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Tau regulates the subcellular localization of calmodulin

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ABSTRACT

Lack of tau expression in neuronal cells results in a change in the expression of few genes. However, little is known about how tau regulates gene expression. Here we show that the presence of tau could alter the subcellular localization of calmodulin, a protein that could be located at the cytoplasm or in the nucleus. Nuclear calmodulin binds to co-transcription factors, regulating the expression of genes like calbindin. In this work, we have found that in neurons containing tau, a higher proportion of calmodulin is present in the cytoplasm compared with neurons lacking tau and that an increase in cytoplasmic calmodulin correlates with a higher expression of calbindin.

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1. Introduction

Tau is a microtubule-associated protein (MAP) that is mainly expressed in neurons [1], where tau is mainly found associated to polymerized tubulin, a cytoplasmic protein that is the major component of brain microtubules. However, tau could be also present free in the cytoplasm [2] or bound to other proteins. Several tau-associated proteins have been described [1], being one of them calmodulin [3–5]. Calmodulin (CaM) is a major calcium sensor in neurons [6] when is present in the cytoplasm. Also, it can bind and regulate diverse target proteins by different mechanisms [7]. In the nucleus, calmodulin binds to some co-transcription factors, like BAF-57 [8], a protein member of a complex involved in the repression of neuronal specific genes [9]. Little is known about how the subcellular localization of calmodulin is regulated, a process that, by it self, could regulate calmodulin functions. In neuronal cells it has been indicated that depolarization could induce the elevation of nuclear CaM in dentate gyrus granule cells and that nuclear CaM accumulation depends on the association of CaM with an endogenous CaM binding protein [10]. In other cases, for specific isoforms of calmodulin, posttranslational modifications of the protein could influence its subcellular localization [11].

Recently, it has been shown that tau protein could regulate the expression of few genes, mainly related to neuron phenotype [12].

However, nothing is known about the mechanism for that regulation for specific genes.

Since tau is mainly a cytoplasmic protein, we have studied if the binding of calmodulin to tau could result in retaining calmodulin at the cytoplasm and if it results in a decrease in the nuclear function of calmodulin, a function that could be related with the expression of neuronal genes like calbindin [8].

In this work, we have found that the lack of tau in neurons changes the subcellular localization of calmodulin and that it correlates with a change in the expression of calbindin.

2. Methods

2.1. Antibodies

The following primary antibodies were used: anti-calmodulin (Millipore); anti-calbindin (Chemicon); anti- β -III-tubulin (Covance); anti- β -actin (Sigma Aldrich); anti-tubulin (Sigma Aldrich); anti-lamin B1 (Santa Cruz Biotechnology). The 7.51 antibody (from Dr. Claude Wischik, UK) was used to detect tau in a phosphorylation-independent manner [13] and detects all soluble tau isoforms in Western blot analysis.

2.2. Animals

Tau^{-/-} mice were generated as previously described [14] and can be obtained from Charles Rivers Co. Heterozygous (Tau^{+/-}) mice were crossed in order to obtain homozygous tau knockout mice (Tau^{-/-}) and control littermates (Tau^{+/+}). Animals were bred at the Centro de Biología Molecular “Severo Ochoa” (Madrid, Spain). Four to five mice were housed per cage with food and water avail-

Abbreviations: MAP, microtubule associated protein; CaM, calmodulin; wt, (wild type); BSA, bovine serum albumin; PBS, phosphate buffered saline.

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Fig. 1. Tau deficient neurons show a higher proportion of calmodulin in the nucleus. (A) Primary hippocampal neurons from wild type (wt) (upper line) and $\text{Tau}^{-/-}$ mice (lower line) were immunolabeled with anti-calmodulin antibody (green). β III-tubulin immunolabeling (red) was used as a neuronal marker and To-Pro-3 (blue) as a nuclear marker. Calmodulin was detected both in the cytoplasm and the nucleus but in different proportion in wt and in $\text{Tau}^{-/-}$ mice. (B) Quantitative fluorescent measurements of calmodulin immunolabeling in the nucleus and the cytoplasm of wt and tau deficient neurons. (C) Western blot analysis confirmed the lack of tau protein in $\text{Tau}^{-/-}$ neurons and the presence of calmodulin in both, wt and $\text{Tau}^{-/-}$, cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

able ad libitum. Mice were maintained in a temperature-controlled environment on a 12/12 h light–dark cycle with light onset at 07:00 h. All experiments were carried out with males.

2.3. Cell cultures

Primary cultures of hippocampal neurons were prepared according to established procedures with slight modifications [15]. Briefly, hippocampal tissue was obtained from E18 mouse embryos, dissected, and dissociated with the Papain Dissociation System (PDS; Worthington Biochemical Corporation, Lakewood, NJ, USA). Neurons were maintained in Neurobasal medium (Gibco, Grand Island, NY, USA) supplemented with 2% B-27, 1% N2, 0.5 mM glutamine, 100 U/ml penicillin, and 100 mg/mL streptomycin, and grown on poly-L-lysine (100 mg/mL) coated plastic dishes. The cells were incubated in 95% air/5% CO_2 in a humidified incubator at 37 °C.

Human neuroblastoma SH-SY5Y cells [16] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin,

100 U/ml streptomycin in a humidified atmosphere of 5% CO_2 /95% air at 37 °C. For differentiation, proliferating SH-SY5Y cells were plated and then cultured in Neurobasal-B27 medium (Gibco, Grand Island, NY) supplemented with 2 mM dibutyryl cyclic AMP and 1 mM glutamine for 7 days.

2.4. Immunofluorescence

Cells were permeabilized in 0.1% Triton X-100 for 15 min and then they were treated with 1 M glycine for 30 min to eliminate their auto fluorescence. After a brief wash with PBS, they were incubated with 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS) for 1 h and then exposed to primary antibody diluted in 1% BSA/PBS for 1 h. Primary antibodies anti- β -III-tubulin (1:2000; Covance) and anti-calmodulin (1:200; Millipore) were used. Cultures were extensively washed and then incubated with the appropriate secondary antibody, conjugated either with Alexa 488 or with Alexa 555 (1:200; Invitrogen), plus To-Pro-3 (1:500; Invitrogen) (nucleic acid stain) for 45 min. After washing,

Fig. 2. Differentiated SH-SY5Y neuroblastoma cells show increased tau protein and decreased nuclear calmodulin levels. (A) Differentiated and non-differentiated (control) SH-SY5Y cells were immunolabeled with anti-calmodulin antibody (green). Calmodulin was detected both in the cytoplasm and the nucleus. To-Pro-3 stain (blue) was used as nuclear marker. (B) Quantitative fluorescent measurements of calmodulin immunolabeling in the nucleus and the cytoplasm of differentiated and non-differentiated SH-SY5Y cells. (C) Western blot analysis of differentiated and non-differentiated SH-SY5Y whole cell extracts showed increased tau protein levels in differentiated cells whereas total calmodulin levels were similar. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

they were immediately mounted with Fluorosave Reagent (Calbiochem).

2.5. Western blot analysis

Cells were lysed in buffer consisting of 20 mM HEPES, pH 7.4, 100 mM NaCl, 20 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 5 mM EDTA, and 2 mM PMSF and a protease inhibitor cocktail (Roche). Samples were electrophoresed on 12% SDS-poly-

acrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The experiments were performed using the following primary antibodies: 7.51 anti-tau (1:100), anti-calmodulin (1:1000), anti-β-actin (1:5000), anti-lamin B (1:200), anti-α-tubulin (1:5000) and anti-calbindin (1:1000). The filters were incubated with the antibody at 4 °C overnight in 5% non-fat dried milk. Secondary goat anti-mouse (1:1000) (Dako), goat anti-rabbit (1:1000) (Dako) and donkey anti-goat (1:1000) (Santa Cruz Biotechnology) antibodies and ECL detection reagents

Fig. 3. Differentiated neuroblastoma SH-SY5Y cells show decreased nuclear calmodulin levels. (A) Cytoplasmic and nuclear fractions from differentiated and non-differentiated (control) SH-SY5Y cells were analyzed by Western blotting to detect calmodulin and tau. Lamin-B was used as nuclear load marker and α -tubulin as cytoplasmic load marker. (B) Protein quantization in cytoplasm and nucleus of differentiated and non-differentiated cells (** $p < 0.005$; * $p < 0.05$).

(Amersham Biosciences, Arlington Heights, IL) were used for immunodetection.

2.6. Subcellular fractionation

Cells were collected in M-SHE buffer (210 mM manitol, 70 mM sucrose, 10 mM HEPES pH 7.4, 1 mM EDTA, 1 mM EGTA) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin), phosphatase inhibitors (1 mM sodium orthovanadate, 30 mM glycerol phosphate, 5 mM pyrophosphate, 1 μ M okadaic acid) and 1 mM dithiothreitol. Cytoplasmic and nuclear fractions were extracted as previously described [12]. Lysis buffer (50 mM Tris-HCl pH 6.8, 10 mM β -mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was added to both fractions. Samples were boiled for 10 min and then analyzed by SDS-PAGE.

2.7. RNA isolation and quantitative real-time PCR

RNA was isolated from differentiated and non-differentiated SH-SY5Y cells using the Absolutely RNA MiniPrep Kit (Stratagene). This RNA was used as a template for the first strand cDNA synthesis (Roche), which was then analyzed by quantitative real-time PCR with an Applied Biosystems ABI PRISM 7900HT real-time detection system with the SYBR green PCR master mix (Applied Biosystems), interpreting the results with the SDS 2.1 software. The primers used to amplify each gene were: 5'-ACAGCATGTCTACTGCT TGGATCCA-3' and 5'-TGGTGCTCCCTTCCCTTGTC ACA-3' for human calbindin; 5'-CACACTGTGCCCATCTACGA-3' and 5'-CTCCTTAATGT-CACGCACGA-3' for human β -actin.

3. Results

In previous studies it was reported a direct binding between tau and calmodulin [3–5]. Since tau is mainly a cytoplasmic protein, its interaction with calmodulin could affect to the subcellular localization of calmodulin. Thus, we have tested that possibility.

Fig. 4. (A) Interaction of nuclear calmodulin (CaM) with BAF complex results in a decrease of calbindin expression [6]. In the presence of tau, the amount of nuclear CaM should decrease and the expression of calbindin should increase. (B) Cortical neurons lacking tau express less calbindin than wild type neurons. (C) Differentiated SH-SY5Y cells show increased calbindin mRNA expression. Quantitative real-time PCR for calbindin gene in differentiated and non-differentiated (control) mRNA samples. (** $p < 0.001$). (D) Differentiated and non-differentiated cell extracts were analyzed by Western blotting to determine calbindin levels. Calbindin quantization was normalized to β -actin levels (* $p < 0.05$) (E).

3.1. The presence of tau increases cytoplasmic localization of calmodulin

By comparing hippocampal neuron cultures from wild type mouse or from a mouse lacking tau we found, by immunofluorescence analysis, increased levels of nuclear calmodulin in neurons from Tau^{-/-} mice (Fig. 1A) compared to wild type mice. The presence of total tau and calmodulin was quantitated in both wild type and Tau^{-/-} neurons (Fig. 1B). As expected a dramatic difference was found in the amount of tau from both mice but not in the amount of calmodulin (Fig. 1C).

A similar result was found when undifferentiated and differentiated human neuroblastoma SY5Y cells were tested. In undifferentiated cells a lower tau level was found compared with differentiated cells as determined by Western blot analyses (Fig. 2C, see also Fig. 3A), whereas no changes in the amount of calmodulin were observed (Fig. 2). Again, a higher nuclear localization for calmodulin was found in non-differentiated cells compared to differentiated neuroblastoma cells (Fig. 2A and B).

3.2. Quantization of calmodulin in the cytoplasm and cell nucleus

To test, biochemically, the subcellular localization of calmodulin, subcellular fractionation to obtain cytoplasmic and nuclear fractions was performed, followed by Western blot analysis to identify calmodulin and tau in those fractions. Fig. 3 shows an increase in nuclear calmodulin in cells lacking tau. Thus, the presence of tau correlates with a decrease in calmodulin localization at the cell nucleus.

3.3. In the absence of tau there is an increase in calbindin expression

A described function for nuclear calmodulin is its interaction with transcriptional factors [8,10] being one of them BAF57, a protein of the BAF complex. Upon binding of calmodulin, the function of BAF complex is activated [6] and the expression of neuronal proteins like calbindin is decreased [12,17] (Fig. 4A). Thus, we have tested if a decrease in the amount of nuclear calmodulin, due to the absence of tau could result in an increase in calbindin expression. First, we tested the expression in cortical neurons containing or lacking tau. Fig. 4B shows an increase in calbindin expression in neurons containing tau protein. Also, we observed that differentiated SY5Y cells, with higher tau expression than undifferentiated cells, showed an increase in calbindin expression (Fig. 4A and B), as determined by calbindin mRNA and protein (Fig. 4C and D).

4. Discussion

In a previous report [12], we showed that tau deficiency lead to the upregulation of BAF57, a protein that is involved in down regulation of neuron-specific gene expression [6]. In addition, in this work, we have found that tau deficiency also regulates calbindin expression. It can take place by activation of BAF complex through its interaction with nuclear calmodulin, as previously reported [8].

Indeed, in previous studies it has been described that nuclear accumulation of calmodulin could be due to its interaction with some nuclear proteins [10]. Now we suggest that cytoplasmic accumulation of calmodulin could be due to its interaction with tau protein.

Thus, our results suggest that tau protein may regulate the subcellular localization and function of calmodulin in neurons. Tau protein binds to calmodulin through an ionic interaction in a sim-

ilar fashion to the binding between tau and other calcium binding proteins like tubulin [18,19]. After that interaction, the presence of calmodulin at the cell nucleus decreases, probably due to the fact that tau protein is mainly a cytoplasmic protein and that the complex tau-calmodulin should be mainly located at the cytoplasm. In the absence of tau or the presence of low tau levels, the level of calmodulin at the cell nucleus increases. Nuclear calmodulin could act as a co-transcription factor regulating the expression of neuronal proteins, like calbindin, facilitating the process of neuron differentiation, in which tau expression may play a role [12,14].

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