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EFFECT OF PHOSPHORYLATION AND AGGREGATION ON TAU BINDING TO DNA

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ABSTRACT: The potential function of neuronal tau was found by our recent studies on the effect of tau on the melting temperature of both calf thymus DNA and plasmid pBluescript-II SK (*Hua and He, Chin. Sci. Bull. 2000, 45:999-1001*). Herein we examined whether or not the interaction of tau with DNA was related to phosphorylation and aggregation. Tau, phosphorylated by neuronal cdc2-like kinase, associated with DNA as shown by electrophoretic mobility shift assay. Similar to native tau, phosphorylated tau could increase the melting temperature of calf thymus DNA. When tau was aggregated or treated with formaldehyde, neither native tau nor phosphorylated tau kept its ability to interact with DNA, suggesting that binding of tau to DNA was in an aggregation-dependent, and a phosphorylation-independent, manner.

KEY WORDS: human neuronal tau, conformation, DNA, aggregation, phosphorylation

INTRODUCTION

Tau is a major microtubule associated protein of neurons [1]. A major known function of tau is to promote and maintain the assembly of the microtubules system, which plays an important role in axonal transport and morphogenesis [2]. The discovery of tau as the major protein subunit of paired helical filaments (PHFs) in Alzheimer disease has markedly increased interest in understanding the structure and function of this protein [3]. In a normal neuron, tau is localized in the axons and neuronal soma [4]. In addition to its association with microtubules, a major location, tau has been found to be associated with ribosomes [5] and localized in nuclei of several cell lines, such as those from human neuroblastoma, human cervical carcinoma, human macrophages and monkey kidney [6,7]. The localization of tau has been observed at the nucleolar regions of the acrocentric chromosomes of human neuroblastoma cells, associated with both fibrillar regions of interphase nucleoli and the nucleolar organizer regions [6,8], which implies some new roles of tau in

these regions.

In recent years, a growing number of studies indicate that the assembly and stabilization of microtubules may not encompass all functions of tau, for example, plasma membrane association, interaction with protein phosphatases and other enzymes [9]. And multiple activities for tau appear to be regulated by phosphorylation and affected by tau aggregation [10]. In this lab, we found that human neuronal tau could increase the melting temperature (Tm) of both calf thymus DNA (CTDNA) and plasmid pBluescript-II SK [11]. Furthermore, the binding of tau to DNA (plasmid and polynucleotide) was exhibited by electrophoretic mobility shift assay. Thus, it is important to further understand the role of tau phosphorylation and aggregation in its interaction with DNA. In present study, using phosphorylation of tau catalyzed by neuronal cdc2-like kinase (NCLK), we show that phosphorylated tau (Ptau) keeps its activity to interact with DNA, but aggregated tau could not interact with DNA.

MATERIALS AND METHODS

Sephadex G50, Q-Sepharose and SP-Sepharose were from Pharmacia. CTDNA (readily soluble, suitable as substrate in DNase assays), Calf thymus DNA-cellulose (double strands) came from Sigma (St. Louis). Rabbit antibody R134d to tau was kindly provided by Dr Iqbal. I^{125}-labeled secondary goat anti-rabbit IgG was from NEM Life Science Products, Inc USA. pET-15b plasmid was from Promega. Other reagents used were of analytic grade without further purification.

Expression, purification, phosphorylation and aggregation of protein

Tau protein was obtained by *in vitro* expression of a recombinant human neuronal tau_{441} clone that contains full length of tau_{441} gene and was kindly provided by Dr. Goedert, University of Cambridge, UK. The construct was expressed and protein was purified as described [12]. Tau was purified in queues with Q-Sepharose, SP-Sepharose and Sephadex-G75 columns and checked by SDS-PAGE as a single major protein band. The concentrations of the purified protein were determined spectrophotometrically by using $E^{280}_{mg/ml} = 0.27$ [13].

Tau was phosphorylated and checked with SDS-PAGE as described [12,13]. After phosphorylation, ultrafiltration (Amicon Centricon-10) was used to concentrate the protein and to remove low-MW solutes. The absorbance (260 nm) of Ptau at the concentrations used in the experiments was less than 0.05.

Tau polymers were prepared by two different ways as: 1) incubating the protein in 25 mM phosphate buffer (pH 7.2), 10 mM DTT, at 37 °C for 48 hrs, as described by Wilson and Binder [14]. According to Luo et al [15], the formation of the fluorescence at 333 nm was used to monitor the aggregation until the emission intensity did not change. 2) tau was incubated with 0.2% and 0.5% formaldehyde at room temperature overnight. An Amicon Microcon-10 column was used to remove excess formaldehyde before use.

In addition, for thermal denaturation of DNA, samples were incubated with the protein at desired temperatures (20, 25, 40, 50, 60, 70, 80 and 90°C) for 10 min, followed by measurements of the absorbance (260 nm) of the mixture. The data were analyzed by Origin 5.0 application software and differential method.

Micro-chromatography of DNA-cellulose and radioimmunodot-blot

Calf thymus DNA-cellulose (0.1 g) was resuspended in standard buffer containing 10% (v/v) glycerol, 10% (w/v) BSA, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, and 20 mM Tris-HCl (pH 7.5). Prior to use, it was kept at 4°C overnight. After that, it was washed with the same buffer for 5 times (1 ml each) in an Eppendorf tube with spinning (1000 rpm, 4°C, 2 min). Approximately, 8 μg tau was added to 25 μl of DNA-cellulose (1 mg) suspension, 50 μl as a final volume of the mixture, with a frequent shake to mix tau with DNA at 37°C for 30 min. The DNA-cellulose was spun down and washed with the standard Tris-HCl buffer for 4 times (1 ml each) after the supernatant was removed. Aliquots were taken and assayed by radioimmunodot-blot [16]. 5 μl of sample (containing 0.1-2.0 ng tau) was applied onto the nitrocellulose membrane before drying at 37° for 1 hr. Then it was blocked by 5% BSA in 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. Rabbit antibody R134d to tau (1:5000) was employed as the primary antibody, sequentially reacted with an I^{125}-labeled secondary antibody (anti-rabbit, 1:100). The radioimmunoreactivity of the blots was visualized and quantitated by using a Phosphorimager (Fujifilm BAS-1500) and TINA 2.0 software (Raytest Isotopenmeβ geräte GmbH).

Synthetic polynucleotides and electrophoretic mobility shift assay (EMSA)

A polynucleotide was arbitrarily synthesized according to the DNA motif of mouse N-Oct-3 [17] by Sangon Company. The sequence is the specific target of the transcript factor Brn2. In order to anneal effectively, 5 more nucleotides were added at the two ends. Polynucleotide a: 5'-AGCT CTCCG TGCAT AAATA ATAGG C-3', and 5' labeled with digoxin. Polynucleotide b: 5'-TCGAG CCTAT TATTT ATGCA CGGAG-3'. The two strands were annealed as a double-stranded DNA probe. An aliquot of the dig-labeled probe (0.6 μM) was used for a binding reaction with protein. The probe and protein were incubated in a 10-μl reaction mixture that contained 20 mM Hepes (pH 7.6), 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2 %, (W/V) Tween20 and 30 mM KCl at room temperature for 20 min. The DNA-protein complexes were resolved in a nondenaturing 6% polyacrylamide gel in 0.25 × TBE running buffer (4°C, 8V/cm). After electrophoresis, the gels were electroblotted (250 mA, 1 hr, 4°C) onto nylon membrane, then fixed in a UV Crosslinker FB-UVXL-1000 of Fisher Scientific Co. NBT and BCIP (Boehringer Mannheim) were used as the colorimetric detection reagents. In EMSA of plasmid, pET-15b was incubated with protein in 25 mM Na_2HPO_4-NaH_2PO_4, pH 7.2 (RT, 30 min). Samples were loaded directly onto 1% (W/V) agarose gel in 0.5 × TAE buffer and electrophoresed at 55V for 1.5 hr.

RESULTS

Tau binding to DNA

In a previous study, we showed that tau increased the T_m of CTDNA and plasmid pBluescript-II SK [11]. Furthermore, the binding of tau to DNA was exhibited by electrophoretic mobility shift assay. Here, calf thymus DNA-cellulose [18] as an affinity chromatography was used to confirm the association of tau with DNA. Tau was overloaded to a DNA-cellulose column. As we have found that the minimum time required for tau to interact with polynucleotide probe was less than 10 sec in EMSA (data not shown), we mixed tau and DNA-cellulose column for 30 min to make sure the binding reached completion. Then we collected the fractions of both flow-through and unspecific elutions by the standard Tris-HCl buffer (pH 7.5). Aliquots of fractions were taken for detection of tau by radioimmuno-dot-blot [16]. The total percentage of the unbound tau was about 70% of the whole loaded tau (Table 1) under the experimental conditions, that is to say, approx. 30% of tau was associated with DNA-cellulose.

Table 1. Tau_{441} binding to DNA-cellulose detected by radio immuno-dot-blot *[16]

Original (%)	Flow through (%)	Unbound (%)**	Bound (%)***
100 ± 9.57	33.57 ± 5.28	36.67 ± 6.37	29.72 ± 5.73

Tau_{441} (3.4×10^{-7}M, as final concentration) was incubated to 1 mg of DNA-cellulose (approx. 4μg DNA), and then washed with the standard 25mM Tris-HCl buffer (pH 7.5) until the washed tau was less than 0.5%. ** The data came from a sum of the four-time wash. *** Calculation showed that bound tau was approximately 1.1×10^{-7}M. Data in the table are means ± S.D.

Tau phosphorylation and aggregation

Since either nuclear tau or cytosolic tau was phosphorylated approximately to the same extent [19], it was an interesting issue whether or not the interaction of tau with DNA was affected by phosphorylation. *In vitro*, NCLK phosphorylated tau on the sites that were hyperphosphorylated in PHF-tau (tau found in PHFs) [20]. SDS-PAGE was a useful method to check whether the reaction of phosphorylation catalyzed by NCLK had reached completion [12]. As shown in Figure 1 (lane 2), tau has undergone the complete phosphorylation under the same condition as described by Paudel [13].

Tau was markedly aggregated when treated with formaldehyde at a low concentration [21]. Unlike some results on sulphated glycosaminoglycans [22], it was unnecessary to use a chemical cross-linker, for example, disuccinimidyl suberate (DSS), as a homobifunctional reagent [13] when detecting the formation of tau polymers in formaldehyde solution with SDS-PAGE. Here we used it as an agent to induce aggregation of both native tau and Ptau. The excess formaldehyde in solution was removed through an Amicon Microcon-10 column. As Figure 1 (lane 4-5) shows, native tau

was completely aggregated in formaldehyde solution at low concentration (0.2%), as the presence of material at the top of the gel indicating covalent aggregation. Ptau aggregated incompletely at a low concentration of formaldehyde (0.2%), as there is still a band in the region 66 kDa indicating non-aggregation, and completely aggregated around 0.5% formaldehyde. (Figure 1, lane 6-7).

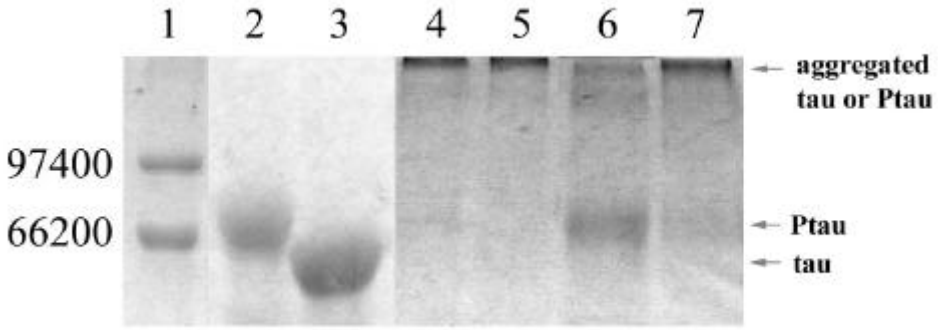

Figure 1. Tau phosphorylation and aggregation. Conditions used to phosphorylate tau by NCLK and to aggregate tau and Ptau were described in the text above. On 10% SDS-PAGE, samples were loaded as molecular mass marker (lane 1), Ptau (lane 2), tau (lane 3), 0.2% (lane 4) and 0.5% formaldehyde-treated tau (lane 5), 0.2% (lane 6) and 0.5% formaldehyde-treated Ptau (lane 7).

Recently, several compounds reported could induce tau aggregation, for instance, sulphated glycosaminoglycans, polyglutamic acid, and neurotoxin [22]. Almost all of the factors presented so far need a homobifunctional cross-linker to stabilize tau polymers induced, making it more convenient for SDS-PAGE detection [13]. Formaldehyde used to induce tau aggregation has some advantages: (1) inducing a complete aggregation of tau, which can be detected on SDS-PAGE without using any cross-linker (DSS) and (2) readily removing the residual unbound formaldehyde through ultrafiltration. Thus it was more reasonable to employ for preparation of aggregated tau, though the aggregation mechanism of formaldehyde-treated tau needs further studying.

Effect of aggregation and phosphorylation on tau binding to DNA

Electrophoretic mobility shift assay is one of the methods to investigate protein-nucleic acid interactions. As shown in Figure 2A, when assayed by gel electrophoresis, native tau retarded the mobility of both supercoiled and nicked-circular forms of plasmid DNA (pET-15b, 5708 bp) distinctly (lane 2). At the same time, Ptau retarded the mobility of DNA in agarose gel, to the similar extent with native tau (Figure 2A, lane 4), suggesting that phosphorylation of tau by NCLK did not influence its binding to DNA. When the formaldehyde-treated tau was incubated with DNA, it was demonstrated that DNA could not be retarded in agarose gel (Figure 2A, lane 3), which showed the same mobility with the control plasmid (Figure 2A, lane 1), indicating a distinct suppression of tau binding to DNA after aggregated.

As mentioned above, Ptau could bind to DNA, similar to its native state. It should be confirmed whether Ptau

could associate with DNA when aggregated. No retardation of the DNA could be detected in agarose gel when incubated with aggregated Ptau (Figure 2A, lane 5). To summarize, aggregation could inhibit the interaction of tau with DNA, however, phosphorylation had little effect on the interaction.

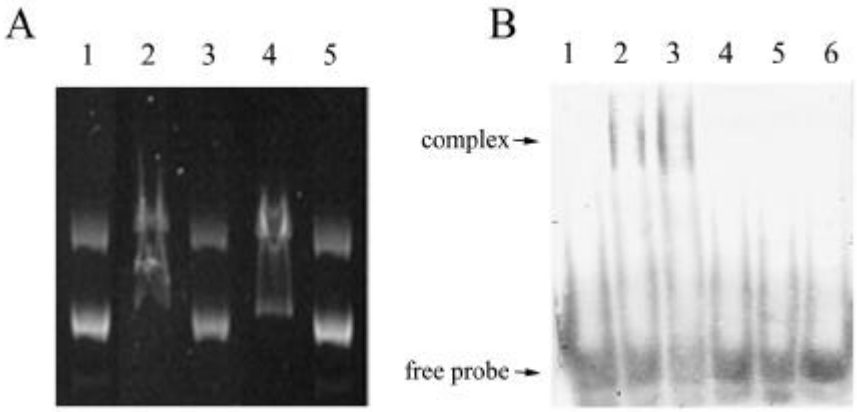

Figure 2. EMSA of DNA in the presence of tau at different states. A. DNA (pET-15b) was incubated with tau (lane 2), Ptau (lane 4), formaldehyde-treated tau (lane 3) and formaldehyde-treated Ptau (lane 5) at molar ratio of DNA/protein = 1/125. Aliquots were loaded directly onto 1% agarose gel. pET-15b alone was used as control (1ane 1). B. A polynucleotide as a probe was arbitrarily synthesized. The labeled one was 5'-AGCT CTCCG TGCAT AAATA ATAGG C-3' (digoxin labeled on 5'), and the two complementary single-stranded polynucleotides were made double stranded by annealing both strands. Lane 1 through 6, represented probe alone (0.6 μM), probe incubated with tau, with Ptau, with formaldehyde-treated Ptau, with formaldehyde-treated tau and with BSA, respectively. All protein samples were used at the same concentration (1.2 μM).

The size of the DNA fragment is normally kept below ~250 base pairs to enable clear distinction of the probe from any complexes in EMSA. The shorter the fragment, the higher the resolution [23]. Since the plasmids were larger than the size suitable for the assay, we employed a labeled polynucleotide probe to confirm the effect of phosphorylation and aggregation on tau binding to DNA. Similar results were obtained as mentioned above. Ptau and native tau were able to interact with the probe and form the tau-DNA complex (Figure 2B, lane 2-3), and neither was the aggregated tau (both formaldehyde-treated Ptau and tau, Figure 2B, lane 4-5). Meanwhile, the complex was not observed in case of BSA control (Figure 2B, lane 6). It was suggested that aggregation of tau inhibited its ability to bind to DNA.

Effect of Phosphorylation and Aggregation of tau on DNA thermal denaturation

Since tau could affect the T_m of CTDNA [11], we were interested to see the effect of phosphorylation and aggregation of tau on this activity. The thermal denaturation profiles of CTDNA with either native tau or Ptau displayed almost the same shape (Figure 3A, curve 4 and 5). The melting temperature of CTDNA was shifted to a higher temperature when adding tau or Ptau to the solution (Figure 3B, curve 4 and 5). It seemed that phosphorylation of tau by NCLK did not affect the interaction between tau and DNA. In previous reports [20] the phosphorylation catalyzed by NCLK was related

to the amino acid residues S-195, S-202, T-205, T-231, S-235, S-396 and S-404, therefore, it was suggested that those residues could not be the essential groups involved in binding to DNA.

In general, the preferred targets of formaldehyde are lysine residues. In order to exclude the possibility that simply lysine charge neutralization decreased the ability of tau binding to DNA, and also to exclude the interference of methyl group (introduced by formaldehyde) on interaction between tau and DNA, we prepared tau polymers in a different way. As described by Wilson and Binder [14], tau could aggregate itself gradually when incubated in 25 mM phosphate buffer (pH 7.2), 10mM DTT, at 37°C over 24 hrs. The fluorescence at 333 nm was used to monitor tau aggregation, according to Luo et al [15]. The aggregated tau was added to DNA when further changes in the emission intensity could not be detected. Again, changes of Tm were used to monitor whether aggregated tau bound to DNA. As shown in Fig 3A (curve 3), the melting transition was only slightly shifted and T_m was not significantly affected when aggregated tau was added to the solution of CTDNA (Figure 3B). Since the conformation of tau would change when it was aggregated [24], it is possible that the interaction of tau with DNA was related to the native conformation of tau.

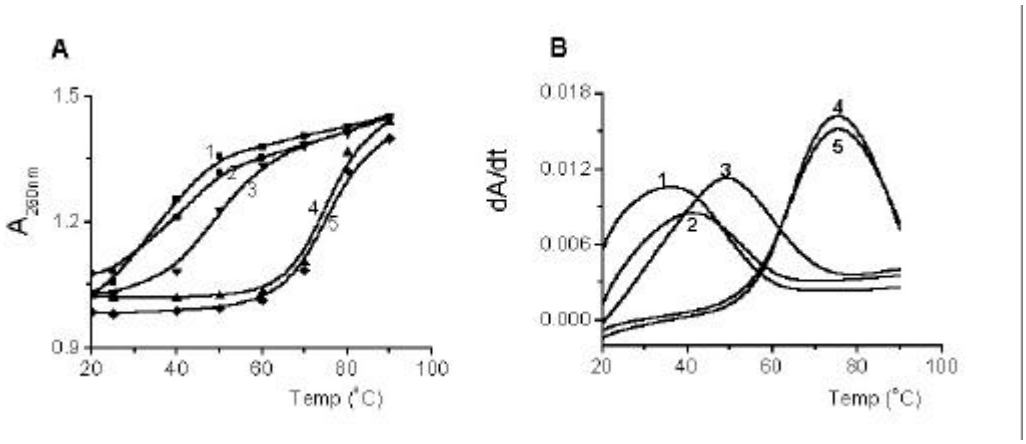

Figure 3. The melting behavior of CTDNA in the presence of tau. A. Thermal denaturation of CTDNA in Tris-EDTA buffer was performed with Ptau by NCLK (curve 5), with tau (curve 4), with aggregated tau (curve 3), with BSA (curve 2) and alone as control (curve 1), respectively. DNA, BSA, tau, aggregated tau and Ptau were at the same concentration (50 μg/ml). B. The same data were showed in differential. The ordinate (dA/dt) represented the differential of the absorbance before and after heat treatment at 260 nm.

DISCUSSION

In recent years, several laboratories reported that tau exists in nuclei of various cell strains, such as neuroblastoma, human lung fibroblast, African green monkey kidney [6] and PC12 [7]. Nuclear tau is specifically localized to the nucleolus [19], especially to the nucleolar organizer regions of the acrocentric chromosomes and to their interphase counterpart [6]. It has been demonstrated that tau has a higher affinity to DNA than to microtubules [25]. In the present study, native tau was found to

bind to plasmid DNA, increasing the melting temperature of CTDNA. Moreover, phosphorylated tau catalyzed by NCLK could associate with DNA and increase the T_m of CTDNA. When tau was aggregated, neither native tau nor phosphorylated tau kept its ability to interact with DNA.

Tau tends to form aggregated polymers both *in vitro* and *in vivo*, such as PHFs and deposits [26] in the brain of AD patients. Phosphorylation and aggregation cause tau to lose its function in the assembly and stabilization of the microtubule system [10]. Papasozomenos and Su, however, found that abnormally phosphorylated tau from AD was associated with purified ribosome [27], implying that Ptau could bind to nucleic acid *in vivo*, similar to our result that the phosphorylation did not interfere with the association of tau with DNA *in vitro*. The effects of Ptau on the melting temperature and electrophoretic mobility of DNA were observed to be the same as native tau. It suggested that the amino acid residues phosphorylated by NCLK, as mentioned above, could not be involved in binding to nucleotides. The essential amino acid residues related to the association with DNA might be some positively-charged ones. Our current experiments (Hua et al., unpublished result) show that tau in tau-DNA complex could be replaced by poly-Lys, however, whether or not Lys residue is the essential group needs further study. The aggregation almost completely inhibited the binding of tau to DNA, indicating that the C-terminal extensions may play a role in binding to DNA, because these extensions are believed to relate to tau aggregation [28].

In the present study, DNA-cellulose chromatography further demonstrated that tau could associate with DNA. It was found that Ptau still associated with DNA in EMSA. Melting temperature experiments indicated that both native tau and Ptau could promote the melting temperature of calf thymus DNA. Nevertheless, when tau was aggregated under the near physiological conditions [14] or treated with formaldehyde, neither native tau nor phosphorylated tau kept its ability to interact with DNA. It is suggested that the association of tau with DNA is in an aggregation-dependent manner, but phosphorylation-independent.

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