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Methylation of human elongation factor eEF1A2 is not essential for eEF1A2-eEF1B interaction

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Aim. Study on a possible role of methylation of lysine residues of eEF1A2 in its interactions with the elongation translation complex eEF1B. **Methods.** Mutagenesis, cloning, 293 human cell culture, BRET (bioluminescence resonance energy transfer), cell transfection, HaloTag pull down of protein complexes, Western blot, densitometry. **Results.** Five mutants of eEF1A2 with a single substitution of methylable lysine residue for arginine and the mutant with all five lysine residues mutated were generated. BRET analysis and HaloTag pull down experiments of isolated protein complexes have shown no differences in interactions of eEF1B subunits with eEF1A2 and its unmethylable mutants. **Conclusion.** Methylation of eEF1A2 apparently does not influence the interactions of eEF1A2 with eEF1B subunits.

Keywords: eEF1A2, eEA1Bα, eEA1Bβ, eEA1Bγ, methylation, BRET, HaloTag.

Introduction

Translation elongation factor eEF1A*GTP carries aminoacyl-tRNA to the A-site of the 80S ribosome facilitating the process of ribosomal protein synthesis. GTP hydrolysis finalizes the codon-anticodon recognition, then eEF1A*GDP leaves the ribosome [1]. The eEF1B complex comprising Bα, Bβ and Bγ subunits helps to exchange GDP for new GTP in eEF1A molecule [2, 3], that is why the eEF1A-eEF1B interaction is very important for translation to proceed.

There are two tissue-specific isoforms of eEF1A with mutually exclusive expression patterns in the organism [4], which are characterized by different spatial organization [5, 6] and different ability to bind protein partners [7–9]. Both isoforms show the significant level of post-translational modifications [10–12].

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eEF1A2 revealed oncogenic properties [13, 14]. An increased expression of eEF1B subunits was also found in different cancers [15, 16].

Methylation of lysine residues is an important modification involved in the regulation of cell activity. For instance, methylation of specific lysine residues in histones indorses binding of the proteins and induces transcriptional silencing [17]. On the other hand, methylation of lysine residues in histones can impede binding of effector molecules [18]. Methylation of lysine residues has been found in several non-histone proteins, including transcription factors, receptors, ribosomal proteins, and translation factors [19–22]. A significant regulatory potential of this modification is emphasized by the discovery of demethylases, the enzymes that can remove a methyl group or several methyl groups from the already modified proteins [23]. Recent studies indicate the involvement of methylation/demethylation processes in both histone and non-histone proteins into oncogenesis [24–27].

Methylation of five conservative lysine residues of eEF1A (K36, K55, K79, K165 and K318) is known for more than 20 years [28]. A role of eEF1A methylation in tumorigenesis was recently suggested [29], however, the molecular mechanisms involved are not clear. Since all lysine residues of eEF1A capable of methylation are situated outside the protein globule [30, 31], we reasoned that methylation of these residues might affect the interaction of eEF1A with other proteins. eEF1B is one of the main translational partners of eEF1A during the elongation step of protein synthesis. Here, we study the effect of methylation of the proto-oncogenic eEF1A2 isoform on its interaction with eEF1B complex.

Materials and Methods

Cloning of eEF1A2 into plasmid pFC14K encoding HaloTag at the C-terminus of the target protein was performed using reagents and materials from the "HaloTag® Complete Pull-Down System" kit (Promega, USA). Amplification primers: 5′-GGCTGCGATCG-CCATGGGAAAGGAAAAGACTCATAT-3' (direct) and 5′-GTCGGTTTAAACTTTAGC-CTTCTGAGCTTTCT-3' (reverse). The composition of the PCR reaction mixture: matrix pcDNA3.1 (+)/eEF1A1 2 ng, 1x Phusion HF buffer, dNTP mixture 200 μM, primers 25 pmol, Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA) 1 unit. PCR amplification conditions: initial denaturation 98° C 30 s, denaturation 98° C 30 s, annealing 61° C 10 s, elongation 72° C 21 s, 30 cycles, final elongation 72° C 10 min. The PCR product was transformed into competent E. coli XL10 Gold cells. The plasmid was isolated using the GeneJet™ MiniPrep Purification Kit (Thermo Scientific, USA) according to the manufacturer's method. The identity of the obtained plasmid was verified by restriction using FD HindIII (Thermo Scientific, USA).

Directed mutagenesis was performed by PCR by the Quick change method (Stratagene, [32]). The primers for replacement of lysine residues by arginine in eEF1A2:

1A2-K36R-Direct: 5'CAAATGCGGAGGT-ATTGACA**G**AAGGACCATTGAGAAGTTC 3'

1A2-K36R-Reverse: 5' GAACTTCTCAAT-GGTCCTTC**T**GTCAATACCTCCGCATTTG 3'

1A2-K55R-Direct: 5' GAAGGGATCCT-TCA**G**GTATGCCTGGGTG 3'

1A2-K55R-Reverse: 5' CACCCAGGC-ATACC**T**GAAGGATCCCTTC 3'

1A2-K79R-Direct: 5' GACATCTCCCTCT-GGA**G**GTTCGAGACCACCAAG 3'

1A2-K79R-Reverse: 5' CTTGGTGGTCTC-GAAC**C**TCCAGAGGGAGATGTC 3'

1A2-K165R-Direct: 5' CCTACAGCGAG-A**G**GCGCTACGACG 3'

1A2-K165R-Reverse: 5' CGTCGTAGCG-CC**T**CTCGCTGTAGG 3'

1A2-K318R-Direct: 5' CGTGTCGGTGA-**G**GGACATCCGGC 3'

1A2-K318R-Reverse: 5' GCCGGATGTC-C**C**TCACCGACACG 3'

The composition of the PCR reaction mixture: matrix pFC14K/eEF1A2 10 ng, 1x Phusion HF buffer, dNTPs 200 μM, $MgCl_2$ 2.3 mM, primers 25 pmol, Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA) 2 units. PCR amplification conditions: initial denaturation 98° C 3 min, denaturation 98° C 12 s, annealing 72° C 30 s, elongation 72° C 3 min, 18 cycles, final elongation 72° C 10 min. For matrix restriction, the PCR mixture was incubated with 1μl of restriction enzyme DpnI at 37° C overnight and deactivated by heating at 65° C for 20 min.

Cells 293 were grown at 37° C, 5 % CO_2, 100 % humidity in DMEM containing 5 % FBS and antibiotics penicillin and streptomycin and glutamine. Transfection of 293 cells with plasmids pFC14K/eEF1A2, pFC14K/eEF1A2(KxxR) and control vector HaloTag was performed with TurboFect reagent (Thermo Scientific, USA) for 24 h, after which cells were pelleted, lysed using mammalian lysis buffer (Promega, USA) at — 80° C until use. Affinity purification of partner proteins was performed using the HaloTag® Complete Pull-Down System (Promega, USA) according to the manufacturer's instructions. Proteins were separated in 10 % PAGE for expression control of HaloTag-eEF1A2(KxxR). Proteins from lysates separated by SDS-PAGE were transferred to PVDF membrane (Hybond-P) at 30 V for 30 min. The membrane was then blocked for 1 h at room temperature in 5 % dried nonfat milk in Tris-buffered saline. After blocking, the membranes were incubated with primary antibodies (1:10000 rabbit anti-HaloTag, Promega, USA) in 3 % dried non-fat milk in 1 X Tris-buffered saline with 0.1 % Tween 20 (v/v, TBST) overnight at 4°C. After washing with 1X TBST, the membrane was incubated with anti-rabbit IgG-HRP (1:5000) secondary antibody in 3 % dried nonfat milk in 1 X TBST or anti-mouse antibody (1:10000) in 3 % dried nonfat milk in 1 X TBST for 1 h at room temperature. ECL was used to visualize bands probed with HRP secondary antibody (Amersham Biosciences ECL Prime). The gel was photographed on a ChemiDoc tool (BioRad, USA). Densitometry was performed using ImageLab software (BioRad, USA).

Interactions of eEF1A2 and its mutants with eEF1Bα by BRET method were analyzed with NanoBRET™ Protein:Protein Interaction System (Promega, USA) [33]. eEF1Bα was cloned into pNLF1 plasmid (Promega, USA). The used primers are: 5'AGTAGAATTCGT-AATGGGTTTCGGAGACCTGAAAAG 3' (direct) and 5' AGTGTCTAGATTAGATCT-TGTTGAAAGCAGCCAC 3'(reverse). The composition of the PCR reaction mixture: matrix pET/eEF1Bα 2 ng, 1x Phusion HF buffer, dNTP mixture 200 μM, primers 25 pmol, Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA) 1 unit. PCR amplification conditions: initial denaturation 98 ° C 30 s, denaturation 98 ° C 12 s, annealing 64° C

15 s, elongation 72° C 12 s, 30 cycles, final elongation 72° C 10 min. The PCR product and pNLF1 plasmid were restricted with FD EcoRI and FD XbaI (Thermo Scientific, USA), ligated with T4 DNA Ligase (HC) (Promega, USA). The product of ligation was transformed into competent E. coli XL10 Gold cells. The plasmid was isolated using the GeneJet ™ MiniPrep Purification Kit (Thermo Scientific, USA) according to the manufacturer's method. The identity of the obtained plasmid was verified by restriction using FD EcoRI and FD XbaI (Thermo Scientific, USA).

The Control Vector plasmid (Promega, USA) expressing the HaloTag alone was used as a negative control. 300 000 cells 293 were plated into a well of a 12-well plate and were grown overnight in DMEM medium at 37°C, 5 % CO. After the attachment to wells the cells were transfected with TurboFect reagent (Thermo Scientific, USA) by plasmids 2 μg HaloTag plasmid — pFC14K/eEF1A2 or its mutants or HaloTag Control Vector and 0.02 μg NanoLuc Plasmid pNLF1/eEF1Bα (ratio 100 : 1). In 24 hours the cells were replanted into 96-well plate (2×10^5cells/well) in Opti-MEM ® I Reduced Serum Medium, no phenol red (Life Technologies, USA) + 4 % FBS, containing 100 nM HaloTag®NanoBRET™ 618 Ligand or 0.1 % DMSO. Cells were incubated at 37°C, 5 % CO_2 overnight (18–24 hours). 5X solution of NanoBRET™ Nano-Glo® Substrate in Opti-MEM®I Reduced Serum Medium without phenol red was added to each well up to 1 x final dilution immediately before measurement. Donor emission (460nm) and acceptor emission (618nm) were measured using plate reader Synergy HT (Bio-Tek Instrument, USA).

Results and Discussion

To investigate the role of methylation of several specific Lys residues in eEF1A2 we produced the mutant proteins where these residues were replaced by Arg. Five mutants with a single substitution, K36R, K55R, K79R, K165R or K318R, were generated. "Full" mutant (FM) was also produced, in which all five indicated Lys residues were replaced by Arg. Arg cannot be methylated by methyltrasferases specific for Lys and the replacement of Lys by Arg should not cause significant conformational changes in the protein, since Arg retains a positive charge of the Lys side chain. Here, the energy transfer and pull down approaches were applied to investigate the interaction of these mutants with different subunits of the eEF1B complex.

The mutant and wild-type eEF1A2 proteins were cloned into plasmid pFC14K encoding HaloTag. The tag was located at the C-terminus of eEF1A2, since a majority of the Lys residues of interest were confined to the N-terminal part of the protein. In this case, HaloTag would not create steric hindrance to the interaction with the partner proteins. The resulting plasmid encoded eEF1A2-HaloTag with a molecular weight of about 85 kDa (50 kDa eEF1A2 and 35 kDa HaloTag).

To estimate a possible effect of the eEF1A2 methylation on its interaction with eEF1B in intracellular environment we used a bioluminescence resonance energy transfer (BRET) system. The interaction between the two fusion proteins, one of which carries luminescent label and another contains fluorescent label, can make these labels close enough for the resonance energy transfer to occur. In this case, we used bioluminescent NanoLuc luciferase

(Promega, USA) fused with eEF1Bα and a fluorescent ligand linked to HaloTag in eEF1A2.

293 cells were transfected with the plasmids encoding wild-type eEF1A2 and its mutants containing HaloTags and eEF1Bα fused with the NanoLuc domain. Fig. 1 shows that BRET between eEF1A2-HaloTag and nanoLuc-eEF1Bα is easily detected evidencing the existence of eEF1A2-eEF1B interaction in 293 cells. Importantly, no methylation-deficient mutants showed a difference at the BRET level as compared to wild-type eEF1A2. It implies that methylation of the Lys residues in positions 36, 55, 79, 165 and 318 of eEF1A2 may have no significant impact on its interaction with the eEF1B complex in human cells.

However, it remains possible that we could not detect the methylation effect due to some limitations of the BRET method, which provides measurements directly in cells. Additionally, we used only one subunit of eEF1B for BRET experiments. So, we employed the HaloTag–based pull down procedure to test if we could observe a difference in the wild type and mutant eEF1A2 proteins binding to different subunits of eEF1B in cell lysates by Western blotting.

The HaloTag® Pull-Down system was used for pull down experiments. The amount of Halo label in the lysates of cells expressing different proteins with HaloTag was estimated by Western blotting with anti-HaloTag antibodies. Then the HaloTag affinity purification system for protein complexes was used to pull down the protein partners of eEF1A2. The proteins were separated by polyacrylamide gel electrophoresis. Noteworthy, before starting the pull down procedure the volume of each electrophoresis sample was adjusted to correspond to the normalized amount of eEF1A-HaloTag, measured by Western blot of the cell lysates with anti-HaloTag antibodies (Fig. 2). Besides, to control the level of binding of HaloTag protein to the resin, the portions of cell lysates after exhausting by pull down were

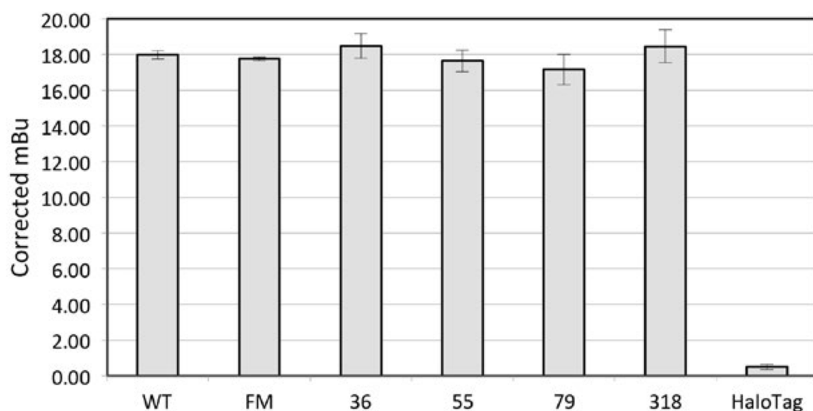


Fig. 1. BRET analysis of interactions NanoLuc-eEF1Bα and eEF1A2-HaloTag or its mutants. BRET data were analyzed with MaxStat Lite software. The diagram represents an average of three experiments + standard deviation. Signatures: WT: eEF1A2 (WT); 36: eEF1A2(K36R); 55: eEF1A2(K55R); 79: eEF1A2(K79R); 318: eEF1A2(K318R); FM: full mutant eEF1A2; HaloTag: HaloTag Control Vector.

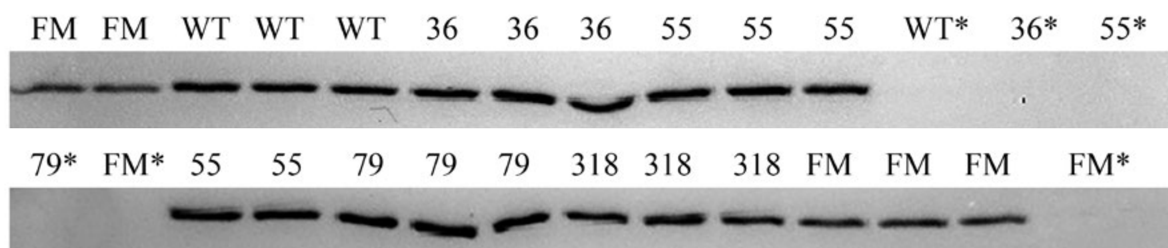
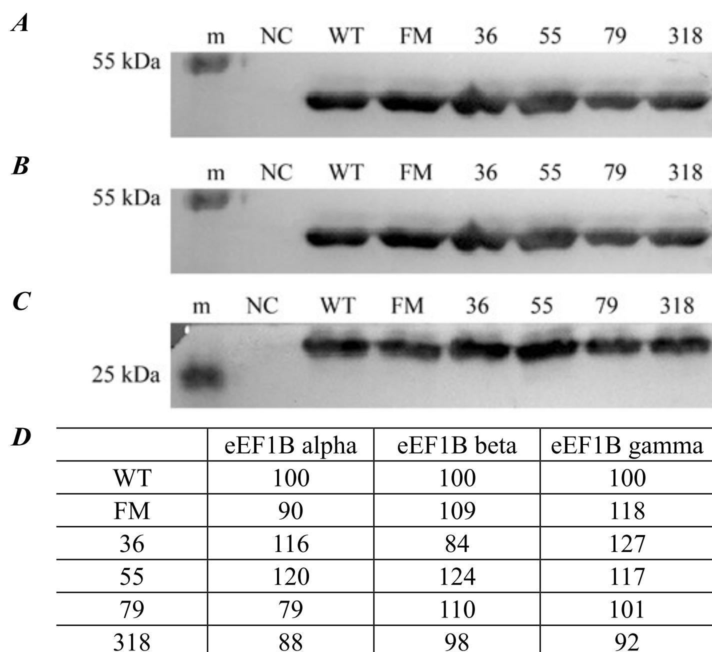


Fig. 2. Comparative analysis of the proteins expression levels in 293 cells after transfection with plasmids carrying WT eEF1A2 or its mutants fused to HaloTags. 10 μl of each cell lysate were loaded on PAAG in triplicate. Average values of the densities were used for calculation of eluate volumes to be loaded on PAAG for Western blot analysis with HaloTag antibodies. eEF1A2(K318R)-HaloTag; FM: full mutant eEF1A2-HaloTag. WT*, 36*, 55*,79*, FM* — same as before but the samples were taken after pull down procedure.

loaded on the same gel. This was necessary to ensure the correct comparison of the protein levels in each sample. Western blots with the antibodies recognizing eEF1Bα, eEF1Bβ and eEF1Bγ subunits were subjected to densitometry to quantitate the subunits levels in the eluates of the pulled down proteins.

Densitometry analysis of the blots confirmed the interaction of eEF1B subunits with eEF1A2 (Fig. 3). It should be kept in mind that eEF1A is capable to interact directly with the eEF1Bα and eEF1Bβ subunits whereas an apparent interaction of eEF1A with eEF1Bγ, observed on Western blots, in fact just reflects



	eEF1B alpha	eEF1B beta	eEF1B gamma
WT	100	100	100
FM	90	109	118
36	116	84	127
55	120	124	117
79	79	110	101
318	88	98	92

Fig. 3. Western blots of pull-down eluates with antibodies against eEF1Bγ (A), eEF1Bβ (B), eEF1Bα (C). (D) — densitometry data. m — Protein weight markers, NC — negative control, HaloTag Control Vector; WT — eEF1A2(WT)-HaloTag; 36 — eEF1A2 (K36R)-HaloTag; 55 — eEF1A2(K55R)-HaloTag; 79 — eEF1A2(K79R)-HaloTag; 318 — eEF1A2(K318R)-HaloTag; FM — full mutant eEF1A-HaloTag. As the levels of expression of HaloTag-linked eEF1A2 or its mutants after transfection could be slightly different, the loading volumes of pulled down fractions were adjusted to correspond to an equal amount of HaloTag protein bait, according to Fig. 2.

eEF1A binding to other subunits of the eEF1B complex [34]. Thus, in the case of eEF1Bγ, we actually observed the interaction of eEF1A2 with the eEF1B complex. Again, no essential changes in the amount of the eEF1Bα, eEF1Bβ and eEF1Bγ subunits pulled down by the methylation-free mutants were detected as compared to wild-type eEF1A2 (Fig. 2). The mutant K165R was not studied in this case, as K165 methylation did not influence the interactions of eEF1A with other elongation factors in pull down procedure [35]. Thus, methylation of eEF1A2 apparently has no substantial effect on its interaction with the eEF1B complex both *in vitro* and in cellular.

After identification of five methyltransferases of eEF1A that are unique for every lysine residue involved [29, 36–38] it became clear that eEF1A is the only known so far target of these enzymes. It is known that methylation of K55 in eEF1A may be important for tumorigenesis [29, 39] but a potential mechanism of the impact of K55 as well as of K36, K79, K165 and K318 methylation remains to be elucidated. As the methylated Lys residues are localized on the surface of the eEF1A proteins one may suggest that this modification may influence the interaction of eEF1A with the protein partners. eEF1A has a number of confirmed translational [40, 41] and non-translational [7, 8, 42–44] protein partners, thus the search for methylation-impacted protein-protein contacts is at the initial stage.

Our data suggest a removal of the altered eEF1A2-eEF1B interaction from the list of potential molecular mechanisms explaining how the methylation influences a tumorigenic action of eEF1A2. As far as translation is concerned, methylation of eEF1A2 may be still important for its interaction with ribosomal proteins [45, 46] and/or aminoacyl-tRNA synthetases [47]. Besides, eEF1A2 fulfills a number of moonlighting functions [48], so methylation may have an impact on the eEF1A2 interaction with different non-translational proteins in cancer cells.

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Метилування фактора елонгації трансляції людини eEF1A2 не впливає на взаємодію eEF1A2-eEF1B.

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Мета. З'ясування впливу метилування лізинових залишків фактора елонгації трансляції eEF1A2 на взаємодію із субодиницями елонгаційного комплексу eEF1B. **Методи.** Мутагенез, клонування, культивування клітин людини 293, трансфекція клітин, BRET (резонанс трансферу біолюмінісцентної енергії), афінна очистка білкових комплексів, Вестерн блот, денситометрія. **Результати.** Отримані п'ять мутантів eEF1A2, в яких один з п'яти консервативних лізинових залишків, здатних до метилування, замінений на аргінін, та один мутант, в якого мутовані всі п'ять лізинів. **Результати** BRET та Вестерн блот аналізу виділених білків-партнерівeEF1A2 показали відсутність суттєвих відмінностей у взаємодії eEF1A2 і його мутантів, не здатних до метилування, із субодиницями елонгаційного комплексу eEF1B. **Висновки.** Метилування лізинових залишків eEF1A2, вочевидь, не впливає на взаємодію із субодиницями елонгаційного комплексу eEF1B.

Ключові слова: eEF1A2, eEA1Bα, eEA1Bβ, eEA1Bγ, метилування, BRET, HaloTag.

Метилирование фактора элонгации трансляции человека EEF1A2 не влияет на взаимодействие eEF1A2-eEF1B.

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Цель. Выяснение влияния метилирования лизиновых остатков фактора элонгации трансляции eEF1A2 на взаимодействие с субъединицами элонгационного комплекса eEF1B. **Методы.** Мутагенез, клонирование, культивирование клеток человека 293, трансфекция клеток, BRET (резонанс трансфера биолюминисцентной энергии), аффинная очистка белковых комплексов, Вестерн блот, денситометрия. **Результаты.** Получены пять мутантов eEF1A2, в которых один из пяти метилируемых консервативных лизиновых остатков, заменен на аргинин, и один мутант, у которого мутированы все пять лизинов. **Результаты** BRET и Вестерн блот анализа выделенных белков-партнеров eEF1A2 не показали существенных различий во взаимодействии eEF1A2 и его неметилируемых мутантов с субъединицами элонгационного комплексаeEF1B. **Выводы.** Метилирование лизиновых остатков eEF1A2, по-видимому, не влияет на взаимодействие с субъединицами элонгационного комплекса eEF1B.

Ключевые слова: eEF1A2, eEA1Bα, eEA1Bβ, eEA1Bγ, метилирование, BRET, HaloTag.

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