Eukaryotic translation initiation factor 5A (eIF5A) is essential for HIF-1 α activation in hypoxia

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Abstract

The eukaryotic initiation factor 5A (eIF5A) is an essential protein involved in translation elongation and cell proliferation. eIF5A undergoes several post-translational modifications including hypusination and acetylation. Hypusination is indispensable for the function of eIF5A. On the other hand, the precise function of acetylation remains unknown, but it may render the protein inactive since hypusination blocks acetylation. Here, we report that acetylation of eIF5A increases under hypoxia. During extended hypoxic periods an increase in the level of eIF5A acetylation correlated with a decrease in HIF1 α , suggesting involvement of eIF5A activity in HIF1 α expression under hypoxia. Indeed, suppression of eIF5A by siRNA oligo-mediated knockdown or treatment with GC7, a deoxyhypusine synthase inhibitor, led to significant reduction of HIF-1 α activity. Furthermore, knockdown of eIF5A or GC7 treatment reduced tumor spheroid formation with a concomitant decrease in HIF-1 α expression. Our results suggest that functional, hypusinated eIF5A is necessary for HIF-1 α expression during hypoxia and that eIF5A is an attractive target for cancer therapy.

Keywords

Acetylation; eIF5A; HIF-1 α ; Hypoxia; Hypusination

Introduction

Eukaryotic Initiation Factor 5A (eIF5A) is a small (~17 kDa) protein that is essential and highly conserved from bacteria to humans [1, 2]. Initial identification of eIF5A from reticulocyte lysate as a protein stimulating methionyl-puromycin synthesis prompted the idea that eIF5A is a translation initiation factor [3]. However, further studies showed that eIF5A plays a role in translation elongation [1, 4]. Recent findings suggest that eIF5A and its bacterial ortholog, EF-P (translation elongation factor P), play a critical role in translation elongation of proteins containing consecutive proline residues [5, 6].

eIF5A is the only known cellular protein containing the unusual amino acid, hypusine [Nε-(4-amino 2-hydroxybutyl) lysine] [7]. The hypusine residue is created by a modification of a specific lysine residue in eIF5A (hypusination), which is catalyzed by a two-step enzymatic cascade. In the first step, an aminobutyl moiety of spermidine is transferred to the conserved lysine residue (K50 in human eIF5A) by deoxyhypusine synthase (DHS), converting it to deoxyhypusine. Deoxyhypusine in eIF5A is subsequently hydroxylated by deoxyhypusine hydroxylase (DOHH) to form mature eIF5A (Hypusinated eIF5A) [8, 9]. This hypusine modification is essential for the function of eIF5A since knockout of either eIF5A or its hypusination enzymes leads to embryonic lethality in mice [10, 11]. Along with hypusination, eIF5A undergoes acetylation at another conserved lysine residue (K47) [12]. The acetylation and hypusination sites K47 and K50, respectively, exist in close proximity in the N-terminal domain [13], suggesting the physical and functional interaction between these two modifications. Indeed, hypusination of eIF5A blocks its acetylation in both yeast and humans [14, 15]. Although the precise physiological function of acetylation remains to be elucidated, our previous results suggest that eIF5A acetylation

facilitates its nuclear localization [15], while eIF5A is believed to work in the cytoplasm as a translational elongation factor. Thus, acetylation appears to render the protein inactive.

Hypoxia is a common feature of solid tumors and promotes many adaptive biological processes in tumors including angiogenesis [16], the epithelial-to-mesenchymal transition [17], invasiveness [18], and metastasis [19]. These adaptive responses to hypoxia are mediated by hypoxia inducible factor-1 α (HIF-1 α), a transcription factor. In normoxia, two proline residues in the oxygen dependent degradation domain (ODDD) of HIF-1 α are hydroxylated by prolyl hydroxylase 2 (PHD2), resulting in polyubiquitylation by pVHL and proteasome-mediated degradation. Upon hypoxia, the activity of PHD2 is inhibited and sequentially HIF-1 α is stabilized and translocates into the nucleus, where HIF-1 α binds to HIF-1 β to form a heterodimer complex and activates the transcription of a number of hypoxia-responsive genes [20, 21].

In this study, we report the first evidence for the link between eIF5A and HIF-1 α activation in hypoxia. Our results indicate that hypusinated eIF5A is essential for HIF-1 α expression and transcriptional activity in response to hypoxia. In addition, we found that hypusinated eIF5A is crucial for tumor spheroid formation. Our results suggest that functional, hypusinated eIF5A is important for cancer cell survival in hypoxia.

Materials and methods

Compounds, antibodies, and cell culture

GC7 was purchased from Biosearch Technologies, Inc., MG132 from Sigma, Deferoxamine mesylate salt (DFX) from Enzo life Sciences, Inc. Anti-acetylated eIF5A (Ac-eIF5A) rabbit polyclonal and anti-hypusinated eIF5A (Hyp-eIF5A) mouse monoclonal antibodies were raised as described previously [15]. Antibodies against α -tubulin (B-5-1-2) and Ac- α -tubulin (6-11B-1) were purchased from Sigma. Monoclonal antibodies against eIF5A and HIF-1 α were obtained from BD Bioscience. All cell lines were maintained in Dulbecco's modified Eagle's medium (Wako) containing 10% heat-inactivated Fetal Bovine Serum (FBS) (Invitrogen) and antibiotics at 37°C, 5% CO₂ in a humidified incubator.

RNA interference and transfection

Small interfering RNAs (siRNAs) against eIF5A were obtained from NipponGene (siRNA #1), QIAGEN (siRNA # 2,3,4,5), and Ambion (Life technologies: siRNA # 6), respectively. Each eIF5A siRNA target sequence is described in Table S1. Non-targeting control siRNA was purchased from NipponGene. All transfections were carried out using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to manufacture's instructions.

Immunoblotting

Immunoblotting was performed as described previously [22].

Quantitative PCR

Total RNA was prepared with RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocols and cDNA was synthesized by reverse transcription with oligo dT primers using RNA PCR Kit (AMV) Ver.3.0 (Takara Bio Inc.). Expression

of HIF-1 target genes was analyzed by quantitative PCR using SYBR Premix Ex Taq II (Takara Bio Inc.) in a LightCycler 480 (Roche). Each primer sequence used in this study is shown in Table S2.

Luciferase assay

Luciferase reporter assay was performed as described previously [23]. Briefly, HT1080 cells stably expressing x5HRE-luciferase reporter were lysed in NETN buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.1% NP-40) and 50 μ L of whole cell lysate was used for luciferase assay. Luciferase activity was measured by adding 50 μ L of luciferase assay reagent (Promega, Cat# E1483) using Synergy H4 hybrid reader (BioTek).

Spheroid growth

A549 cells (3×10^3 cells) were cultured in U-bottom 96-well plate (PrimeSurface 96U from Sumitomo Bakelite Co., LTD) for forming spheroids. After culture for 24 h, images of spheroids were captured using a Celigo Imaging Cytometer (Nexcelom Bioscience) (Day 0). Images were captured every two days for two weeks. Total spheroid area was measured using image J software.

Statistical analysis

To determine the significance of differences between two groups, the Student's t-test was performed for all experiments.

Results and Discussion

Hypoxia induces acetylation of eIF5A

DOHH, the second enzyme in the eIF5A hypusination pathway, is an iron-dependent monooxygenase that commonly requires molecular oxygen for its enzymatic reaction [24]. Because our previous observations indicate that hypusination of eIF5A blocks its acetylation [14, 15], we hypothesized that low oxygen tension increases eIF5A acetylation possibly by inhibiting DOHH-catalyzed hypusination. As shown in Fig. 1A, exposure to hypoxia increased the level of eIF5A acetylation in both HeLa and A549 cells without affecting α -tubulin acetylation. Because deacetylation of α -tubulin is catalyzed by the same enzymes, HDAC6 and SIRT2 [22, 25-27], as those for eIF5A [15, 28], this observation suggests that the increased level of eIF5A acetylation under hypoxia is due to suppression of hypusination by inhibiting DOHH rather than inhibition of deacetylation. This idea was further supported by the observation that eIF5A acetylation was dramatically increased by exposure to a hypoxia mimetic, deferoxamine mesylate (DFX) (Fig. 1B), which has also been reported to inhibit DOHH [29]. An additional treatment with both trichostatin A (TSA), a pan-inhibitor for Zn-dependent lysine deacetylases including HDAC6, and nicotinamide (NA), a pan-inhibitor for NAD-dependent lysine deacetylases including SIRT2, were unable to elicit a further increase in acetylation (Fig. S1), suggesting that the level of hypoxia-induced eIF5A acetylation is comparable to that which can be accomplished by the full inhibition of eIF5A deacetylation.

eIF5A is essential for HIF-1 α expression induced by exposure to hypoxia

To investigate a possible link between eIF5A acetylation and HIF-1 α expression under hypoxic conditions, we performed time course experiments for

monitoring eIF5A acetylation and HIF-1 α expression during hypoxia or with DFX, a hypoxia-mimicking agent. HIF-1 α expression induced by hypoxia or DFX was transient. Importantly, cells with high levels of eIF5A acetylation showed a decrease in the level of HIF1 α during an extended hypoxic period (Fig. 1B and C, and Fig. S2).

Because the increase in acetylation of eIF5A reflects the decrease in hypusination, it is suggested that HIF-1 α becomes destabilized even under hypoxic conditions when hypusinated, active eIF5A is downregulated. To test this possibility, we examined the effect of eIF5A knockdown on HIF-1 α expression using a series of siRNA oligos. As shown in Fig. 2A, induction of HIF-1 α protein expression upon hypoxia in A549 cells was dramatically diminished by treatment with all siRNA oligos against eIF5A tested. Importantly, the degree of reduction of HIF-1 α protein levels was well correlated with the efficiency of the eIF5A knockdown (Fig. 2A). This observation is not cell-type specific, because eIF5A knockdown in different types of cancer cells including HT1080, HeLa, and PC-3 also displayed reduced HIF-1 α expression under hypoxia (Fig. S3A-C). These results indicate that eIF5A is necessary for HIF-1 α expression in response to hypoxia. Of note, both PHD2 and DOHH are oxygen-sensitive enzymes, however; induction of HIF-1 α expression by inhibiting PHD2 preceded that of eIF5A acetylation possibly by inhibiting DOHH under hypoxic conditions (Fig. 1B and C, and Fig. S2). This delayed induction of eIF5A acetylation may be due to the possibility that only the newly synthesized unhypusinated form of eIF5A is acetylated since hypusination of eIF5A is irreversible.

Because hypusine modification of eIF5A is essential for its physiological activity [30], we next tested if the active hypusinated form of eIF5A is required for HIF-1 α expression in hypoxia. To this end, we used *N*1-guanyl-1, 7-diaminoheptane

(GC7), an inhibitor of DHS, to inhibit hypusination of eIF5A. Similarly to the effect of eIF5A knockdown, treatment with GC7 suppressed the expression of HIF-1 α in response to hypoxia, concomitant with a reduction of the hypusinated form of eIF5A (Fig. 2B). As expected, not only hypoxia but also GC7 treatment increased acetylation of eIF5A. Importantly, GC7 reduced both the levels of HIF-1 α expression and eIF5A hypusination in hypoxic cells in a dose-dependent manner (Figure 2C). Of note, hypoxia did not apparently affect the level of hypusination of eIF5A. This may be simply due to the cross-reactivity of our antibody for hypusinated eIF5A to the deoxyhypusinated form [15]. These results strongly suggest that hypusinated eIF5A is indispensable for induction of HIF-1 α expression upon hypoxia. The role of eIF5A in expression of HIF-1 α appears to be posttranscriptional since the HIF-1 α mRNA level was not changed in eIF5A-knockdown cells (Fig. 2D). Treatment of the knocked-down cells with the proteasome inhibitor MG132 recovered the level of HIF-1 α protein (Fig. 2E), suggesting that eIF5A affects protein stability of HIF-1 α in hypoxia.

eIF5A is essential for the transcriptional activity of HIF-1 α

We next examined the effect of inhibition of eIF5A on the transcriptional activity of HIF-1 α using HT1080 cells stably expressing the x5HRE-luciferase reporter [23]. Low oxygen tension increased HIF-1 α transcriptional activity, but this increase was suppressed by eIF5A knockdown (Fig. 3A) or treatment with GC7 (Fig. 3B). The effect of eIF5A inhibition on HIF-1 α transcriptional activity was further examined by measuring mRNA levels of HIF-1 α target genes. Upregulation of mRNA levels of HIF-1 target genes in response to hypoxia was almost completely abolished in eIF5A-knockdown or GC7-treated cells (Figure 3C and D). These results

further support the importance of eIF5A and its hypusination in the activity of HIF-1 α in response to hypoxia.

Functional eIF5A is important for tumor sphere formation

Tumor spheroid culture is an *in vitro* tumor model more physiologically relevant than conventional 2D culture models. Like solid tumors, the environment inside of spheroids is hypoxic, where HIF-1 α -mediated adaptive response can be observed. Indeed, consistent with previous reports [31, 32], HIF-1 α knockdown significantly reduced the size of tumor spheroids (Fig. S4). To test whether eIF5A is involved in HIF-1 α activation in tumor spheroids under normoxia (Fig. 2 and 3), we investigated the effect of impaired eIF5A function on tumor spheroid formation by monitoring the size of spheroids. A significant decrease in the size of spheroids was observed in eIF5A-knockdown spheroids cultured for over 10 days (Fig. 4A). In addition, GC7 treatment also significantly reduced the size of spheroids, which correlated with decreases in the levels of both eIF5A hypusination and HIF-1 α protein (Fig. 4B and C), suggesting that inhibition of tumor spheroid formation is due to HIF-1 α inactivation by inhibiting hypusination of eIF5A. These results imply that eIF5A functions to keep tumor spheroids growing by enhancing HIF-1 α expression.

In this study, we uncovered a novel function of eIF5A in the regulation of HIF-1 α expression in response to hypoxia. Our findings suggest that eIF5A enhances HIF-1 α expression at the posttranscriptional level, as eIF5A knockdown did not affect mRNA levels of HIF-1 α (Fig. 2E). However, the precise molecular mechanism underlying eIF5A-mediated HIF-1 α expression remains to be elucidated. Involvement of eIF5A in translation elongation [1, 4] suggests that eIF5A is directly involved in translation of HIF-1 α . However, this possibility seems unlikely because the amino

acid sequence of HIF-1 α protein does not contain consecutive proline motifs, translation of which requires eIF5A [5, 6]. Alternatively, eIF5A may regulate HIF-1 α stability. Proteasomal degradation of HIF-1 α under hypoxia is known to be regulated by several factors other than PHD, including SUMOylation [33], hypoxia-associated factor (HAF) [34], RACK1 [35], and SHARP1 or DEC2 [36]. eIF5A may promote HIF-1 α stability through the regulation of the translation of proteins involved in these pathways. Our observation that reduction of HIF-1 α expression by eIF5A knockdown was rescued by proteasome inhibitors (Fig. 2D) supports this hypothesis. Further studies should be performed for unveiling the molecular mechanism by which eIF5A regulates HIF-1 α expression.

Involvement of eIF5A in tumorigenesis appears to be controversial. Screening using a short hairpin RNA library in human lymphomas identified eIF5A as a tumor suppressor gene [37]. On the other hand, several reports show that eIF5A possesses oncogenic activities in some types of cancers including pancreatic ductal adenocarcinoma and colorectal cancer [38, 39]. The reason for this inconsistency is currently unknown, but it may depend on cell types or microenvironmental conditions. As our results clearly showed that eIF5A is essential for HIF-1 α activation in response to hypoxia, eIF5A may act as an oncogene under hypoxic microenvironmental conditions. Because HIF-1 α -mediated adaptive responses in hypoxia influence the efficacy of cancer therapy [40], the regulation of eIF5A-mediated HIF-1 α activation is an attractive therapeutic target. Indeed, eIF5A knockdown or inhibition of hypusination by GC7 significantly reduced tumor spheroid formation (Fig. 4). Thus, hypusination of eIF5A is a unique post-translational modification, which provides a good means for targeted therapy.

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Figure legends

Fig. 1. Hypoxia induces acetylation of eIF5A. (A) HeLa and A549 cells were grown in hypoxia (1 % O₂) or normoxia (20 % O₂) for 24 h. The level of eIF5A acetylation was detected by immunoblotting with an anti-Ac-eIF5A antibody. (B and C) Induction of HIF-1 α expression precedes eIF5A acetylation in response to hypoxic conditions. A549 cells were exposed to 200 μ M DFX (B) or hypoxia (C) for the indicated periods of time. Cell lysates were immunoblotted with the indicated antibodies.

Fig. 2. Hypusinated eIF5A is required for HIF-1 α expression in hypoxia. (A and B) HIF-1 α expression in hypoxia is reduced by inhibition of eIF5A. A549 cells were transfected with control or siRNA oligonucleotides targeting eIF5A mRNA (A) or treated with 30 μ M GC7 for 12 h (B), followed by exposure to hypoxia for 12 h. Cell

lysates were immunoblotted with the indicated antibodies. (C) Dose-dependent effects of GC7 on HIF-1 α expression in hypoxia. HT1080 cells were treated with different concentrations of GC7 for 12 h, followed by exposure to hypoxia for 12 h. Cell lysates were immunoblotted with the indicated antibodies. (D) mRNA level of HIF-1 α is not changed by eIF5A knockdown. A549 cells were transfected with control or eIF5A siRNA oligonucleotides, followed by exposure to hypoxia for 12 h. The level of HIF-1 α mRNA was measured by quantitative real time PCR. (E) MG132 recovers reduction of HIF-1 α expression in eIF5A knockdown cells. A549 cells were transfected with control or eIF5A siRNA oligonucleotides, followed by exposure to 10 μ M MG132 and hypoxia for 12 hours. Cell lysates were immunoblotted with the indicated antibodies.

Fig. 3. Hypusinated eIF5A is essential for the transcriptional activity of HIF-1 α in hypoxia. (A and B) Transcriptional activity of HIF-1 α in hypoxia is decreased by inhibition of eIF5A. HT1080 cells stably expressing HRE-luciferase were transfected with control or eIF5A siRNA oligonucleotides (A), or treated with the indicated concentrations of GC7 for 12 h (B), followed by exposure to hypoxia for 12 h. Luciferase activity in cells was measured using a Luminometer. (C and D) Increase in HIF-1 α target-gene expression in response to hypoxia is decreased by inhibition of eIF5A. A549 cells were transfected with control or eIF5A siRNA oligonucleotides (C), or treated with 30 μ M GC7 for 12 h (D), followed by exposure to hypoxia for 12 h. Levels of mRNAs of the indicated HIF-1 α target genes were analyzed by quantitative real time PCR.

Fig. 4. Functional eIF5A is important for tumor spheroid growth. (A) Tumor spheroid formation is reduced by eIF5A knockdown. A549 cells were transfected with control or eIF5A siRNA oligonucleotides, followed by culture in a U-bottom 96 well plate for two weeks. Data are the means $\pm$ S.E. of three independent experiments. *P<0.05. Pictures show representative examples. (B) Tumor spheroid formation is reduced by treatment with GC7. A549 cells cultured in a U-bottom 96-well plate were treated with the indicated concentrations of GC7 every three days. Data are the means $\pm$ S.E. of three independent experiments. *P<0.05. Pictures show representative examples. (C) HIF-1 α expression level is reduced in GC7-treated spheroids. Spheroids cultured in a U-bottom 96 well plate at days 10 and 12 were collected and the expression level of HIF-1 α was detected by western blotting.

Fig. 1

Fig. 2

Fig. 3

A **B** **C** **Fig. 4**