Biochimica et Biophysica Acta 1833 (2013) 1378–1387

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

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

Aggeliki Lyberopoulou, Ilias Mylonis, George Papachristos, Dimitrios Sagris, Alkmini Kalousi, Christina Befani, Panagiotis Liakos, George Simos, Eleni Georgatsou *

Laboratory of Biochemistry, School of Medicine, University of Thessaly, Biopolis 41110, Larissa, Greece

ARTICLE INFO

Article history:
Received 10 July 2012
Received in revised form 19 February 2013
Accepted 21 February 2013
Available online 28 February 2013

Keywords:
HIF-1α
HIF-2
MgcRacGAP
PAS-B
Hypoxia
Cytoskeleton

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

* Corresponding author. Tel.: +30 2410 685581, +30 2410 685584; fax: +30 2410 685545.
E-mail address: egeorgat@med.uth.gr (E. Georgatsou).

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.02.025

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)