Gene 664 (2018) 1–11

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Phosphorylated nucleolar Tau protein is related to the neuronal *in vitro* differentiation

Concetta Federico[a,1], Laura Gil[b,1], Francesca Bruno[a], Agata Grazia D'Amico[c,d], Velia D'Agata[c], Salvatore Saccone[a,*]

[a] Department of Biological, Geological and Environmental Sciences, University of Catania, Italy
[b] Department of Genetics, Medical School, University "Alfonso X el Sabio", Madrid, Spain
[c] Department of Biomedical and Biotechnological Sciences, University of Catania, Italy
[d] Department of Human Science and Promotion of Quality of Life, San Raffaele Open University of Rome, Italy

ARTICLE INFO

Keywords:
Tau-1
AT8
MAPT gene
Cell differentiation
rRNA genes
Cell nucleus
Neurons

ABSTRACT

Tau is a multifunctional protein, originally identified as a cytoplasmic protein associated with microtubules. It is codified by the *MAPT* gene, and the alternative splicing, in the neuronal cells, results in six different isoforms. Tau was subsequently observed in the cell nucleus, where its function is not yet clearly understood. Here, we studied the *MAPT* gene and the cellular localization of the AT8 and Tau-1 epitopes of Tau protein, in the SK-N-BE cell line, which differentiates in neuronal-like cells after retinoic acid treatment. These epitopes correspond to the phosphorylated Ser202/Thr205 and unphosphorylated Pro189/Gly207 amino acid residues, respectively, possibly involved in conformational changes of the protein. Our results demonstrated the presence of the smaller Tau isoform (352 amino acids), whose amount increases in differentiated SK-N-BE cells, with Tau-1/AT8 nuclear distribution related to the differentiation process. Tau-1 showed a spot-like nucleolar localization, in both replicative and differentiated cells, while AT8 was only detected in the differentiated cells, diffusely occupying the entire nucleolar region. Moreover, in the replicative cells exposed to actinomycin-D, AT8 and Tau-1 move to the nucleolar periphery and colocalize, in few spots, with the upstream binding transcription factor (UBTF). Our results, also obtained with lymphocytes exposed to the mitogenic compound phytohaemagglutinin, indicate the AT8 epitope of Tau as a marker of neuronal cell differentiation, whose presence in the nucleolus appears to be related to rDNA transcriptional inactivation.

1. Introduction

Tau is a native unfolded protein with a flexible structure, discovered forty years ago as a protein associated with microtubules (Weingarten et al., 1975). It plays an important role not only in the cytoplasm, stabilizing microtubules and participating in axonal metabolism (Goode et al., 1997; Dixit et al., 2008), but also in the cell nucleus (Loomis et al., 1990), where interaction with chromatin has been shown (Sjöberg et al., 2006; Sultan et al., 2011; Hernàndez-Ortega et al., 2015; Mansuroglu et al., 2016). It is encoded by the microtubule-associated protein Tau (*MAPT*) gene, which yields three transcripts of 2, 6 and 9 kb, with the 2 kb transcript observed in the cell nucleus (Bukar Maina et al., 2016).

In the central nervous system (CNS), Tau protein is largely present in six different isoforms, due to alternative splicing of exon 2 (E2), exon 3 (E3), and exon 10 (E10), with the largest composed of 441 amino acids. It is structurally subdivided into four parts: the N-terminal acidic region, the proline-rich domain (PRD), the microtubule binding domain

Abbreviations: Act-D, actinomycin-D; ACTB, actin-b; AD, Alzheimer's disease; AT8, Tau epitope with phosphorylated Ser202/Thr205 residues; CNS, central nervous system; DAPI, 4′,6-diamidino-2-phenylindole; DFC, dense fibrillar component (of the nucleolus); EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ETS, external transcribed spacer (in the rRNA gene cluster); FBL, fibrillarin; FC, fibrillar centre (of the nucleolus); FITC, fluorescein isothiocyanate; GC, granular component (of the nucleolus); IF, immunofluorescence; ITS1, internal transcribed spacer-1 (in the rRNA gene cluster); *MAPT*, microtubule-associated protein Tau gene; MBD, microtubule binding domain (of the Tau protein); NFT, meurofibrillary tangles; NOR, Nucleolar Organizing Regions; PBS, phosphate buffered saline; PCR, Polymerase Chain Reaction; PHA, phytohaemagglutinin; PNS, peripheral nervous system; PRD, proline-rich domain (of the Tau protein); qRT-PCR, Quantitative Reverse Transcribed PCR; RA, retinoic acid; rDNA, ribosomal gene cluster; RPMI 1640, Roswell Park Memorial Institute medium; RQ, relative quantification; Tau-1, Tau epitope with non-phosphorylated Pro189/Gly207 residues; TRITC, tetramethylrhodamine; UBTF, upstream binding transcription factor

* Corresponding author at: Department of Biological, Geological and Environmental Sciences, Via Androne, 81, 95124 Catania, Italy.
E-mail address: saccosal@unict.it (S. Saccone).
[1] These authors contributed equally.

https://doi.org/10.1016/j.gene.2018.04.051
Received 15 October 2017; Received in revised form 6 April 2018; Accepted 18 April 2018
Available online 21 April 2018
0378-1119/

(MBD) and the C-terminal region. The different uses of E2 and E3 determine the formation of a protein with 0, 1 or 2 repeats (called 0N, 1N and 2N) in the amino-terminal acidic region and the use of E10 determines the presence of three or four repeats (called 3R and 4R) in the MBD. The different combination of E2, E3, and E10 defines six Tau isoforms called 0N3R, 1N3R, 2N3R, 0N4R, 1N4R, and 2N4R (Buée et al., 2000; Andreadis, 2005; Wang and Mandelkow, 2016; Bukar Maina et al., 2016). The alternative splicing of the *MAPT* gene, in human brain and neuroblastoma cells (Goedert et al., 1989; Loomis et al., 1990), results in protein isoforms visible in a western blot as a series of closely spaced bands, with a size greater than about 50 kDa.

Conformational changes of Tau protein are determined by site-specific phosphorylations (Avila, 2009), allowing the acquisition of transitory structures (Von Bergen et al., 2005; Jeganathan et al., 2008) able to interact with multiple different proteins as well as with DNA and RNA (Qi et al., 2015; Wang et al., 2006). Tau protein is phosphorylated by several serine/threonine kinase proteins, some of them related to abnormal Tau hyperphosphorylation, such as PKA, CaMKII, GSK-3β and cdk5. In particular among these proteins, GSK-3β plays an important role in both physiological and pathological phosphorylation (Wang et al., 2007; Hanger and Noble, 2011). In addition, deregulation of protein phosphatases, such as PP2A, is of extreme importance because their activity is often regulated by kinase-dependent phosphorylation (Wang et al., 2007).

Phosphorylations are involved in the formation of neurofibrillary tangles (NFT) that clinically define Alzheimer's disease (AD) and its various stages (Braak and Braak, 1991). However, only a few epitopes of Tau have been described in the nucleus, such as the phosphorylated AT8 (pSer202/Thr205) and AT100 (pThr212/Ser214) (Rossi et al., 2008; Hernàndez-Ortega et al., 2015; Gil et al., 2017), and the non-phosphorylated Tau-1 (Pro189/Gly207 residues), with the latter specifically immunolocalized in the nucleolus of neuronal and non-neuronal cells (Loomis et al., 1990; Thurston et al., 1996, 1997; Sjöberg et al., 2006; Rossi et al., 2008). Indeed, the non-phosphorylated epitope Pro189/Gly207 of Tau was localized in the nucleolus of human fibroblast and HeLa cells, where it partially colocalized with alpha satellite DNA from pericentromeric chromatin, suggesting a role in the organization of the nucleolar heterochromatin (Sjöberg et al., 2006).

The nucleolus is the sub-nuclear region where rRNA synthesis and ribosomal assembly takes place. It is composed of three different regions, namely the fibrillar centre (FC) surrounded by the dense fibrillar component (DFC), and the granular component (GC) that, in turn, surrounds the FC/DFC region (Boisvert et al., 2007; Bártová et al., 2010; Denissov et al., 2011; Smirnov et al., 2016). The FC corresponds to the Nucleolar Organizing Regions (NORs), also detectable in the mitotic chromosomes and defining the chromatin with transcriptionally-active rDNA repeats (McStay, 2016), endowed with a non-nucleosomal euchromatic state in the interior of the nucleolus, as demonstrated by psoralen photocrosslinking (Conconi et al., 1989). In addition to these three functional subregions, the nucleolus is characterized by the presence of large heterochromatic blocks, generally located at the periphery of the GC, composed by the centromeric regions of the chromosomes involved in nucleolus formation, and by the heterochromatic stably silenced rRNA genes (Lucchini and Sogo, 1992; Thiry and Lafontaine, 2005; Grummt, 2007; Sanij and Hannan, 2009; Mangan et al., 2017). Indeed, the nucleolus contains rRNA genes, but a number of these genes are constitutively unexpressed, being silenced by epigenetic modifications involving methylation of the cytosine in the rDNA repeats (Akhmanova et al., 2000; Sanij and Hannan, 2009; Parlato and Kreiner, 2013; D'Aquila et al., 2017). Replicative cells are endowed by a high number of rRNA genes (about 50%) transcribed by RNA polymerase I, contrary to the differentiated cells, endowed by a low number (about 10%) of active rRNA genes (Sanij and Hannan, 2009; Takada and Kurisaki, 2015). It was recently demonstrated that a downregulation of rDNA transcription is one of the mechanisms strictly related to the cell differentiation program (Hayashi et al., 2014).

In the present work, we studied nuclear Tau in the human neuroblastoma cell line SK-N-BE, which is able to differentiate in neuronal-like cells when induced by retinoic acid (Leotta et al., 2014). We analysed the non-phosphorylated Pro189/Gly207 and the phosphorylated pSer202/Thr205 epitopes, detected with the antibodies Tau-1 and AT8, respectively. Moreover, we identified and quantified the Tau isoforms expressed in the replicative and differentiated SK-N-BE cells. The relevance of the obtained results, with respect to the nucleolar activity and cell differentiation, is discussed.

2. Materials and methods

2.1. Cell cultures

The human neuroblastoma cell line SK-N-BE (Biedler et al., 1978) and human peripheral blood lymphocytes were cultured in RPMI 1640 medium, supplemented with 10% foetal bovine serum (FBS) (20% for lymphocytes), 1% antibiotic Penicillin/Streptomycin (100 U/ml; 100 μg/ml) and 1% L-Glutamine, at 37 °C, with 5% CO_2. To obtain replicative lymphocytes, the culture was performed with medium containing 3% phytohaemagglutinin (PHA) (GIBCO cat. n. 10576-015) for 72 h. Human lymphocytes were obtained, from healthy volunteers, by venous blood sampling and then put into heparinised tubes. All procedures performed in the present study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Signed informed consent forms were obtained from all the volunteer subjects.

SK-N-BE cells were differentiated in neuronal-like cells with a treatment of 10 μM retinoic acid (RA) (Sigma–Aldrich Cat. n. R2625) added to the culture medium every 72 h (day 0, 3, 6, 9) to obtain differentiated cells on the 12th day of treatment (D'Alessio et al., 1995; Andres et al., 2013; Leotta et al., 2014). Replicative SK-N-BE cells were incubated for 1 h with 0.05 μg/ml of actinomycin-D (Act-D), before harvesting, to obtain the block of transcription (Perry and Kelley, 1970; Bensaude, 2011).

2.2. Cell preparation and immunofluorescence analysis

Immunofluorescence (IF) experiments were performed as previously described (Maugeri et al., 2016). Briefly, SK-N-BE cells were cultured on glass chamber-slides and fixed with 4% paraformaldehyde for 20 min at room temperature. Human lymphocytes were cytocentrifuged on glass slides and then fixed as above.

Cells were washed with phosphate buffered saline (PBS) and incubated for 15 min in PBS containing 0.5% Triton X-100. Immunodetections were performed by incubation with the specific antibody at the dilution suggested by the manufacturers. Before the incubation with primary antibody, a preincubation step of 1 h at 37 °C with blocking solution (non-fat dry milk or bovine serum albumin 1%) was carried out. The antibodies used were Tau-1 (Millipore Cat. MAB3420), to detect Pro189/Gly207 residues, and AT8 (Thermo Scientific Cat. MN1020) to detect pSer202/Thr205. Moreover, antibodies against UBTF (NBP1-82545), GAP-43 (Millipore Cat. AB5312) and fibrillarin LS-C204517 (Clone 38F3) were used to evaluate the different status of the SK-N-BE cells. Dual colour IF was also performed by using mouse anti-Tau-1 or AT8 antibodies together with the rabbit anti-UBTF antibody.

After overnight incubation and additional PBS washes, cells were incubated at 37 °C for 1 h with FITC-conjugated sheep anti-mouse secondary antibody (Sigma-Aldrich, 1:100 dilution in blocking solution) together, in the case of the dual colour IF, with the TRITC-conjugated goat anti-rabbit (Sigma-Aldrich, 1:400 dilution in blocking solution).

Experiments were repeated at least three times. The results were analysed with confocal laser scanning microscopy (CLSM) (Zeiss LSM700) and images were captured at 400× and 630× magnification,