Acetylation regulates subcellular localization of eukaryotic translation initiation factor 5A (eIF5A)

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\textbf{Abstract}

Eukaryotic translation initiation factor 5A (eIF5A) is a protein subject to hypusination, which is essential for its function. eIF5A is also acetylated, but the role of that modification is unknown. Here, we report that acetylation regulates the subcellular localization of eIF5A. We identified PCAF as the major cellular acetyltransferase of eIF5A, and HDAC6 and SIRT2 as its major deacetylases. Inhibition of the deacetylases or impaired hypusination increased acetylation of eIF5A, leading to nuclear accumulation. As eIF5A is constitutively hypusinated under physiological conditions, we suggest that reversible acetylation plays a major role in controlling the subcellular localization of eIF5A.

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1. Introduction

eIF5A is a small (~17 KDa) acidic protein that is essential for cell proliferation in many eukaryotes. Mammals have two paralogs, eIF5A1 and eIF5A2. eIF5A1 is ubiquitously expressed in all tissues [1–3] and is modified post-translationally with an unusual amino acid, hypusine [\textsuperscript{4}]. Hypusine is synthesized from the polyamine spermidine in two consecutive enzymatic steps: first, deoxyhypusine synthase (DHS) catalyzes the transfer of the aminobutyl moiety from spermidine to a specific lysine residue (Lys50 in human eIF5A) to form the deoxyhypusine intermediate, which is ultimately hydroxylated by deoxyhypusine hydroxylase (DOHH) to produce active hypusinated eIF5A [5]. eIF5A and its hypusine modification are essential in cellular physiology: hypusination mutants exhibit slowed cell growth in mammals [6]; in yeast, a hypusination site mutant (K51R) fails to rescue the eIF5A knockout [3]. Although eIF5A is evidently essential for eukaryotic cell proliferation [2,7,8], its precise function has remained unknown. eIF5A was originally identified, along with other initiation factors, from high-salt washes of rabbit reticulocytes lysate ribosomes [9], and was shown to stimulate methionyl-puromycin synthesis [10]; therefore, eIF5A was considered to be a translational initiation factor. However, recent findings suggest that it plays a role in translation elongation [11] and other aspects of RNA metabolism such as RNA export [12,13].

The subcellular localization of eIF5A and its involvement in nuclear export have remained controversial. eIF5A was initially demonstrated to be localized at the nuclear pore complex, where it is involved in Rev-mediated nuclear export of unspliced HIV-1 mRNAs [12]. However, Shi et al. showed that eIF5A is cytoplasmic in a variety of cell types, and that HIV-1 Rev overexpression does not affect its cellular distribution [14]. Subsequently, Rosorius et al. demonstrated that eIF5A undergoes nucleo-cytoplasmic shuttling, and proposed that CRM1 acts as its nuclear export factor [15]. Although eIF5A lacks a conventional nuclear import signal, experiments with GFP-tagged truncated eIF5A suggested that the ~19 N-terminal residues are responsible for eIF5A nuclear localization [16]. eIF5A regulates diverse cellular processes, including induction of p53-dependent apoptosis and cytokine-mediated islet β cell...
dysfunction [17,18], suggesting a functional role for eIF5A in the nucleus. Recently, Lee et al. demonstrated that exogenously produced eIF5A is primarily expressed as an unhyposinuated precursor, and can only be hyposinuated when co-expressed with DHS and DOHH. Moreover, unhyposinuated GFP-eIF5A is primarily localized in the nucleus, whereas the hyposinuated form is cytoplasmic, suggesting that hyposinuation determines the subcellular distribution of eIF5A [19]. However, because hyposinuation occurs on almost all eIF5A proteins, co-translationally [20] and irreversibly, hyposinuation seems unlikely to be responsible for the dynamic regulation of nucleo-cytoplasmic transport under physiological conditions. eIF5A residues K47 and K68 are post-translationally modified by acetylation [20,21]. The crystal structure of human eIF5A consists of two domains of predominantly β-sheet character [22]. The acetylation and hyposinuation sites (i.e. K47 and K50, respectively) are in close proximity on a disordered loop structure, the hypusine loop, in the N-terminal domain. The positive charge at K47 is essential for eIF5A activity, suggesting that acetylation at this position negatively regulates eIF5A function in protein synthesis [23]. However, the role of eIF5A acetylation in other functions remains elusive.

In this study, we found that the acetylated form of eIF5A is primarily enriched in the nucleus, whereas unacetylated eIF5A is primarily cytoplasmic; this distinction is even more pronounced in the presence of histone deacetylase (HDAC) inhibitors. These results suggest that acetylation acts as a molecular switch for eIF5A, allowing it to exert distinct functions in the cytoplasm and nucleus.

2. Materials and methods

2.1. Compounds, cell culture and transfection

Tricostetan A (TSA) and nicotinamide (NA) were purchased from Wako Chemicals. GC7 was purchased from BIOSEARCH TECHNOLOGIES, INC., SCOP402 was kindly provided by Dr. Norikazu Nishino (Kyushu Institute of Technology, Japan). All compounds were prepared as stock solutions in dimethyl sulfoxide (DMSO) and stored at −20 °C. 293T and HeLa cells were transiently transfected with expression vectors using the FuGENE HD reagent (Roche) and the Lipofectamine™ LTX reagent (Invitrogen), respectively. RNAi oligos used in this study were obtained from Dharmacon, with the exception of the eIF5A and HDAC1 oligos, which were obtained from Nippon Gene. All sequence information of siRNA oligos is described in Supplemental Table 1.

2.2. Plasmids construction

The cDNA encoding human eIF5A was PCR-amplified from a human brain cDNA library and sub-cloned into either GFP-pEGFP (BD Biosciences) or to the C-terminus of Flag-tag in pcDNA3 (Invitrogen). Plasmids for expression of GFP-eIF5A and Flag-eIF5A mutant proteins (K47R, K47Q, K50R, and K47,50R) were generated by site-directed mutagenesis using primer sets containing the desired mutations. DNA encoding human DHS was PCR-amplified from a human brain cDNA library and sub-cloned into pcDNA3-Flag. The pcEFL-DOHH construct was kindly provided by Dr. Myung Hee Park (National Institute of Health, USA); the coding sequence was PCR-amplified and sub-cloned into the pcDNA3-Myc vector.

2.3. Antibodies and immunoblotting

A rabbit polyclonal antibody against acetylated eIF5A was raised using an acetylated eIF5A peptide (VDMSTSK(Ac)TG(HGHC) with the aid of the Support Unit for Bio-material Analysis and Animal Resources Development at the RIKEN BSI Research Resources Center (RRC). A mouse monoclonal antibody against hyposinuated eIF5A was raised using a hyposinuated eIF5A peptide (STSKTGK(Hyp)HGAKC). Mouse monoclonal anti-Flag M2, anti-α-tubulin (B-5-1-2), and anti-Ac-α-tubulin (6-11B-1) antibodies were purchased from Sigma. Monoclonal anti-c-Myc (9E10) and monoclonal anti-eIF5A antibodies were obtained from Santa Cruz Biotechnology and BD Bioscience, respectively. Rabbit polyclonal anti-CBP, anti-PCAF, anti-GCN5, anti-SIRT1, and anti-SIRT2 antibodies were purchased from Cell Signaling Technology. Immunoblotting was performed using whole cell lysates, prepared by collecting washed cells directly in 1× SDS–PAGE sample loading buffer and heating at 95 °C for 5 min. Equal volumes of each whole cell lysate was resolved by SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting. Membranes were incubated with primary and secondary antibodies; immune complexes were detected using the Immobilon™ Western Chemiluminescent HRP substrate (Millipore); and luminescence was analyzed using a LAS-3000 image analyzer (GE Healthcare).

2.4. Immunostaining and fluorescence microscopy

Cells grown on coverslips were fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.2% Triton X-100 in PBS (PBT). Fixed cells were blocked with 5% normal goat serum in 0.1% PBT for 15 min and incubated with primary antibodies against eIF5A (1:200 dilution), Ac-eIF5A (1:100), Flag (1:500), or Myc (1:500) for 1–2 h followed by the Alexa Fluor 594, Alexa Fluor 488, or Alexa Fluor 350 secondary antibodies (Invitrogen) for 20 min. Slides were mounted with mounting medium (Vector Laboratories), and images were taken using a Delta Vision Microscope (SEKI Technontron Corp.).

2.5. In vitro histone acetyltransferases (HATs) assay

Recombinant His-tagged eIF5A was purified as described previously [24]. In vitro HAT assays were performed as described previously [24] with minor modifications: briefly, 8 μg of His-eIF5A was incubated with 8.5 μg of purified Flag-PCAF in the presence of 0.33 mM acetyl CoA. The reaction was performed at 37 °C for 1 h. The samples were mixed with reducing/loading buffer and resolved by SDS–PAGE on 13% polyacrylamide gels followed by immunoblotting with the indicated antibodies.

3. Results and discussion

3.1. Hyposinuation inhibits acetylation

Using an anti-acetylated lysine (Ac-Lys) antibody and radioactively labeled spermidine, Lee et al. observed that co-transfection with DHS and DOHH enables hyposinuation of eIF5A exogenously expressed in mammalian cells; furthermore, unhyposinuated eIF5A expressed in the absence of DHS and DOHH is heavily acetylated at K47 but is dramatically deacetylated upon hyposinuation, suggesting crosstalk between hyposinuation and acetylation [19]. We first confirmed these observations using a newly developed anti-acetylated eIF5A (Ac-eIF5A), and an anti-hyposinuated eIF5A (Hyp-eIF5A) antibody. Exogenously expressed eIF5A could be hyposinuated only when co-expressed with DHS and DOHH (Fig. 1A). In addition, DHS and DOHH expression reduced the acetylation at K47 (Fig. 1A). The specificity of the antibodies was confirmed by detecting acetylation and hyposinuation of K47R and K50R mutants as well as wild-type eIF5A overexpressed in 293T cells with or without DHS/DOHH (Fig. 1B). In the absence of DHS and DOHH, wild-type Flag-eIF5A was heavily acetylated, but the acetylation signal was completely lost in K47R, indicating that the antibody can specifically recognize eIF5A acetylated at K47. The hyposinuation signal
was detected in exogenously expressed eIF5A proteins in the presence of DHS and DOHH, but not in the K50R mutant lacking the hypusination site. Hypusination of the K47R mutant, which is defective in acetylation, suggests that acetylation is not required for hypusination. To further observe the relationship between hypusination and acetylation, we treated HeLa cells with different concentrations of GC7, a DHS inhibitor, for 16 h. Immunoblot analysis showed that GC7 inhibited eIF5A hypusination at a concentration as low as 2 μM. While the eIF5A acetylation level was low in cells treated with GC7 at a low concentration (0.5 μM), it was dramatically increased by 2 μM or higher concentrations, confirming that hypusination negatively regulates acetylation (Fig. 1C). Endogenous eIF5A in the steady state is poorly acetylated because eIF5A is fully hypusinated in the absence of GC7 (Fig. 1C). Because crystal structure analysis revealed that the two lysine residues for acetylation and hypusination are closed [22], hypusination may hinder the access of the eIF5A acetyltransferase, PCAF (see below).

### 3.2. Identification of HATs and HDACs regulating eIF5A acetylation status

To identify the cellular acetyltransferase responsible for eIF5A acetylation, we next performed siRNA-mediated knockdown experiments using 293T cells (Fig. 2A). Of four acetyltransferases tested, only PCAF knockdown significantly reduced the acetylation level of endogenous eIF5A. Furthermore, in an in vitro HAT assay using bacterially produced recombinant eIF5A, we found that PCAF indeed acetylates eIF5A in an acetyl-CoA-dependent manner (Fig. 2B). To identify the class of HDACs involved in deacetylation of eIF5A, we treated 293T cells with three HDAC inhibitors of differing specificity: TSA, NA, and SCOP402. TSA inhibits all class I/II enzymes, whereas NA inhibits class III; SCOP402 inhibits class I/II enzymes except HDAC6 [25]. Immunoblot analysis of the cell lysates collected after 16 h incubation with the HDAC inhibitors showed that acetylated eIF5A was increased in both TSA- and NA-treated cells but not in SCOP402-treated cells (Fig. 2C). Because the only difference in target enzyme specificity between TSA and SCOP401 is HDAC6 inhibition, we conclude that at least HDAC6 is involved in deacetylation of eIF5A. The acetylation signal was significantly enhanced in cells treated with both TSA and NA simultaneously, suggesting that a class III HDAC in addition to HDAC6 is also involved in the eIF5A deacetylation. Importantly, the level of eIF5A hypusination was not changed in response to treatment with HDAC inhibitors (Fig. 2C), suggesting that acetylation does not affect its hypusination. Next, we expressed ten major cellular deacetylase enzymes together with Flag-eIF5A in 293T cells (Fig. 2D, S1A). Only overexpression of HDAC6 or SIRT2, but not their catalytically dead mutants, significantly reduced the acetylation level of eIF5A. Consistent with this, the acetylation level of endogenous eIF5A was greatly increased by HDAC6 and SIRT2 knockdown in 293T cells (Fig. 2E), indicating that these enzymes actually work as deacetylases under physiological conditions. Additionally, SIRT1 overexpression slightly but reproducibly reduced eIF5A acetylation. Indeed, both recombinant SIRT1 and SIRT2 catalyzed the deacetylation of eIF5A in an in vitro deacetylase assay (Fig. S1B–C). However, the SIRT1 knockdown failed to increase endogenous eIF5A acetylation in 293T cells (Fig. 2E). These results indicate that HDAC6 and SIRT2 are the major cellular deacetylases of eIF5A.

### 3.3. Effect of acetylation and hypusination on subcellular distribution of eIF5A

Lee et al. reported that hypusination is important for the localization of eIF5A to the cytoplasm, where translation occurs [19]. In general, reversible acetylation plays a role in regulating protein–protein interactions and subcellular localization. As the acetylation site is located within a loop close to the hypusination site (K50), it seemed possible that eIF5A acetylation plays some role in the subcellular localization of eIF5A. Therefore, we investigated the subcellular localization of acetylated species of endogenous eIF5A by indirect immunofluorescence. Although total eIF5A was primarily cytoplasmic in nature, acetylated eIF5A was predominately localized to the nucleus (Fig. 3A). The fluorescent signals obtained with eIF5A and Ac-eIF5A antibodies are specific, because these signals were diminished, respectively, by eIF5A knockdown and by competition with the acetylated peptide used as the epitope (Fig. 3A). Furthermore, treatment with either HDAC inhibitors (TSA/NA) or DHS inhibitor (GC7) induced a dramatic shift of subcellular distribution from primarily cytoplasmic to nuclear (Fig. 3B). These results clearly demonstrate that cytoplasmic eIF5A is not...
acetylated, whereas nuclear eIF5A is acetylated; furthermore, acetylation directs nuclear localization.

Next we tested whether eIF5A K47 acetylation is indeed important for controlling the subcellular localization of eIF5A, by expressing GFP-eIF5A and its mutants. As shown in Fig. 4A, unhypusinated GFP-eIF5A expressed in the absence of DHS/DOHH tended to localize to the nucleus. On the other hand, both wild-type eIF5A and a mutant incapable of being acetylated (K47R) were successfully localized to the cytoplasm when hypusinated by DHS/DOHH (Fig. 4A). Importantly, the subcellular distribution of a mutant mimicking acetylation (K47Q) was constitutively nuclear even after hypusination by DHS/DOHH (Fig. 4A), suggesting that acetylation interferes with the eIF5A nuclear import. Similarly, the K50R mutant, which is unhypusinated but heavily acetylated, also accumulated in the nucleus. The K47,50R mutant, which can be neither acetylated nor hypusinated, was also localized to the nucleus (Fig. 4A–B). These results indicate that cytoplasmic localization of eIF5A requires not only hypusination but also hypoacetylation.

Various groups studying the subcellular localization of eIF5A have reported somewhat inconsistent results. For instance, Xiao et al. reported that eIF5A is cytoplasmic and co-localizes with the endoplasmic reticulum in multiple mammalian cell lines [26]. In contrast, Rosorius et al. proposed that eIF5A enters the nucleus through passive diffusion and may be exported from the nucleus in a CRM1-dependent manner [27]. On the other hand, other groups showed that exportin 4, an importin β family receptor, is responsible for the nuclear export of eIF5A [13,28]. Recently, Lee et al. reported that compared to fully hypusinated cytoplasmic, endogenous eIF5A, exogenously expressed eIF5A lacking hypusination tends to localize in the nucleus, but can be relocalized to the cytoplasm when fully hypusinated [19]. Compared to the stable hypusine modification, acetylation is reversible and may play an important role in regulating eIF5A cellular activity by altering the conformation of the hypusine loop or eIF5A’s affinity for nuclear transport factors. Thus, control of subcellular localization of eIF5A appears to be dependent more on acetylation than on hypusination under the physiological condition. Our data indicate that co-translationally hypusinated eIF5A is acetylated by PCAF and deacetylated by HDAC6 and SIRT2, which facilitates nucleo-cytoplasmic

![Fig. 2. Identification of the acetyltransferases (HATs) and deacetylases (HDACs) of eIF5A.](image-url)
shuttling of eIF5A. Although the nuclear function of eIF5A is still unclear, the evolutionarily conserved acetylation occurring near the site of hypusination may regulate its nuclear localization and its function in the nucleus.

4. Conclusion

We have elucidated the enzymes responsible for determining the acetylation status of eIF5A K47: PCAF performs acetylation, while HDAC6 and SIRT2 perform deacetylation. Our results suggest that eIF5A acetylation status regulates its subcellular localization. This knowledge will enable us to study the possible functional role of this important protein in the nuclear compartment under physiological conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.06.042.

References and notes