Identification of novel TDRD7 isoforms

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Aim. The aim of our study was to investigate the tudor domain-containing protein 7 (TDRD7) subcellular localization, which could be linked to diverse functions of this protein within the cell. Methods. In this study we employed cell imaging technique for detecting TDRD7 subcellular localization, Western blot analysis of HEK293 cell fractions with anti-TDRD7 monoclonal antibodies and bioinformatical search of possible TDRD7 isoforms in Uniprot, Ensemble, UCSC databases. Results. We have observed specific TDRD7-containing structures in cytoplasm as well as in the nucleus in HEK293 cells. The Western blot analysis of subcellular fractions (cytoplasm, mitochondria, nucleus) allowed us to detect three lower immunoreactive bands, with the approximate molecular weight of 130, 110 and 60 kDa (we termed them as TDRD7β, TDRD7γ and TDRD7δ) and specific subcellular localization. The bioinformatical analysis of TDRD7 primary structure allowed us to determine two alternative transcripts from TDRD7 gene coding for proteins with calculated molecular weight of 130 and 60 kDa. Conclusion. The presented data demonstrate the existence at protein level of potential TDRD7 isoforms: TDRD7β, TDRD7γ and TDRD7δ. The expression profile of these splice variants and their role in cells remains to be elucidated.

Keywords: TDRD7, S6 kinase, isoform, piRNAs, translation.

Introduction. Cell growth is a complex and at the same time tightly coordinated process. It requires the presence of different stimulatory molecules and sufficient quantities of nutrients, an appropriate work of translational apparatus and consistent cytoskeleton dynamics [1]. The key regulators of cell growth are mTOR and ribosomal protein S6 kinases (S6Ks), involved in the PI3K-mTOR signaling pathway. The activity of S6Ks and mTOR is regulated by phosphorylation/dephosphorylation events and requires different protein-protein interactions either with up-stream effectors or with specific substrates, which are facilitated/regulated by scaffold proteins [2].

One of a such possible regulators is TDRD7 (tudor domain containing 7) protein, referred also as PCTAIRE2BP (PCTAIRE2 binding protein) or Trap (tudor repeat associator with PCTAIRE 2) or KIAA1529. Recently it was identified by yeast two-hybrid system as a S6K1-binding partner [3]. TDRD7 is a scaffold protein with molecular weight of about 130 kDa (160 kDa in SDS-PA-GE), first detected in a complex of Ser/Thr PCTAIRE2 kinase [4]. TDRD7 was found to associate with proteins which regulate microtubule dynamics, mRNA transport and protein translation apparatus, and thus could indirectly affect the mTOR/S6K signaling pathway. It is a member of the tudor domains containing proteins group, highly enriched in germ cells and involved in the formation of polar/germinal granules (or nuage), the electron-dense organelles, abundant in RNA and proteins, crucial for spermatogenesis and some of small RNA pathways in the germline of many organisms [5, 6].

In human cells TDRD7 is predominantly expressed in brain and testis [4]. The TDRD7 gene is conserved in...
chimpanzee, dog, cow, rat, chicken, and zebra-fish. The gene consists of 18 exons and its nucleotide sequence in human displays 80–99 % identity with Mus musculus, Rattus norvegicus, Canis familiaris, Pan troglodytes (www.genecards.org). It has been reported recently that human TDRD7 mutations result in the cataract formation via misregulation of specific developmentally critical lens transcripts, and the TDRD7 null mutant mice develop cataract as well as glaucoma, the latter defined by elevated intraocular pressure (IOP) and optic nerve damage [7].

It was shown that in HeLa, COS7 cells TDRD7 is observed in the cytoplasm: it may localize on the periphery of mitochondria through electrostatic interaction and together with some other members of tudor-family (TDRD1/MTR-1, TDRD6) specifically localized in nuage (chromatoid body), which contains Dicer and microRNAs, suggesting that this structure is involved in a microRNA pathway and form a ribonucleoprotein complex in spermatids [4, 8–10]. It was also reported that after overexpression of TDRD7 in COS7 cells a small portion of this protein was detected in nucleus [11].

According to the recent data, in cytoplasm TDRD7 is involved in complexes with ik3/Cables, a cyclin-dependent kinase binding protein, and with TACC1-chTOG-Aurora kinase A, which could control mRNA movement depending on microtubule organization in the establishment or maintenance of cell polarity [11–13].

An essential feature of TDRD7 is the presence of Tudor repeats, which form a conserved 60-amino acid large domain, initially described in proteins associated with nucleic acids [14–16]. The structural analysis of the Tudor fold reveals a barrel-like structure composed of β-sheets forming a hydrophobic pocket surrounded by negatively charged residues that more likely constitute a protein-protein interaction surface [14, 17]. It has been established recently, that Tudor domains are specialized in specific recognition of methylated lysine or methylated arginine residues, and are involved in various epigenetic functions, such as chromatin remodeling through histones demethylation, and RNA processing via regulation mRNA splicing machinery and RISC activity [14, 18–20]. Moreover, it is suggested that a possible function of Tudor domain repeats in proteins, which carry out several such motives in their structure, is to act like chaperones and assemble molecules that associate with each Tudor domain into macromolecular complexes [8, 21].

The bioinformatical studies performed recently allowed identification and description of new domains in the N-terminal region of TDRD7, named LOTUS (Limkain, Oskar and Tudor containing proteins 5 and 7) [22]. TDRD7 has three such domains: LOTUS 1 (1–115 aa), LOTUS 2 (160–315 aa) and LOTUS 3 (335–420 aa). LOTUS domains are non-conserved (17–30 % homology) globular structures found in various proteins from metazoans and plants. They are often associated with RNA-specific modules and are likely to adopt a winged helix fold, but the exact molecular role of LOTUS domain remains to be discovered.

The involvement of TDRD7 in different cellular complexes and its role in specific classes of RNA transport, processing, translation regulation, microtubule organization, indicate the importance of TDRD7 functions in cell and the necessity of further investigation.

Recently, a potential link between the mTOR/S6K signaling pathway and TDRD7 has been established, as far as this protein has been found among S6K1 partners in yeast two-hybrid screen [3]. In this study, we present the data indicating the existence of potential novel TDRD7 isoforms termed TDRD7β, TDRD7γ and TDRD7δ. All these forms are shorter than the main TDRD7α (160 kDa) form and possess molecular weight of 130, 110 and 60 kDa correspondingly.

We have observed a different subcellular localization of the TDRD7 variants by Western blot analysis of HEK293 cellular fractions. According to our data TDRD7α and TDRD7β isoforms predominantly exis in cytosol, TDRD7γ in mitochondrial and TDRD7δ in nuclear fractions respectively. These data are in agreement with the results of confocal study indicating the presence of TDRD7 not only in cytoplasm, but also in nucleus.

Materials and methods. Bioinformatics. A new TDRD7 splice variant was identified using Uniprot (www.uniprot.org) and Ensemble (www.ensembl.org) databases and ASAP program [23]. This web-tool is based on an automated method for discovering human tissue-specific regulation of alternative splicing through a genome-wide analysis of expressed sequence tags (ESTs) that involves classifying human EST libraries according to tissue categories and Bayesian statistical analysis.
The BLAST analysis for 290–411 aa fragment (F2) of TDRD7 was performed using BLAST Network Service on ExPASy (http://web.expasy.org) and SCANPS (Similarity searches using Barton’s algorithm – http://www.ebi.ac.uk).

**Western blot analysis.** Cell lysate was prepared in a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 50 mM NaF, 1 mM EDTA, and a mixture of protease inhibitors (Roche Molecular Diagnostics). A portion of total lysate (40 µg) was separated by SDS-PAGE and immunoblotted with anti-TDRD7 antibodies as described previously [24]. Secondary HRP-labelled anti-mouse antibodies were from «Cell Signaling» (USA).

**Cell culture and immunocytochemistry.** HEK293 cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10 % fetal bovine serum («HyClone», UK), penicillin (200 U/ml) and streptomycin (200 mg/ml). For immunofluorescent staining HEK293 cells were grown in tissue culture chambers («Nunc», Denmark), fixed with 3.7 % formaldehyde 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 5 % FBS diluted in PBS-T. Afterwards staining with primary and secondary antisera was performed as described previously [25], anti-mouse Alexa-Fluor 546-conjugated secondary antibodies were from «Invitrogen» (USA). Fluorescently labelled proteins were visualized with a Zeiss LSM510 confocal microscope, and the images were analyzed using the LSM510 image browser software [26]. Nuclei were stained with Hoechst 33258 («Sigma», USA).

**Isolation and preparation of cell fractions.** Cell fractions were obtained using different centrifugation conditions according to the protocol [27]. Briefly, HEK 293 cells were washed with phosphate buffered saline two times. Cells were scraped in 400 µl ice-cold IB-1 buffer (225 mM manitol, 75 mM sucrose, 30 mM Tris-HCl, pH 7.4, and 0.1 mM EGTA), homogenized and then centrifuged at 600 g for 5 min at 4 °C. The pellet, which contains nuclear fraction was freeze and stored at –20 °C. Supernatant was collected and centrifuged at 7000 g for 10 min at 4 °C. Supernatant was separated, freeze and stored at –20 °C. The pellet (containing mitochondrial fraction) was gently resuspended with IB-2 buffer (225 mM manitol, 75 mM sucrose, 30 mM Tris-HCl, pH 7.4), frozen at –20 °C.

**Results and discussion.** Previous molecular cloning of TDRD7 fragments allowed us to develop specific monoclonal antibodies against this protein directed to 290–411 aa part of TDRD7 [23]. This fragment contains a part of LOTUS domain identified recently using the sequence profile analysis. BLAST Network Service on ExPASy and SCANPS for this amino acid sequence revealed the presence of this fragment exclusively in TDRD7 protein in human cells.

It has been already demonstrated that TDRD7 is localized on the mitochondria outer membrane and in cytoplasmic structures called chromatoid bodies [5, 6]. We employed an immunofluorescent-confocal microscopy and anti-TDRD7 (E6) monoclonal antibody for more precise studying TDRD7 intracellular localization. The data of confocal studies (Fig. 1, see inset) indicate that a red fluorescent signal corresponding to TDRD7 was detected 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 (marked with arrows). These data show that some fraction of endogenous TDRD7 is present in the nucleus of wild HEK293 cells, the phenomenon, which has been observed previously only for COS7 cells overexpressing TDRD7 [11].

To get an additional evidence supporting the TDRD7 nuclear localization we have performed a subcellular fractionation of HEK293 cells with subsequent Western blot analysis. Surprisingly, we have detected the presence of not only 160 kDa TDRD7 protein band (TDRD7a), but also additional bands of about 130 kDa (TDRD7b) in cytosolic fraction. At the same time protein bands of 110 kDa (TDRD7γ) and 60 kDa (TDRD7δ) recognized by TDRD7 mAbs were detected in mitochondrial and nuclear fractions (Fig. 2). These data indicate the existence of additional isoforms of TDRD7 named as TDRD7β, TDRD7γ and TDRD7δ correspondingly.

To analyse a possibility of TDRD7 isoforms existence we made an extensive bioinformatical analysis. The performed search confirmed the presence of only one gene coding for TDRD7 in the mammalian genome. However, the screening of UniProt and Ensemble databases led to the identification of two alternative TDRD7 transcripts (short and long) with open
reading frame coding for hypothetical 130 and 60 kDa TDRD7 isoforms. The sequence analysis revealed the absence of exon 2 in the long transcript and several exons (1–9, 11) in the short one. Fig. 3, a, shows the intron/exon boundary of known TDRD7 form (TDRD7α) and new splicing variants. The existence of possible TDRD7 alternative transcripts was further confirmed with ASAP program and UCSC database (http://genome.ucsc.edu/).

Analysing the data received we may predict that TDRD7β detected by Western blot analysis represents 130 kDa TDRD7 isoform expressed from the long alternative TDRD7 transcript revealed by bioinformatical search. The short alternative TDRD7 transcript (Fig. 3) coding for 60 kDa protein represents different from TDRD7α isofrom with similar molecular weight. The specific epitope for anti-TDRD7 mAbs (290–411 aa) used in WB is absent in the short transcript protein product (Fig. 3).

In UCSC database there are some TDRD7 EST clones corresponding to the protein, which contains N-terminal sequences and lacks the C-terminal sequence of TDRD7. Hence, it is possible that the predicted TDRD7β is a product of specific C-terminal splicing of TDRD7α. However, additional studies should be performed to analyse either they represent just fragments of known TDRD7α or novel isoforms of TDRD7.

Taking together, our results demonstrated the possible existence of novel isoforms of TDRD7 named TDRD7β, TDRD7γ and TDRD7δ, which are characterized by different subcellular localization. But etc., an additional research, and first of all PCR analysis of cDNA from HEK293 cells, has to be performed to prove that the identified protein bands represent novel TDRD7 isoforms. Moreover, the screening of the TDRD7 isoforms in different cell lines and tissues at RNA and protein levels is necessary to determine the physiological importance of the new isoforms. The presence of N-terminal LOTUS domains which can bring TDRD7 into the complexes with RNA-protein modules could explain the appearance of TDRD7δ isoform in perinuclear and nuclear zones. Using a panel of TDRD7 siRNAs or shRNAs it would be possible to get an additional evidence of novel TDRD7 isoform existence.

Conclusions. In summary, we have provided evidence for the existence of novel, most probably alternatively spliced isoforms of TDRD7, named TDRD7β, TDRD7γ and TDRD7δ. According to the confocal microscopy studies and Western blot analysis of subcellular fractions of HEK293 cells we have suggested the presence of TDRD7β isoform in cytoplasm (together
Fig. 1. TDRD7 localization in HEK293 cells. Cells were fixed with 3.7 % formaldehyde and stained with anti-TDRD7 antibodies as secondary antibodies Alexa-Fluore 546-conjugated anti-rabbit antibodies were used (a); nuclei were stained with Hoechst (b). Picture (c) represents a merge of signals ×100.

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with the canonical TDRD7α form), TDRD7γ in mitochondrial and TDRD7δ in nuclear fractions respectively. The expression profile of these splice variants and their role in cells remain to be elucidated.

Acknowledgements. The work was supported with grants from the National Academy of Sciences of Ukraine. We thank Dr. V. Gorchev and Dr. S. Karahim for help in confocal studies.

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UDC 577.217
Received 20.09.11