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The use of the *in situ* proximity ligation assay for validating S6 kinase 1 CoAlation under oxidative stress

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Aim. To verify CoAlation of ribosomal protein S6 kinase 1 (S6K1) in the HEK293/Pank1b cells exposed to oxidative stress using proximity ligation assay (PLA). **Methods.** *In situ* proximity ligation assay. **Results.** S6 kinase 1 undergoes CoA covalent modification in cellular response to oxidative stress. **Conclusions.** The previously developed mass spectrometry-based methodology allowed us to identify over 2100 CoAlated proteins in the cells exposed to oxidative or metabolic stress. Many protein kinases were found to be CoAlated, including S6K1. In this study, the proximity ligation assay was used to demonstrate a significant increase of S6K1 CoAlation under oxidative stress compared to untreated cells.

Keywords: CoA, S6 kinase 1, *in situ* proximity ligation assay, post-translation modification, oxidative stress, signal transduction, confocal microscopy, cell growth, antibodies.

Introduction

Coenzyme A (CoA) is a fundamental cofactor in all living cells. CoA and its thioesters have been implicated in diverse metabolic processes and biosynthetic pathways, as well as in the regulation of gene expression [1]. We have recently discovered a novel function of CoA in redox regulation. Using in-house developed anti-CoA monoclonal antibodies [2, 3] and novel methodologies, we demonstrated for the first time that CoA can function as a major cellular antioxidant through a novel post-translational modification, termed protein CoAlation (covalent binding of CoA to oxidized cysteine residues under metabolic or oxidative stress). To date, using MS-based methodology we have identified 2100 CoAlated proteins in the bacterial and mammalian cells and tissues, exposed to oxidizing agents or nutrient depri-

vation [4]. The majority of CoAlated proteins, accounting for over 65 %, are known to be associated with metabolic processes. Additionally, a large number of proteins involved in stress response and protein synthesis were also found to be CoAlated. CoA-modified cysteine residues are commonly located at functionally and structurally crucial sites, resulting in changes to enzyme activity, subcellular localization, conformation changes and the protection of oxidized cystein residues from irreversible overoxidation [5–7]. Recently, a key enzyme in mitotic regulation, Aurora A kinase, was shown to be CoAlated in response to the oxidative stress. Biochemical, biophysical and X-ray crystallography studies revealed a unique mode of CoA binding to Aurora A kinase. It is mediated by covalent modification of Cys290 located in the activation loop by CoA as well as selective binding of the ADP moiety of CoA to the ATP binding pocket of Aurora A kinase and results in the reversible inhibition of its kinase activity [8]. These novel findings suggest that other protein kinases, which possess cysteine residues in the activation segment, can be also modified by CoA when the cells are exposed to oxidative or metabolic stress.

A key player of the PI3K/mTOR/S6K signaling pathway, S6K1, was identified among CoAlated proteins in HEK293/Pank1B cells treated with diamide [unpublished data]. S6Ks have a plethora of functions in signaling pathways, including the regulation of protein synthesis, cell growth and energy metabolism in response to various signals, including amino acids, AMP/ATP levels and insulin [9]. The S6K1 gene encodes several alternatively translated isoforms: p85S6K1, p70S6K1 and p60S6K1. The activity of S6K1 is regulated by multiple phosphorylations mediated by mTOR and PDK1 kinases (Fig. 1). The LC-MS/MS analysis of CoAlated proteins in the HEK293/Pank1b cells treated with diamide showed CoAlation of cysteine 217 (Cys217) in S6K1. Cys217 (using hereafter the numbering based on a shorter 502-residue protein corresponding to p70-S6K1 isoform) is located in the activa-

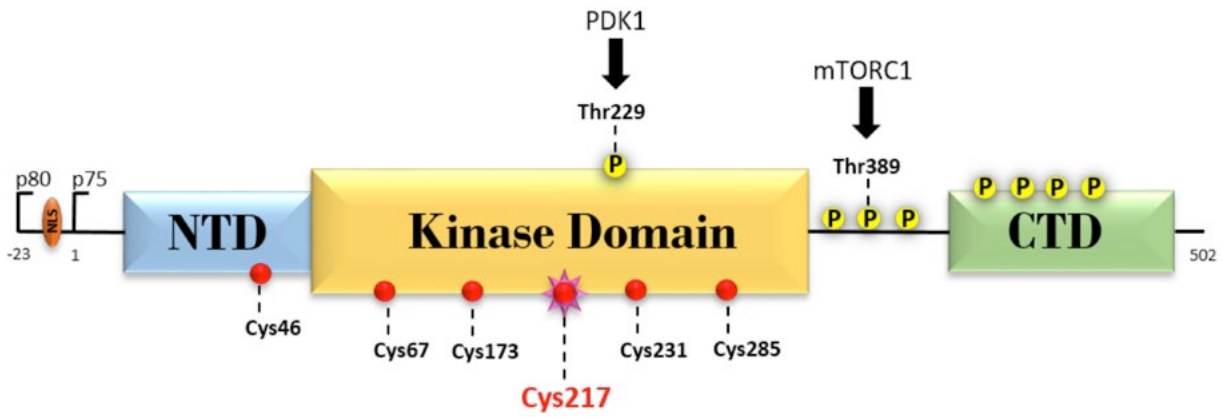

Fig. 1. Schematic structure and domain organization of S6K1. The kinase domain, N- and C-terminal regulatory domains (NTD and CTD), the sites of mTOR and PDK1 phosphorylation, as well as location of cysteine residues are indicated, Cys217 is highlighted in red.

tion loop of the kinase domain (Fig. 1) which plays an important role in the regulation of the kinase activity.

The aim of this work was to validate S6K1 CoAlation in cellular response to oxidative stress using *in situ* proximity ligation assay.

Materials and Methods

Mammalian cell culture

HEK293/Pank1β model cell line (human embryonic kidney 293) with stable overexpression of Pantothenate kinase 1β (Pank1β) was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) supplemented with 10 % fetal bovine serum (FBS) (Hyclone), 50 U/ml penicillin and 0.25 μg/m streptomycin (Lonza). The generated cell line was tested and shown to be free of mycoplasma infection. HEK293/Pank1β cells were grown on coverslips placed in 24-well culture dishes in complete DMEM with 10 % FBS until ~70 % confluency. Then, the media were replaced with pyruvate-free DMEM supplemented with 5 mM glucose and 10 % FBS, and the cells were incubated for other 24 h. To induce the oxidative stress, the cells were treated with 0.5 mM H_2O_2 for 30 min at 37 °C in the same media.

Proximity Ligation Assay

Proximity ligation assay (PLA) was performed by Duolink *In Situ* Red Starter Kit Mouse/Rabbit (DUO92101, Sigma-Aldrich, Dorset, UK), accordingly to the manufacture manual. Cells were fixed with 10 % neutral buffered formalin (Sigma, F5554) supplemented with 100 mM NEM (from 1M stock in ethanol, Sigma, E3876) for 15 min RT. The addition of NEM was necessary to prevent disulfide bond formation by blocking sulfhydryls (e.g., reduced cysteines) forming stable, covalent thioether bonds. Cell membranes permeabilization and antigen demasking were performed by adding 0.2 % Triton X-100/PBS for 15 min at room temperature (RT). To eliminate autofluorescence, the samples were incubated for 15 min with 10 mM cupric sulphate and 50 mM ammonium acetate, pH 5.0. Non-specific binding of antibodies was blocked by incubating samples in TBS with Duolink™ Blocking buffer for 30 min at +30 °C. Primary mouse monoclonal antibodies anti-CoA 1F10/B11 (1 μg/ml, [2]) and rabbit polyclonal anti-S6K1 (1 μg/ml) were added to the Duolink Antibody Diluent (1:100 dilution), and then to each reaction well. For negative control, Duolink Antibody Diluent only was used. The slides were incubated in a humidified chamber at +4 °C overnight. Then, the slides were washed in buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05 % tween-20 before the incubation with PLA PLUS and PLA MINUS probes of secondary antibodies (diluted in the same buffer at 1:5 ratio) for 1 h at 37 °C in dark humidified chamber. After series of rinsing, samples were incubated in the hybridization solution for 15 min. For the ligation step, ligase (previously diluted in Duolink ligation solution, 1:40) was added to slides for 30 min to allow the ligation and circularization of the DNA-oligos. The amplification was performed by introducing DNA-polymerase diluted in Duolink amplification solution (1:80) into each reaction well, the slides were incubated for 100 min. Following incubation, the slides were washed again with a wash buffer without Tween-20 and dried overnight. Next, the samples were washed with TBS and

embedded into Duolink *in situ* mounting medium containing DAPI to stain the nuclei. Analysis was performed using Leica TCS SPE Confocal system with coded DMi8 inverted microscope (Leica, Germany).

High-resolution images from three independent experiments were analyzed by ImageJ (National Institutes of Health (NIH)) to quantify the density of PLA signal per cell (fluorescence intensity divided by number of DAPI nuclei). Statistical analysis and data graph were performed by Microsoft Excel. For comparison of PLA data, non-parametrical two-tailed Student's t-test was used. The values less than 0.05 were considered to be statistically significant.

Results and Discussion

CoAlome analysis with the developed LC-MS/MS methodology allowed us to identify diverse ranges of CoAlated proteins in mammalian cells and tissues [5]. Notably, many protein kinases were found to be CoA-modified in response to oxidative stress, including S6 kinase 1. The site of CoAlation was mapped to Cys217, located in the activation loop of kinase domain. In this study, we applied the proximity ligation assay to validate CoAlation of S6K1 in HEK293/Pank1b cells exposed to oxidative stress. By measuring close proximity, PLA allows us to detect the post-translation modification (PTMs) that cannot be established with simple co-localization studies (Fig. 2).

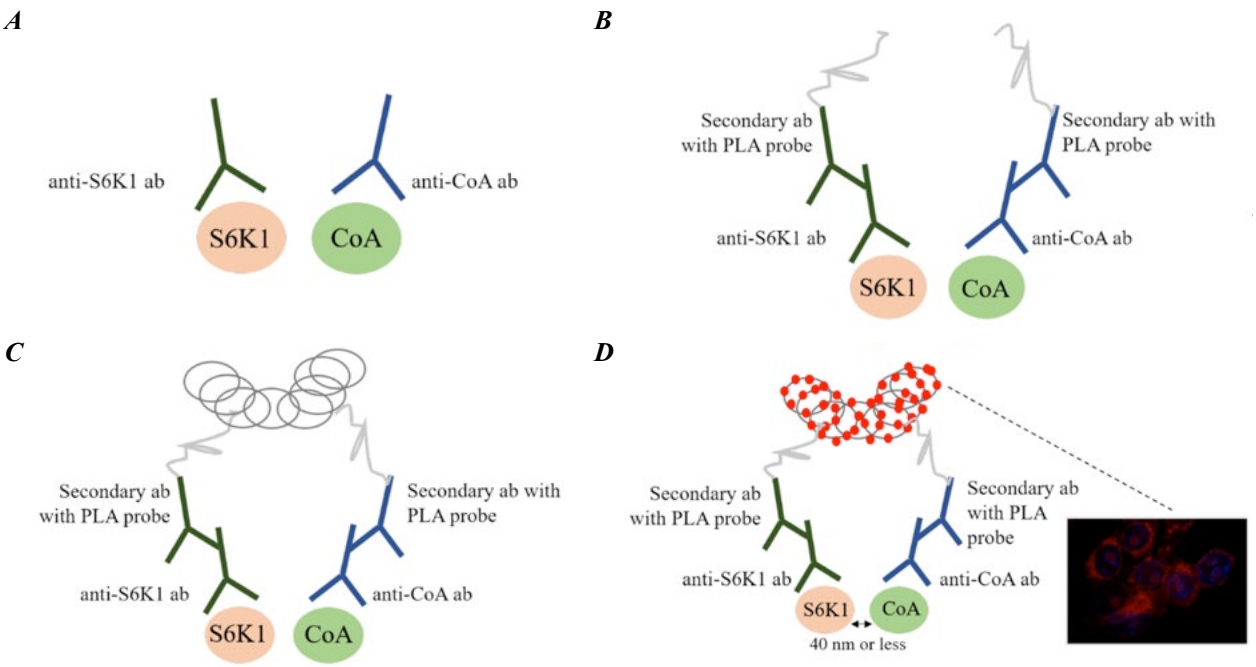

Fig. 2. Scheme of the PLA to study S6K1 CoAlation. *A* — Addition of primary antibodies against S6K1 and CoA, respectively, are added. *B* — Secondary antibodies with PLA probes of PLUS DNA and MINUS DNA, respectively, are added. *C* — Two circle-forming DNA oligonucleotides and the ligation enzyme are added. *D* — Rolling-circle amplification. Fluorescently labelled, complementary oligonucleotide probes bind to the amplified DNA. Signal appears as a distinct dot and can be visualized by fluorescent microscopy.

Two primary antibodies, raised in different species, are required for the detection of PTMs in cells. These include an antibody specific for a protein of interest and the other recognizing PTM. The proximity probes include secondary antibodies of different species with conjugated nucleotide tags (Fig. 2). When the probes are in close proximity to each other in cells, complementary sequences hybridize, allowing the ligation. The presence of DNA polymerase promotes the production of circular DNA, which contains fluorescently label oligonucleotides. If the modification is present on the protein, fluorescent signal is generated [10]. The distance between both probes has to be 40 nm or less for generating detectable immunofluorescent signal.

The data presented in Fig. 3 reveal a low immunofluorecent signal of CoA modification present on the S6K1, detected in negative control (A) and in HEK293/Pank1b cells not treated with H_2O_2. A significant increase of the immunoreactive signal was observed in the cells exposed to oxidative stress (Fig. 3C). The quantitation analysis of the PLA immunofluoreactivity (Fig. 3D) shows the increasing number of dots per cell for the samples undergoing H_2O_2-induced oxidative stress, implying the higher level of S6K1-CoA association. Generated results are in agreement with the LC-MS/MS analysis of CoAlated proteins, which showed covalent modification of S6K1 by CoA.

In overall, our data provide additional evidence of S6K1 modification by CoA *in cellular.* Taking into account the anti-oxidative function of protein CoAlation we propose that CoAlation of S6K1 may protect surface exposed Cys217

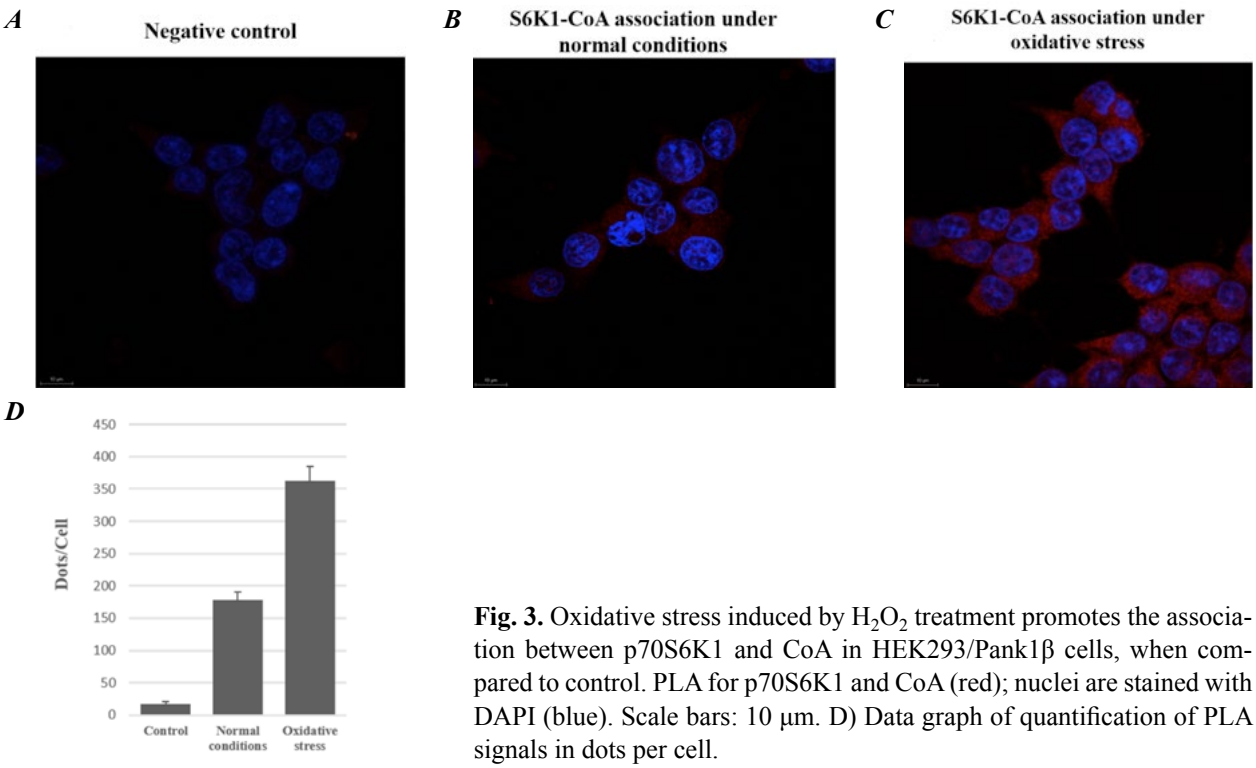

Fig. 3. Oxidative stress induced by H_2O_2 treatment promotes the association between p70S6K1 and CoA in HEK293/Pank1β cells, when compared to control. PLA for p70S6K1 and CoA (red); nuclei are stained with DAPI (blue). Scale bars: 10 μm. D) Data graph of quantification of PLA signals in dots per cell.

from over-oxidation, preventing irreversible loss of function and subsequent degradation. Additionally, by analogy of Aurora A kinase regulation by CoAlation of Cys290 (located in the activation loop), we anticipate that the activity of S6K1 could be affected as well by covalent modification of Cys217, which is located in S6K1 catalytic domain in close proximity to the key for kinase activity Thr229 phosphorylation site, and by the possible interaction of ADP moiety of CoA with the kinase ATP binding site [8]. Testing these hypotheses will be the subject of our further studies.

Acknowledgments

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Використання *in situ* proximity ligation методу для підтвердження КоАлювання S6 кінази 1 в умовах оксидативного стресу

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Мета. Підтвердити КоАлювання кінази 1 рибосомального білка S6 (S6K1) в клітинах HEK293/Pank1b в умовах оксидативного стресу, використовуючи *in situ* proximity ligation метод (PLA). **Методи.** *In situ* proximity ligation метод. **Результати.** S6 кіназа 1 КоАлюється у відповідь на оксидативний стрес в клітинах. **Висновки.** Попередньо розроблена методика мас-спектрометрального аналізу дозволила ідентифікувати понад 2100 КоАльованих білків у клітинах, що підлягали дії оксидативного або метаболічного стресу. Виявлено, що багато протеїнкіназ є КоАльованими, включно з S6K1. У цьому дослідженні було використано *in situ* proximity ligation метод для демонстрації значного збільшення рівня КоАлювання S6K1 за умов оксидативного стресу порівняно з клітинами за нормальних умов.

Ключові слова: КоА, S6 кіназа 1, *in situ* proximity ligation метод, пост-трансляційна модифікація, оксидативний стрес, передача сигналу, конфокальна мікроскопія, ріст клітин, антитіла.

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