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Adaptor protein TDRD7 is co-localized with ribosomal protein S6 kinases S6K1 and S6K2 in cell lines of different tissue origin

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Our previous studies have shown that S6K1 and S6K2 protein kinases form the complexes with newly identified adaptor protein TDRD7, which is involved in regulation of cytoskeleton dynamics, mRNA transport, protein translation, piRNAs processing and transposons silensing. ***Aim****.Determination the subcellular localization of S6K1-TDRD7 and S6K2-TDRD7 protein complexes.* ***Methods****. Immunofluorescense microscopy was used to study co-localization of S6K1/S6K2 and TDRD7 in HEK293, HEPG2 cell lines as well as in rat primary hippocampal neurons using primary polyclonal anti-S6K1 antibodies, monoclonal anti-S6K2 and anti- TDRD7 antibodies.* ***Results****. It was found that S6K1 is co-localized with TDRD7 in perinuclear region of HEK293 cells. S6K1 and S6K2 were also co-localized with TDRD7 in perinuclear region of HEPG2 cells and in soma of primary rat hippocampal neurons.* ***Conclusions****. In this report we provide an additional experimental evidences of possible S6K1-TDRD7 and S6K2-TDRD7 complexes formation in cells of different tissue origins that may reflect their potential physiological importance. However, elucidation of the exact composition of these complexes and their role in cell physiology requires additional studies.*

Keywords: S6K1, S6K2, TDRD7, immunocytochemical analysis.

Introduction. The ribosomal protein S6 kinases (S6Ks) are serine/threonine kinases, which belong to the family of AGC kinases [1]. The activation of S6Ks is coordinated via the mitogen-stimulated PI3K and the nutrient sensing mTOR pathways [2]. S6Ks are involved in the regulation of diverse cellular processes and functions including protein synthesis, RNA splicing, transcription, cell growth and survival [1, 2]. Mammalian cells express two S6K forms: S6K1 and S6K2, and each of them exists in long (S6K1/I and S6K2/I) and short (S6K1/II and S6K2/II) splicing variants.

The predominantly expressed in cells short (70 kDa) isoform of S6K1 is localized mainly but not exclusively within the cytoplasm. A nuclear localization sequence (NLS) found within the N-terminal (23 aa) extension of long S6K1 isoform (85 kDa) may target it to the nucleus [3, 4]. Similarly, the long (56 kDa) isoform of S6K2 differs from the short (54 kDa) splicing version by the presence of the NLS [1, 4] located within the N-terminal (13 aa) extension. At the same time, both S6K2 isoforms contain additional NLS at the C-terminus, which can determine their nuclear localization [5, 6].

While S6K1 and S6K2 share a very high degree of similarity in their kinase domains (more then 80 %), the N- and C-terminal regulatory regions exhibit only 20 % identity. These regions contain different binding motifs and regulatory sites, which may recruit S6K isoforms to different cellular complexes/compartments and/or modulate their stability and enzymatic activity. At present, more than twenty substrates have been identified for S6Ks, but only few of them have well defined functional significance [2].

A number of S6Ks-binding partners have been implicated in the regulating of their kinase activity, inclu-

ding small GTPases Rac1 and cdc42, protein kinases PDK1 and PKCγ, protein phosphatases PP2A and PP1 and cytoskeleton protein neurabin [2, 7–9].

Previously, in the course of yeast-two hybrid screening of HeLa cDNA library by S6K1 as a bite we have identified a novel S6K1/2 binding partner – Tudor domain containing 7 protein (TDRD7) [10]. Further studies confirmed the formation of S6K1/TDRD7 and S6K2/TDRD7 complexes *in vivo* [11]. In addition we have revealed the existence of several S6K-mediated phosphorylation sites within TDRD7 protein sequence that was confirmed by *in vitro* kinase assay [11]. TDRD7 protein is known as a scaffold protein with molecular weight about 130 kDa. It was originally identified as a binding partner of Ser/Thr PCTAIRE2 kinase [12]. Subsequent investigations have led to the discovery of TDRD7 in a complex with ik3/Cables – cyclin-dependent kinase binding protein [13].

It was demonstrated recently that loss-of-function mutations in TDRD7 as well as Tdrd7 nullizygosity may cause cataracts or glaucoma formation in mammals and also cause an arrest in spermatogenesis [14].

TDRD7 is localized in cytoplasm and together with other members of Tudor-family (TDRD1/MTR-1, TDRD6) has been implicated in the formation of polar/germinal granules (nuage), which contain Dicer and microRNAs. These structures are involved in the micro RNA pathway and the formation of a ribonucleoprotein complex in spermatids [12, 15–17]. In addition it was suggested the involvement of TDRD7 in the complex with TACC1-chTOG-Aurora kinase A, which controls mRNA fate in conjunction with microtubule organization and the maintenance of cell polarity [18].

Our previous studies allowed us to determine TDRD7 distribution in HEK293 cells [19]. Using anti-TDRD7 monoclonal antibodies (E6) we had demonstrated that a large fraction of this protein was localized in the nucleus and perinuclear region of HEK293 cells.

In this report, we present the data regarding possible co-localization of S6K1 and S6K2 with TDRD7 protein in specific subcellular compartments of HEK293, HEPG2 cells, and rat primary hippocampal neurons.

Materials and methods. *Antibodies*. Monoclonal anti-TDRD7 (specific to 181–411 aa sequence), anti-S6K2 polyclonal (specific to the C-terminal region of S6K2) and anti-S6K1 polyclonal antibodies (specific to the C-terminal region of S6K1) have been described previously [20, 21].

Anti-mouse and anti-rabbit secondary antibodies labelled with FITC Alexa Fluor 488 (green) and Alexa Fluor 568 (red) («Invitrogen», USA) respectively were used for immunocytochemical analysis. To decrease the autofluorescence background the preparations were incubated for 30 min in 10 mM $CuSO_4$ and 50 mM of CH_3COONH_4 at pH 5.0. Control preparations were incubated without primary antibodies.

Cell culture and immunocytochemical analysis. HEK293 (human embryonic kidney) and HEPG2 (human hepatocellular carcinoma) cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10 % foetal bovine serum («HyClone», UK), penicillin (200 U/ml) and streptomycin (200 mg/ml).

Culture of rat primary hippocampal neurons was kindly gifted from laboratory of molecular biophysics, Bogomoletz Institute of Physiology.

For immunofluorescent staining, HEK293 cells were grown in tissue culture chambers («Nunc», Denmark), fixed with 3.7 % paraformaldehyde in PBS, and permeabilized with PBS-T (0.2 % Tween-20) three times for 5 min. Unspecific binding was blocked by 45 min incubation of cells with 10 % FBS diluted in PBS-T. Afterwards, incubation with primary and secondary antibodies was performed as described previously [22]. Fluorescently labelled proteins were visualized using a Zeiss LSM510 confocal microscope, and the images were analyzed using the LSM510 image browser software [23]. Nuclei were stained with Hoechst 33258 («Sigma», USA).

Results and discussion. It has been already demonstrated that the 23- and 13-amino-acid extensions at the N-termini of S6K1/I and S6K2/I contain nuclear localization signals (NLSs) that target these isoforms constitutively to the nucleus [24, 25]. It is known that S6K1/II isoform is predominantly cytosolic, but it can also be accumulated in the nucleus when cells are treated with inhibitor of nuclear export in human cells leptomycin B (LMB) [26]. It is believed that the presence of an additional NLS at the C terminus of the S6K2/II determines its predominantly nuclear localization. Nucleocytoplasmic shuttling has been reported for both cytoplasmic forms of S6K, but the mechanisms of its regulation have not been elucidated [27–29].

It was reported previously that TDRD7 was localized on the mitochondria outer membrane and in cytoplasmic structures called chromatoid bodies [16, 28]. Our recent studies with employment of immunofluorescent-confocal microscopy and anti-TDRD7 (E6) monoclonal antibody indicated the presence of TDRD7 not only in the cytoplasm, but in the nucleus as well. Especially strong TDRD7 positive signal was observed in the perinuclear zone and in the region of mitotic spindle formation [19].

The obtained data are in accordance with the observation of another research group which had demonstr ated that the pool of endogenous TDRD7 is present in the nucleus of COS 7 cells overexpressing TDRD7 [13].

To determine possible co-localization sites of TDRD7 with S6K1 and S6K2 we applied an immunocytofluorescent analysis. For the first time, we detected co-localization of TDRD7 with S6K1 kinase in HEK293 cells. According to our observations S6K1/TDRD7 complexes are distributed within cytoplasm and nuclei of the cells and a large amount of S6K1 and TDRD7 are concentrated in perinuclear region and peripheral zone of HEK293 cells (Fig. 1). Such co-localization of S6K1 and TDRD7 could reflect physiological impor- tance of possible S6K1-TDRD7 complex formation.

The results obtained encouraged us to enquire whether TDRD7 and S6K1 are co-localized in the other cell types. It was revealed that endogenous S6K1 is co-localized with TDRD7 protein and demonstrates weak level of co-localization in the cytoplasm of HEPG2 cells, partly in perinuclear region, but not so apparently as in HEK293 (Fig. 2, *A*, see inset).

Further, taking into account that TDRD7 also forms complex with S6K2 [11], we decided to check whether TDRD7 is co-localized with S6K2 kinase.

It has been already mentioned that both S6K2 isoforms contain additional NLS at the C-terminus, which determines their predominant nuclear localization in quiescent cells [6]. So, the next question was whether co-localization of TDRD7 with S6K2 kinase will be different from that observed for S6K1.

According to our data, TDRD7 may be co-localized with S6K2 in HEPG2 cells, predominantly in perinuclear region (Fig. 2, *B*, see inset). Such co-localization was similar to that observed in HEK293 cells for S6K1.

Finally, as far as TDRD7 is known to be overexpressed in neural tissue [12], we decided to examine the possible subcellular co-localization of S6K1-TDRD7 and S6K2-TDRD7 in rat hippocampal primary neurons culture (Fig. 3, see inset).

Immunocytochemical analysis revealed that S6K1-TDRD7 and S6K2-TDRD7 complexes are localized predominantly in soma of neurons rather than in neurits.

It has been reported previously, that in quiescent cells S6K1 is mostly concentrated in cytoplasm [5, 9, 27, 29]. Nevertheless, keeping in mind that S6K1 long isoform contains NLS, some fractions of S6K1 were expected to be present in nucleus. According to our observation a large amount of S6K1 and TDRD7 are concentrated in perinuclear region of HEK293. Co-localization of S6K1 and TDRD7 in HEPG2 was weak, whereas co-localization of TDRD7 and S6K2 in these cells was much more stronger.

Our observation suggests some physiological importance of the S6K1/TDRD7 and S6K2/TDRD7 comp-

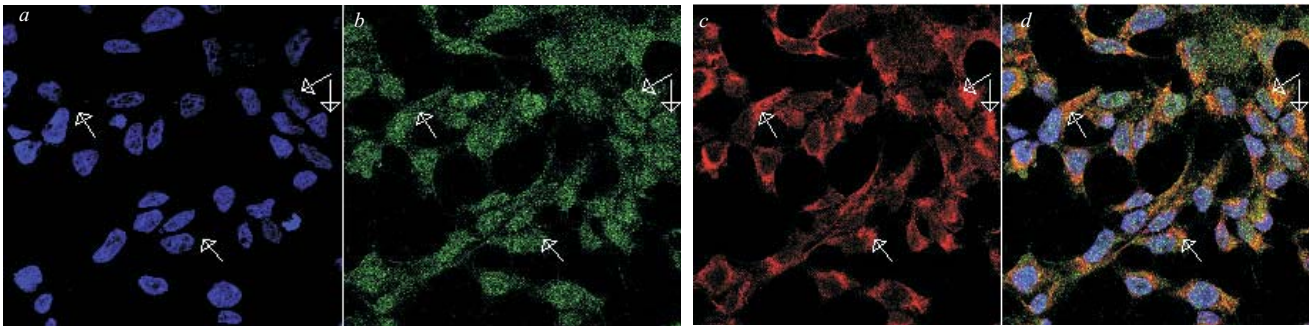

Fig. 1. TDRD7 co-localized with S6K1 in HEK293 cells. HEK293 cells were fixed with 3.7 % FA; S6K1 (green) (*B*) and TDRD7 (red) (*C*) content (subcellular localization) was revealed using corresponding antibodies. As secondary antibodies Alexa-Fluore 488 (green) and Alexa Fluor 568 (red) antibodies were used. Nuclei were stained with Hoechst 33258 (*A*). Picture (*D*) represents a merge of signals. White arrows indicate co-localization signals of TDRD7 and S6K1. Oc.10×; ob. 100×

Figures to article by O. M. Skorokhod et al.

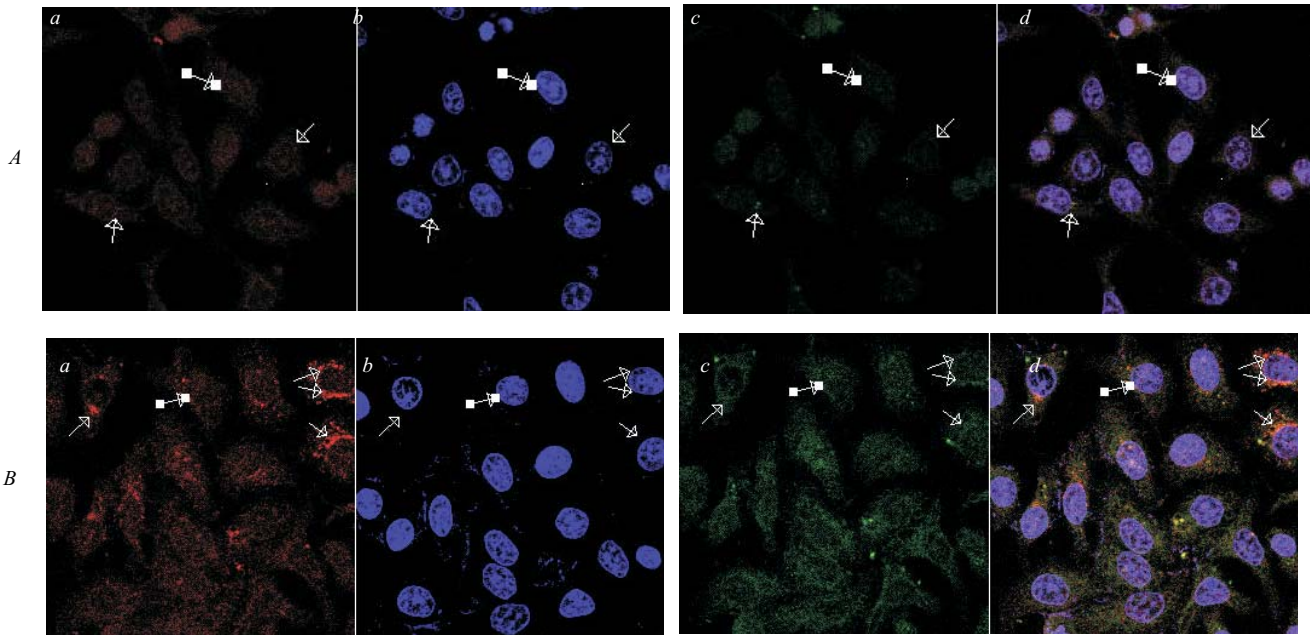

Fig. 2. TDRD7 co-localized with S6K1 (*A*) and S6K2 (*B*) in HEPG2 cells. HEPG2 cells were fixed with 3.7 % FA. S6K1 and S6K2 (red in *A* and *B*, respectively) (*a*) and TDRD7 (green) (*c*) content was revealed using corresponding antibodies; as secondary antibodies Alexa-Fluore 488 and Alexa Fluor 568 (green and red, respectively) antibodies were used. Nuclei were stained with Hoechst 33258 (*b*); *d* – a merge of signals; white arrows indicate co-localization signals of TDRD7 and S6K1 (*A*) and TDRD7 and S6K2 (*B*); oc.10×; ob. 100×

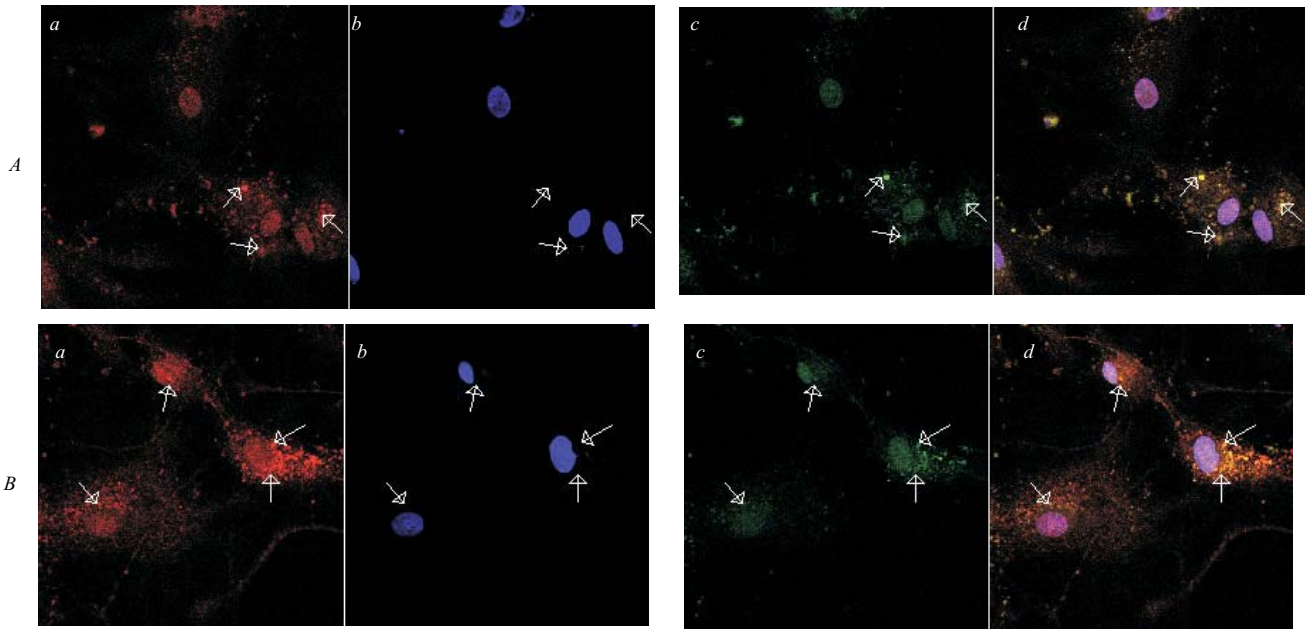

Fig. 3. TDRD7 co-localized with S6K1 (*A*) and S6K2 (*B*) in rat hyppocampal primary neurons culture. Cells were fixed with 3.7 % FA. S6K1 and S6K2 (red in *A* and *B*, respectively) (*a*) and TDRD7 (green) (*c*) content was revealed using corresponding antibodies; as secondary antibodies Alexa-Fluore 488 and Alexa Fluor 568 (green and red respectively) antibodies were used. Nuclei were stained with Hoechst 33258 (*b*); *d* – a merge of signals; white arrows indicate co-localization signals of TDRD7 and S6K1 (*A*) and TDRD7 and S6K2 (*B*); oc.10×; ob. 100×

lexes formation. We could assume that perinuclear localization of both complexes may reflect a possible role of TDRD7 in the regulation of S6K1 and/or S6K2 nucleo-cytoplsmic shuttling.

In addition, taking into account that TDRD7 serves as a substrate for S6K1 as well as for S6K2 *in vitro* [11], we may speculate that S6Ks also can influence TDRD7 intracellular localization and its functions *in vivo*.

In this report we provide the additional experimental evidences of possible S6K1-TDRD7 and S6K2-TDRD7 complexes formation in cells of different tissue origin. Taking into consideration our previous results [10, 11] the obtained data also support the idea of possible physiological importance of these complexes formation. However, determination of the exact composition of complexes and elucidation of their role in cells require additional studies.

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О. М. Скороход, А. І. Хоруженко, В. В. Філоненко

Адапторний білок TDRD7 співлокалізується з кіназами S6 рибосомного білка S6K1 і S6K2 у клітинних лініях різного тканинного походження

Резюме

Раніше нами показано, що протеїнові кінази S6K1 і S6K2 формують комплекси з нещодавно ідентифікованим адапторним білком TDRD7, залученим до регуляції динаміки цитоскелету, транспорту мРНК, трансляції білків, процесингу піРНК та сайленсингу транспозонів. ***Мета****. Визначення субклітинної локалізації можливих комплексів S6K1– TDRD7 і S6K2–TDRD7.* ***Методи****. Клітинні лінії HEK293, HEPG2 і первинна культура нейронів гіпокампу щура використано для імунофлуоресцентного аналізу із застосуванням поліклональних анти-S6K1 антитіл та моноклональних анти-S6K2 і анти-TDRD7 антитіл.* ***Результати****. Виявлено, що S6K1 співлокалізується з TDRD7 переважно в навколоядерній зоні клітин HEK293. Окрім того, S6K1 і S6K2 співлокалізуються з TDRD7 також переважно у навколоядерній ділянці клітин HEPG2 та в сомі первинної культури нейронів гіпокампу щура.* ***Висновки****. Отримано додаткові експериментальні свідчення щодо формування комплексів S6K1– TDRD7 і S6K2–TDRD7 у клітинах різного тканинного походження, що, ймовірно, підтверджує їхню фізіологічну значущість. Втім, для визначення точного складу комплексів і з'ясування їхньої ролі у клітинах необхідні додаткові дослідження.*

Ключові слова: S6K1, S6K2, TDRD7, імуноцитофлуоресцентний аналіз.

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Адапторный белок TDRD7 колокализируется с киназами S6K1 и S6K2 рибосомного белка S6 в клеточных линиях различного тканевого происхождения

Резюме

Ранее нами показано, что протеиновые киназы S6K1 и S6K2 формируют комплексы с недавно идентифицированным адапторным белком TDRD7, участвующим в регуляции динамики цитоскелета, транспорте мРНК, трансляции белка, процессинге пиРНК и сайленсинге транспозонов. ***Цель****. Определение субклеточний локализации возможных комплексов S6K1–TDRD7 и S6K2–TDRD7.* ***Методы****. Клеточные линии HEK293, HEPG2 и первичная культура нейронов гиппокампа крысы использованы для иммунофлуоресцентного анализа с применением поликлональных анти-S6K1 антител и моноклональных анти-S6K2 и анти-TDRD7 антител.* ***Результаты****. Обнаружено, что S6K1 колокализуется с TDRD7 преимущественно в околоядерной зоне клеток HEK293. Кроме того, S6K1 и S6K2 колокализуются с TDRD7 также преимущественно в околоядерной области клеток HEPG2 и в соме первичной культуры нейронов гиппокампа крысы.* ***Выводы****. Получены дополнительные экспериментальные доказательства формирования комплексов S6K1– TDRD7 и S6K2–TDRD7 в клетках разного тканевого происхождения, что, возможно, подтверждает их физиологическую значимость. Однако для определения точного состава комплексов и выяснения их роли в клетках необходимы дополнительные исследования.*

Ключевые слова: S6K1, S6K2, TDRD7, иммуноцитофлуоресцентный анализ.

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