Atypical CRM1-dependent Nuclear Export Signal Mediates Regulation of Hypoxia-inducible Factor-1α by MAPK*

Received for publication, April 22, 2008, and in revised form, August 5, 2008 Published, JBC Papers in Press, August 7, 2008, DOI 10.1074/jbc.M803081200

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Hypoxia-inducible factor 1 (HIF-1) is the key transcriptional activator of hypoxia-inducible genes and an important anti-cancer target. Its regulated subunit, HIF-1α, is controlled by oxygen levels and major signaling pathways. We reported previously that phosphorylation of Ser641/643 by p42/44 MAPK is essential for HIF-1α nuclear accumulation and activity. We now show that a fragment of HIF-1α (amino acids 616–658), termed MAPK target domain, contains a nuclear export signal (NES), which has atypical hydrophobic residue spacing. Localization, reporter gene, and co-immunoprecipitation assays demonstrate that the identified NES interacts with CRM1 in a phosphorylation-sensitive manner. Furthermore, disruption of the NES (amino acids 637/1638A/639A) restores nuclear localization and activity of nonphosphorylated HIF-1α and renders it largely resistant to inhibition of MAPK, an effect reproduced by a phosphomimetic mutation (S641E). As these data predict, overexpression of wild-type or mutant (S641A/S643A) MAPK target domain in HeLa cells modulates the activity and subcellular distribution of endogenous HIF-1α. We suggest that control of HIF-1α nuclear transport represents an important MAPK-dependent regulatory mechanism.

Under normal oxygen concentration, HIF-1α protein levels are kept low by von Hippel-Lindau-mediated polyubiquitination and subsequent degradation. Interaction with von Hippel-Lindau requires the hydroxylation of two proline residues in the oxygen-dependent degradation domain of HIF-1α (3). The proline hydroxylases that participate in this process depend on iron and require molecular oxygen and 2-oxoglutarate as substrates. When oxygen concentration is low, hydroxylation is impaired allowing HIF-1α to be stabilized, enter the nucleus, bind to ARNT and DNA, and induce the expression of target genes. This latter process is also regulated by oxygen tension as HIF-1α hydroxylation at Asn803 by FIH-1 compromises its association with the transcriptional co-activator CBP/p300 (4). Reactive oxygen species produced under hypoxia as well as intermediate products of cell metabolism can also affect hydroxylation and, consequently, modulate HIF-1 activity (5–7).

HIF-1α expression and transcriptional activity are additionally controlled, irrespective of oxygen levels, by major signaling pathways such as phosphatidylinositol 3-kinase and MAPK, which are induced by growth factors, cytokines, and oncogenes (8, 9). Activation of the phosphatidylinositol 3-kinase pathway leads to enhanced levels of HIF-1α protein synthesis (10), whereas MAPK activation promotes predominantly HIF-1 transcriptional activity via direct phosphorylation of HIF-1α (11). We have recently revealed that p44/42 MAPK (ERK1/2) modifies HIF-1α residues Ser641 and Ser643 and showed that inhibition of this phosphorylation by mutagenesis, MAPK pathway inhibitors, or treatment with flavonoids impairs activity and nuclear accumulation of HIF-1α (12, 13). The degree of nuclear accumulation of a protein depends on its relative nuclear import and export rates (14). Nuclear import of HIF-1α has been suggested previously to be regulated (15, 16). Although the ability of human HIF-1α to also exit the nucleus and be a shuttling protein has been documented (17, 18), the transport signals or factors regulating its nuclear export have remained so far unknown. In this work we characterize an atypical but CRM1-dependent NES in human HIF-1α. We further show that the identified NES responds to MAPK-dependent phosphorylation and therefore provides additional means of HIF-1α regulation.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—HIF-1α point mutants were constructed with the QuikChange® II site-directed mutagenesis kit (Stratagene) using as templates pGEX-HIF-1α (19) or pGEX-HIF-1α(S→A) (12) and suitable primers (sequence available...
upon request) and were then subcloned as BamHI inserts into the mammalian expression vector pEGFP-C1 (Clontech). The cDNA fragments corresponding to HIF-1α MTD (amino acids 616–658) were obtained by PCR using as template pGEX-HIF-1α (wild-type or mutant forms). The derived inserts were subcloned as BamHI fragments into pEGFP-C1 and pCMV-FLAG vectors. The DNA sequence of the point mutants was confirmed by sequencing performed by Lark Technologies Inc.

Cell Culture and Transfection—Human HeLa, HEK293-T, and Huh7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. Transient transfections were performed as described previously (12). When required, 20 h after transfection cells were treated for 4 h with 50 µM PD98059, 10 ng/ml LMB (both from Sigma), or 1 mM DMOG (Alexis Biochemicals).

Reporter Gene Assays—Luciferase assays were performed in cells transiently co-transfected with plasmids expressing different forms of full-length or MTD of HIF-1α (or the empty pEGFP-C1 parental vector as control) as reported previously (12).

Protein Purification and Phosphorylation Assays—GST-HIF-1α, GST-MTD, and GST-MTD(S→A) were expressed in E. coli and purified as GST-HIF-1α (19). Phosphorylation reactions were carried out according to procedures published previously (12).

Immunoprecipitation—HeLa cells treated with DMOG or transfected with GFP-HIF-1α, GFP-MTD, or their mutant forms were washed with cold PBS and lysed (20 min, 4 °C) in buffer containing 25 mM Hepes, pH 7.6, 150 mM NaCl, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 50 mM β-glycerol phosphate, and 10 mM Na₃VO₄. Immunoprecipitation of endogenous or GFP-tagged proteins was performed as described previously using an affinity-purified polyclonal anti-HIF-1α antibody raised in rabbits against GST-HIF-1α (348–826) (20) or an anti-GFP antibody (12), respectively, and precipitates were analyzed by Western blotting using anti-FLAG antibody (Sigma), washed twice with PBS, and incubated with 50 µM PD98059, 10 ng/ml LMB (both from Sigma), or 1 mM DMOG (Alexis Biochemicals).

Fluorescence Microscopy—HeLa, HEK293-T, and Huh7 cells grown on coverslips were transiently transfected with GFP-tagged full-length HIF-1α, its MTD fragment, or their mutant forms, 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 DAPI for 2 min and mounted on slides. Images were collected with a Leica DFC480 camera (LAS software version V2.3.1.R1) on an Axioscope 40 Zeiss microscope equipped with a 40× objective at room temperature.

Immunofluorescence—HeLa cells were grown on coverslips and transiently transfected with pCMV-FLAG vector expressing the MTD fragment or its S→A mutant. 20 h post-transfection cells were treated for 4 h with 1 mM DMOG and if required with 50 µM PD98059, washed once with PBS, and fixed with 3% formaldehyde in PBS for 5 min at room temperature. Cells were washed twice with PBS and permeabilized with PBS containing 0.1% Triton X-100 for 15 min. After washing twice with PBS, cells were treated with 3% bovine serum albumin in PBS for 1 h. Coverslips were incubated for 1 h at room temperature with a polyclonal anti-HIF-1α antibody (20) and a monoclonal anti-MAPK (sigma), washed twice with PBS, and incubated for 30 min at room temperature with fluorescein isothiocyanate-conjugated anti-rabbit and Cy3-conjugated anti-mouse secondary antibodies (Jackson Immunoresearch). After washing twice with PBS, cells were counterstained with DAPI, mounted on slides, and viewed by a Leica DFC480 camera (LAS software version V2.3.1.R1) on an Axioscope 40 Zeiss microscope equipped with a 40× objective at room temperature.

RESULTS

Phosphomimetic S641E Mutation Stimulates HIF-1α Activity and Renders It Resistant to MAPK Inhibition—We have recently reported that mutation of HIF-1α Ser641 and/or Ser643 inhibited its phosphorylation by p42/44 MAPK and impaired its activity and nuclear accumulation by stimulating its nuclear export, suggesting that these sites are the predominant, if not the only, MAPK targets on HIF-1α (12). To positively demonstrate the role of this modification in the regulation of HIF-1α nuclear transport, we used site-directed mutagenesis to convert Ser641 to Glu (S→E), which mimics the charge of phosphorylated serine. Ser641 was chosen randomly between Ser641 and Ser643 because mutations in either of them caused identical phenotypes in terms of inhibition of transcriptional activity and nuclear accumulation (12). Wild-type as well as mutant HIF-1α(S→E) were expressed as GFP fusions in HeLa cells. As expected, both wild-type GFP-HIF-1α and GFP-HIF-1α(S→E) were nuclear in all cells examined (Fig. 1A, panels a and c). Incubation with the MEK inhibitor PD98059 caused partial redistribution of GFP-HIF-1α to the cytoplasm (Fig. 1A, panel b), but it did not affect the localization of GFP-HIF-1α(S→E), which remained exclusively inside the nucleus of all the cells that expressed it (Fig. 1A, panel d). Determination of transcriptional activities by reporter gene assays showed that GFP-HIF-1α(S→E) exhibited significantly enhanced transcriptional activity compared with the wild-type form (Fig. 1B). Moreover, GFP-HIF-1α(S→E) retained most of its activity in the presence of PD98059, whereas the same conditions reduced dramatically the activity of the wild-type form (Fig. 1B). We conclude that phosphorylation at Ser641 is both essential and sufficient for MAPK-dependent stimulation of HIF-1α nuclear accumulation and activity. However, the significant, albeit minor, effect of PD98059 on the S→E mutant suggests an additional effect of MAPK on HIF-1α-mediated transcription (see also “Discussion”).

Identification of a Phosphorylation- and CRM1-dependent NES in HIF-1α—To gain mechanistic insight into the phosphorylation-dependent subcellular localization of HIF-1α, we examined the amino acid sequences neighboring the MAPK sites for the presence of nuclear transport signals. We noticed a number of conserved hydrophobic residues (Fig. 2A) that, despite their atypical spacing and composition, were reminiscent of a leucine-rich NES (21). To study the function of this sequence and its possible reliance on phosphorylation, we cloned a fragment of HIF-1α encompassing residues 616–658.
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A

|     | GFP | DAPI | GFP | DAPI |
|-----|-----|------|-----|------|
| wt  | a   | b    | c   | d    |
| S→E | e   | f    | g   | h    |

B

![Graph](image)

FIGURE 1. Phosphomimetic mutation S641E stimulates HIF-1α activity and renders it resistant to MAPK inhibition. A, localization of wild-type (wt) GFP-HIF-1α or mutant form GFP-HIF-1α(S→E) bearing the phosphomimetic S641E mutation (as indicated) in HeLa cells without (panels a and c) or after (panels b and d) treatment with 50 μM PD98059 for 4 h. Panels a–d indicate nuclei stained with DAPI; white arrowheads indicate the nuclei of transfected cells. In all cases the cells shown in the micrographs of this figure are representative of greater than 95% of the cells examined in at least three repetitions of the experiment. B, transcriptional activity of wild-type (wt) GFP-HIF-1α or GFP-HIF-1α(S→E) in HeLa cells, 24 h post-transfection, determined by reporter gene assay as described under “Experimental Procedures.” Where indicated (+), cells were treated with 50 μM PD98059 for 4 h prior lysis. Values are expressed in relation to the values obtained from the empty pEGFP vector and represent the mean of three independent experiments performed in triplicate (± S.E.).

(As shown in Fig. 2B (lanes 3, 7, and 11), this 43-amino acid-long fragment of HIF-1α retained the ability to be efficiently phosphorylated in vitro by either p42 MAPK or HeLa cell nuclear extracts and was, therefore, termed MAPK Target Domain (MTD). We also created mutant MTD forms lacking the phosphorylation sites (conversion of both Ser641 and Ser643 to Ala; S→A) or the three hydrophobic residues of the putative NES (triple mutation I637A/L638A/I639A; I→A) or both (I→A/S→A) (Fig. 2A, lower panel). As anticipated, MTD(S→A) could no longer be phosphorylated by p42 MAPK or HeLa nuclear extracts (Fig. 2B, lanes 4, 8, and 12).

We next examined the possibility that MTD contains an autonomous NES. Wild-type and mutant forms of MTD were fused to GFP and expressed in HeLa cells. In all cells examined, wild-type GFP-MTD was mostly nuclear (Fig. 2C, panel b), whereas the S→A mutant was found almost exclusively in the cytoplasm (Fig. 2C, panel c), which was suggestive of efficient nuclear export in the absence of phosphorylation. Concomitant mutation of the putative NES in the I→A/S→A form re-localized most of the protein inside the nucleus (Fig. 2C, panel d) showing that residues 637I638I639 are required for efficient nuclear export. Finally, destruction of the NES alone in the I→A mutant, as expected, retained the nuclear localization of the protein (Fig. 2C, panel e). These data suggest that the conserved hydrophobic sequence in MTD can indeed function as an NES, but only when the neighboring phosphoacceptor sites remain unmodified.

To test whether the identified NES directs HIF-1α-MTD to the major nuclear export pathway involving CRM1 (21), HeLa cells expressing GFP-MTD(S→A) were treated with the specific CRM1 inhibitor leptomycin B (LMB). Incubation with LMB reversed the cytoplasmic localization of GFP-MTD(S→A) in all of the expressing cells and caused its accumulation inside the nucleus (Fig. 3A). To investigate whether MTD interacts with CRM1, HeLa cells expressing wild-type or mutant forms of GFP-MTD were subjected to immunoprecipitation with an anti-GFP serum. Analysis of the immunoprecipitates revealed a weak interaction of endogenous CRM1 with wild-type MTD (Fig. 3B, lane 1). However, this interaction was considerably strengthened in the case of the S→A mutant (Fig. 3B, lane 2). On the other hand, destruction of the NES in the I→A and I→A/S→A mutants completely abolished the interaction with CRM1 (Fig. 3B, lanes 3 and 4). Immunoblotting with the same anti-GFP serum used for the immunoprecipitation confirmed similar recoveries of GFP-MTD forms in the precipitates (Fig. 3B, bottom panel).

In the same way, we examined the interaction between full-length HIF-1α and CRM1 in HeLa cells expressing wild-type and mutant forms of GFP-HIF-1α. Although we could not detect an interaction of CRM1 with wild-type HIF-1α, binding of CRM1 to the phosphorylation-deficient mutant S→A was readily detectable (Fig. 3C, lanes 1 and 2). As in the case of MTD, interaction to CRM1 was blocked when the NES sequence was additionally mutated in the I→A/S→A form (Fig. 3C, lane 4). Immunoblotting with an anti-HIF-1α antibody verified similar recoveries of GFP-HIF1α forms in the immunoprecipitates (Fig. 3C, bottom panel). To confirm these findings with endogenous wild-type HIF-1α, HeLa cells were treated with dimethyloxalylglycine (DMOG), a hypoxia-mimetic chemical that induces HIF-1α by inhibiting its hydroxylation (3), exposed to the MEK inhibitor PD98059, and subjected to immunoprecipitation using an anti-HIF-1α antibody. Analysis of the immunoprecipitates showed that CRM1 associated with endogenous wild-type HIF-1α only under conditions that inhibit the MAPK pathway (Fig. 3D, top panel, lane 4), verifying that phosphorylation of HIF-1α by MAPK inhibits its interaction with CRM1. Collectively, these data demonstrate that the MTD of HIF-1α contains an atypical hydrophobic NES that interacts with CRM1 in a MAPK-phosphorylation sensitive manner. As we were not able to convincingly demonstrate stable binding of CRM1 to MTD using recombinant proteins in vitro (data not shown), the interaction between CRM1 and the identified atypical NES may be of low affinity or require additional cellular factors (see also “Discussion”).

Identified NES Mediates Sensitivity of HIF-1α to MAPK-dependent Phosphorylation—As shown in Fig. 1, both localization and activity of HIF-1α are sensitive to MAPK pathway inhibi-
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Therefore, we tested if this sensitivity involves the identified NES by analyzing the subcellular distribution and transcriptional activity of GFP-tagged wild-type or mutant forms of HIF-1α expressed in HeLa cells. As we have already reported, wild-type GFP-HIF-1α was localized inside the nucleus (Fig. 4A, panel b), whereas the phosphorylation-deficient S→A mutant was predominantly cytoplasmic (Fig. 4A, panel c). However, parallel destruction of the NES resulted in the redistribution of the phosphorylation-deficient I→A/S→A mutant inside the nucleus in all transfected cells (Fig. 4A, panel d).

Treating the cells with the MEK inhibitor PD98059 impaired the nuclear accumulation of wild-type GFP-HIF-1α (Fig. 4A, panel g) but did not affect the nuclear localization of the I→A HIF-1α or its mutant forms and examined by fluorescence microscopy. In full agreement with the results previously obtained in HeLa cells, Fig. 5 shows that wild-type GFP-HIF-1α was predominantly nuclear (panels b), the phosphorylation-deficient form S→A was mislocalized into the cytoplasm (panels c), and the I→A/S→A mutant form, lacking both the NES and the MAPK-phosphorylation sites, re-accumulated inside the nucleus (panels d) in all transfected cells of both cell lines.

Taken together, these data show that disruption of the HIF-1α NES suppresses the mutation at the MAPK receptor site and activates the phosphorylation-deficient mutants of HIF-1α. Moreover, the HIF-1α NES mutants become largely resistant to elimination of phosphorylation resulting from constitutive activation of MAPK in HeLa cells (see Fig. 5C in Ref. 12).

In agreement with the localization studies at the single cell level, analysis of the whole transformed cell population by a reporter gene assay demonstrated that the phosphorylation-deficient cytoplasmic S→A form showed significantly reduced transcriptional activity, whereas the NES-defective nuclear I→A/S→A and I→A forms preserved their activity at levels similar to that of wild-type GFP-HIF-1α (Fig. 4B). Inhibiting MAPK activation by PD98059 minimized wild-type GFP-HIF-1α activity to the level of the mutant S→A form but had only a small effect on the transcriptional activity of the I→A/S→A and I→A mutants (Fig. 4B). These data show that disruption of the NES (the I→A mutation) can rescue the lack of phosphorylation (the S→A mutation). In accordance, the I→A mutation also phenocopied the phosphomimetic S→E mutation as far as resistance of HIF-1α to PD98059 is concerned (compare Figs. 1 and 4).

To substantiate these findings in additional cell types, human embryonic kidney (HEK293-T) and hepatoma (Huh7) cells were transiently transfected with full-length GFP-HIF-1α or its mutant forms and examined by fluorescence microscopy. In full agreement with the results previously obtained in HeLa cells, Fig. 5 shows that wild-type GFP-HIF-1α was predominantly nuclear (panels b), the phosphorylation-deficient form S→A was mislocalized into the cytoplasm (panels c), and the I→A/S→A mutant form, lacking both the NES and the MAPK-phosphorylation sites, re-accumulated inside the nucleus (panels d) in all transfected cells of both cell lines.

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![Image](image_url)

FIGURE 3. NES of HIF-1α-MTD depends on and interacts with CRM1. A, 24 h post-transfection, HeLa cells expressing GFP-MTD(S→A) were further incubated without (panel a) or with (panel b) 10 ng/ml LMB for 4 h, fixed, stained with DAPI (panels a' and b', white arrowheads indicate the nuclei of transfected cells), and subjected to fluorescence microscopy. In all cases, the cells shown in the micrographs of this figure are representative of greater than 95% of the cells examined in at least three repetitions of the experiment. B and C, total cell extracts of HeLa cells expressing wild-type (lane 1) or mutant forms (lanes 2–4 as indicated) of GFP-MTD (B) or full-length GFP-HIF-1α (C) were subjected to immunoprecipitation with an anti-GFP serum. Extracts (input; upper two panels) and precipitated proteins (I.P., lower two panels) were analyzed by Western blotting using anti-CRM1 and anti-GFP (B) or anti-CRM1 and anti-HIF-1α (C) antibodies as indicated. Only the relevant parts of the blots are shown. D, total cell extracts of HeLa cells treated for 4 h with 1 mm DMOG alone (−) or in combination with 50 μM PD98059 (+) were subjected to immunoprecipitation with a rabbit anti-HIF-1α polyclonal antibody. Extracts (input) and precipitated proteins (I.P.) were analyzed by Western blotting using anti-CRM1 mouse monoclonal and an affinity-purified anti-HIF-1α rabbit polyclonal antibodies.

either mutation in HIF-1α or MAPK pathway inhibition. This strongly suggests that the phosphorylation-sensitive HIF-1α NES characterized in this work is an important mediator of the control that the MAPK pathway exerts on HIF-1α activity.

Overexpression of Wild-type and Mutant MTD Modulates the Localization and Activity of Endogenous HeLa Cell HIF-1α—To extend our analysis, we studied the effect of wild-type or mutant MTD on the localization and activity of endogenous HeLa cell HIF-1α. As wild-type MTD is a good phosphorylation substrate for MAPK but a weak interaction partner of CRM1 (Fig. 2B and Fig. 3B), our results would predict that, when overexpressed, it should compete with endogenous HIF-1α for MAPK-mediated phosphorylation, thus impairing nuclear accumulation and transcriptional activity of HIF-1α. On the other hand, overexpression of MTD(S→A), which is a bad MAPK substrate but interacts readily with CRM1, should inhibit the interaction between unmodified endogenous HIF-1α and CRM1, thus causing nuclear retention and stimulation of HIF-1α under conditions that eliminate MAPK activity (i.e. upon treatment with PD98059).

To test these predictions, HeLa cells overexpressing FLAG-tagged MTD or MTD(S→A) were induced to express endogenous HIF-1α by DMOG and were then subjected to double immunofluorescence staining with anti-HIF-1α and anti-
cells to PD98059 caused the partial redistribution of endogenous HIF-1α to the cytoplasm, except in the cells that overexpressed FLAG-MTD(S→A), in which HIF-1α remained exclusively nuclear (Fig. 6A, panels m–p). In agreement, when MTD(S→A) was overexpressed, endogenous HIF-1 activity was not only mostly preserved but also became largely insensitive to inhibition of the MAPK pathway (Fig. 6B). This supports our hypothesis that MTD(S→A), by its ability to interact with CRM1, should inhibit nuclear export of unphosphorylated HIF-1α. Thus, overexpression of MTD or MTD(S→A) affects HIF-1α localization and modulates HIF-1 activity by interfering with MAPK- or CRM1-dependent functions, respectively.

**DISCUSSION**

Two major ways to control HIF-1α have been previously widely described. The first relies on hydroxylation-dependent degradation/inactivation and appears to be the preferential means of matching HIF-1 activity to oxygen and metabolite levels (6, 22). The second does not depend directly on oxygen levels, involves control of HIF-1α protein synthesis via the phosphatidylinositol 3-kinase/AKT pathway, and may link HIF-1 activity to the growth state of the cell (10, 23). Our work now suggests an additional oxygen-independent mechanism that operates downstream of the synthesis and stabilization steps, involves regulated nuclear export of HIF-1α, responds to MAPK pathway activation, and may thus connect HIF-1 activity to cell proliferation. Its principal component lies in the MTD of HIF-1α in a form of an atypical hydrophobic CRM1-dependent NES which is “masked” when one or two proximal serine residues are phosphorylated by p42/44 MAPK. As also shown for a few other shuttling transcription factors (24–27), inhibition of nuclear export by phosphorylation can be a very efficient way to ensure exclusive nuclear localization and full activation of HIF-1α. This may occur after growth factor stimulation at normoxia or under hypoxia which can also activate itself the inositol 3-kinase/AKT pathway, and may link HIF-1 activity to oxygen and metabolite levels (6, 22). The second does not depend directly on oxygen levels, involves control of HIF-1α protein synthesis via the phosphatidylinositol 3-kinase/AKT pathway, and may link HIF-1 activity to the growth state of the cell (10, 23). Our work now suggests an additional oxygen-independent mechanism

FLAG antibodies or analyzed in reporter gene assays. In nontransfected cells, endogenous DMOG-induced HIF-1α was found exclusively inside the nucleus, whereas in cells overexpressing FLAG-MTD, HIF-1α was distributed in both the nucleus and cytoplasm (Fig. 6A, panels a–d). Accordingly, overexpression of FLAG-MTD caused dramatic reduction of HIF-1 transcriptional activity (Fig. 6B), corroborating our prediction that MTD should inhibit both phosphorylation and nuclear accumulation of HIF-1α. When the cells were treated with PD98059, DMOG-induced HIF-1α and FLAG-MTD were distributed in both the nucleus and cytoplasm (Fig. 6A, panels e–i), indicating that inhibition of the MAPK pathway caused nuclear export not only of endogenous HIF-1α but also of FLAG-MTD.

In cells overexpressing the phosphorylation-deficient FLAG-MTD(S→A) form, the nuclear accumulation of endogenous HIF-1α was not affected, whereas FLAG-MTD(S→A) was itself largely cytoplasmic (Fig. 6A, panels i–l). Exposing the

**FIGURE 5.** Mutation of the NES causes the nuclear accumulation of phosphorylation-deficient HIF-1α in HEK293-T and Huh7 cells. Subcellular localization of GFP (panel a), GFP-HIF-1α (panel b), and its mutant forms (panels c–e as indicated) expressed in HEK-293T (A) and Huh7 (B) cells. 24 h post-transfection cells were fixed and subjected to fluorescence microscopy. Nuclei are stained with DAPI (panels a–e), and white arrowheads indicate the nuclei of transfected cells. In all cases, the cells shown in the micrographs of this figure are representative of greater than 95% of the cells examined in at least two repetitions of the experiment.
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A

MTD wt

MTD wt

MTD (S→A)

MTD (S→A)

B

FIGURE 6. Expression of MTD or MTD(S→A) in HeLa cells modulates the activity of endogenous HIF-1α. A, HeLa cells expressing FLAG-MTD (panels a–h) or FLAG-MTD(S→A) (panels i–p) were treated for 4 h with 1 mM DMOG alone (–) or in combination with 50 μM PD98059 (+). The cells were then fixed, immunostained with affinity-purified anti-HIF-1α rabbit polyclonal (panels a, e, i, and m) and anti-FLAG mouse monoclonal (panels b, f, j, and n) antibodies, counterstained with DAPI (panels d, h, l, and p), and subjected to immunofluorescence microscopy. Images of endogenous HIF-1α localization using a fluorescein isothiocyanate-conjugated secondary antibody are shown in green, whereas images of FLAG-MTD localization using a Cy3-conjugated secondary antibody are shown in red. Merged pictures (merge is represented by yellow) are shown in panels c, g, k, and o. White arrowheads indicate the nuclei of transfected cells. In all cases, the cells shown in the micrographs in this figure are representative of greater than 95% of the cells examined in two repetitions of the experiment. B, determination of HIF-1α transcriptional activity in HeLa cells expressing FLAG, FLAG-MTD, or FLAG-MTD(S→A) and treated with 1 mM DMOG, 50 μM PD98059, or their combination for 4 h (as indicated). Values are in abstract units (RLU, relative luciferase activity) and represent the mean of two independent experiments performed in triplicate (± S.E.).

Composition and spacing. Nevertheless, localization experiments in the presence of leptomycin B (Fig. 3A) as well as immunoprecipitation analysis (Fig. 3, B–D) suggest that this NES physically interacts with CRM1 when the neighboring SP sites are not phosphorylated. We were, however, unable to provide evidence for direct binding of CRM1 to the HIF-1α NES in the presence of Ran-GTP using recombinant bacterially expressed proteins in vitro (data not shown). It is known that many NESs that depend on CRM1 bind to it with low affinity making in vitro reconstitution of nuclear export complexes extremely difficult (21, 30). This may also be the case for the atypical HIF-1α NES. Alternatively, its interaction with CRM1 may require additional cellular factors, such as RanBP3 (21, 30), or be indirect through an unknown adaptor protein that recognizes the conserved hydrophobic residues.

Our work does not exclude additional effects of MAPK-mediated phosphorylation on the HIF-1α pathway. Although the transcriptional inactivity of the HIF-1α S→A mutant matches closely its nuclear exclusion, the reduction in the activity of wild-type HIF-1α in the presence of PD98059 or FLAG-MTD (Fig. 1B, Fig. 4B, and Fig. 6B) is disproportionate to its cytoplasmic mis-localization (Fig. 1A, panel b, Fig. 4A, panel g, and Fig. 6A, panels a–d; respectively). Steady-state distribution of HIF-1α should normally represent a snapshot of a dynamic transport process that involves continuous and rapid shuttling in and out of the nucleus. It is therefore possible that when nuclear export of HIF-1α is stimulated by MAPK inhibition, the nuclear resident time of HIF-1α may still be long enough for optical detection but too short for efficient heterodimerization, binding to DNA, and transcriptional activation. Alternatively, it is also possible that inhibition of HIF-1α phosphorylation by PD98059 or FLAG-MTD negatively affects additional steps such as the interaction of HIF-1α with co-transcriptional activators. In support of this second hypothesis, both FLAG-MTD and GFP-MTD accumulate inside the nucleus (Fig. 2C, panel b, and Fig. 6A, panel b), although their small size should allow free diffusion between nuclear and cytoplasmic compartments. This indicates that HIF-1α MTD may contain, in addition to the NES, an uncharted sequence that upon phosphorylation promotes docking to an unknown nuclear factor required for optimal HIF-1α activity. Finally, treatment with PD98059 causes mild but significant inhibition of activity even in the cases of the phosphomimetic S→E (Fig. 1B) or NES I→A (Fig. 4B) HIF-1α mutants suggesting indirect effects of MAPK on HIF-1-mediated transcription, such as phosphorylation of CBP/p300 and stimulation of its interaction with HIF-1α (31).

In any case, the characterization of the MAPK-targeted NES on HIF-1α opens up the way for further investigation of HIF-1α nuclear transport and its significance for regulating the hypoxia response pathway, especially under conditions that stimulate the MAPK pathway and cellular proliferation. Our findings also offer proof-of-principle that nucleocytoplasmic trafficking may be an intriguing target for controlling HIF-1 activity under pathological conditions involving tissue hypoxia.

Acknowledgments—We are grateful to the other members of our laboratories for their help and useful comments.

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