MgcRacGAP Interacts with HIF-1 α and Regulates its Transcriptional Activity

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Key Words

Hypoxia inducible factor 1 • HIF-1 α • MgcRacGAP • Myo domain • GTPase activating proteins • GAP

Abstract

HIF-1 α is the inducible subunit of the dimeric transcription factor HIF-1 (Hypoxia Inducible Factor 1). It is induced by hypoxia and hypoxia-mimetics in most cell types, as well as non-hypoxic signals such as growth factors, cytokines and oncogenes, often in a cell specific manner. HIF-1 is present in virtually all cells of higher eukaryotes and its function is of great biomedical relevance since it is highly involved in development, tumor progression and tissue ischemia. Intracellular signaling to HIF-1 α , as well as its further action, involves its participation in numerous protein complexes. Using the yeast two-hybrid system we have identified MgcRacGAP (male germ cell Rac GTPase Activating Protein) as a HIF-1 α interacting protein. The MgcRacGAP protein is a regulator of Rho proteins, which are principally involved in cytoskeletal organization. We have verified specific binding of HIF-1 α and MgcRacGAP *in vitro* and *in vivo* in mammalian cells. We have additionally shown that MgcRacGAP overexpression inhibits HIF-1 α

transcriptional activity, without lowering HIF-1 α protein levels, or altering its subcellular localization. Moreover, this inhibition is dependent on the MgcRacGAP domain that interacts with HIF-1 α . In conclusion, our findings demonstrate that HIF-1 α function is negatively affected by its interaction with MgcRacGAP.

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Introduction

HIF-1 is the key molecule for the cellular response to hypoxia. As a transcription factor, it controls the expression of several classes of genes that help the cell adapt to hypoxic stress. HIF-1 affects several cellular and systemic activities such as glucose metabolism, erythropoiesis, angiogenesis, cell motility, proliferation and apoptosis. HIF-1 is comprised of two polypeptide subunits, HIF-1 α , which is itself induced by hypoxia, and HIF-1 β or aryl hydrocarbon receptor nuclear translocator (ARNT), which is constitutively expressed. Both subunits contain basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domains at their N-terminal regions which mediate their dimerisation and binding to hypoxia response elements (HREs) at the promoters of their target genes [1, 2].

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HIF-1 α is induced by hypoxia through an oxygen-dependent post-transcriptional mechanism. Under normoxia, HIF-1 α is continuously degraded by the proteasome because its oxygen dependent degradation (ODD) domain is “marked” by hydroxylation on two prolines, as the result of oxygen availability acting as a substrate of the hydroxylation reaction [3, 4]. Degradation is mediated by interaction of the hydroxylated domain with the Von Hippel-Lindau protein (pVHL), a constituent of an E3 ubiquitin ligase complex [5, 6]. When O₂ levels drop, the prolyl hydroxylases (PHDs) responsible for HIF-1 α hydroxylation are inactive, HIF-1 is stabilized and rapidly accumulates in the nucleus [7, 8]. Phosphorylation by MAPKs of serine residues residing at the inhibitory domain (ID), flanked by the N- and C- terminal transcription activation domains (N-TAD and C-TAD respectively), is responsible for blocking its export from the nucleus [9]. A second type of hydroxylation in the C-TAD by an O₂ dependent asparaginyl hydroxylase named Factor Inhibiting HIF-1 (FIH-1) is responsible for the impairment of HIF-1 transcriptional activity in normoxia, since only the non-hydroxylated C-TAD is able to associate with the transcriptional co-activator CBP/p300 [10]. At least 70 genes have been shown to be directly targeted by HIF-1 [8] and several microarray based studies have suggested that the number of HIF-1 regulated genes is of the order of several hundred [11, 12]. The oxygen dependent responsiveness of HIF-1 elicited intense interest in every aspect of its regulation and function, since hypoxia is involved in several pathological conditions, such as ischemias and cancer [13, 14].

Accumulating evidence over the past few years has shown that physiological stimuli other than hypoxia can result in the induction of HIF-1 α and suggest that HIF-1 may play a wider role in energy metabolism and cellular proliferation than previously appreciated. HIF-1 is induced under normoxic conditions by signals that affect major cellular pathways such as those involving PI3K/AKT, or MAPK/ERK, both of which can be stimulated by growth factors, cytokines, reactive oxygen species (ROS), oncogenes, or hypoxia-mimetic chemicals [15-19]. In many cases and in spite of the ubiquitous expression of HIF-1 α , these effects have been proven to be cell-type specific implying involvement of HIF-1 α in different tissue-specific cellular functions.

While in action, HIF-1 α forms complexes with several different cellular proteins which interact with specific regions of HIF-1 α , such as FIH-1 [20], ARD1 [21], Jab1 [22], OS-9 [23] and necdin [24]. Many of them

have been identified by yeast two-hybrid screenings and in several cases, new aspects of HIF-1 function have been revealed. We have also used the yeast two-hybrid system to discover yet unknown molecular partners of HIF-1 α . We show in this study that HIF-1 α interacts *in vitro* and *in vivo* with MgcRacGAP, a Rac1 GTPase activating protein, which downregulates HIF-1 transcriptional activity.

Materials and Methods

Plasmids

Plasmid pGBT9-HIF-1 α (244-532) was constructed as follows: the cDNA of full length HIF-1 α was amplified by PCR from plasmid pcDNA3-HIF-1 [25], using as primers:

forward: TTTTTC^{CCCGG}CCGATTCACCATGGAG

reverse: TTTTGGATCCGTTAACTTGATCCAAAGCTCTG

(SmaI and BamHI sites underlined, start and stop codons are in italics respectively). The PCR product was cloned into the SmaI and BamHI sites of the pGBT9 vector (BD Biosciences Clontech, Palo Alto, CA U.S.A.), sequenced, digested with SalI and the SalI fragment was subcloned into the SalI site of pGBT9. Plasmid pGEX4T1-MgcRacGAP(1-138) was constructed as follows: the insert of the murine clone isolated from the two-hybrid screening encoding for the amino-terminal domain of MgcRacGAP(1-138) was inserted in the NotI site of the bacterial expression vector pGEX4T1 (Amersham Biosciences, Piscataway, NJ U.S.A.) in frame with the Glutathione S-transferase (GST) open reading frame. Plasmid pHisGFP-HIF-1 α (244-532) was constructed as follows: the BamHI fragment of pGBT9-HIF-1 α (244-532) was subcloned in the BamHI site of the pHisGFP plasmid [26]. The following plasmids have been previously described: pEGFP-HIF-1 α [18], pME18S-FLAG, pME18S-FLAG-MgcRacGAP and pME18S-FLAG- Δ Myo-MgcRacGAP [27], pGL3-5HRE-VEGF [28], pGLHIF1.3 and pGLHIF1mt.3 [29]. pGL3-basic was from Promega (Promega Corporation, Madison, USA).

Yeast Two-Hybrid Screening

S. cerevisiae strain pJ69-4A [30] was co-transformed with the pGBT9-HIF-1 α (244-532) plasmid and an equimolar mixture of E9.5 and E10.5 mouse embryonic cDNA library which has been constructed in the pVP16 fusion vector [31]. Positive clones were selected on the appropriate synthetic complete (SC) medium without tryptophan, leucine, histidine and adenine, containing 5 mM AT (3-amino-1,2,4-triazole) (Sigma-Aldrich) and consequently assayed for activation of the *LacZ* gene on X-gal plates. The prey plasmids from the selected clones were rescued in *E. coli* and used to retransform the original yeast strain containing the bait plasmid. Clones that recovered the original phenotype (survival on AT, β -galactosidase expression) were further analyzed by sequencing performed by the Microchemistry Laboratory of the Institute of Molecular Biology and Biotechnology (IMBB) of the Research Center of Crete (RCC) (Heraklion, Crete, Greece).

Cell cultures and Transfections

Human cervical carcinoma cells (HeLa) or human embryonic kidney 293 cells (293T) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) containing 10% fetal bovine serum (FBS, Biochrom KG Seromed), antibiotic-antimycotic solution (Gibco BRL) and 2 mM L-glutamine (Biochrom KG Seromed). Cells were maintained at 37°C in a 5% CO₂/95% air incubator. For hypoxic exposures, cells were placed in a sealed chamber (Billups-Rothenberg) and flooded with 1% O₂/5% CO₂/94% N₂. When required, desferrioxamine (DFO) or MG132 (Sigma Chemicals) were added to a final concentration of 150 µM and 10 µM respectively, for 4 h.

Transient transfections were carried out in 10 cm dishes or 12-well plates using the Transpass™ D2 Transfection Reagent (New England Biolabs Inc.). In each experiment the total amount of plasmid DNA was adjusted to 10 µg/dish or 2 µg/well, according to the manufacturer's instructions.

SDS-PAGE and Western analysis

Protein samples were resolved by 8% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Coomassie blue staining or Western blotting using an anti-HIF-1α mouse monoclonal antibody (1:500; BD Transduction Laboratories, Franklin Lakes, NJ, U.S.A.), or a rabbit anti-VEGF polyclonal antibody (1:200, Santa Cruz Biotechnology, California, U.S.A.) or an anti-F-actin mouse monoclonal antibody (1:500; Serotec, Kidlington, Oxford, UK.) or a goat anti-GST polyclonal antibody (1:1000; Amersham Biosciences Piscataway, NJ, U.S.A.). Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse IgG goat (1:3000; Bio-Rad Laboratories, Hercules, CA U.S.A.) or anti-rabbit IgG (1:3000, Cell Signaling, Beverly, MA U.S.A.) or anti-goat IgG mouse (1:3000; Jackson Immunoresearch, Baltimore Pike, U.S.A.) antibody, followed by ECL detection (Amersham).

Protein expression and purification

To prepare GST fusion proteins, E. coli BL21 (RIL) cells harbouring the relevant recombinant expression plasmids were cultured in the presence of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 28°C for 4 h. Cells were suspended in ice-cold buffer A (1x PBS, 5 mM DTT, 0.1 mM PMSF) containing EDTA-free protease inhibitor cocktail (Roche), the cell suspension was sonicated and insoluble debris was pelleted by centrifugation (12,000g for 15 min at 4°C). The supernatants were mixed with Glutathione (GSH) Sepharose Beads (Amersham Biosciences, Piscataway, NJ U.S.A.) at 4°C for 30 min and bound proteins were eluted with GSH Elution Buffer (10 mM GSH, 50 mM Tris-HCl pH 8.0). The purity and quantity of isolated GST-fusion proteins was examined using SDS-PAGE followed by Coomassie Blue staining or detection with the anti-GST antibody.

To prepare His-tagged proteins, E. coli BL21 (RIL) cells harbouring the relevant recombinant expression plasmids were lysed by sonication in ice cold buffer B (50 mM Tris-Cl, pH 7.4, 20 mM NaCl, 5 mM MgCl₂, 5 mM β-mercaptoethanol, 5% glycerol, 1 mM PMSF, 0.1% Triton X-100) containing EDTA-free protease inhibitor cocktail. The cell suspension was sonicated

and insoluble debris was pelleted by centrifugation (12,000g for 15 min at 4°C). The supernatants were mixed with Ni-NTA-agarose beads (Qiagen) at 4°C for 60 min and bound proteins were eluted with 250 mM imidazole. The purity and quantity of isolated His-tagged proteins was examined using SDS-PAGE followed by Coomassie Blue staining or detection with anti-HIF-1α or anti-GFP monoclonal antibodies (1:1000; Roche, Indianapolis, U.S.A.).

Pull down binding assays

For the GST-pull down binding assay, ~50 µg of GST or GST-MgcRacGAP(1-138) immobilized on GSH Sepharose beads were incubated in reaction buffer A with ~85 µg of purified HisGFP-HIF-1α(244-532) protein. After 4 h of incubation at 4°C, the beads were washed three times with cold buffer A. The bound proteins were eluted in SDS sample buffer and analyzed by SDS-PAGE and bound proteins were visualized by Western blotting using the relevant antibody.

For the His-pull down binding assay, ~25 µg Ni-NTA immobilized HisGFP, HisGFP-HIF-1α(244-532) or HisGFP-HIF-1α were incubated in reaction buffer B with ~50 µg of purified GST-MgcRacGAP(1-138) protein. The following steps were as in the GST-pull down assay, except for buffer B in the washes.

Immunoprecipitation

HeLa (or 293T) cells were cultured in 10 cm dishes, transfected with equal quantities of plasmids expressing GFP or GFP-HIF-1α and FLAG or FLAG-MgcRacGAP or FLAG-ΔMyo-MgcRacGAP, and lysed 24 h after transfection in lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 5 mM β-mercaptoethanol, 1 mM PMSF, 1% Triton X-100, EDTA-free protease inhibitor cocktail). The cell suspension was centrifuged at 12,000g for 15 min at 4°C, the volume of the supernatant was adjusted to 500 µl with lysis buffer and quantified by the Bio-Rad Protein assay Dye Reagent (Bio-Rad Laboratories GmbH). Samples were incubated with 0.6 µg mouse anti-FLAG M2 monoclonal antibody (Sigma, Saint Louis, Missouri, U.S.A.) or 1 µl of a rabbit polyclonal anti-GFP serum, generously provided by Dr. H. Boleti (Hellenic Pasteur Institute, Athens, Greece) for 3 h at 4°C. Twenty µl of Protein G-Sepharose beads (Sigma-Aldrich, St. Louis, MO U.S.A.) or Protein A-Sepharose beads (Amersham Biosciences, Piscataway, NJ U.S.A.) were added respectively and incubation was continued for 12 h at 4°C. Beads were collected by centrifugation, washed three times with lysis buffer and bound proteins were eluted in SDS sample buffer, followed by SDS-PAGE and Western blotting with the anti-HIF-1α mouse monoclonal antibody.

Luciferase assays

HeLa (or 293 T) cells were co-transfected with 0.6 µg of each plasmid, except the β-galactosidase expressing plasmid pCMV-lacZ, which was always 0.3 µg per well. Transfected cells were cultured in 12-well plates for 24 h and subsequently incubated for 4 h under hypoxia (1% O₂) or in the presence of 150 µM DFO. Luciferase activity was determined in the extract by a chemiluminescence assay kit (Promega, Madison, Wis U.S.A.) as described by the manufacturer, with a luminometer (TD20/20, Turner Designs) and values were normalized to their

Fig. 1. HIF-1 α interacts with MgcRacGAP in *S. cerevisiae*: (A) Schematic representation of the HIF-1 α protein domains. The underlying bar indicates the region used as bait in the two-hybrid screening. (B) Replica plating of *S. cerevisiae* pJ69-4A strain transformed with plasmids as indicated in the schematic representation (right): 1. pGBT9-HIF-1 α (244-532) and pVP16-MgcRacGAP (1-138) (four different colonies), 2. pGBT9 and pVP16 (two different colonies, negative control), 3. pGBT9-SRPK1 α Nt and pVP16-SAF-B (two different colonies, positive control) [63]. The strains were grown on SC medium -Trp, -Leu, -Ade, -His, +50 mM AT (left) or SC medium -Trp, -Leu, +X-gal (right).

β -galactosidase activity and expressed as fold-increase in relation to cells transfected with the empty GFP or FLAG vectors.

Immunofluorescence

HeLa cells grown on coverslips were transiently transfected with FLAG or FLAG-MgcRacGAP or FLAG- Δ Myo-MgcRacGAP expressing plasmids and subjected to hypoxia or treated with DFO or MG132. Twenty four hours posttransfection the cells were washed once with PBS and fixed with 3% formaldehyde in PBS for 5 min at room temperature. Cells were then washed twice with PBS and permeabilized with PBS containing 0.1% Triton X-100 for 15 min. After two washes with PBS, cells were treated with 3% BSA in PBS for 1 h. Coverslips were incubated for 1 h at room temperature with an affinity purified monoclonal anti-FLAG antibody (Sigma, 1:250 dilution in PBS containing 1% BSA), washed twice with PBS and subsequently incubated for 1 h at room temperature with a rabbit polyclonal anti-HIF-1 α antibody. This antibody was raised in rabbits using as antigen recombinant GST-HIF-1 α ₃₄₈₋₈₂₆ [9]. Anti-GST antibodies were removed by passing the serum over a GST-Sepharose column. The antibody was subsequently recovered by affinity purification over nitrocellulose membrane-immobilized antigen and used in a final concentration of 2 μ g/ml in PBS containing 1% BSA. Primary antibodies were detected by incubation with FITC-conjugated anti-mouse secondary antibody (1:50 dilution in PBS containing 1% BSA) and CY3-conjugated anti-rabbit secondary antibody (1:50 dilution in PBS containing 1% BSA) respectively. After washing twice with PBS, cells were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 2 min and mounted on slides. Images were collected on an Axioscope 40 Zeiss microscope.

Results

HIF-1 α interacts with MgcRacGAP in yeast cells and in vitro

To identify new candidate proteins that interact with HIF-1 α we performed a yeast two-hybrid screen using as bait a part of HIF-1 α corresponding to amino acid residues 244-532. This polypeptide encompasses a part of the PAS B domain and the region between the PAS B and N-TAD domains of HIF-1 α (Fig. 1A), to which no functions or interactions have been attributed so far. All previous studies applying the yeast two-hybrid system to identify HIF-1 α partners (see Introduction) have used as bait the C-terminal part of the protein. We thus reasoned that novel interactions might emerge by using as bait the central part of HIF-1 α .

The cDNA corresponding to amino acid residues 244 to 532 of human HIF-1 α was fused to the DNA-binding domain of the yeast transcription factor GAL4 and used as bait to screen a mouse embryonic (days E9.5 and E10.5) cDNA library. A total of 12x10⁶ transformants was screened, and from the 120 different clones selected initially, twelve recovered the original phenotype. Among the finally characterized clones, DNA sequencing revealed that the nucleotide sequence of one of them encoded for mMgcRacGAP (AB030252). More precisely, this cDNA clone contains nucleotides -20 to +414, encoding for the amino acid residues 1-138 of mMgcRacGAP, containing the Myo (myosin like coiled coil) domain of the protein

Fig. 2. HIF-1 α binds to MgcRacGAP in vitro: (A) In vitro pull down assay of purified HisGFP-HIF1 α (244-532) protein with GST or GST-MgcRacGAP(1-138) immobilized on GSH-Sepharose beads. Samples were resolved by SDS-PAGE and immunoblotted with the anti-GFP antibody. Input was 1/10 of the quantity of HisGFP-HIF1 α (244-532) used for the binding assay. Flowthrough was 1/10 of the quantity recovered. (B) In vitro pull down assay of purified GST-MgcRacGAP(1-138) protein with HisGFP or HisGFP-HIF1 α (244-532) immobilized on Ni-NTA Sepharose beads. Samples were resolved by SDS-PAGE and immunoblotted with the anti-GST antibody. Input was 1/10 of the quantity of GST-MgcRacGAP(1-138) used for the binding assay. Flowthrough was 1/10 of the quantity recovered. (C) In vitro pull down assay of purified GST-MgcRacGAP(1-138) protein with HisGFP or HisGFP-HIF1 α (244-532) immobilized on Ni-NTA Sepharose beads. Samples were resolved by SDS-PAGE and immunoblotted with the anti-GST antibody. Input was 1/10 of the quantity of GST-MgcRacGAP(1-138) used for the binding assay. Flowthrough was 1/10 of the quantity recovered. FT: flowthrough, E: eluate.

(amino acids 41-124) [32]. Fig. 1B shows the high expression of the reporter genes HIS3 and LacZ when the bait region of HIF-1 α is co-expressed with the selected mMgcRacGAP clone in the original *S. cerevisiae* strain. The transformed strain is resistant to 50 mM AT, suggesting a strong interaction between the two proteins in yeast cells.

To demonstrate a direct interaction between HIF-1 α and MgcRacGAP we performed *in vitro* pull down binding assays with bacterially expressed HIF-1 α and MgcRacGAP. The fragment of HIF-1 α used as bait in the two-hybrid system was expressed in *E. coli* as a HisGFP-fusion protein (HisGFP-HIF-1 α (244-532)) and purified. Accordingly, the domain of mMgcRacGAP identified by the yeast two-hybrid assay (MgcRacGAP(1-

138)) was also expressed in *E. coli* as a GST-fusion protein. Fig. 2A shows that HisGFP-HIF-1 α (244-532) binds to GST-MgcRacGAP(1-138), since it is specifically retained by beads carrying GST-MgcRacGAP(1-138), but not GST alone (Fig. 2A, compare lanes 3 and 5). Conversely, when HisGFP-HIF-1 α (244-532) was immobilized on Ni-NTA-Sepharose beads, these beads could specifically retain GST-MgcRacGAP(1-138) as shown in Fig. 2B (compare lanes 3 and 5). To verify this interaction in the context of full-length HIF-1 α , bacterially expressed full length HisGFP-HIF-1 α was immobilized on Ni-NTA-Sepharose beads. These beads could also retain specifically GST-MgcRacGAP(1-138) (Fig. 2C). These results demonstrate that the Myo domain of MgcRacGAP interacts *in vitro* with HIF-1 α .

Fig. 3. HIF-1 α interacts with MgcRacGAP *in vivo*: HeLa cells were transfected with GFP-HIF-1 α (pEGFP-HIF-1 α) and FLAG (pME18S-FLAG), or FLAG- Δ Myo-MgcRacGAP (pME18S-FLAG- Δ Myo-MgcRacGAP) or FLAG-MgcRacGAP (pME18S-FLAG-MgcRacGAP) expressing plasmids, as indicated. Upper panel: Western blotting with an anti-HIF-1 α antibody of the 1/10 of the quantity of the total cell extracts that were used for the immunoprecipitation. Lower panel: Total cell extracts were immunoprecipitated with an anti-FLAG antibody and immunoprecipitates were immunoblotted with an anti-HIF-1 α antibody. The heavy chain of the anti-FLAG antibody is shown as antibody quantity control. In three cases (lanes 2, 4 and 6) MG132 was added in the cell culture 4 h prior to lysis.

HIF-1 α interacts with MgcRacGAP in human cells

To verify that HIF-1 α and MgcRacGAP also interact in living cells, human HeLa cells were co-transfected with expression vectors encoding GFP-tagged full length human HIF-1 α and FLAG-tagged full length human MgcRacGAP. Immunoprecipitation with an anti-FLAG antibody from whole cell extracts and subsequent immunoblotting assays performed with an anti-HIF-1 α antibody, revealed association of GFP-HIF-1 α with FLAG-MgcRacGAP (Fig. 3, lane 5, lower panel). Interaction of HIF-1 α with MgcRacGAP under these conditions was specific, since no HIF-1 α was detected in the immunoprecipitates of cells transfected with the empty vector expressing only the FLAG epitope (Fig. 3, lane 1, lower panel). Moreover, HIF-1 α was not detected in the immunoprecipitates of cells transfected with the plasmid expressing FLAG- Δ Myo-MgcRacGAP, a deletion mutant of MgcRacGAP lacking amino acids 1-106 [32] (Fig. 3, lane 3, lower panel). When MG132, a proteasome inhibitor stabilizing HIF-1 α , was added to the cell culture, the quantity of HIF-1 α bound to MgcRacGAP was accordingly increased (Fig. 3, lane 6, lower panel), but still no HIF-1 α protein was detected in the control immunoprecipitates (Fig. 3, lanes 2 and 4, lower panel). Similar experiments were performed in 293T cells producing the same results (data not shown).

These results demonstrate that HIF-1 α interacts with MgcRacGAP *in vitro* as well as *in vivo*, and suggest that the Myo domain of MgcRacGAP is responsible for this interaction.

Overexpression of MgcRacGAP inhibits HIF-1 transcriptional activity

To investigate whether the MgcRacGAP/HIF-1 α interaction affects HIF-1 function, we examined the hypoxia—or DFO—induced HIF-1 transcriptional activity in the presence or absence of overexpressed human MgcRacGAP. Transcriptional activity of HIF-1 was measured by the use of an HRE-driven luciferase reporter gene. HeLa cells were co-transfected with the pGL3-basic or pGL3-5HRE-VEGF plasmids and the FLAG, FLAG-MgcRacGAP or FLAG- Δ Myo-MgcRacGAP expressing plasmids. Twenty four hours after transfection, cells were either subjected to hypoxia (1% O₂) or treated with 150 μ M DFO for 4 hours before determination of luciferase activity. As shown in Fig. 4A, induction of endogenous HIF-1 α by hypoxia or DFO, increased HIF-1 transcriptional activity by approximately 10 and 16 fold respectively. However, co-expression of full length MgcRacGAP dramatically inhibited the activity of HIF-1 in both cases. Interestingly, the activity of HIF-1 was not significantly reduced in the presence of the Δ Myo-MgcRacGAP mutant protein, confirming the involvement of the Myo domain in the interaction with HIF-1 α (Fig. 4A).

To confirm the inhibitory effect of MgcRacGAP on HIF-1 α , we transfected HeLa cells with plasmids expressing GFP-HIF-1 α and FLAG-MgcRacGAP and assayed for luciferase activity. As shown in Fig. 4B, expression of GFP-HIF-1 α alone specifically increased HRE driven luciferase activity by approximately 15 fold under normoxia. Co-expression of full length

Fig. 4. HIF-1 α mediated luciferase activity and endogenous VEGF expression is suppressed by MgcRacGAP overexpression in HeLa cells: (A) Reporter gene assays of the transcriptional activity of HIF-1 determined 24 hours after transfection of HeLa cells with plasmids producing the indicated proteins (under the graph), under the indicated conditions (in the legend at the right of the graph). (B) Reporter gene assays of the transcriptional activity of HIF-1 determined 24 h after transfection of HeLa cells with the plasmids expressing the indicated proteins (under the graph and in the legend at the right of the graph). The values shown were determined as the mean of three different experiments performed in duplicate, normalized to the corresponding β -galactosidase value and expressed as fold increase over the values obtained with cells transfected with the empty vectors and the luciferase plasmids. (C) HeLa cells were transfected with FLAG (pME18S-FLAG) or FLAG-MgcRacGAP (pME18S-FLAG-MgcRacGAP) and GFP (pEGFP-C1) or GFP-HIF-1 α (pEGFP-HIF-1 α) expressing plasmids as indicated, or treated with DFO for 4 h prior to lysis. Total cell extracts were immunoblotted with an anti-VEGF antibody (upper panel). Equal protein loading was confirmed by reprobing the membrane for actin expression (lower panel).

MgcRacGAP inhibited this activity by at least 50%, whereas Δ Myo-MgcRacGAP reduced HIF-1 α activity by less than 10%. Similar results were obtained in experiments performed with a luciferase reporter gene plasmid (pGLHIF1.3) containing HREs from the erythropoietin (EPO) promoter, confirming that the luciferase activity measured was dependent on HIF-1 driven transcription (data not shown).

The inhibitory effect of MgcRacGAP on HIF-1 transcriptional activity was further confirmed when the levels of endogenous VEGF protein were examined in cells co-expressing HIF-1 α with MgcRacGAP. As shown in Fig. 4C, when GFP-HIF-1 α was expressed in HeLa cells its target gene *VEGF* was induced and the VEGF protein was detected in whole cell extracts (lane 4) in the same position as in untransfected cells treated with DFO

Fig. 5. HIF-1 α protein levels and subcellular localization are not affected by MgcRacGAP overexpression. (A) HeLa cells were transfected with FLAG (pME18S-FLAG) or FLAG-MgcRacGAP (pME18S-FLAG-MgcRacGAP) and GFP (pEGFP-C1) or GFP-HIF-1 α (pEGFP-HIF-1 α) expressing plasmids, as indicated. Total cell extracts were immunoprecipitated with an anti-GFP antibody and immunoprecipitates were immunoblotted with an anti-HIF-1 α antibody. The heavy chain of the anti-GFP antibody is shown as antibody quantity control (lower panel). In three cases (lanes 4, 5 and 6) MG132 was added in the cell culture 4 h prior to lysis. (B) HeLa cells grown on coverslips were transfected with FLAG (pME18S-FLAG) or FLAG-MgcRacGAP (pME18S-FLAG-MgcRacGAP) or FLAG- Δ Myo-MgcRacGAP (pME18S-FLAG- Δ Myo-MgcRacGAP) expressing plasmids, as indicated. Twenty four hours after transfection the cells were subjected for 4 h at 1% O₂ or left at normoxic conditions (20% O₂) and were subsequently stained with the rabbit anti-HIF-1 α polyclonal antibody (red), the mouse anti-FLAG monoclonal antibody (green) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for DNA staining (blue) and subjected to immunofluorescence microscopy.

(lane 2). Expression of FLAG-MgcRacGAP alone did not affect VEGF expression (lane 3 compared to lane 1), whereas its presence in GFP-HIF-1 α overexpressing cells reduces VEGF levels by at least 50% (lane 5, compare with lane 4).

Taken together, our experiments show that MgcRacGAP overexpression severely impairs HIF-1 transcriptional activity and this inhibition is largely dependent on the presence of the Myo domain which mediates the physical interaction between HIF-1 α and MgcRacGAP.

Inhibition of HIF-1 activity by MgcRacGAP is not due to altered localization or stability of HIF-1 α

The data shown in Fig. 3 suggest that the expression of HIF-1 α is not affected by the simultaneous overexpression of MgcRacGAP (Fig. 3, compare lanes 1, 3 and 5, upper panel). To verify this observation we immunoprecipitated GFP-HIF-1 α from cells co-transfected with or without MgcRacGAP. Fig. 5A clearly shows that HIF-1 α protein levels are not detectably affected by the overexpression of MgcRacGAP (Fig. 5A,

compare lanes 2 and 3). When the HIF-1 α stabilizing agent MG132 was added in the cell cultures, the amount of HIF-1 α in the immunoprecipitate was increased, irrespective of the absence or presence of overexpressed MgcRacGAP (Fig. 5A, lanes 5 and 6). Thus, the inhibition of HIF-1 activity in MgcRacGAP overexpressing cells (Fig. 4) is not due to a downregulation of HIF-1 α protein levels.

This is also confirmed by immunofluorescence experiments performed, in order to examine the subcellular localization of endogenous HIF-1 α upon MgcRacGAP overexpression. HeLa cells transfected with FLAG, FLAG-MgcRacGAP, or FLAG- Δ Myo-MgcRacGAP expressing plasmids, were treated by hypoxia, DFO or MG132. As shown in Figure 5B, hypoxia induces HIF-1 α which accumulates in the nucleus, as expected. FLAG-MgcRacGAP localizes also exclusively in the nucleus both under normoxia and hypoxia. In cells overexpressing MgcRacGAP or Δ Myo-MgcRacGAP the level of HIF-1 α induction is apparently not affected (compared to untransfected cells) and HIF-1 α remains inside the nucleus. We obtained the same results when the cells were treated with DFO or MG132 (data not shown).

Taken together, our results suggest that the interaction between MgcRacGAP and HIF-1 α impairs the transcriptional activity of HIF-1 without significantly affecting the localization or HIF-1 α protein expression level.

Discussion

In this study, we have used the central part of the HIF-1 α molecule as bait in a yeast two-hybrid screen in order to identify new interacting molecules and consequently, novel HIF-1 functions. This screen, led to the identification of the Myo domain of MgcRacGAP as a potential binding partner of HIF-1 α . HIF-1 α can also interact with MgcRacGAP *in vitro*, as shown by pull down binding assays and in mammalian cells, as demonstrated by co-immunoprecipitation experiments. The region of MgcRacGAP that we have shown to interact with HIF-1 α is situated in its most N-terminal part and harbors a coiled coil motif, called Myo domain. This domain of MgcRacGAP has been shown to directly interact with α -, β - and γ -tubulins, mediating association of MgcRacGAP with the microtubules [27].

MgcRacGAP belongs to the family of GTPase activating proteins (GAPs) which interact with the GTP-

bound small G proteins of the Rho family and stimulate GTP hydrolysis. Thus, they negatively regulate the function of Rho proteins, accelerating transition to their inactive GDP-bound form. RhoA, Rac1 and Cdc42, the prototypes of the Rho family GTPases, regulate signaling pathways that control cytoskeletal organization [33, 34]. MgcRacGAP has specificity for Rac1 and Cdc42 [32, 35, 36] and when phosphorylated, for RhoA [37]. It has been initially isolated as a Rac GTPase interacting protein primarily expressed in male germ cells [35], as a differentiation regulated transcript in adipocytes [38] and as a protein involved in growth and differentiation of leukaemic cells [32]. However, the most prominent and well studied role of MgcRacGAP is in the M phase of the cell cycle, where it is required for correct cytokinesis, affecting, in conjunction with many proteins, RhoA and Cdc42 function [27, 37, 39-43]. As a subunit of the centralspindlin complex it controls the assembly of the contractile ring and the ingression of the cleavage furrow during cytokinesis [43]. Accordingly, MgcRacGAP null mutant mice die during preimplantation development due to defective cell divisions [44].

We have examined whether the HIF-1 α /MgcRacGAP interaction interferes with HIF-1 function and we have indeed demonstrated by luciferase reporter assays and by the examination of the levels of expression of an endogenous HIF-1 target gene, that overexpression of MgcRacGAP impairs the transcription activity of HIF-1. Interestingly, the mutant form of MgcRacGAP lacking the Myo domain practically does not affect HIF-1 activity, implying that this domain is necessary for the inhibition. Since the same domain also specifically interacts with HIF-1 α , it is very likely that the inhibition is a result of the direct association of HIF-1 α with MgcRacGAP. However, this association does not result in destabilization or mislocalisation of HIF-1 α . In cells overexpressing MgcRacGAP no reduction in the protein levels of HIF-1 α is observed and HIF-1 α remains nuclear.

Our reporter gene assays have shown that HIF-1 barely retains a fragment of its transcriptional activity (or that only a minor fragment of HIF-1 molecules retain activity) upon overexpression of MgcRacGAP. The region of HIF-1 α found to interact with MgcRacGAP contains a large part of the PAS B domain known to be required for its interaction with ARNT [45]. It is possible that intranuclear complexing of MgcRacGAP with HIF-1 α might sequester it from the HIF-1 heterodimers, thereby effectively reducing transcriptionally active HIF-1. Similarly, intranuclear interaction of HIF-1 α with MgcRacGAP could affect its association with

transcriptional effectors, influencing some step leading to transcriptional regulation of its target genes. MgcRacGAP has been shown to affect transcriptional activity of another transcription factor, STAT3, in a leukemic cell line [46]. In a following study, it has been shown that MgcRacGAP is necessary, together with the GTP-bound form of Rac1, for the nuclear translocation of STAT transcription factors [47]. Such a mechanism does not seem to be the reason for the inhibition of HIF-1 α activity, since in our experiments FLAG-MgcRacGAP is localized in the nucleus under all conditions and does not affect the nuclear accumulation of HIF-1 α .

The functional relationship between HIF-1 α and MgcRacGAP revealed in our studies may reflect an involvement of the cytoskeleton in the cellular response to hypoxia. Indeed, alterations of cytoskeletal structures in response to hypoxia have been documented in many different cellular systems such as renal cells, neurons, hepatic endothelial, pulmonary endothelial, and alveolar epithelial cells [48-53]. Regulation of actin cytoskeleton organization is mediated by members of the Rho GTPase family and Rho protein function has been shown in several cases to be implicated in hypoxia induced cytoskeletal changes [52] [54-58]. However, there are very few previous studies relating HIF-1 function directly to Rho protein function. In the first case, Rac1, the prototype substrate of MgcRacGAP was shown to be activated by hypoxia and to be required for hypoxic activation and, more precisely, HIF-1 α N-TAD function [59]. In a second study with renal carcinoma cells, RhoA has been shown to be required for HIF-1 α mRNA and protein up-regulation [60]. Finally, it has been shown that attenuation of Rac1 expression by siRNA, or dominant negative mutants of Rac1, inhibit HIF-1 α expression in different cell lines [59, 61, 62]. In the present study, we have shown that MgcRacGAP, an inhibitor of Rho protein activity, interacts with and impairs the function of HIF-1 α . Our data are therefore in accordance with the above mentioned studies, suggest a possible link between Rho/Rac

inactivation and HIF-1 inhibition via MgcRacGAP and support the requirement of active Rho/Rac protein for HIF-1 transcriptional activation.

In conclusion, although the *in vivo* function of the HIF-1 α /MgcRacGAP complex is hard to address at this point and its physiological significance still remains to be elucidated, our overexpression studies clearly demonstrate inhibition of HIF-1 α activity by its specific interaction with MgcRacGAP. We are currently interested in investigating the exact molecular mechanism by which MgcRacGAP interferes with HIF-1 α activity and to identify under which conditions the complex is natively formed in the cells. Our data suggest a mechanism involving a cross-talk between signalling pathways regulating cytoskeletal organization and the hypoxia response. Such a pathway convergence might be frequent either under physiological conditions during embryonic development or under pathological conditions like metastatic cancers where migratory mechanisms are often in action under hypoxic conditions.

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