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MgcRacGAP, a cytoskeleton regulator, inhibits HIF-1 transcriptional activity by blocking its dimerization

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ABSTRACT

Hypoxia inducible factor-1 (HIF-1), a dimeric transcription factor of the bHLH-PAS family, is comprised of HIF-1α, which is inducible by hypoxia and ARNT or HIF-1β, which is constitutively expressed. HIF-1 is involved in cellular homeostasis under hypoxia, in development and in several diseases affected by oxygen availability, particularly cancer. Since its expression is positively correlated with poor outcome prognosis for cancer patients, HIF-1 is a target for pharmaceutical therapy. We have previously shown that male germ cell Rac GTPase activating protein (MgcRacGAP), a regulator of Rho proteins which are principally involved in cytoskeletal organization, binds to HIF-1α and inhibits its transcriptional activity. In this work, we have explored the mechanism of the MgcRacGAP-mediated HIF-1 inactivation. We show that the Myo domain of MgcRacGAP, which is both necessary and sufficient for HIF-1 repression, binds to the PAS-B domain of HIF-1α. Furthermore MgcRacGAP competes with ARNT for binding to the HIF-1α PAS-B domain, as shown by *in vitro* binding pull down assays. In mammalian cells, ARNT overexpression can overcome the MgcRacGAP-mediated inhibition and MgcRacGAP binding to HIF-1α *in vivo* inhibits its dimerization with ARNT. We additionally present results indicating that MgcRacGAP binding to HIF-1α is specific, since it does not affect the transcriptional activity of HIF-2, a close evolutionary relative of HIF-1 also involved in hypoxia regulation and cancer. Our results reveal a new mechanism for HIF-1 transcriptional activity regulation, suggest a novel hypoxia-cytoskeleton link and provide new tools for selective HIF-1 inhibition.

1. Introduction

HIF-1 is a transcriptional activator that regulates the transcription of several hundreds of genes. Changes in the transcriptional activity of HIF-1 target genes promote survival of cells under low oxygen tension (hypoxia) or other stimuli. HIF-1 acts in all mammalian cells, in adult as well as in embryonic organisms under physiological or pathological conditions. It is a heterodimer comprising the inducible regulatory HIF-1α subunit and the constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT) subunit, both members of the basic helix–loop–helix (bHLH) Per-ARNT-Sim (PAS) family of transcription factors [1]. This family is characterized by the presence of the PAS dimerization domain that contains two conserved core repeats, PAS-A and PAS-B [2]. HIF-1α is induced by hypoxia through an oxygen dependent post-transcriptional mechanism: under normoxia, HIF-1α is continuously degraded by the proteasome because its oxygen dependent degradation (ODD) domain is hydroxylated on two prolines, as the result of oxygen availability (used as a substrate in the hydroxylation reaction) [3,4]. Degradation is mediated by interaction of the hydroxylated domain of HIF-1α with the von Hippel–Lindau protein (pVHL), a constituent of an E3 ubiquitin ligase complex [5]. When O_2 levels drop, the prolyl hydroxylases (PHDs) responsible for HIF-1α hydroxylation are inactive, HIF-1α is stabilized, rapidly accumulates in the nucleus, heterodimerizes with ARNT via the PAS domain and the heterodimer binds to hypoxia responsive elements (HREs) residing on the promoters of its target genes, altering the transcriptome of the cell according to the stimulus that induced HIF-1α. The non-inducible subunit, ARNT, heterodimerizes not only with HIF-1α and HIF-2α but also with aryl hydrocarbon receptor (AhR) and other molecules such as estrogen receptor (ER) α and β [6], Rel/B, CD30 [7] playing a role in responses such as xenobiotic metabolism, immune response and diabetes [8]. HIF-1α expression and activity are additionally

Abbreviations: HIF-1, hypoxia inducible factor 1; ARNT, aryl hydrocarbon receptor nuclear translocator; MgcRacGAP, male germ cell Rac GTPase activating protein; bHLH, basic helix–loop–helix; PAS, Per-ARNT-Sim; ODD, oxygen dependent degradation; pVHL, von Hippel–Lindau protein; PHDs, prolyl hydroxylases; HREs, hypoxia responsive elements; AhR, aryl hydrocarbon receptor; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; MKLP1, mitotic kinesin-like protein 1; TACC3, transforming acidic coiled-coil protein 3; Ect2, epithelial cell-transforming sequence 2; CK1, casein kinase 1; Ainp1, ARNT interacting protein 1; RACK1, receptor of activated protein kinase C 1; Hsp90, heat shock protein 90

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regulated by oxygen-independent mechanisms: HIF-1α 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, metabolic signals, reactive oxygen species (ROS), oncogenes, or hypoxia-mimetic chemicals [9,10]. HIF-2α, α protein 48% identical to HIF-1α, but less studied, also participates to the cellular response to hypoxia being globally regulated similarly to HIF-1α. However, there are several aspects that differentiate between the two paralogues in tissue specificity, regulation, target gene specificity and participation in tumoral growth [11].

The cause of HIF activation, hypoxia and/or deregulation of signaling pathways is often observed in cancer cells. Intratumoral hypoxia, loss-of-function of tumor-suppressor genes and gain of function of oncogenes and viral transforming genes, induce HIF-1α. Levels of HIF-1α correlate with tumor growth, vascularization and metastasis both in animal models and in clinical studies. Induction of HIF-1α leads to stimulation of angiogenesis and metabolic reprogramming of cancer cells, promoting their proliferation, migration, metastasis and resistance to therapy [12]. HIF-1 is, therefore, an established validated therapeutic target in anti-cancer treatment. Many anticancer drugs have been shown to inhibit HIF-1α activity and many anticancer efforts are aimed at discovering new and selective inhibitors of HIF-1α activity [13,14].

In a previous study we have used the yeast two hybrid system to identify interacting proteins with the central part of HIF-1α (aminoacids 244–532). This region encompasses most of the PAS-B domain and the region preceding the N-TAD domain. We have focused our study to the interacting protein MgcRacGAP that as we have demonstrated, binds to HIF-1α via its N-terminal region (aa 1–138) containing the Myo (myosin like coiled-coil) domain of the protein (aa 41–124) [15].

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 [16]. MgcRacGAP has specificity for Rac1 and Cdc42 [17,18] and when phosphorylated at Ser387 during mitosis by Aurora B kinase, it changes specificity, being inactive versus Rac1, but active towards RhoA [19]. It contains three functional domains: the coiled coil myosin-like domain residing at the N-terminus, the PKC-like cysteine rich domain and the catalytic domain, responsible for its GAP activity. MgcRacGAP plays different roles in interphase cells and in mitotic cells. During interphase it inactivates Rac1, involved in the formation of lamellipodia and the activation of NADPH oxidase, whereas during mitosis it is required for cytokinesis as a scaffolding factor and/or a RhoA GAP. In fact, requirement or dispensability of its catalytic activity for cytokinesis have both been reported for different cell lines [20,21] and it seems indeed that RhoA activation requirement for cytokinesis is cell type specific [22]. However, the requirement of MgcRacGAP for normal cytokinesis for all types of cells underlines its essential function as a scaffold protein for cytokinesis effectors. Finally, new roles of MgcRacGAP have emerged the last few years, such as its chaperone function for the nuclear translocation of the transcription factor STAT3 [23] and its involvement in v-src induced transformation of NIH3T3 cells [24]. The HIF-1α binding domain of MgcRacGAP (Myo) is not involved in Rac1 binding or STAT3 activation and transport. It has however been shown to be necessary for MgcRacGAP function during cytokinesis. It binds tubulins [25], Ect2, a RhoA GEF [26] and, to the 40 N-terminal domain amino acids preceding it, binds the kinesin MKLP1 [27].

In the present study we unravel the mechanism by which MgcRacGAP represses HIF-1 function: it associates with the PAS-B domain of HIF-1α and displaces ARNT, thereby reducing the transcriptionally active HIF-1α-ARNT heterodimers.

2. Materials and methods

2.1. Plasmid constructions

pBEVY-GU-GFP-MgcRacGAP(1–138) was constructed by PCR amplification of MgcRacGAP(1–138) and cloned into the *Bam*HI site of pBEVY-GU-GFP [28] using the following primers:

sense 5′-TTTTTGGATCCATGGATACTATGATGCTGA-3′
antisense 5′-TTTTTGGATCCTTAATTGCTGCTGGATGG-3′, (*Bam*HI sites are underlined).
pHisGFP-HIF-1α(240–353) was constructed by PCR amplification of HIF-1α(240–353) and cloned into the *Bam*HI site of pHisGFP [29] using the following primers:

sense 5′-TTTTGGATCCAAGACTTTCCTCAGTCG-3′

antisense 5′-TTTTGGATCCGAGAAAATCAAGTCGTGCT-3′, (*Bam*HI sites are underlined). Similarly, pHisGFP-HIF-1α(336–529) was constructed by PCR amplification of HIF-1α(336–529) and cloned into the *Bam*HI site of pHisGFP using the following primers:

sense 5′-TTTTGGATCCGTATGTGTGAATTACGTTGTG-3′
antisense 5′-TTTTGGATCCATTGACCATATCACTATCC-3′, (*Bam*HI sites are underlined).

pFLAG-ARNT was constructed by subcloning the *Bam*HI fragment of full length human ARNT from pET-GST-ARNT [29] in pFLAG-CMV-2 (Sigma).

The pAC28-ARNT-bHLH-PAS, a bacterial expression plasmid that codes for the amino terminal part of human ARNT (amino acids 1–474), in fusion to a thioredoxine six histidine (TrxH6) epitope [30] was kindly provided by Murray L. Whitelaw (Discipline of Biochemistry, University of Adelaide, Australia).

The following plasmids were previously reported: pARNT-HIF-1α and pHRE*lac*Z [31], pGEX-HIF-1α(1–530) [32], pHisGFP-HIF-1α(244–532) and pGEX-4T1-MgcRacGAP(1–138) [15], pEGFP-HIF-1α [33], pEGFP-HIF-2α [34], pME18S-FLAG-MgcRacGAP, pME18S-FLAG-ΔMyo-MgcRacGAP, pME18S-FLAG-R386A*MgcRacGAP [25].

The firefly luciferase pGL3-5HRE-VEGF plasmid was kindly provided by Dr. A. J. Giacia (Stanford University, U.S.A), the pGL3-Sod2 and pGL3-PGK plasmids were kindly provided by Dr. Joseph A. Garcia (Department of Medicine, University of Texas Southwestern Medical Center, Dallas) and Dr. Celeste Simon (Abramson Family Cancer Research Institute, Philadelphia), respectively. The pCI-*Renilla* luciferase plasmid was a gift by Dr. M. U. Muckenthaler (University of Heidelberg, Germany).

2.2. Cell cultures and transfections

Human HeLa, HEK293T or Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco UK) containing 10% fetal bovine serum (FBS, Gibco UK) and 100 U/ml penicillin–streptomycin (Euroclone, UK). Cells were maintained at 37 °C in a 5% CO_2/95% air incubator. For hypoxic exposure, cells were incubated for 4 hours in 1% O_2/5% CO_2/94% N_2 in an IN VIVO$_2$ 200 hypoxia work-station (Ruskinn, Life Sciences). Transient transfections were carried out as already described [15].

2.3. SDS–PAGE and Western blot

Protein samples were resolved by 8% SDS–PAGE and analyzed by Coomassie Blue, Silver Staining or Western blotting using a rabbit anti-HIF-1α polyclonal antibody [15] or a rabbit anti-GFP polyclonal antibody generously provided by Dr. H. Boleti (Hellenic Pasteur Institute, Athens, Greece) or a mouse anti-ARNT monoclonal antibody (BD Trasduction Laboratories) or a mouse anti-pentaHis-HRP-conjugated antibody (Qiagen) or a goat anti-GST polyclonal antibody (Amersham)

or a mouse anti-FLAG monoclonal antibody (Sigma) or a goat anti-RacGAP1 polyclonal antibody (Santa Cruz Biotechnology) or a mouse anti-tubulin monoclonal antibody (Millipore, Billerica, MA, USA). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse or mouse anti-goat or goat anti-rabbit IgG antibody (all from Cell Signalling) followed by ECL. Visualization was performed by developing the signal on film or by using a transilluminator (UVItec Alliance 4.7., Cambridge, UK).

2.4. Protein expression and purification

The expression and purification of GST-fusion and His-tagged proteins were carried out as already described [15], with minor modifications. In buffers A and B, 5 μM pepstatin and 1 μM aprotinin were used instead of EDTA-free protease inhibitors (all from Sigma). Buffer B was adjusted to: 25 mM Tris-Cl pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF and 1% Triton X-100. The quantity of isolated GST-fusion or His-tagged proteins was assessed by SDS–PAGE followed by Coomassie Blue or silver staining or detection with the relevant antibodies by western blotting.

2.5. In vitro binding assays

Approximately 15 μg of purified GST or GST-MgcRacGAP(1–138) were immobilized on 25 μl of glutathione-sepharose beads (Amersham) and incubated with 25 μg of purified His-GFP-HIF-1α(244–532), His-GFP-HIF-1α(240–353) or His-GFP-HIF-1α(336–529) for 4 hours at 4 °C in buffer B. At the end of the incubation the beads were washed three times in ice cold buffer B and bound proteins were eluted in 1× SDS–PAGE loading buffer. Samples were analyzed by SDS–PAGE and Western blotting using the relevant antibodies.

2.6. Immunoprecipitation

Huh7 or HEK293T cells grown in 10 cm culture dishes were transfected with equal quantities of plasmids or subjected to 1% O_2 for 4 hours when needed. Twenty four hours post-transfection the cells were washed with cold buffer PBS and lysed in ice cold buffer C containing 25 mM HEPES pH 7.6, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF, 5 mM DTT, 1% Triton X-100, 5 μM pepstatin, 1 μM aprotinin (10 min at 4 °C). The cell suspension was centrifuged at 12.000 g for 15 min at 4 °C and the volume of the supernatant was adjusted to 0.7 ml with buffer C. Samples were incubated with 1 μg of polyclonal anti-HIF-1α or anti-GFP antibody for 3 hours at 4 °C under gentle shaking. Twenty microliters of protein-A bead slurry was added and the incubation continued for 16 hours under the same conditions. After the incubation, beads were collected by centrifugation, washed three times with buffer C and bound proteins were eluted in 1× SDS–PAGE loading buffer. Samples were analyzed by SDS–PAGE and Western blotting using the relevant antibodies.

2.7. In vitro competitive binding assays

Approximately 5 μg of purified GST-HIF-1α(1–530) was incubated with 0 (only buffer C) or 2.5 or 5 or 10 μg of purified His-ARNT(1–474) and 5 μg of purified GST-MgcRacGAP(1–138) in 0.7 ml of buffer C for 2 hours at 4 °C under gentle shaking. Anti-HIF-1α polyclonal antibody (1 μg) was added and the incubation continued for 3 hours under the same conditions. Subsequently, 20 μl of protein-A bead slurry was added and the incubation continued for 3 more hours under the same conditions. At the end of the incubation, the samples were centrifuged, beads were collected and washed three times with buffer C.

Bound proteins were eluted in 1× SDS–PAGE loading buffer and were analyzed by SDS–PAGE and silver staining or Western blotting using the relevant antibodies.

For the reverse experiment, 5 μg of purified GST-HIF-1α(1–530) was incubated with 0 (only buffer C) or 2.5 or 5 or 10 μg of purified GST-MgcRacGAP(1–138) and 5 μg of purified His-ARNT(1–474) in 0.7 ml of buffer C under the same conditions and incubation time, following the same analysis.

2.8. Luciferase assays

Huh7 (or HeLa or HEK293T) cells were co-transfected with 0.4 μg of each plasmid described in the text, with the pCI *Renilla* plasmid (0.25 μg per well) and the pGL3-5HRE-VEGF plasmid (0.75 μg per well) was used in all assays except when, pGL3-PGK or pGL3-Sod2 plasmids (0.75 μg per well) were used as described. Transfected cells were cultured in 12-well plates for 24 hours or for 20 hours and subsequently incubated for 4 hours in hypoxia (1% O_2). Luciferase activity was determined in the cell extracts using a dual luciferase chemiluminescence assay kit (Promega U.S.A) in a luminometer (TD20/20 Turner Designs).

2.9. Yeast transformation and β-galactosidase assays

Yeast strain RS453 carrying the reporter plasmid pHRE-*lacZ* and expressing human ARNT and HIF-1α was transformed with pBEVY-GU derived plasmids. Independent transformants were selected, cultured and assayed at certain time points for β-galactosidase, as previously described [31].

2.10. Statistical analysis

Results are expressed as mean ± S.E. Statistical analysis was assessed using the unpaired *t*-test in the OpenOffice.org version 3.0 Software (Oracle); $p < 0.05$ was considered to be significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

3. Results

3.1. Inhibition of HIF-1 by MgcRacGAP involves its Myo domain but not its catalytic activity

We have shown previously that the Myo domain of MgcRacGAP is necessary for HIF-1 transcriptional inhibition since, when deleted, inhibition is abolished [15]. Since, however, MgcRacGAP is a GTPase activating protein functioning in both interphase and mitosis, we asked if its catalytic activity is needed for HIF-1 inhibition. We used a mutant form of MgcRacGAP lacking catalytic activity (FLAG-R386A*MgcRacGAP) [18]. HeLa cells were transfected with vectors expressing the wild type FLAG-MgcRacGAP, FLAG-ΔMyo-MgcRacGAP, or FLAG-R386A* MgcRacGAP and transcriptional activity of hypoxia induced HIF was measured. As shown in Fig. 1A, FLAG-MgcRacGAP represses HIF-1 (as previously shown) and the mutant FLAG-R386A*MgcRacGAP also represses HIF-1 at practically the same extend, demonstrating that the catalytic activity of MgcRacGAP is not required for transcriptional repression of HIF-1. The mutant form of MgcRacGAP lacking the region containing the Myo domain (FLAG-ΔMyo-MgcRacGAP) was unable to inhibit HIF-1 induction under the same conditions, although FLAG-ΔMyo-MgcRacGAP was efficiently expressed, compared to the protein levels of the different MgcRacGAP isoforms both in normoxia (Fig. 1B left panel) and under endogenous HIF-1α expression (Fig. 1B right panel). Once more, our results confirm the necessity of the presence of the Myo region for HIF-1 transcriptional repression by MgcRacGAP. We further sought to verify that the Myo domain can independently repress HIF-1 activity so we subcloned the MgcRacGAP cDNA encoding the Myo domain (amino acids 1–138) in mammalian expression vectors in order to express the Myo region of MgcRacGAP in human cells (GFP-Myo, FLAG-Myo or Myo). In all cases, peptides would create large aggregates

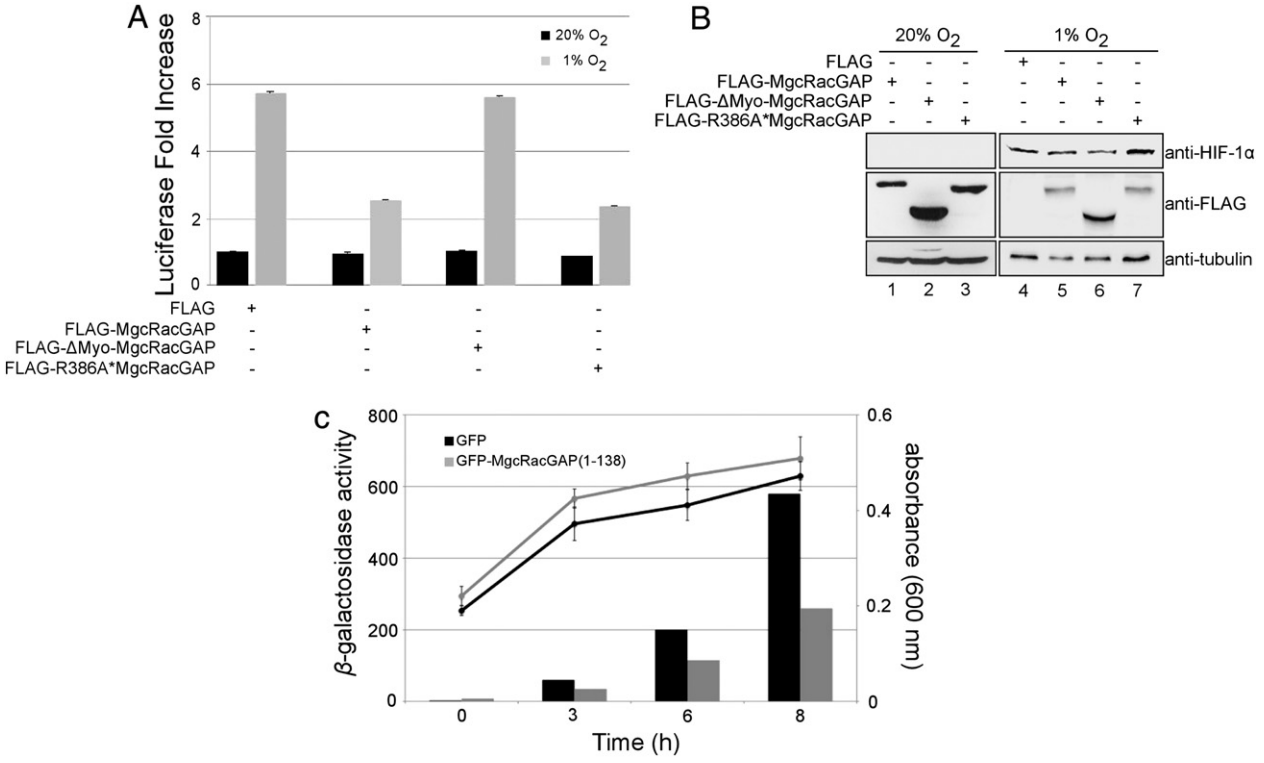

Fig. 1. The Myo domain of MgcRacGAP is sufficient for HIF-1 transcriptional activity repression. (A) HIF-1 transcriptional activity was determined 24 hours after transfection of HeLa cells with plasmids expressing the indicated proteins. Incubation under hypoxia was for 4 hours. Values are presented as a ratio of firefly over renilla luciferase activity divided by the value obtained from cells transfected with the empty vector and maintained in normoxia. Results represent the mean of three independent experiments performed in triplicates (±S.E.). (B) Western blot analysis of whole cell extracts of HeLa cells transfected with the plasmids expressing the indicated proteins under normoxia (left panel) and hypoxia (right panel), revealed with the indicated antibodies (C) HIF-1 activity determination in the yeast HIF-1 reconstitution system (see text). Yeast cells were transformed either with pBEVY-GU-GFP or pBEVY-GU-GFP-MgcRacGAP(1–138) and independent transformants were selected, cultured and aliquots expressing the indicated proteins were analyzed at the indicated time points for β-galactosidase activity. Culture growth was monitored by OD_{600} measuring at the specified time points. Results are shown as average of absolute values and represent the mean of the results obtained from three independent transformants performed in triplicates.

precipitating in several sites of HeLa, HEK293T or Huh7 cells, as revealed by microscope examination (data not shown). We managed to circumvent this problem by expressing inducibly the GFP-MgcRacGAP(1–138) peptide in a simple *in vivo* reconstituted HIF-1 system in yeast: the HIF-1α and ARNT subunits are expressed in *Saccharomyces cerevisiae* under the control of the same bidirectional inducible promoter and the *E scherichia coli* β-galactosidase gene expression is under the control of HREs. This way β-galactosidase quantity produced by yeast cells reflects HIF-1 activity [31]. As shown in Fig. 1C, when GFP-MgcRacGAP(1–138) was expressed in the yeast HIF-1 reconstitution system under the control of the same inducible promoter as the HIF-1 subunits, HIF-1 induction was inhibited. The inhibition was evident as soon as HIF-1 induction was detectable (3 hours of culture) and by 8 hours of culture it attained more than 50%. Yeast culture growth rates were unaffected by the presence of MgcRacGAP (Fig. 1C). We repeated the experiment expressing the Myo region of MgcRacGAP in fusion with GST (GST-MgcRacGAP(1–138)) and obtained practically the same results (data not shown). We thus can conclude that the region of MgcRacGAP encompassing amino acids 1–138 is sufficient for repression of HIF-1 transcriptional activity in the yeast HIF-1 reconstitution system.

3.2. MgcRacGAP(1–138) binds in vitro specifically to the PAS-B domain of HIF-1α

The region of HIF-1α interaction with MgcRacGAP (aminoacids 244–532) encompasses almost the whole PAS-B domain, responsible for HIF-1α-ARNT dimerization. Since the PAS-B domain of HIF-1α extends up to amino acid 343, we constructed two separate peptides covering the region in question in fusion with GFP (HisGFP-HIF-1α(240–353) and HisGFP-HIF-1α(336–529)) (Fig. 2A) and tested their binding on the Myo region of MgcRacGAP by in vitro pull down experiments. As shown in Fig. 2B, HisGFP-HIF-1α(240–353), encompassing the PAS-B domain, binds to the MgcRacGAP Myo domain with practically the same efficiency as the original HisGFP-HIF-1α(244–532), whereas HisGFP-HIF-1α(336–529) binds only weakly. These results show that MgcRacGAP binds to HIF-1α predominantly via the PAS-B domain, suggesting that it may interfere with HIF-1α-ARNT heterodimerization.

3.3. MgcRacGAP-mediated HIF-1 transcriptional activity inhibition is impaired by ARNT overexpression

In order to study the involvement of the constitutively expressed subunit of HIF-1, ARNT, in the repression of HIF-1 activity by MgcRacGAP, we examined the expression of HIF-1α in Huh7 human hepatoma cells in the presence of overexpressed ARNT. HIF-1 expression was achieved either by overexpressing HIF-1α (transfection with pEGFP-HIF-1α), or by inducing HIF-1α under low O_2 (1%). More specifically, cells transfected with pEGFP-HIF-1α in the presence of pFLAG-MgcRacGAP were co-transfected with pFLAG-ARNT and HIF-1 transcriptional activity was measured. As shown in the left panel of Fig. 3A, whereas HIF-1 activity is repressed to ~60% in the presence of pFLAG-MgcRacGAP (see also Ref. [15]), co-transfection with pFLAG-ARNT restores HIF-1 activity to practically initial levels. GFP-HIF-1α shows equal expression levels under all conditions and MgcRacGAP expression levels remain unaffected by FLAG-ARNT overexpression (Fig. 3A right panel). Accordingly, when endogenous HIF-1 activity was measured under hypoxia (Fig. 3B, left panel), cells transfected with pFLAG-ARNT in the presence of pFLAG-MgcRacGAP had a

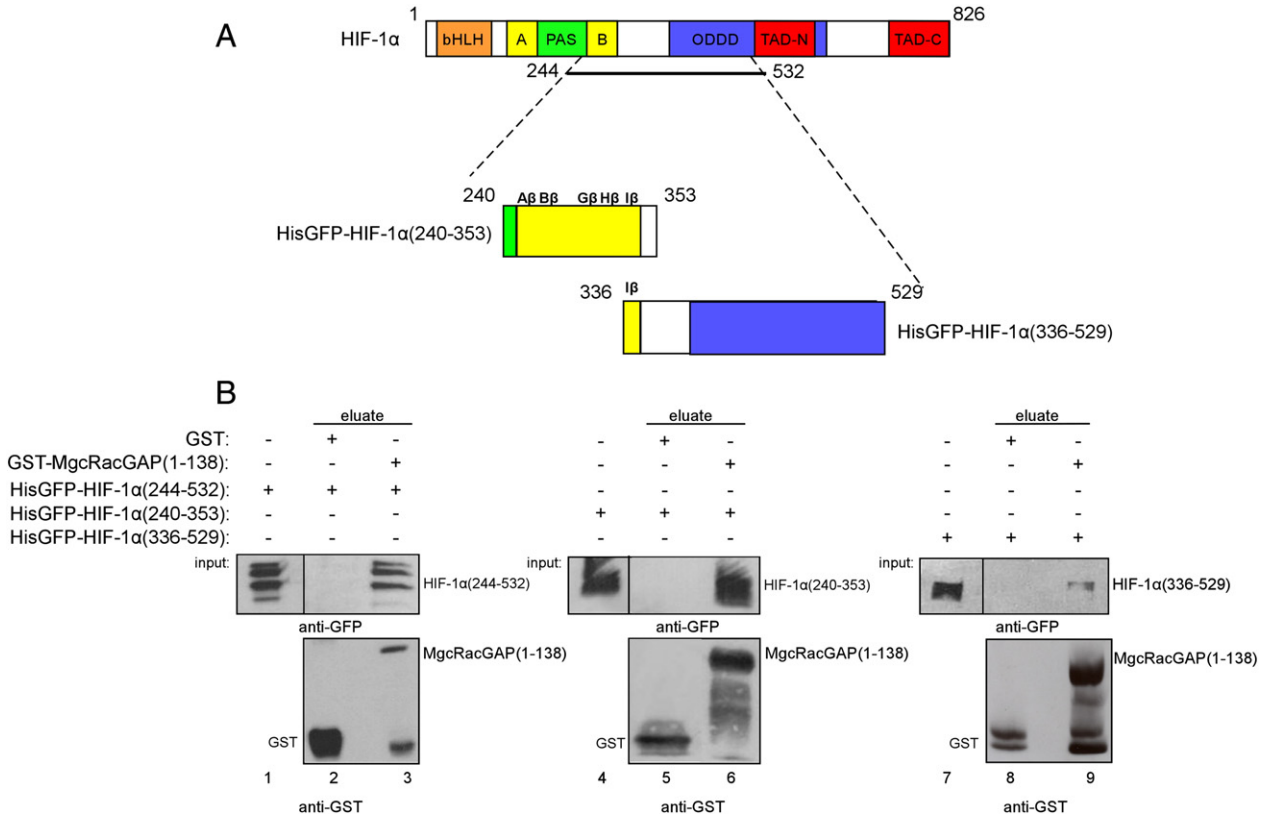

Fig. 2. MgcRacGAP(1–138) binds *in vitro* to the PASB domain of HIF-1α. (A) Schematic representation of the HIF-1 fragments used for the *in vitro* binding assays. Aβ–Iβ indicate the corresponding β-strands of the PAS-B domain. The horizontal line corresponds to the fragment used in the two hybrid assay. (B) *In vitro* binding assay of the HisGFP-HIF-1α(244–532) (lanes 1, 2, 3), HisGFP-HIF-1α(240–353) (lanes 4, 5, 6) or HisGFP-HIF-1α(336–529) (lanes 7, 8, 9) protein with GST (lanes 2, 5, 8) and GST-MgcRacGAP(1–138) (lanes 3, 6, 9) followed by western blot with the anti-GFP (upper panel) and anti-GST (lower panel) antibodies. Input (lanes 1, 4, 7) was 1/10 of the quantity of each one of the HisGFP-HIF-1α peptides used for the assay.

significantly increased activity of HIF-1 compared to cells transfected with only pFLAG-MgcRacGAP. Again, MgcRacGAP protein levels remain unaffected by FLAG-ARNT overexpression (Fig. 3B, right panel). These results suggest that the MgcRacGAP-mediated repression of HIF-1 transcriptional activity involves ARNT.

3.4. MgcRacGAP competes with ARNT for HIF-1α binding in vitro

According to the data showing that 1) MgcRacGAP binds to HIF-1α via the PAS-B domain and that 2) the MgcRacGAP mediated repression of HIF-1 transcriptional activity involves ARNT, we hypothesized that MgcRacGAP binding to HIF-1α excludes ARNT from dimerizing with HIF-1α. We thus performed *in vitro* competition assays between bacterially produced GST-HIF-1α (1–530), His-ARNT(1–474) and GST-MgcRacGAP (1–138) (Fig. 4A). GST-HIF-1α (1–530) was either incubated with equal amounts of MgcRacGAP (1–138) in the presence of increasing quantities of His-ARNT(1–474), or, inversely, with His-ARNT(1–474) and increasing quantities of MgcRacGAP (1–138). Subsequently, HIF-1α was immunoprecipitated with an anti-HIF-1α polyclonal antibody and bound ARNT or MgcRacGAP were detected in the immunoprecipitates by Western blot, using the relevant antibodies. As shown in Fig. 4B, both ARNT and MgcRacGAP co-immunoprecipitate with HIF-1α under the conditions used, in the absence of MgcRacGAP or ARNT, respectively (lanes 1, 2). When, however, increasing quantities of ARNT are added to the HIF-1α-MgcRacGAP incubation mixture, less MgcRacGAP co-immunoprecipitates with HIF-1α and this decrease inversely correlates with the increase of ARNT amounts (lanes 3–5). Accordingly, in the reverse experiment, where increasing amounts of MgcRacGAP are added in the HIF-1α-ARNT incubation mixture, the amount of ARNT bound to HIF-1α decreases according to MgcRacGAP quantity increase (lanes 6–8). These results show that MgcRacGAP binding to HIF-1α interferes with HIF-1α-ARNT dimerization.

3.5. In vivo overexpression of MgcRacGAP displaces ARNT from HIF-1α-ARNT dimers

In order to confirm that MgcRacGAP competes with ARNT for HIF-1α binding *in vivo*, we examined the presence of ARNT and MgcRacGAP in HIF-1α complexes immunoprecipitated from human cells. HIF-1α was expressed exogenously in normoxia, or induced under hypoxic conditions in Huh7 cells, in the presence or absence of MgcRacGAP. As shown in Fig. 5, immunoprecipitates of HIF-1α either with the anti-GFP antibody (overexpressed HIF-1α) or the anti-HIF-1α antibody (HIF-1α expressed under hypoxia) contained significantly lower quantities of ARNT when MgcRacGAP was co-expressed. More specifically, in cells exogenously expressing GFP-HIF-1α, the amount of ARNT that co-immunoprecipitates with HIF-1α is clearly decreased when MgcRacGAP is present (Fig. 5A, compare lanes 5 and 6). Accordingly, immunoprecipitates of HIF-1α from cells subjected to hypoxia contain significantly less ARNT when MgcRacGAP is present (Fig. 5B, compare lanes 3 and 4). Interestingly, as indicated by the results in lanes 2 and 6, endogenous MgcRacGAP co-immunoprecipitates with HIF-1α.These results show that MgcRacGAP binding to HIF-1α decreases the amount of HIF-1α molecules found in dimers with ARNT.

3.6. MgcRacGAP overexpression does not affect HIF-2 transcriptional activity

The PAS-B domain is one of the most conserved between HIF-1α and HIF-2α (74.5% identity and 87.3% similarity). In an effort to obtain

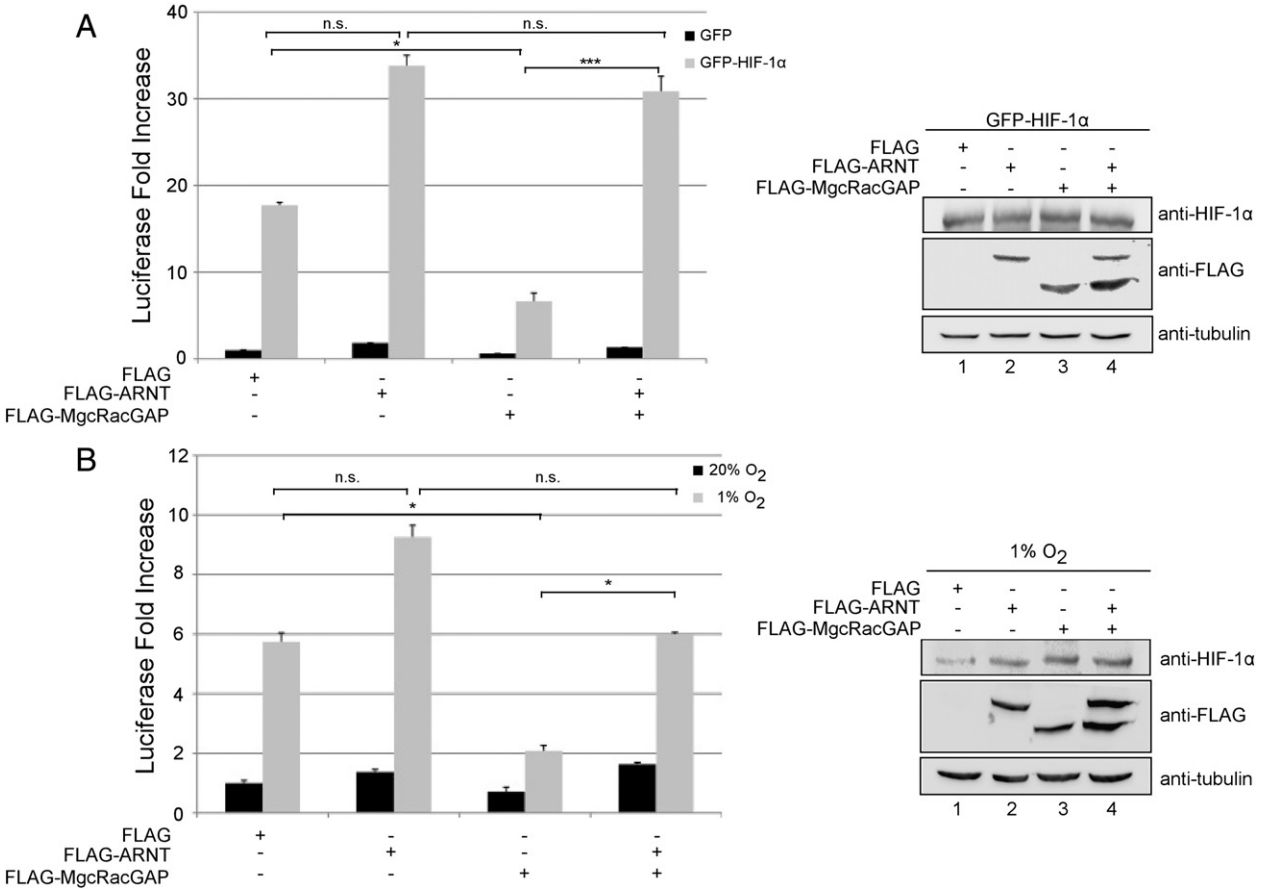

Fig. 3. MgcRacGAP mediated HIF-1 transcriptional inhibition is impaired by ARNT overexpression. (A) HIF-1 transcriptional activity was determined 24 hours after transfection (left panel). Huh7 cells were transfected with plasmids expressing either GFP (black bars) or GFP-HIF-1α (grey bars) and plasmids expressing the indicated proteins. Values are presented as the ratio of firefly over renilla luciferase activity divided by the value obtained by cells transfected only with the empty vectors. Results represent the mean of three independent experiments performed in triplicates (±S.E.). Expression levels of GFP-HIF-1α (lanes 1 to 4), FLAG-MgcRacGAP (lanes 3, 4), and FLAG-ARNT (lanes 2, 4), were monitored by Western blot analysis with the indicated antibodies (right panel). (B) HIF-1 transcriptional activity was determined 24 hours after transfection of Huh7 cells with plasmids expressing the indicated proteins (left panel). Incubation under hypoxia was for 4 hours. Values are presented as a ratio of firefly over renilla luciferase activity divided by the value obtained from cells transfected with the empty vector and maintained in normoxia. Results represent the mean of three independent experiments performed in triplicates (±S.E.). Expression levels of endogenous HIF-1α (lanes 1 to 4), FLAG-MgcRacGAP (lanes 3, 4), and FLAG-ARNT (lanes 2, 4), were monitored by Western blot analysis with the indicated antibodies (right panel). Statistical comparisons between two groups of data in luciferase assays are indicated with horizontal lines. Asterisk denotes statistical significance. Only values $p < 0.05$ are considered significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). n.s.: not significant.

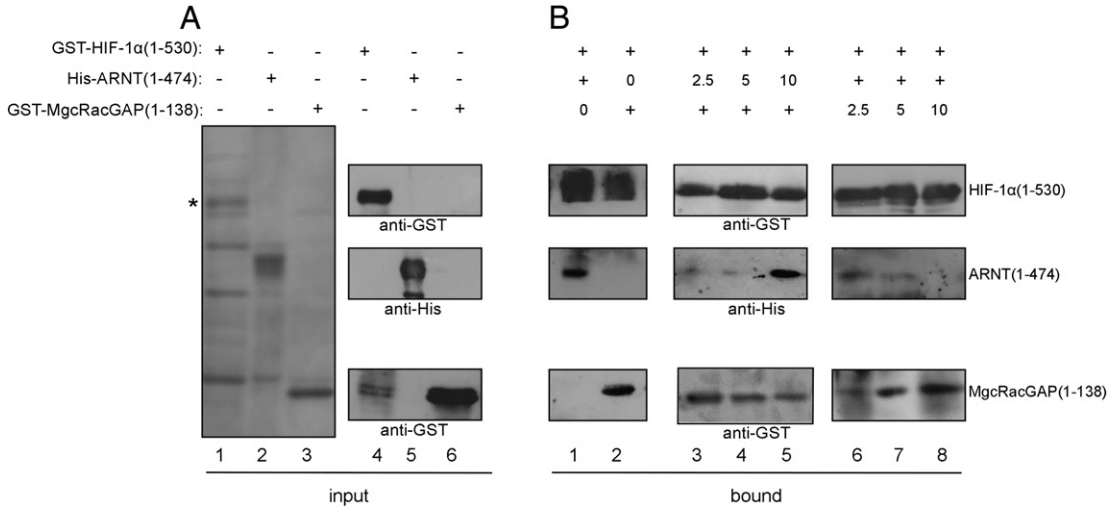

Fig. 4. MgcRacGAP(1–138) competes with bacterially expressed ARNT and *vice versa*, on HIF-1α *in vitro* binding. (A) Silver staining (left panel) and western blot analysis (right panel) of bacterially expressed and purified GST-HIF-1α(1–530) (lanes 1, 4), His-ARNT(1–474) (lanes 2, 5) and GST-MgcRacGAP(1–138) (lanes 3, 6) with the indicated antibodies. Input quantity was 5 μg for each protein. Asterisk denotes the full-length bacterially expressed protein. (B) *In vitro* competitive binding assay, using anti-HIF-1α antibody for the immunoprecipitation of 5 μg of purified GST-HIF-1α(1–530) (upper panel, lanes 1 to 8), pre-incubated with 0 (only buffer), 2.5, 5, 10 μg of His-ARNT(1–474) and 5 μg GST-MgcRacGAP(1–138) (lanes 2 to 5) or 0, 2.5, 5, 10 μg of GST-MgcRacGAP(1–138) and 5 μg of His-ARNT(1–474) (lanes 1, 6, 7, 8). Anti-GST and anti-His antibodies were used for the detection of the recombinant proteins, as indicated. Only the relevant parts of the blots are shown.

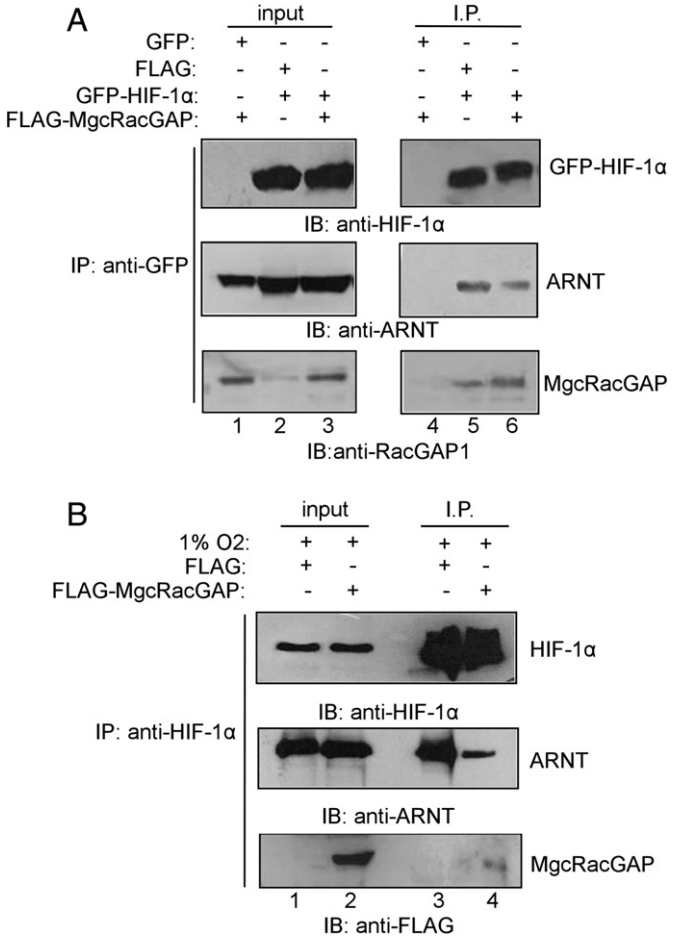

Fig. 5. Overexpression of MgcRacGAP prevents ARNT from dimerizing with HIF-1α. (A) Western blot analysis of inputs (left panels) and immunoprecipitates (right panels) obtained from HEK293T cells transfected with plasmids encoding GFP (lanes 1, 4) or GFP-HIF-1α (lanes 2, 3, 5, 6,), FLAG (lanes 2, 5), or FLAG-MgcRacGAP (lanes 1, 3, 4, 6). Anti-GFP antibody was used for immunoprecipitation and anti-ARNT, anti-HIF-1α and anti-RacGAP1 antibodies for immunoblotting as indicated. (B) Western blot analysis of inputs (left panel) and immunoprecipitates (right panel) obtained from Huh7 cells transfected with plasmids encoding FLAG (lane 1, 3) or FLAG-MgcRacGAP (lanes 2, 4) and subjected to hypoxia for 4 hours. Anti-HIF-1α antibody was used for immunoprecipitation and anti-ARNT, anti-HIF-1α and anti-FLAG antibodies for immunoblotting, as indicated.

hints about the structural requirements of the MgcRacGAP-HIF-1α interaction, we assayed the impact of MgcRacGAP overexpression on HIF-2 transcriptional activity. HEK293T cells were transfected with pEGFP-HIF-2α in the presence or absence of pFLAG-MgcRacGAP and HRE-driven luciferase activity was measured. As shown in the left panel of Fig. 6A, HIF-2 transcriptional activity was not affected by MgcRacGAP co-expression, whereas the inhibitory effect of MgcRacGAP on HIF-1 transcriptional activity was once again evident, although expression levels of GFP-HIF-1, GFP-HIF-2 and MgcRacGAP were practically unaffected by the conditions used (Fig. 6A, right panel). We subsequently measured endogenous HIF-2 activity in the presence of MgcRacGAP under hypoxia. Since both HIF-1α and HIF-2α are expressed in these cells, we used native promoter-driven luciferase reporter plasmids (pGL3-PGK and pGL3-Sod2), in order to simultaneously and separately monitor for transcriptional activity of the two molecules. The two reporter plasmids have been shown to be specifically activated by either HIF-1 (pGL3-PGK) or HIF-2 (pGL3-Sod2) [35,36] and this is also evident in Huh7 cells under our experimental conditions (Befani et al., manuscript submitted). Huh7 cells were transfected with pFLAG-MgcRacGAP or pFLAG-ARNT or both, in the presence of either pGL3-PGK or pGL3-Sod2 and subjected to hypoxia. Presented in Fig. 6B, our results show a clear difference of the influence of MgcRacGAP on HIF-1 or HIF-2 transcriptional activity: Whereas HIF-1 activity is shown to be repressed in the presence of MgcRacGAP and recover by the addition of ARNT (left panel), HIF-2 activity remains unaffected (right panel). The above results suggest that MgcRacGAP does not bind on the HIF-2α PAS-B domain, structurally and functionally distinguishing between the two HIF isoforms.

4. Discussion

HIF-1 is today one of the most intensively explored molecules, because of its importance in development and its major role in diseases such as ischemia and cancer.

In fact, research for novel, HIF specific inhibitors, is highly intense [13,14].

We have shown previously that MgcRacGAP inhibits HIF-1 activity by binding to the inducible subunit of HIF-1, HIF-1α. Repression of HIF-1 transcriptional activity is not due to altered HIF-1α protein levels or subcellular localization [15]. Binding of MgcRacGAP to HIF-1α is mediated through its Myo domain, a coiled-coil domain resembling myosins and mainly tropomyosins [18]. In this work we have explored the mechanism of MgcRacGAP-mediated HIF-1 activity inhibition. We present evidence that the above phenomenon is due to the competition of MgcRacGAP with ARNT for binding to the PAS-B domain of HIF-1α, diminishing, thus, the effective HIF-1α-ARNT dimer concentration and inhibiting HIF-1 transcriptional efficiency.

We first show that the catalytic activity of MgcRacGAP is not required for MgcRacGAP-mediated repression and that the Myo domain of MgcRacGAP is not only necessary [15] but also sufficient for the transcriptional inactivation of HIF-1. This observation suggests that the other domains of MgcRacGAP are not required for its effect on HIF-1 activity, implying that direct binding of the Myo domain on HIF-1α is sufficient for HIF-1 activity inhibition, without intervention from other molecules. We further establish that the Myo domain binds specifically to the PAS-B domain contained in the HIF-1α fragment that was initially used to detect the interaction with MgcRacGAP. Since PAS-B is required for the heterodimerization of HIF-1α with ARNT, this finding supports the MgcRacGAP-ARNT competition hypothesis. Accordingly, overexpression of ARNT in mammalian cells alleviates the MgcRacGAP-mediated repression of HIF-1 activity. We have confirmed the competition between MgcRacGAP and ARNT for HIF-1α binding by *in vitro* pull down experiments and we have further confirmed by co-immunoprecipitation experiments that overexpression of MgcRacGAP in cultured cells displaces ARNT from HIF-1 dimers *in vivo*. We additionally show that the effect of MgcRacGAP on HIF-1 transcriptional activity is not exerted on HIF-2, underscoring the specificity of the MgcRacGAP binding on HIF-1α and functionally differentiating between HIF-1 and HIF-2.

Regulation of transcription factor activity by differential dimerization is not an uncommon mechanism. Several proteins act as inhibitors of transcription factor activity by binding and replacing a functional subunit of the transcription factor complex. Proteins escorting or retaining transcription factors at specific loci into the cell are also known regulators of transcription factor activity. In fact, regulation of HIF-1 activity by control of its dimerization is a mechanism already shown to be used by the cell: Hsp90 competes with ARNT for HIF-1α binding [37], casein kinase 1 (CK1) phosphorylates a Ser at the PAS-B domain inhibiting dimerization [38], ARNT interacting peptide (Ainp1) binds on the PAS-B domain of ARNT similarly reducing the HIF-1 dimers [39] and septin9_i1 binds on the bHLH domain of HIF-1α, stabilizing the dimer [40].

Dimerization of HIF-1α with ARNT is a prerequisite for DNA binding and subsequent transcriptional activation by HIF-1 [41]. The PAS-B domain core motif folds in a tertiary structure containing a β-sheet flanked on one face by a-helices. Studies on the structural requirements for HIF-2α-ARNT PAS-B domain heterodimerization show

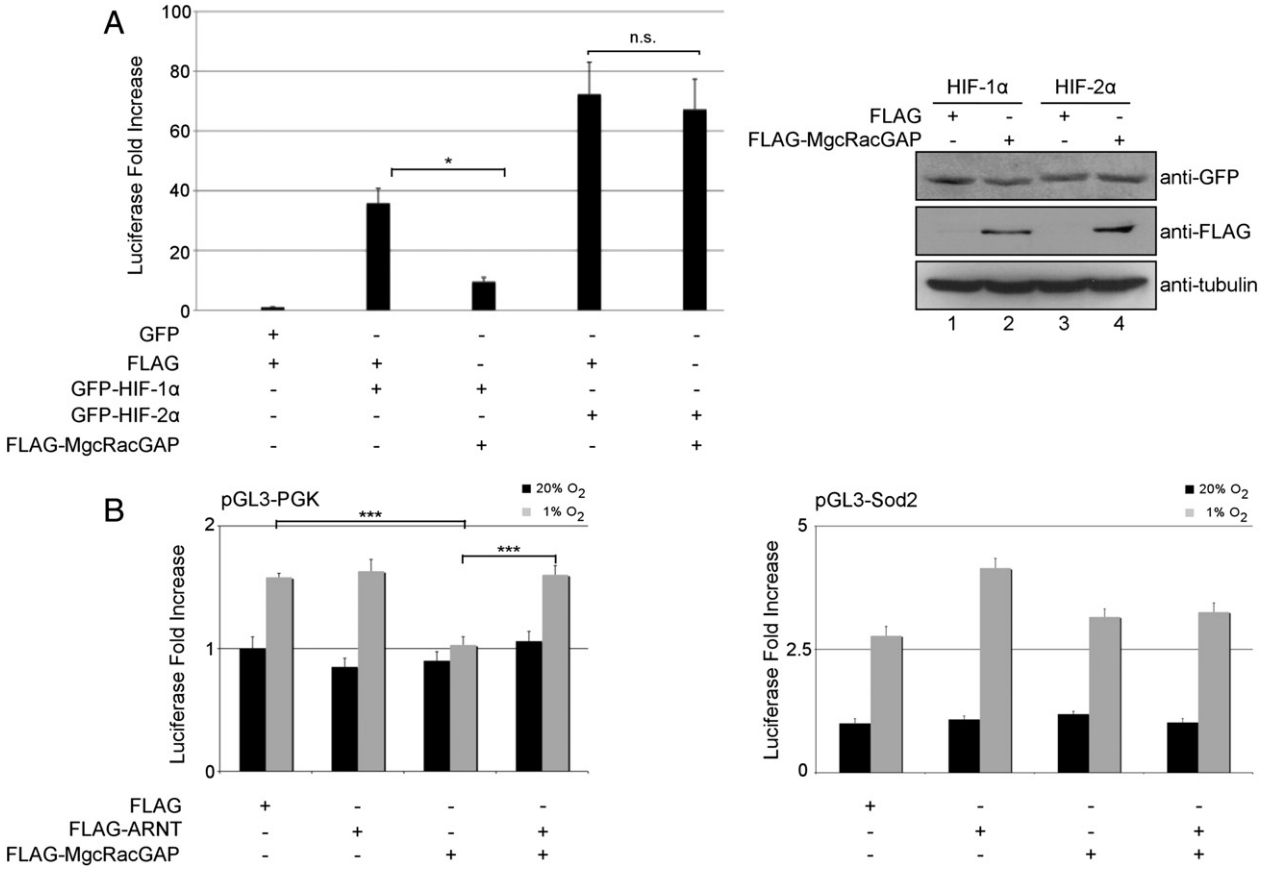

Fig. 6. MgcRacGAP overexpression does not affect HIF-2 transcriptional activity. (A) HIF-1 and HIF-2 transcriptional activity was determined 24 hours after transfection (left panel). HEK293T cells were transfected with plasmids expressing the indicated proteins. Expression levels of FLAG-MgcRacGAP (lanes 2, 4), GFP-HIF-1α (lanes 1, 2), and GFP-HIF-2α (lanes 3, 4), were monitored by Western blot analysis with the indicated antibodies (right panel). Values are presented as the ratio of firefly over renilla luciferase activity divided by the value obtained by cells transfected only with the empty vectors. Results represent the mean of two independent experiments performed in triplicates (±S.E.). (B) HIF-1 (left panel) and HIF-2 (right panel) transcriptional activity was determined 24 hours after transfection of Huh7 cells with plasmids expressing the indicated proteins and the pGL3-PGK and pGL3-Sod2 reporter plasmids respectively. Incubation under hypoxia was for 4 hours. Values are presented as a ratio of firefly over renilla luciferase activity divided by the value obtained from cells transfected with the empty vector and maintained in normoxia. Results represent the mean of two independent experiments performed in triplicates (±S.E.). Statistical comparisons between two groups of data are indicated with horizontal lines. Asterisk denotes statistical significance. Only values $p < 0.05$ are considered significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Only significant comparisons are indicated.

that the interaction takes place via an antiparallel association of the solvent exposed surface of their central β-sheets [42]. The central sheets Hβ and Iβ are located at the C-terminal part of the PAS-B region and mutagenesis of specific residues in this region abolishes dimerization. The HIF-1α PAS-B domain is suggested to use the same interphase as HIF-2 and the same modules are also used for the weaker ARNT homodimerization [43].

We have recently reported that when Ser247, located at the Aβ strand of PAS-B HIF-1α, is phosphorylated by CK1, dimerization is impaired, as well as HIF-1 transcriptional activity [38]. Our experiments with S247A HIF-1α (no phosphorylation) and S247D HIF-1α (phosphorylation mimicking) suggest that MgcRacGAP-mediated inhibition of HIF-1 activity is not affected by phosphorylation since both mutants show lower transcriptional activity in the presence of MgcRacGAP, similar to the wild type molecule (data not shown). Recently, a new role of the PAS-B domain of bHLH-PAS transcription factors has emerged, in regulating coactivator recruitment and oligomerization status. It was shown that coactivators bearing α-helices and especially coiled-coil domains bind to the α-helical part of the PAS-B domain without compromising interactions mediated by the β-sheet [44]. In fact, the transcriptional coactivator TACC3 of HIF-1 binds to the PAS-B domain of ARNT and enhances HIF-1 transcriptional activity [45].

The fragment of HIF-1α that we have used in our *in vitro* binding experiments (aminoacids 240–353) contains the whole PAS-B domain and was shown to bind MgcRacGAP specifically and with practically equal efficiency as the HisGFP-HIF-1α(244–532) fragment. We do not know at present which region of the PAS-B domain is involved in MgcRacGAP binding. However, our results suggest that MgcRacGAP binds on the β-sheet part of the HIF-1α PAS-B domain rather than the a-helix, since it displaces ARNT. Supporting this, we found a weak binding of MgcRacGAP on the fragment containing only the central β-sheet strand Iβ (critical for PAS-B domain dimerization) (Fig. 2A). Moreover, since the phosphorylation at the Aβ strand does not disturb the MgcRacGAP effect on HIF-1 activity, this β-strand is probably not part of the MgcRacGAP-HIF-1α interaction surface.

It is very interesting that although the PAS-B domains of HIF-1α and HIF-2α are highly conserved, MgcRacGAP does not bind to HIF-2α as suggested by our transcriptional activity studies. Accordingly, and contrary to HIF-1, the activity of HIF-2 was unaffected by MgcRacGAP when expressed endogenously under hypoxia, as monitored by HIF-1 and HIF-2-specific reporter plasmids. The specific binding of MgcRacGAP on the PAS-B domain of HIF-1α and not that of HIF-2α suggests that the interaction relies on HIF-1α-specific residues and not simply on residues defining the general features of PAS-B domains. Deletion and site-specific mutagenesis studies will identify the residues involved in the specific HIF-1α-MgcRacGAP interaction.

Given that the interaction of MgcRacGAP with HIF-1α disturbs HIF-1α-ARNT dimerization, its action should liberate monomeric HIF-1α and ARNT molecules that would otherwise be complexed

together. HIF-1α has been shown to interact with other molecules such as Max or Sp1 via the PAS-B domain in ARNT-free complexes [46,47] frequently resulting in repression of gene expression under hypoxia. On the other hand, ARNT has many different partners that implicate it in several different cellular control pathways. It has even been shown to be redistributed between the cytoplasm and the nucleus under the effect of Ainp1, a peptide that binds on its PAS-B domain [39]. It is possible that monomeric ARNT molecules that would otherwise be bound to HIF-1α, in the presence of MgcRacGAP bind to other molecules such as HIF-2α or AhR, or homodimerize, depending on the relative concentration of the different peptides and the status of the cell. This way, MgcRacGAP interference might simultaneously diminish HIF-1 activity and affect the action of another ARNT-binding molecule. It would thus be interesting to monitor the activity of ARNT dependent pathways in the presence of MgcRacGAP.

It is not clear in the case of the MgcRacGAP mediated HIF-1 inhibition if this is a novel function of MgcRacGAP or an action that is connected with one of the functions of MgcRacGAP in cytoskeletal organization. It is interesting that another protein functioning in cytoskeletal organization, a septin, has been recently shown to bind to HIF-1α (but not HIF-2α) and regulate its activity. Septins are evolutionary conserved GTP-binding cytoskeletal proteins that form filaments associated with cytoskeleton organization, membrane dynamics, mitosis, cytokinesis and cell cycle progression [48,49]. They play an important role in cytokinesis by rigidifying the cleavage furrow but they are also shown to scaffold, anchor or stabilize other proteins [50,51]. Septin9_i1 binds to the bHLH domain of HIF-1α and prevents its degradation by displacing RACK1. RACK1 binds on the PAS-A domain and competes with the stabilizing protein HSP90 promoting thus the degradation of HIF-1α at the proteasome, in an oxygen-independent regulatory mechanism [40,52]. The binding of Sept9_i1 on HIF-1α also stabilizes the HIF-1α-ARNT complex, further increasing HIF-1 activity. Septins have been shown to be upregulated in cancer cells and Sept9_iv knock out in prostate cells reduces cellular proliferation, tumor growth and angiogenesis in a HIF-1 dependent manner. Hence, both proteins (Septin9_i1 and MgcRacGAP) can bind on the bHLH-PAS domain of HIF-1α and modulate its dimerization with ARNT either positively (Septin9_i1) or negatively (MgcRacGAP).

A cross-talk between signaling pathways regulating cytoskeletal organization and the hypoxia response has already been documented: Hypoxia can induce alterations to cytoskeletal structures and Rho protein function has been implicated in hypoxia induced cytoskeletal changes. Furthermore, Rac1 has been shown to be activated during hypoxia and also to be required for HIF-1 activation [53–57]. The inhibition of HIF-1 by the inhibitor of Rac1 activity, MgcRacGAP, could be part of this cross-talk, functioning for the down-regulation of HIF-1 activity either via Rac1, or directly, by binding to HIF-1α. A recent study showing involvement of MgcRacGAP in v-Src oncogene induced transformation suggests that the virus induces constitutive (during all the phases of the cell cycle) phosphorylation at Ser387 in the catalytic domain, inactivating its Rac1 directed activity, resulting in active Rac1 which is required for v-Src induced oncogenic transformation [24,58].

Why and when MgcRacGAP, a cytoskeleton function regulator, inhibits HIF-1 activity *in vivo* under physiological conditions, remains to be explored. MgcRacGAP expression has been shown to be cell-cycle regulated [59] and this property could be important for HIF-1 activity or compartmentalization in the cell under physiological conditions, and most importantly, under tumoral growth conditions. HIF-1 has been shown to affect, except from growth and metabolic regulation of cancer cells, epithelial to mesenchymal transition, invasion and metastasis [13], processes involving cell differentiation and motility, evidently requiring a fine-tuned cross-talk between hypoxia and cytoskeleton function. Our results suggest that peptides derived from the region of the MgcRacGAP Myo domain that binds to HIF-1α could be exploited as selective inhibitors of HIF-1 activity.

In conclusion, we have delineated the principles of the mechanism by which MgcRacGAP inhibits HIF-1 activity. Further exploration of the MgcRacGAP mediated inhibition of HIF-1 will provide precious information on basic processes of the cell such as the hypoxia response and cytoskeleton function, but also tools for pharmaceutical intervention on HIF-1 function.

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