5, and 6) or nuclear (lanes 3, 4, 7, and 8) protein extracts for 30 min at 30 °C in phosphorylation buffer with [γ-32P]ATP, resolved by SDS-PAGE, and stained with Coomassie Blue (lanes 1–4) or analyzed by autoradiography (lanes 5–8). Dots indicate the position of GST-HIF-1α, also shown by an arrowhead. The position of GST is also indicated by an arrowhead. B, 100 μg of HeLa nuclear protein extract was separated by SDS-PAGE on gels containing either 0.1 mg/ml GST-HIF-1α (right panel) or 0.1 mg/ml GST (left panel). After the end of the electrophoresis, the proteins in the gel were subjected to immunoblotting analysis with anti-HIF-1α antibodies. Numbers correspond to the positions of molecular mass markers (in kDa). Arrowheads indicate three radioactive bands corresponding to kinases modifying GST-HIF-1α.

**FIGURE 2.** Recombinant GST-HIF-1α is phosphorylated in vitro by p42 MAPK. A, recombinant GST-HIF-1α was incubated without (lanes 1 and 3) or with (lanes 2 and 4) 100 units of recombinant p42 MAPK kinase for 1 h at 30 °C in phosphorylation buffer without (lanes 1 and 2) or with [γ-32P]ATP (lanes 3 and 4) and analyzed by 10% SDS-PAGE followed by silver staining (lanes 1 and 2) or autoradiography (lanes 3 and 4). The slower migrating GST-HIF-1α band in lane 2 coincides with the radioactive band in lane 4. Numbers indicate the positions of molecular mass markers (in kDa). B, lanes 1 and 2 same as in A but analyzed by 8% SDS-PAGE followed by Western blot with anti-HIF1α.

As shown in Fig. 2A, lane 2, the GST moiety was phosphorylated by two distinct kinases, suggesting that modification of bacterially produced HIF-1α by p42 MAPK reproduces a physiologically relevant event.

To verify this, we used pure recombinant p42 MAPK. As shown in Fig. 2A, lane 4, p42 MAPK could indeed phosphorylate GST-HIF-1α, whereas no phosphorylation of the GST moiety by p42 MAPK could be detected (data not shown). Furthermore, incubation of GST-HIF-1α with p42 MAPK in the presence of ATP caused the appearance of a slower migrating form of GST-HIF-1α that apparently corresponds to the phosphorylated species (Fig. 2A, lane 2). The shift in the SDS gel mobility, better shown by autoradiography after analysis on a lower acrylamide percentage gel (Fig. 2B, lane 2), has also been previously demonstrated after phosphorylation of endogenous or in vitro translated HIF-1α (18) suggesting that modification of bacterially produced HIF-1α by p42 MAPK reproduces a physiologically relevant event.

To further compare the HIF-1α kinase activity present in HeLa nuclear extracts and p42 MAPK, a set of HIF-1α deletion mutants was constructed (Fig. 3A). These mutants were expressed in bacteria as GST fusion proteins, purified (Fig. 3B), and used in parallel with full-length GST-HIF-1α as substrates for phosphorylation by either p42 MAPK or HeLa nuclear extract. As shown in Fig. 3, A and C, the amino- and carboxy-terminal fragments of HIF-1α (i.e. fragments 1–251 and 652–826) were very poor substrates for p42 MAPK compared with full-length HIF-1α. Fragment 1–530 was also a relatively poor substrate, whereas fragments 1–652 and 348–826 retained most of the capacity of the full-length form to be modified by p42 MAPK. The same fragments were also the best substrates for the kinase activity in the nuclear extract (Fig. 3, A and D). However, in this case also the other fragments, and especially the amino-terminal one, 1–251, exhibited significant phosphorylation levels compared with full-length HIF-1α. Taking all the data into account, the major phosphorylation sites by MAPK appear to lie between residues 530 and 652 of HIF-1α, an area containing the TAD-N and part of the ODD and inhibitory domains. On the other hand and assuming that the kinase activity of the nuclear extract toward HIF-1α is roughly the sum of the activities of the p42/44 MAPK and a second unidentified kinase (probably the 100 kDa protein), the latter targets the amino-terminal part of HIF-1α (residues 1–251) comprising the basic helix loop helix and most part of the PAS domain (Fig. 3A).

**Serine Residues 641 and 643 in the Inhibitory Domain of HIF-1α Are Targeted by p42 MAPK**—To map precisely the phosphorylation sites, recombinant GST-HIF-1α was detected in the gel containing GST-HIF-1α (but not in the gel with GST only), suggesting that the HIF-1α kinase activity in the HeLa nuclear extracts can be attributed to at least three kinases, with apparent molecular masses of ~100, 44, and 42 kDa. The two lower M_r bands are reminiscent of the electrophoretic mobility of the p42/44 MAPK, which has been previously shown to be implicated in HIF-1α phosphorylation (18).

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HIF-1α phosphorylation by recombinant p42 MAPK, subjected to SDS-PAGE electrophoresis, and the band corresponding to the lower gel mobility modified form of HIF-1α (see Fig. 2) was excised, digested by trypsin, and subjected to analysis with mass spectrometry. Phosphorylation of a protein normally results in a net mass addition of 80 Da to a modified serine, threonine, or tyrosine residue. During fragmentation of a phosphorylated peptide, the loss of the phosphate group as phosphoric acid (H₃PO₄, 98 Da), is characteristic for peptides containing a phosphoserine or phosphothreonine (28). Phosphoserine becomes converted to dehydroalanine after the loss of H₃PO₄ (69 Da). Using the procedure outlined under “Experimental Procedures,” we identified a double-charged ion (M + 2H)⁺ (707.4 Da) or 2 μg of HeLa nuclear protein extract (D) for 30 min at 30 °C. Dots indicate the position of the recombinant GST-tagged HIF-1α fragments.

Analysis of MS/MS b' and y' fragments or further fragmentation was inconclusive regarding the exact site of phosphorylation, although some evidence was found for both serine sites. For example, the ion with m/z 916.4 (Fig. 4A) represents the y₉⁺ fragment PSPTHIHK, suggesting phosphorylation of Ser-641. On the other hand, the presence of b₅⁺ (m/z 498.6) indicated that Ser-643 could also be phosphorylated. No evidence was found for Thr-645 phosphorylation. These results identified a candidate region for HIF-1α phosphorylation, although it was not possible to determine the relative stoichiometry of phosphorylation on the two serine residues.

Serine residues 641 and 643, suggested by the mass spectroscopy analysis to be phosphorylated by MAPK, are both found in SP dipeptides, which fit, at least partly, the p42/44 MAPK consensus site PXX(T)P (29). Furthermore, sequence analysis reveals the presence of at least one of these two serine residues in a conserved position in all known vertebrate HIF-1α homologues (Fig. 4B). To independently confirm the mass spectroscopy data, either or both of these serine residues were converted into alanine by site-directed mutagenesis (Fig. 4B). The three mutant proteins, namely HS641A (HIF-1α with Ala at position 641), HS643A (HIF-1α with Ala at position 643), and HSDMA (HIF-1α double mutant with Ala at positions 641 and 643) were expressed as GST fusions in bacteria, purified, and used as substrates for recombinant p42 MAPK and HeLa nuclear extract in parallel with wild-type GST-HIF-1α. As shown in Fig. 4C, phosphorylation by MAPK was reduced by ~60% in relation to wild-type HIF-1α when either Ser-641 or Ser-643 was mutated and practically abolished (reduction by 85%) when both were substituted by alanine. When nuclear extract was used as a kinase source, mutation of either of Ser-641 or Ser-643 caused an approximate 40% reduction in the phosphorylation level that went up to 60% in the case of the double mutant. Taken together, these data identify serine residues 641 and 643 in the inhibitory domain of human HIF-1α as the predominant, and probably only, p42 MAPK in vitro phosphorylation sites. Phosphorylation by the nuclear extract is also mainly directed to these sites, indicating that the major part of the HIF-1α kinase activity present in HeLa cells is due to p42/44 MAPK. To further verify this, we used as the source of kinase activity extracts...
prepared from cells treated with the MAPK pathway inhibitor PD98059. As shown in Fig. 4D, the phosphorylation level of wild-type HIF-1α was reduced to ~46% upon inhibition of MAPK activation and was similar to the level of phosphorylation of the HSDMA mutant by untreated cell extracts. Furthermore, treatment with PD98059 did not influence the phosphorylation level of the HSDMA mutant, confirming that modification of this mutant is mediated by a kinase distinct from MAPK.

Our data were not conclusive on the exact stoichiometry of HIF-1α phosphorylation by MAPK, i.e. on whether both or only one of the two Ser residues can be modified in the same HIF-1α protein molecule. Because the two sites are similar, adjacent and only one of them conserved, it is likely that one or the other is indiscriminately chosen by p42 MAPK in human HIF-1α. If this is indeed the case, mutation of either one reduces the extent of the modification of the other, probably by disrupting the “context” of the phosphorylation site.

**Mutations in the MAPK Phosphorylation Sites of HIF-1α Render It Inactive in HeLa Cells**—It has been previously suggested that phosphorylation of HIF-1α by p42/44 MAPK is required for its in vivo transcriptional activity (16). Because the MAPK phosphorylation sites identified in vitro may also represent in vivo modification sites, we wanted to test if their mutation affected the expression or activity of HIF-1α in living cells. For this purpose, wild-type HIF-1α as well as the HS641A, HS643A, and HSDMA mutants were subcloned into the pEGFP-C1 mammalian expression vector and intro-
Free GFP could only be detected in the immunoprecipitate from the control cells transfected with the empty pEFGP-C1 vector (Fig. 5A, lane 1), showing that the integrity of the GFP-HIF-1α was maintained. Interestingly, the single as well as the double mutant forms of GFP-HIF-1α migrated slightly faster than the wild-type form, indicating a reduced phosphorylation status (compare lanes 4–6 with lane 2). To investigate this, cells expressing wild-type GFP-HIF-1α were also treated prior to their lysis with PD98059, which should reduce the phosphorylation level of HIF-1α. In this case, wild-type GFP-HIF-1α also exhibited faster gel mobility (Fig. 5A, lane 3). The fact that inhibition of p42/44 MAPK and mutation of Ser-641 and/or Ser-643 of HIF-1α lead to the same effect supports the notion that these serine residues identified in vitro as p42 MAPK sites are also major in vivo targets.

The transcriptional activity of the mutants was compared with that of the wild-type protein following transient transfection of HeLa cells together with the luciferase reporter construct pGL3–5HRE-VEGF and the β-galactosidase construct CMV-lacZ, which was used as an internal control. As negative control the empty vector pEGFP-C1 was used. The results shown in Fig. 5B demonstrate that wild-type GFP-HIF-1α shows a 7-fold increase in transcriptional activity compared with GFP alone. This was significantly reduced when the cells expressing wild-type GFP-HIF-1α were incubated with PD98059 in agreement with previous reports (18, 19, 30, 31). An even further reduction was observed when the single (HS641A and HS643A) or double (HSDMA) mutants substituted for the wild-type protein. The effectiveness of PD98059 to inhibit the activation of MAPK under our conditions was measured by immunodetection of total as well as phosphorylated (i.e. active) MAPK in cell fractions. As shown in Fig. 5C, treatment with PD98059 both reduced the amount of MAPK in the nucleus and inhibited its phosphorylation. However, this inhibition was not complete, in agreement with the partial inactivation effect of PD98059 on HIF-1α shown in Fig. 5B (and its partial loss of nuclear accumulation; see below, Fig. 7). Taken together, our data show that either inhibition of MAPK or disruption of the MAPK phosphorylation sites on HIF-1α can impair the function of HIF-1 in living human cells.

The mutations that abolish MAPK phosphorylation of HIF-1α may specifically inhibit one or more of the steps required for HRE-dependent transcriptional activation in human cells such as import of HIF-1α into the nucleus, heterodimerization with ARNT, binding to DNA, and interaction with the transcriptional co-activators or they may even nonspecifically affect the overall stability and folding conformation of HIF-1α. To investigate these possibilities, the transcriptional activity of HIF-1α and its MAPK site mutants was measured using a recently developed yeast-based method (23). In brief, wild-type human HIF-1α and the three MAPK site mutants were cloned into suitable yeast expression vectors and introduced into Saccharomyces cerevisiae cells, each together with a plasmid expressing human ARNT and a pHRE-lacZ reporter plasmid. After induction of their expression, the activity of β-galactosidase was determined as...
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FIGURE 6. Mutations in the MAPK phosphorylation sites of HIF-1α do not affect its transactivating capacity in yeast but impair it in HeLa cells. A, transactivation activity of wild-type HIF-1α and its mutant forms in yeast. Results are shown as % of activity in relation to wild-type HIF-1α and represent the mean of three independent experiments performed in triplicate (± S.E.). B, transactivation activity of fusion constructs between the GAL4-DBD and the carboxyl-terminal part (amino acids 348–826) of wild-type and mutant HIF-1α after transfection of HeLa cells together with a gal4-tk-luc reporter and Renilla luciferase control plasmids. Values, determined as a ratio of firefly over Renilla luciferase activity, are expressed in relation to the results obtained from cells expressing GAL4-DBD alone and represent the mean of two independent experiments performed in triplicate (± S.E.).

a measure of the transcriptional activity of each protein. Surprisingly, as shown in Fig. 6A, all three mutants exhibited in yeast almost the same activity as wild-type HIF-1α. Analysis by Western blot also revealed no defect in their expression or stability (data not shown). These results strongly indicate that mutations of Ser-641 and/or Ser-643 into Ala do not destroy the capacity of HIF-1α to act as a transcriptional regulator per se but rather specifically interfere with a process required for HIF-1α activation in human cells. To identify this process, mammalian expression plasmids were constructed encoding the DBD of the yeast transcription factor GAL4 (amino acids 1–147) fused in-frame with the carboxy-terminal part (amino acids 348–826) of the wild-type or mutant forms of HIF-1α. The encoded fusion proteins GAL4-DBD-HIF-1α348–826 (termed in short GAL-HΔN), GAL-HΔN641A, GAL-HΔN643A, and GAL-HΔN5DMA could then be assayed for transcriptional activity in HeLa cells by co-transfection with a GAL4 reporter construct (gal4-tk-luc), i.e. without interference of the HIF-1α NLS-terminal DNA-binding and dimerization domains. As shown in Fig. 6B, GAL-HΔN exhibited 8-fold higher reporter gene activation compared with GAL4-DBD alone in agreement with previous reports showing that the transactivation domains of HIF-1α reside in its carboxy-terminal part (10, 32, 33). In contrast, mutations in either or both of Ser-641 and Ser-643 reduced transcriptional activity of the fusion proteins to less than 2-fold in comparison to GAL4-DBD. Taking into account that the GAL-DBD harbors an nuclear localization signal (NLS) motif capable of taking fusion proteins into the nucleus (34) and also targets them to the promoter of the reporter gene via its own DNA binding capacity, the mutations in the MAPK sites of HIF-1α should either impair its interaction with the human transcriptional co-activators or decrease its accumulation inside the human nucleus by affecting a mechanism other than nuclear import.

The MAPK Phosphorylation-defective Forms of HIF-1α Are Mislocalized in the Cytoplasm of HeLa Cells by CRM1-dependent Nuclear Export—To address the issue of nuclear accumulation we examined the subcellular distribution of GFP-HIF-1α and its mutant forms in transiently transfected HeLa cells. As shown in Fig. 7A, panel a, examination of control cells expressing GFP by fluorescence microscopy revealed that GFP was found distributed in both cytoplasms and, due to its small size, also nucleus. In contrast, in cells expressing wild-type GFP-HIF-1α, the GFP-tagged protein could only be detected inside the nucleus (Fig. 7A, panel b). When the same cells were incubated in the presence of PD98059 for 5 h before observation, GFP-HIF-1α was shown distributed in both nucleus and cytoplasm (Fig. 7A, panel c), suggesting that inhibition of phosphorylation by MAPK impairs its nuclear accumulation. Examination of the cells expressing one of the three mutant HIF-1α forms (HS641A, HS643A, and HSDMA) fused to GFP, showed that in all three cases the mutant protein were found largely excluded from the nucleus and localized predominantly in the cytoplasmic space (Fig. 7A, panels d–f). This nuclear exclusion could be due to either impaired nuclear import or overactive nuclear export. To test the latter possibility, the localization experiment of the various GFP-HIF-1α forms was repeated but the cells were treated with LMB, a specific inhibitor of the main mammalian nuclear protein export receptor CRM1, for 4 h prior to observation (Fig. 7B). Exposure of LMB did not influence the localization of GFP, which remained distributed in the whole cell (Fig. 7B, panel a) or GFP-HIF-1α, which stayed accumulated inside the nucleus (Fig. 7B, panel b). However, the distribution of the mutant GFP-HIF-1α forms changed dramatically in the presence of LMB: all three fusion proteins were re-localized and accumulated inside the nucleus like wild-type GFP-HIF-1α (Fig. 7B, panels c–e). Unfortunately, we were unable to microscopically examine the cells that were incubated with both PD98059 and LMB because simultaneous exposure to the two inhibitors proved to be detrimental and caused massive cell detachment (data not shown). The rest of the localization data, however, clearly indicate that inhibition of the MAPK-dependent phosphorylation of GFP-HIF-1α obstructs its nuclear accumulation not by blocking import but rather by enhancing export out of the nucleus.

If the reduced transcriptional activity of the MAPK phosphorylation deficient forms of HIF-1α were due to their cytoplasmic dislocation, their restored nuclear localization would be expected to also restore, at least to some extent, their transcriptional activity. Therefore, the transcriptional activities of wild-type and mutant (SDMA) GFP-HIF-1α were measured in the presence and absence of LMB (Fig. 8A). Whereas LMB did not
affect the activity of wild-type GFP-HIF-1, the activity of the HSDMA mutant was increased 2-fold and reached almost 70% of the wild-type activity. Similar results were also obtained when the GAL4-HΔN fusion constructs were analyzed (Fig. 8B). Again, LMB did not significantly influence the activity of wild-type GAL4-HΔN but more than doubled the activity of the GAL-HΔNSDMA mutant. These data show that the predominant reason for the inactivation of HIF-1α by MAPK site mutations is its efficient export and subsequent exclusion from the nucleus. Because LMB treatment does not completely restore the activity of the mutants, it is also possible that the mutations have additional minor effects on the function of HIF-1α. Nevertheless, it is clearly suggested that phosphorylation of HIF-1α Ser-641 or Ser-643 by p42/44 MAPK can block the CRM1-dependent export of HIF-1α to the cytoplasm leading to unimpeded nuclear accumulation and activation of HIF-1 target genes.

**DISCUSSION**

Although, it has been long known that HIF-1α is a target of p44/p42 MAPK (18–20), the sites of phosphorylation remained unidentified. We show here that p42 MAPK modifies HIF-1α residues Ser-641 and Ser-643. These residues were identified by mass spectroscopic analysis of in vitro phosphorylated recombinant HIF-1α and confirmed by deletion analysis and site-directed mutagenesis. Our results are in agreement with previous reports (19–21), which mapped the in vitro MAPK phosphorylation sites in the carboxyl-terminal part (amino acid residues 531–826) of HIF-1α. We further show that mutating Ser-641 and/or Ser-643 into alanines inhibits both the phosphorylation-dependent mobility shift of HIF-1α and its transcriptional activity in human cells. Similar inhibition was also observed for the wild-type protein when MAPK activity was impaired by PD98059. Taken together, these findings strongly suggest that Ser-641 and Ser-643 constitute major in vitro and in vivo MAPK phosphorylation sites important for the function of HIF-1α. At least one of these two serine residues is conserved in the same position in all vertebrate homologues of HIF-1α. However, none of them is found in HIF-2α (EPAS1/HLF/HRF; data not shown), another closely related hypoxia-regulated transcription factor, in agreement with the fact that HIF-2α appears not to be a direct target of MAPK (35).

The reduction of HIF-1α transcriptional activity by inhibition of MAPK was previously reported in many cases of hypoxic as well as normoxic induction of HIF-1α (18–20, 30, 31, 36–40) and it was often shown to be independent of HIF-1α protein levels (18, 19, 30, 31). The correlation of these findings with the fact that HIF-1α can be directly phosphorylated by p44/p42 MAPK has led to the hypothesis that phosphorylation of HIF-1α by MAPK increases its transcriptional activity probably by augmenting its interaction with other components of the tran-
Regulation of nuclear transport of HIF-1α has also been a controversial subject in the literature. First Kallio et al. (10), using transfected cells expressing GFP-HIF-1α, suggested that nuclear accumulation of HIF-1α does not take place by default but is a process regulated by hypoxia or hypoxia mimetics. Subsequent studies supported (39, 40, 43) or contradicted (30, 44) this idea and exogenous or even endogenous HIF-1α could be observed solely in the nucleus or in the cytoplasm or in both nucleus and cytoplasm depending on the cell type and culturing conditions. Our data can help reconcile these apparently conflicting previous findings. The regulation of the subcellular distribution of HIF-1α by MAPK would imply involvement of the modified residues and the responsible kinase.

Regulation of nuclear translocation of inducible transcription factors is an essential element of many signaling pathways (46, 47) and can involve nuclear import, cytoplasmic retention, or nuclear export mechanisms. When nuclear import is stimulated by phosphorylation, the modified residues are normally found close to the NLS, to facilitate recognition by the soluble nuclear import factors or unmask the NLS (47). The sites of MAPK-dependent phosphorylation on HIF-1α (Ser-641, Ser-643) are relatively distant to its characterized functional NLS, located in residues 719–756 (10, 48). This and the fact that providing an exogenous NLS, as in the case of GAL4-DBD fusions, does not “rescue” the activity of the mutant HIF-1α forms make stimulation of HIF-1α nuclear import by MAPK rather unlikely. A second possibility is that phosphorylation of HIF-1α by MAPK stimulates its nuclear accumulation by releasing HIF-1α from cytoplasmic retention sites. In favor of this hypothesis, we have reproducibly observed that the phosphorylation-deficient mutant forms of HIF-1α

scriptional machinery. This was questioned by a more recent report, which showed that the interaction between the C-TAD of HIF-1α and co-activator p300 was facilitated by MAPK-dependent phosphorylation of p300 and not HIF-1α (21). Therefore, the role of HIF-1α phosphorylation remained until now controversial. We now offer direct proof that inhibition of phosphorylation of HIF-1α Ser-641 and/or Ser-643 causes its nuclear exclusion without affecting significantly its stability or its capacity to act as a transcriptional activator. These data strongly suggest that MAPK-dependent phosphorylation of HIF-1α is required for its efficient accumulation inside the nucleus.

Because exclusion of HIF-1α from the nucleus would automatically lead to HIF-1 inactivation, our findings resolve the previous controversies and indicate that an activated MAPK pathway can diverge to more than one direct phosphorylation target (e.g. HIF-1α and p300) increasing thus the response efficiency (Fig. 9). Additionally, it has also been reported that Thr-796 in the C-TAD of HIF-1α might be phosphorylated because its mutation to alanine strongly reduced the transcriptional activity of HIF-1α (22). It is likely that phosphorylation of this residue, by a yet unidentified kinase, inhibits the interaction with factor inhibiting HIF-1, thereby preventing hydroxylation and facilitating binding of C-TAD to CBP (41). Thus, multiple direct phosphorylation events appear to regulate the activity of HIF-1α at different steps along its activation pathway. Multisite phosphorylation is common in transcription factors and may allow sophisticated regulation and signal integration (42). To this respect, we also report on an additional kinase activity, probably attributed to a 100-kDa protein, that targets the amino-terminal part of HIF-1α involved in heterodimerization and DNA binding. Whether these processes are also controlled by phosphorylation remains to be seen and will involve identification of the modified residues and the responsible kinase.

FIGURE 8. Treatment with LMB restores the transcriptional activity of the HIF-1α MAPK site mutants.

Transcriptional activity of GFP- (A) or GAL4-DBD (B) fusion constructs of wild-type HIF-1α or its SDMA mutant determined 24 h after transfection of HeLa cells together with the corresponding reporter and control plasmids and 4 h incubation in the absence or presence of 20 ng/ml LMB as indicated. Values (relative luciferase activity in abstract units, represent the mean of two independent experiments performed in triplicate (±S.E.).
Our data therefore imply that nuclear export of proteins bearing a leucine-rich nuclear export signal. Our data also offer a possible explanation for the inhibitory function of the inhibitory domain of HIF-1α (residues 576–785) (32). This domain is now shown to contain the MAPK phosphorylation sites that regulate the recognition of HIF-1α by the nuclear export machinery. The inhibitory domain may therefore be responsible for the nuclear export of unmodified HIF-1α by mediating direct or indirect association with CRM1. Lack of this domain would restrict HIF-1α inside the nucleus and cause transcriptional activation even in the absence of MAPK pathway stimulation. On the other hand, saturation of the nuclear export machinery by overexpressed HIF-1α or inability to efficiently counteract nuclear import upon stabilization of HIF-1α could account for the presence of HIF-1α in both the nucleus and cytoplasm under non-stimulatory conditions. As shown schematically in Fig. 9, activation of MAPK would then be required for phosphorylation of HIF-1α, termination of nuclear export, efficient accumulation inside the nucleus, interaction with ARNT and DNA, and subsequent manifestation of its full transcriptional activity, further augmented by the previously suggested (21) MAPK-dependent phosphorylation of p300. The identification of the MAPK-phosphorylation sites and their role in the activation of HIF-1α described in this work can now provide additional means for interfering with HIF-1 activity in pathological conditions.

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