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Nuclear Magnetic Resonance Spectroscopy Characterization of Interaction of Tau with DNA and Its Regulation by Phosphorylation

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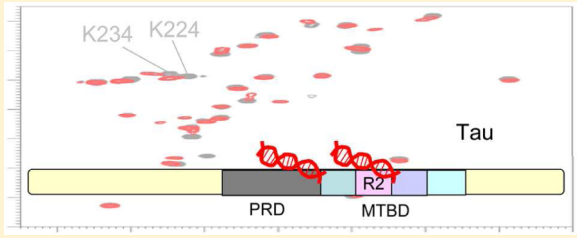
Supporting Information

ABSTRACT: The capacity of endogenous Tau to bind DNA has been recently identified in neurons under physiological or oxidative stress conditions. Characterization of the protein domains involved in Tau−DNA complex formation is an essential first step in clarifying the contribution of Tau−DNA interactions to neurological biological processes. To identify the amino acid residues involved in the interaction of Tau with oligonucleotides, we have characterized a Tau−DNA complex using nuclear magnetic resonance spectroscopy. Interaction of an AT-rich or GC-rich 22 bp oligonucleotide with Tau showed multiple points of anchoring along the intrinsically disordered Tau protein. The main sites of contact characterized here correspond to the second half of the proline-rich domain (PRD) of Tau and the R2 repeat in the microtubule binding domain. This latter interaction site includes the PHF6* sequence known to govern Tau aggregation. The characterization was pursued by studying the binding of phosphorylated forms of Tau, displaying multiple phosphorylation sites mainly in the PRD, to the same oligonucleotide. No interaction of phospho-Tau with the oligonucleotide was detected, suggesting that pathological Tau phosphorylation could affect the physiological function of Tau mediated by DNA binding.



Several neurological disorders have been linked to alterations of protein−DNA interactions potentially leading to perturbation of gene expression, although the proteins involved are not considered transcription factors *per se*.[1] The proteins α-synuclein (αSyn), Prp prion protein, SOD1 (superoxide dismutase 1), Tau, and amyloid Aβ peptides are examples of such proteins. Called amyloid proteins, they have in common, on one hand, their sensitivity to aggregation and, on the other, their capacity to translocate into the nucleus and interact with DNA.[1] The amyloid proteins also share the characteristic of being associated with neurological disorders: Parkinson's disease, spongiform encephalopathy, amyotrophic lateral sclerosis, and Alzheimer's disease (AD). Their interaction with DNA can result in an increase in their aggregation propensity,[2] as was shown, at least *in vitro*, for αSyn or Prp,[3,4] and/or lead to gene expression modification as proposed upon the nuclear translocation of amyloid Aβ1−42.[5−7] This suggests that the interaction of these amyloid proteins with DNA might make an important contribution to the disease. To understand the part of amyloid protein−DNA interactions related to the establishment of such diseases, it is necessary to determine the protein regions involved in DNA complex formation.

The involvement of Tau in the development of AD pathology has been predominantly linked to the loss of its function as a microtubule-associated protein. The molecular mechanisms of Tau-linked pathways associated with neurodegeneration are, however, far from being totally elucidated. Recently, the importance of the multifunctional aspect of Tau has strongly emerged.[8] Tau-dependent global heterochromatin relaxation leading to aberrant gene expression in *Drosophila*[9] and cell cycle reactivation is one example of a mechanism by which Tau has been implicated in the establishment of pathogenesis beyond its role as a microtubule stabilizer.

Even though Tau is mainly described as an axonal protein, it can also be localized in the nucleus of neurons and other cell types[10−14] where it was shown to directly or indirectly bind the DNA.[12,14−16] The DNA binding capacity of Tau appears to be related to its DNA protective function, because Tau has been shown to prevent neuronal genomic DNA from damage under physiological or heat-shock conditions[14,17] and to promote chromosome stability.[16,18] This is in agreement with the *in vitro* observation that Tau binds the minor groove of the DNA

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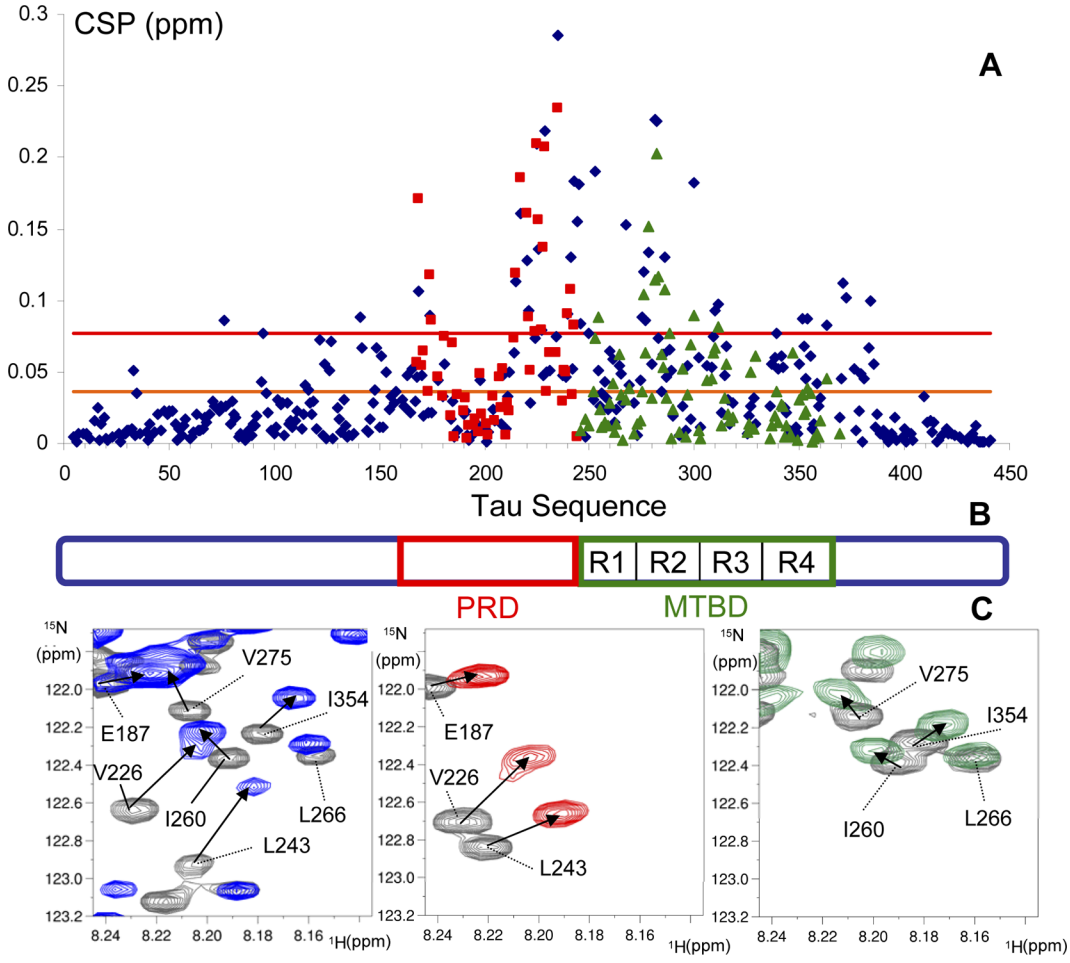

Figure 1. Mapping of Tau interaction sites with the GSAT oligonucleotide. (A) Combined ^{1}H, ^{15}N CSP, calculated as defined in Materials and Methods, of resonances in two-dimensional (2D) ^{1}H−^{15}N HSQC spectra of full-length [^{15}N]Tau (blue diamonds), F[165−245]Tau or PRD (red squares), and F[244−372]Tau or MTBD (green triangles). CSP are differences calculated between the spectra of the protein with a molar ratio of the GSAT oligonucleotide of 2 and of the protein free in solution, for every assigned resonance along the sequence. The orange threshold corresponds to the average CSP and the red one to the average CSP plus one standard deviation for the Tau data. (B) Schematic Tau protein that highlights the PRD (red box) and repeat regions R1−R4 in the MTBD (green box) along the Tau sequence. CSP along the Tau amino acid sequence are shown in Figure S4 of the Supporting Information. (C) Details of overlaid 2D ^{1}H−^{15}N HSQC spectra of [^{15}N]Tau, [^{15}N]F[165−245]Tau, and [^{15}N]F[244−372]Tau (from left to right, respectively) free in solution (gray spectra) and with a molar ratio of GSAT oligonucleotide of 2 (spectra superimposed in blue, red, and green, respectively).

double helix, protecting DNA from oxidative damage or thermal denaturation.[14,19,20]

Tau activity is physiologically regulated by multiple post-translational modifications, and aberrant phosphorylations have been systematically linked to its dysfunction. Hyperphosphorylated Tau is the main component of the paired helical filaments, the aggregated form of Tau found in neurons of patients suffering from AD. Multiphosphorylation was proposed to induce the detachment of Tau from the microtubules and to impair axonal transport, leading eventually to neuronal death. The work on the pathological aspect of Tau phosphorylation has mainly focused on its relationship with Tau aggregation or microtubule stabilization. Very few studies have examined the effect of phosphorylation on the stress-related DNA protective role of Tau. Phosphorylation of Tau has been observed in the case of the nuclear fraction of Tau in neuroblastoma N2a cells and in mouse brains exposed to formaldehyde[21] as well as in neuroblastoma cells infected with herpes simplex virus type 1,[22] showing the relevance of establishing the consequences of Tau phosphorylation on its DNA binding capacity. Additionally, formaldehyde treatment of N2a cells induces the co-occurrence of Tau hyperphosphorylation and DNA damage,[21] and *in vitro*, Tau phosphorylation by GSK3 kinase reduces its capacity to protect DNA against thermal denaturation or reactive oxygen species,[23] suggesting that phosphorylation could be related to the dysfunction of the nuclear fraction of Tau.

In this context, we have focused our interest on the determination of the regions of Tau protein involved in its interaction with DNA. Several regions of Tau have been defined along its primary sequence: an N-terminal region, a proline-rich domain (PRD), a microtubule binding domain (MTBD) consisting of four partially repeated sequences (R1−R4), and a C-terminal region (Figure 1). The sequences described as being responsible of nucleating Tau aggregation,[24] called PHF6* and PHF6, are located at the beginning of the R2 and R3 repeats, respectively, in the MTBD. Both the PRD and the MTBD are involved in the microtubule stabilizing activity of Tau.[25,26]

The amino acid composition of the intrinsically disordered Tau protein is enriched with a few residue types that represent

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most of its primary sequence. This is the case of the 44 lysine residues corresponding to 10% of the amino acid composition of the longest 441-amino acid Tau isoform and the 80 Ser/Thr residues, potential sites of phosphorylation. When combined in a Tau protein phosphorylated at multiple sites, the modification of charge distribution could lead to a very complex regulation of Tau interactions. We have previously shown that the analytical capacity of nuclear magnetic resonance (NMR) spectroscopy allows us to define interaction sites between Tau and a protein or a ligand such as heparin at the level of an amino acid residue.[27] Similarly, the phosphorylation pattern of the Tau samples used in the interaction assays can be defined in a global manner, visualizing all the single-site modifications in one experiment.[28,29] With these analytical capacities, we have used NMR spectroscopy to investigate the interaction of Tau with a 22 bp oligonucleotide, whose sequence was derived from murine γ-satellite DNA, to define the regions of Tau involved in its interaction with DNA. Additionally, we have used phosphorylated forms of Tau protein, characterized by NMR, to investigate the impact of a multiple-phosphorylation pattern on the Tau−DNA interaction.

MATERIALS AND METHODS

Annealing of Oligonucleotides for the NMR Experiments. The forward AT-rich 5′-ATTTAGAAATGTCCACTGTAGG-3′ oligonucleotide (Eurofins MWG Operon, Ebersberg, Germany) and its reverse complement (80 nmol each) were mixed in 1 mL of annealing buffer [65 mM Tris-HCl (pH 7.7), 15 mM $MgCl_2$, and 1.5 mM EDTA]. The oligonucleotide mix was subjected to a 3 min denaturation at 95 °C followed by a slow cooling via a 1 °C step every minute to 10 °C. The annealed oligonucleotides [called double-stranded (ds) AT-rich or GSAT oligonucleotide] were purified on a MonoQ HR 5/5 anion exchange resin equilibrated in TE buffer [10 mM Tris-HCl (pH 7.7) and 1 mM EDTA]. A NaCl salt gradient was used to separate the major annealed fraction from the residual oligonucleotides. The collected fractions were pooled and buffer exchanged against 50 mM ammonium bicarbonate before lyophilization. The lyophilized ds AT-rich oligonucleotide was suspended in NMR buffer [50 mM deuterated Tris-d_{11} (pH 6.6), 25 mM NaCl, and 2.5 mM EDTA]. The same procedure was used to prepare ds GC-rich oligonucleotide from the GC-rich sequence 5′-ATCCAGAGGTGTCCACTGTAGG-3′.

Isotopic Labeling of Recombinant Tau Protein. The Tau protein used in this study is the longest isoform, 441 amino acid residues, expressed as a recombinant protein without any tag from the recombinant pET15b *Escherichia coli* expression vector in Bl21(DE3) strains. Tau fragments were expressed as fusion protein with an N-terminal histidine tag for F[244−372]Tau or without any tag for F[165−245]Tau, from the same expression vector. Tau peptides F[220−240]Tau and F[271−294]Tau were expressed as a fusion with the SUMO protein presenting an N-terminal His tag from a modified pET vector.[30]

For uniform ^{15}N labeling, the protein production was conducted in M9 medium with 1 g of $^{15}NH_4Cl$, a supplement of 300 mg of [^{15}N]ISOGRO complete medium (Isotech), and a MEM vitamin cocktail (Sigma) per liter of growth medium. Two grams of [$^{13}C_6$]glucose was used for the doubly labeled proteins instead of 4 g of glucose for sole ^{15}N labeling. Production of recombinant protein was started with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside, and bacterial growth was then pursued for a 4 h period, at 37 °C. A first purification step was obtained by heating the bacterial protein extract for 15 min at 75 °C. The Tau protein and Tau fragments were recovered in the soluble fraction after centrifugation at 15000*g* for 30 min. Purification of the [^{15}N]Tau protein and [^{15}N]F[165−245]Tau was performed by cation exchange chromatography in 50 mM phosphate buffer (pH 6.3) and 1 mM EDTA (5 mL Hitrap SP Sepharose FF column, GE Healthcare). [^{15}N]F[244−372]Tau, [^{15}N]Sumo-F[220−240]Tau, and [^{15}N]Sumo-F[271−294]Tau were purified on nickel resin (5 mL HisTrap, GE Healthcare) according to the manufacturer's standard protocol. The pooled fractions from the chromatography purification step were transferred to ammonium bicarbonate by desalting on a 15/60 Hiprep Desalting Column (G25 resin, GE Healthcare) and lyophilized. [^{15}N]Tau, [^{15}N]Tau fragments, and [^{15}N]Sumo-fused Tau peptides were suspended in NMR buffer [50 mM deuterated Tris-d_{11}, 30 mM NaCl (pH 6.7), 2.5 mM EDTA, and 2 mM DTT]. The same purification procedures were followed for the doubly ^{15}N- and ^{13}C-labeled proteins. The acquisition of two-dimensional (2D) NMR spectra for the Tau, Tau fragments, and peptides in the presence of the GSAT oligonucleotide, suspended in the same buffer as the proteins, was performed at a protein final concentration in the range of 45−100 μM.

Phosphorylation of Recombinant Tau. The mouse brain extract was prepared by homogenizing a half brain (~0.2 g) in 500 μL of homogeneizing buffer [10 mM Tris-HCl (pH 7.4), 5 mM EGTA, 2 mM DTT, and 1 μM okadaic acide (Sigma)] supplemented with 20 μg/mL leupeptin and 40 mM Pefabloc. The suspension was first crudely homogenized manually with a potter in 1.5 mL tubes followed by several passages through a 20 gauge needle. Ultracentrifugation was next performed at 100000*g* for 1 h in 1.5 mL tubes. The [^{15}N]Tau protein was dissolved at 10 μM in 10 mL of phosphorylation buffer [2 mM ATP, 40 mM Hepes-KOH (pH 7.3), 2 mM $MgCl_2$, 5 mM EGTA, and 2 mM DTT supplemented with a protease inhibitor cocktail (Complete, Roche) and 1 μM okadaïc acid (Sigma)]. The phosphorylation reaction was performed at 37 °C for 24 h with 500 μL of mouse brain extract. The mixture was next heat inactivated at 75 °C for 15 min. After centrifugation, the supernatant was transferred to ammonium bicarbonate by desalting on a 15/60 Hiprep Desalting Column (G25 resin, GE Healthcare) and lyophilized. Phosphorylation by the recombinant CDK2/CycA3 kinase was previously described in detail.[29]

Acquisition of Data. TMSP-d_4 (trimethyl silyl propionate, 1 mM) used as a proton chemical shift internal reference (0 ppm) and 5% D_2O were added to the protein sample. $^1H-^{15}N$ HSQC 2D spectra were recorded at 293 K on a 900 MHz Avance III NMR spectrometer equipped with a triple-resonance cryogenic probehead (Bruker, Karlsruhe, Germany). Three-dimensional (3D) HNCACB spectra were acquired at 600 MHz for assignment of Tau peptide resonances on 300 μM ^{15}N- and ^{13}C-labeled Sumo-F[220−240]Tau and Sumo-F[271−294]Tau. Assignment of the DNA-bound Tau resonances was based on the gradual chemical shift observed in the titration experiments and a 3D HNCACB spectrum acquired on a 300 μM ^{15}N- and ^{13}C-labeled F[165−245]Tau with a 0.5 molar ratio of the GSAT oligonucleotide.

Data Analysis. Spectra were processed using Bruker TOPSPIN 2.1 (Bruker). Peak picking were performed using Sparky (T. D. Goddard and D. G. Kneller, *SPARKY 3*, University of California, San Francisco). The chemical shift perturbations (CSP) of individual amide resonances were

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calculated with the following equation, taking into account the relative dispersion of the proton and nitrogen δ chemical shifts: CSP = $[(\delta^1H_{bound} - \delta^1H_{free})^2 + 0.2(\delta^{15}N_{bound} - \delta^{15}N_{free})^2]^{1/2}$. Bound and free subscripts in the formula stand for the δ in the oligonucleotide-bound protein state and free protein state, respectively.

RESULTS

Interaction of Full-Length Tau Protein with a Double-Stranded AT-Rich Oligonucleotide. A detailed molecular study of the interaction of Tau with DNA was conducted using a double-stranded AT-rich oligonucleotide whose sequence is found present within pericentromeric γ-satellite AT-rich DNA (GSAT). A 936 bp fragment from this chromosomic region was previously shown to interact with Tau by an electrophoretic mobility shift assay (EMSA).[13] An oligonucleotide was chosen instead of this DNA fragment to facilitate experiments by nuclear magnetic resonance spectroscopy (NMR). The capacity of recombinant Tau to form protein−DNA complexes with the AT-rich 22 bp GSAT oligonucleotide *in vitro* was confirmed by an EMSA (Figure S1 of the Supporting Information). The double-stranded GSAT oligonucleotide used in the experiments was purified by anion exchange chromatography to ensure the homogeneity of the preparation. The principle of the mapping experiment by NMR is the comparison of 2D spectra of ^{15}N-labeled Tau acquired in the presence of the GSAT oligonucleotide with respect to the spectra of ^{15}N-labeled Tau alone (Figure S2 of the Supporting Information). The resonances in these spectra correspond to ^{1}H−^{15}N amide correlations and are assigned to an amino acid residue in the protein sequence. The assignment of Tau resonances has previously been completed by us and others.[31−33] These resonances are perturbed in intensity and/or chemical shifts in case their chemical environment or their conformation is modified and can thus be used to monitor the interaction between molecular partners. Addition of the GSAT oligonucleotide to Tau indeed induced many CSP in the [^{15}N]Tau 2D HSQC spectrum (Figure 1). To investigate whether this interaction is dependent on the oligonucleotide sequence, the experiments were similarly performed with a second oligonucleotide with a higher GC content, which was annealed and purified as the GSAT oligonucleotide. [^{15}N]Tau 2D HSQC spectra in the presence of one oligonucleotide or the other can be superimposed (compare red and green signals in Figure S3A,C of the Supporting Information), showing that the interaction with Tau is mediated through the backbone of the nucleic acid, in a manner independent of the nature of the bases. Because of the disordered nature of Tau and its large size, the signal overlap precludes a complete coverage of the CSP. Despite the missing data for some residues (Figure S4 of the Supporting Information), the numerous CSP observed along the Tau sequence revealed a complex interaction consisting of multiple binding sites (Figure 1 and Figure S4 of the Supporting Information).

Interaction of a Double-Stranded GSAT Oligonucleotide with the PRD and MTBD of Tau. To confirm and further define the amino acids of Tau participating in the interaction with the GSAT oligonucleotide, two fragments of Tau sequence that encompass the regions found to be involved in the interaction of Tau with the GSAT oligonucleotide were used. Tau fragments corresponding to the PRD F[165−245]Tau and to the MTBD F[244−372]Tau (also called K18) were complexed with the GSAT oligonucleotide (Figure 1 and Figure S2 of the Supporting Information). Comparison of the extent and direction of the respective CSP (Figures 1 and 2)

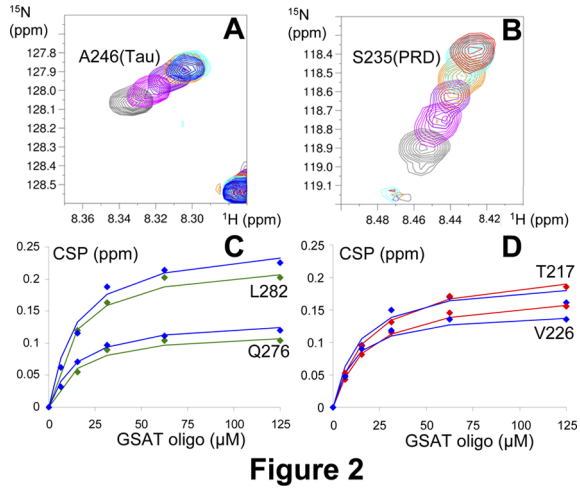

Figure 2. Affinity of Tau for the GSAT oligonucleotide. (A and B) Details of overlaid 2D ^{1}H−^{15}N HSQC spectra of [^{15}N]Tau and F[165−245]Tau, respectively, free in solution (gray spectra) and with 0.1, 0.25, 0.5, and 1 molar ratios of GSAT oligonucleotide (spectra superimposed in pink, purple, orange, and cyan, respectively). The final titration point of a 2 molar ratio of GSAT oligonucleotide is (A) the blue resonance for Tau and (B) the red resonance for F[165−245]Tau. (C and D) Saturation curves were obtained by plotting the gradual CSP observed for resonances corresponding to amino acid residues in the Tau binding sites vs the increasing amount of GSAT oligonucleotide. Diamonds correspond to the experimental data, and the solid curve corresponds to the fit by the equation $(CSP)_{max} \times [oligo]/K_D + [oligo]$, with $(CSP)_{Max}$ being the CSP attained at saturation and [oligo] being the concentration of added GSAT oligonucleotide. (C) CSP of the resonances of L282 and Q276 in Tau (blue) and F[244−372]Tau (green). (D) CSP of the resonances of T217 and V226 in Tau (blue) and F[165−245]Tau (red).

indicated that the PRD and MTBD interacted with the GSAT oligonucleotide in a similar manner isolated or embedded in full-length Tau (Figures 1 and 2). Overall, the data obtained during NMR analysis of Tau−DNA interaction identified the second half of the PRD, from amino acid R209 to A246, as the region of Tau protein predominantly involved in anchoring the 22 bp GSAT oligonucleotide. In addition, the N-terminal part of the R2 repeat containing the PHF6* sequence involved in Tau aggregation,[24] from amino acid K267 to S289, was identified as a second site of interaction with the GSAT oligonucleotide. Titrations of the GSAT oligonucleotide into Tau, the MTBD samples, and the PRD samples were used to calculate dissociation constants (K_D) that characterize these interactions. Gradual CSP along the titration (Figure 2A,B) can be fit to a saturation curve (Figure 2C,D) to derive the K_D values for every residue that shows a CSP above the average for Tau resonances at saturation (above the red threshold in Figure 1A). In the PRD, the average K_D value calculated on the basis of the resonances of eight residues present between L215 and S241 residues of Tau was 10.5 ± 2.4 μM and in the case of the F[165−245]Tau fragment was 19.2 ± 0.8 μM. In the MTBD fragment, the average K_D value calculated on the basis of the resonances of seven residues present between V275 and N286 residues of Tau was 11.3 ± 1.7 μM and in the case of the F[244−372]Tau fragment was 15.7 ± 3.3 μM. The K_D calculated for some residues of Tau located outside of these

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main interaction regions showed lower affinity, with a value of 30.6 ± 8.4 μM for six residues located between Q351 and A384 residues and a value of 19.1 ± 3 μM for T169 and K174 residues. These data showed that the GSAT oligonucleotide binds to the PRD and R2 repeat with similar affinity, confirming both regions as the main binding sites. The data obtained with the isolated domains indicate that both the PRD and the MTBD can bind the GSAT oligonucleotide in an independent manner. The similar affinities observed for the isolated domains compared to that of the full-length Tau confirmed the independence of these sites within the Tau protein for oligonucleotide binding. A combination of those binding sites would indeed have led to a significantly higher affinity of Tau compared to those of the isolated domains. However, the presence of both binding sites within the Tau protein could account for the lower K_D observed for Tau compared to those of both fragments, as the concentration of binding sites is double compared to that of the isolated fragments.

Interaction of the GSAT Oligonucleotide with Tau Peptides. To gain further insight into the minimal regions of Tau protein necessary to establish an interaction with the GSAT oligonucleotide, shorter peptides of Tau were used. These Tau peptides were the F[220−240]Tau peptide consisting of an amino acid sequence present in the PRD and the F[271−294]Tau peptide consisting of an amino acid sequence present in R2 of the MTBD, including the PHF6* sequence. Both peptides were chosen as they showed interaction with the GSAT oligonucleotide when embedded in the Tau sequence (Figure 1 and Figure S4 of the Supporting Information). Because the F[271−294]Tau peptide displayed poor solubility, fusions with the SUMO protein were used, both for recombinant expression and for acquisition of 2D NMR spectra. Because SUMO is a small folded protein, the overlap with the Tau peptide signals was reasonable and therefore allowed a correct analysis of the Tau resonances. Comparison of the 2D HSQC spectra of ^{15}N-labeled Tau SUMO peptides obtained after addition of an equimolar concentration of the GSAT oligonucleotide with the spectra of the peptide alone showed limited CSP (Figure 3). In the case of the SUMO-F[220−240]Tau peptide, CSP were observed between amino acids 222 and 229 (Figure 3A and Figure S5A of the Supporting Information), while for the SUMO-F[271−294]Tau peptide, the spectrum was not modified after addition of the GSAT nucleotide (Figure 3B and Figure S5B of the Supporting Information). The CSP in the spectrum of SUMO-F[220−240]Tau are, however, small compared to those observed for the resonances corresponding to the same sequence embedded in larger fragments (compare Figure 1A with Figure S5A,B of the Supporting Information). The 20-amino acid Tau peptides were thus not able to interact efficiently with the GSAT oligonucleotide on their own, showing that a minimal length is required to anchor the 22-base oligonucleotide.

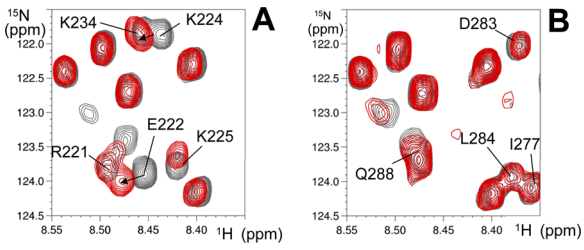

Figure 3. Mapping of Tau interaction sites with the GSAT oligonucleotide. Details of overlaid 2D ^{1}H−^{15}N HSQC spectra of (A) [^{15}N]Sumo-F[220−240]Tau from the Tau PRD and (B) [^{15}N]Sumo-F[271−294]Tau from the Tau MTBD free in solution (gray) and with a 1 molar ratio of GSAT oligonucleotide (superimposed in red). Resonances from the Tau peptides are labeled.

Modulation of Interaction of Tau with the GSAT Oligonucleotide by Tau Phosphorylation. Interactions of Tau with several molecular partners are known to be modulated by phosphorylation. We thus next addressed the question of the influence of Tau phosphorylation on the capacity of Tau to interact with DNA. To investigate this aspect, we first used an *in vitro* phosphorylated Tau protein obtained by incubating [^{15}N]Tau with a mouse brain extract that contains a complex mixture of kinases known to phosphorylate Tau.[34] During these experiments, phosphatase activities were blocked by okadaïc acid. Under these conditions, Tau protein was phosphorylated at multiple sites (Figure 4A).

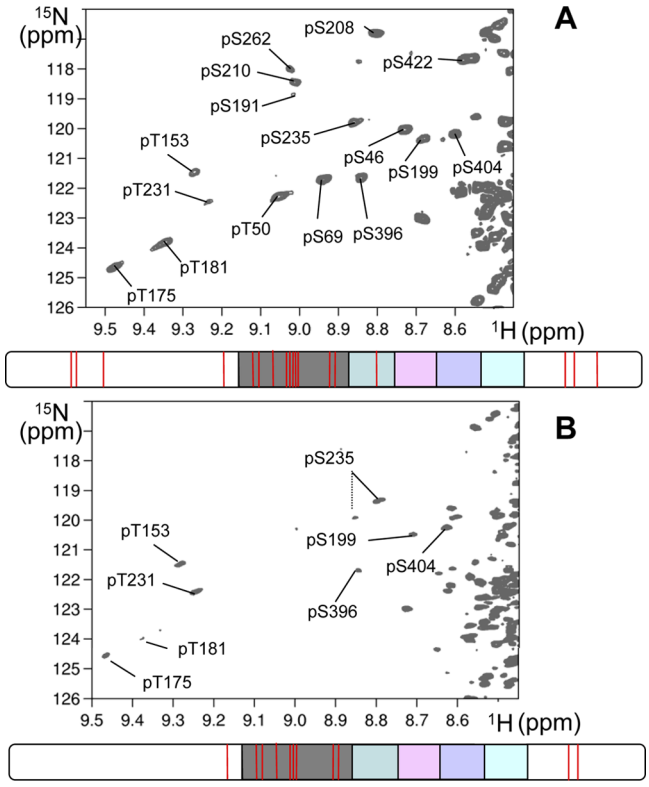

Figure 4. Phosphorylation patterns of Tau. Details of 2D ^{1}H−^{15}N HSQC spectra of [^{15}N]Tau phosphorylated with (A) mouse brain extracts and (B) CDK2/CycA3 recombinant kinase. Resonances corresponding to phosphorylated Ser and Thr residues are labeled. The schematic Tau protein highlights the repeat regions, color-coded light blue, pink, purple, and green. The detected phosphorylation sites are represented by red bars.

Analysis of the phosphorylated forms of Tau using NMR spectroscopy showed new resonances in the region corresponding to pSer and pThr residues that were absent in the spectrum of the unphosphorylated form of Tau. A total of 18 resonances of phosphorylated residues could be detected. The phosphorylation sites obtained here with a mixture of kinases were identified by comparing the chemical shifts of their resonances

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with those of resonances of phosphorylation sites obtained by incubating Tau with individual recombinant kinases that we have previously described.[28,29,35] These phosphorylations affected all the regions of Tau: the N-terminal region (pS46, pT50, pS69, and pT153), the PRD (pT175, pT181, pS191, pS199, pS202, pT205, pS208, pS210, pT231, and pS235), the MTBD (pS262), and the C-terminal region (pS396, pS404, and pS422) (scheme in Figure 4A). Incubation of this phosphorylated form of Tau with the GSAT oligonucleotide resulted in no detectable interaction as translated by the absence of perturbation in the resulting 2D spectrum obtained in the presence of the GSAT oligonucleotide as compared to the spectrum of free phospho-Tau (Figure S3B,D of the Supporting Information). The experiment was repeated with the second oligonucleotide having a higher GC content that also showed no interaction with the phosphorylated Tau sample (Figure S3B,D of the Supporting Information). Phosphorylations of Tau at multiple sites, mainly located in the PRD, thus abolish its interaction with the oligonucleotides. The interaction assay of a phosphorylated Tau isoform with the GSAT oligonucleotide was repeated with a Tau protein displaying a reduced phosphorylation profile, obtained by using the recombinant CDK23/CycA3 kinase (Figure 4B) whose pattern of Tau phosphorylation we have previously thoroughly characterized.[29] The major CDK phosphorylation sites located in the oligonucleotide Tau binding region were limited to pT231 and pS235 residues of the PRD. Despite this lower level of phosphorylation, as compared to that observed after incubation of Tau with the kinase activity of brain extracts, the CDK phospho-Tau was unable to bind to the GSAT oligonucleotide (Figure 5). No binding was observed not only in the PRD in the case of CDK phospho-Tau (Figure 5B) or CDK phospho-F[165−245]Tau (Figure S6 of the Supporting Information) but also in the MTBD in phospho-Tau (Figure 5D). Despite the distance between the GSAT oligonucleotide binding site present in the R2 repeat and the phosphorylation sites in the PRD, the phosphorylation events do globally affect the interaction.

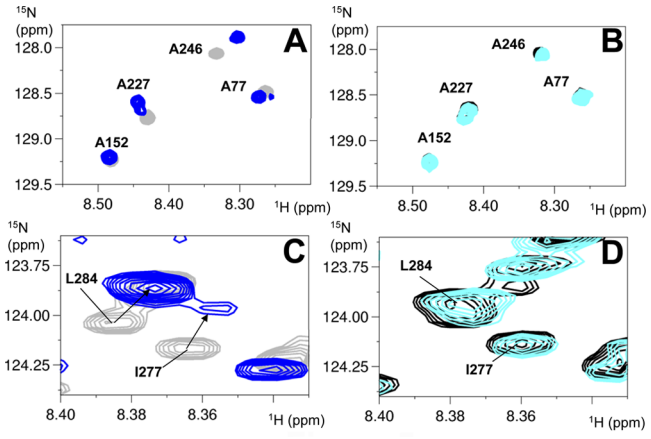

Figure 5. Phosphorylation of Tau with CDK2/CycA3 decreases its capacity to interact with oligonucleotides. Details of overlaid 2D ^{1}H−^{15}N HSQC spectra of (A and B) [^{15}N]Tau free in solution (gray) and with a 1 molar ratio of GSAT oligonucleotide (superimposed in blue) and (B and D) [^{15}N]CDK2 phospho-Tau free in solution (black) and with a 1 molar ratio of GSAT oligonucleotide (superimposed in cyan). Panels A and B show regions of alanine residues; A227 and A246 are located in the PRD. I277 and L284 in panels C and D are located in the R2 repeat.

DISCUSSION

The interaction of Tau with a 22 bp oligonucleotide was investigated using NMR spectroscopy to obtain a detailed description of the region(s) of Tau protein involved in the establishment of Tau−DNA interactions. Our first interrogation was about the specificity of the interaction because the C-terminal part of Tau is very rich in lysine residues that could potentially drive unspecific electrostatic interactions. It seems not to be the case, as we observe a localized interaction of the oligonucleotide predominantly with the second part of the PRD of Tau and the R2 repeat in the MTBD. Tau interacted with the AT-rich as well as with the GC-rich oligonucleotides, suggesting that Tau−DNA interaction is mediated by the DNA backbone.

Protein−nucleic acid interaction involves Lys/Arg basic residues, both being well represented in the Tau−GSAT oligonucleotide interacting domains. Additionally, polar residues such as Ser/Thr or Gln/Asn residues, well represented in the PRD, are favored in establishing contacts with the DNA backbone. The amino acid composition of the PHF6* peptide present in the MTBD is, however, less typical of a DNA-interacting sequence as it contains several hydrophobic residues that are usually under-represented at protein−DNA interfaces.[36] The larger side chains of the hydrophobic residues indeed prevent access of the protein backbone atoms to the DNA.

This result is nevertheless in agreement with recent work on the thermodynamics of the Tau−DNA interaction, based on surface plasmon resonance (SPR), which has identified hydrophobicity as an important contributor to the stabilization of the interaction.[37] Interaction of the GSAT oligonucleotide with the R2 sequence of Tau was clearly detected by CSP, in the case of both the full-length Tau protein and the F[244−372]Tau (K18) fragment, showing that the oligonucleotide is able to interact with the isolated MTBD in a manner independent of the flanking sequences. The involvement of the PRD and MTBD of Tau in oligonucleotide binding, as determined using an EMSA, has been previously reported,[20] as well as the capacity of these two domains to independently interact with oligonucleotide sequences. Isolated peptide sequences located in the interaction regions, F[220−240]Tau from the PRD or F[271−294]Tau from the R2 repeat, only weakly interacted with the GSAT oligonucleotide. This suggests that a minimal protein length is required for the oligonucleotide to anchor, which is not found in the 20-amino acid peptide sequences.

Phosphorylation of the nuclear fraction of Tau has not yet been thoroughly characterized, but a recent study supports the idea that Tau can be phosphorylated on several sites in the nuclear compartment.[21] We here showed that phosphorylation strongly reduces the level of interaction of Tau with the GSAT oligonucleotide, with only very small CSP detected in phospho-Tau spectra upon addition of the oligonucleotide. A bulk electrostatic effect can be invoked to explain the loss of Tau−DNA interaction, because phosphorylations reduce the net positive charge.[38] However, this electrostatic model implies that the phospho sites have to be located in the proximity of the polybasic region.[38] The phospho-Tau proteins used in our study were phosphorylated at numerous Ser/Thr residues mainly located in the PRD, the only phosphorylation site

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detected in the MTBD being pS262 in the case of Tau phosphorylated by the brain extracts. Despite this observation, the interaction is abolished by phosphorylation not only in the PRD but also in the repeats. The large number of acidic residues introduced by phosphorylation of Ser/Thr residues could explain the loss of interaction in the PRD, but the link is not straightforward between multiple phosphorylations in the PRD and loss of interaction in the MTBD. We indeed showed that the isolated MTBD can bind in a manner independent of the PRD to the oligonucleotide. The phosphorylation sites closest to the Tau R2 repeat DNA binding site (F[271−294]Tau) are pT231/pS235 present in the PRD, located some 40 amino acid residues away in the primary sequence, in a disordered protein context. However, several structural studies point to the fact that Tau is not an entirely disordered polymer but shows a complex network of transient long-range interactions between distant domains of the protein mediated by electrostatic effects.[33,39−41] Intramolecular FRET (Forster resonance energy transfer) measurements have suggested that Tau retains some global folding even in its "natively unfolded" state.[40] The conclusion that Tau is not a fully extended polymer was also drawn from PRE (paramagnetic relaxation enhancement) NMR experiments combined with SAXs data[33] and from conformational ensemble approaches.[41] More specifically, the N-terminal and C-terminal domains of Tau are found to dynamically fold back on the central part of the Tau protein,[33,41] in a model called a "paperclip".[40] The PRD was additionally shown to contact repeat R3.[33] These weak contacts between distant domains of the Tau protein, in particular between the PRD and the MTBD, could potentially explain the long-range effect of the phosphorylations in the PRD affecting the DNA binding of the MTBR.

In vitro phosphorylation of Tau by GSK3 was recently shown to decrease the capacity of Tau to interact with DNA in an EMSA.[23] Phosphorylated Tau expressed in Sf9 cells was also shown to lose its capacity to interact with calf thymus DNA by SPR.[37] pT181 and pS396 were immuno-detected in the GSK3 phospho-Tau protein,[23] and pS202/pT205 and pT212/pS214 were detected by the AT8 and AT100 antibodies, respectively, in the sf9-expressed Tau.[37] These phosphorylation patterns do not support the idea that phosphorylations have to be located on sites close to the interaction regions with the DNA to prevent the interaction. As immunodetections do not allow a global survey of the resulting Tau phosphorylation pattern, contrary to the NMR analysis, additional phosphorylations in the GSK3 phospho-Tau or sf9-expressed phospho-Tau might have occurred without detection.

In vivo, the phospho-Tau protein detected in replicating HEK cells by the PHF1 antibody does not colocalize with the condensed chromosomes, suggesting that phosphorylations can prevent the Tau−DNA interaction.[37] Nuclear shuttling of Tau into the nucleus upon heat shock results in a decrease in the level of Tau phosphorylation.[14] Additionally, the capacity of Tau to protect neurons against hyperthermia-induced DNA damage was correlated to an increased level of binding of dephosphorylated Tau to DNA.[14] It is thus possible that the fraction of Tau translocating and transiently remaining in the nucleus under stress conditions is mainly dephosphorylated. In the reverse, recovery of cells after heat shock is associated with an increased level of Tau phosphorylation and a concomitant decrease in the level of nuclear Tau. Nevertheless, nuclear phosphorylated Tau was detected under other stress conditions; the treatment of neuroblastoma cells with formaldehyde and GSK3 kinase was proposed in this case to phosphorylate Tau in the nucleus. Interestingly, accumulation of phosphorylated Tau in the nuclei under formaldehyde treatment co-occurred with DNA alteration.[23] We here describe that the phosphorylation of Tau, mainly in its PRD, results in a loss of its capacity to interact with nucleotide sequences. Such a pattern of phosphorylation would thus result in the inability of Tau to bind DNA and could consequently induce a loss of its DNA protective function. Therefore, hyperphosphorylation could be linked to pathological aspects of Tau function in the nucleus.

■ ASSOCIATED CONTENT

Supporting Information

Recombinant Tau forms a protein−DNA complex with the GSAT oligonucleotide in EMSA experiments (Figure S1). Recombinant Tau forms a protein−DNA complex with the GSAT oligonucleotide in NMR experiments (Figure S2). Comparison of interaction of Tau with ds AT-rich and GC-rich oligonucleotides. Phosphorylation of Tau by rat brain extract decreases its capacity to interact with both oligonucleotides (Figure S3). CSP due to the interaction with the GSAT oligonucleotide mapped onto the amino acid residue sequence of the PRD and repeat regions of Tau (Figure S4). CSP along the sequences of [^{15}N]Sumo-F[220−240]Tau and [^{15}N]Sumo-F[271−294] peptides upon addition of the GSAT oligonucleotide (Figure S5). Comparison of the interaction of ^{15}N F[165−245]Tau and ^{15}N CDK-phospho F[165−245]Tau with the GSAT oligonucleotide (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AD, Alzheimer's disease; CSP, chemical shift perturbations; EMSA, electrophoretic mobility shift assay; GSAT, γ-satellite AT-rich DNA; HSQC, heteronuclear single-quantum spectrum; MTBD, microtubule binding domain of the Tau protein; NMR, nuclear magnetic resonance spectroscopy; SPR, surface plasmon resonance; PRD, proline-rich domain of the Tau protein.

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