Phosphorylated full-length Tau interacts with 14-3-3 proteins via two short phosphorylated sequences, each occupying a binding groove of 14-3-3 dimer

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Abbreviations: AUC, Analytical ultracentrifugation; AUC-SV, Analytical ultracentrifugation-sedimentation velocity; NMR, Nuclear magnetic resonance; PPI, Protein-protein interactions; SPR, Surface plasmon resonance; Tau-PKA, Tau phosphorylated by Protein Kinase A; TROSY-HSQC, Transverse Relaxation Optimized Spectroscopy- Heteronuclear Single Quantum Coherence Spectroscopy; wt-Tau, wild-type Tau.

Conflict of interest: None

Abstract

Protein-protein interactions (PPIs) remain poorly explored targets for the treatment of Alzheimer's disease (AD). The interaction of 14-3-3 proteins with Tau was shown to have detrimental effects on neuronal cells and to be linked to Tau pathology. This PPI is therefore seen as a potential target for AD. When Tau is phosphorylated by PKA (Tau-PKA), two 14-3-3 binding epitopes are generated, surrounding the phosphorylated serines 214 and 324 of Tau. The crystal structures of 14-3-3 in complex with peptides surrounding these Tau phosphosites show that both these motifs are anchored in the amphipathic binding groove of 14-3-3. However, in the absence of structural data with the full-length Tau protein, the stoichiometry of the complex or the interface and affinity of the partners, are still unclear. In this work, we addressed these points, using a broad range of biophysical techniques. The interaction of the long disordered Tau-PKA protein with 14-3-3 σ is restricted to two short sequences, containing phosphorylated serines, which bind in the amphipathic binding groove of 14-3-3. Phosphorylation of Tau is fundamental for the formation of this stable complex, and the affinity of the Tau-PKA/14-3-3 interaction is in the 1-10 micromolar range. Each monomer of the 14-3-3 σ dimer binds one of the two different phosphorylated peptides of Tau-PKA, suggesting a 14-3-3/Tau-PKA stoichiometry of 2:1, confirmed by analytical ultracentrifugation. These results contribute to a better understanding of this PPI and provide useful insights for drug discovery projects aiming at the inhibition of this interaction.

Graphical Abstract

Pharmacological modulation of the 14-3-3/Tau interaction has interest in drug discovery projects related to Alzheimer's disease mitigation. Biophysical methods were used to define the interface, stoichiometry and affinity characterizing the binding of 14-3-3 to full length phosphorylated Tau protein. We concluded that the binding interface is small and the interaction of moderate affinity, making the interface likely druggable for inhibition.

Introduction

The discovery of new disease modifying treatments (DMTs) for Alzheimer's disease (AD) is a major challenge in pharmaceutical research. β -amyloid and Tau pathways are the most targeted by DMTs in clinical development, including the use of aggregation inhibitors, immunotherapies, protease inhibitors and post-translational modification (PTM) enzyme inhibitors [1–5]. In the context of complex regulation of these pathways, most of the protein-protein interactions (PPIs) implicated in the pathophysiology remains poorly explored. Several of these PPIs are potential targets for AD (reviewed in [6]), such as the interaction between 14-3-3 and phosphorylated Tau protein, for which some inhibitors have already been reported [7,8].

Tau is a neuronal protein whose most well-known function is the stabilization of microtubules, contributing to neuronal activity [9,10]. The interactome and multiple functions of Tau are markedly influenced by the many PTMs that this intrinsically disordered protein is subjected to [11–13]. Phosphorylation is the most studied PTM of Tau because it is increased under pathological conditions [14,15]. Phosphorylated Tau is indeed the major component of the intraneuronal neurofibrillary tangles [15,16], one of the hallmarks of AD, the evolution of which is shown to be correlated to the progression of the disease [17,18]. Phosphorylation of specific residues on Tau is also linked to its detachment from microtubules [19], onset of Tau aggregation [11,20] or changes in Tau transport across the cell [21].

14-3-3 proteins are adapter proteins that bind almost exclusively phosphorylated proteins through their amphipathic binding groove. 14-3-3 isoforms, named β , γ , ϵ , η , σ , τ and ζ , are all dimeric 60 kDa proteins composed of 9 helices, named α A-I [22,23]. Recent studies point to the existence of more than 800 protein partners that bind to 14-3-3 [24]. Due to this high number of partners, 14-3-3 proteins have influence on several processes in the cell such as cellular signaling

[25], apoptosis [26] and cell-cycle [27]. Not surprisingly, 14-3-3 proteins are thus linked to several pathologies, notably neurodegenerative diseases [28,29].

14-3-3 are abundantly expressed in the brain and in 1996 Layfield et al, identified 14-3-3 proteins as one of the components of neurofibrillary tangles in AD brain [30], which lead to the hypothesis that these proteins could have a role on Tau pathology. Four years later, the direct interaction of 14-3-3 with Tau was confirmed [31]. Since then further studies have shown that 14-3-3 mediates the phosphorylation of Tau [31–34] by several kinases. For example, 14-3-3 ζ plays a role of scaffold protein by interacting with both GSK3 β and Tau, stimulating phosphorylation of the latter [32]. In addition, 14-3-3 ζ interacts with Tau independently, stimulating phosphorylation by PKA as a substrate effector [31] and increases neuronal Tau-mediated toxicity [33,35,36]. For example, overexpression of 14-3-3 σ promotes Tau detachment from microtubules and consequently disrupts the tubulin cytoskeleton. As a consequence of this severe impairment of microtubule stability, neuritic growth of cells overexpressing 14-3-3 σ is reduced [36]. Some studies also suggest that 14-3-3 has a role on promoting Tau aggregation [33,37–40]. Although more studies need to be performed to understand the role of 14-3-3 on the global Tau pathology in AD, the available information points to the inhibition of the 14-3-3/Tau PPI as a potential therapeutic approach for targeting Tau pathology in AD. The available structural studies (crystallographic studies of peptides surrounding pS214 and pS324 in complex with 14-3-3 σ) [36] on the 14-3-3/Tau PPI suggest that pS214 and pS324 of phosphorylated Tau can bind to the phospho-accepting site of 14-3-3.

Previous work has mainly made use of Tau-derived peptides, and structural data on the interaction of 14-3-3 with full-length Tau are missing. In this work, we used several biophysical techniques, including nuclear magnetic resonance (NMR), analytical ultracentrifugation (AUC)

and surface plasmon resonance (SPR) to obtain further structural insights on the Tau/14-3-3 PPI. In particular, we studied the interaction of full length PKA-phosphorylated Tau (Tau-PKA). PKA has been shown to phosphorylate S214 and S324 of Tau[7,8,41], residues previously shown to be part of 14-3-3 binding motifs[7,8,36,42]. We show that each monomer of the 14-3-3 σ dimer binds one of the phosphorylated serine residues of Tau-PKA, suggesting a 14-3-3/Tau-PKA stoichiometry of 2:1, confirmed by AUC. Our NMR data show that the only interaction between Tau-PKA and 14-3-3 occurs in the amphipathic binding groove of 14-3-3, with no additional contacts between the proteins. We also determined the affinity of this PPI by two different techniques and showed that it is in the μ M range, addressing a non-consensual point in the literature[36,38]. This information contributes to a better understanding of the structural features of this PPI. Identification of the interacting regions of both proteins is a crucial point that needs to be taken into account when designing inhibitors of the 14-3-3/Tau PPI.

Methods

14-3-3 proteins expression and purification

14-3-3 ζ , 14-3-3 σ or 14-3-3 $\sigma\Delta C$ (cleaved after T231 – used for NMR experiments to improve the quality of spectra) were expressed in *E. coli* BL21 (DE3) cells transformed with a pProExHtb vector carrying the cDNA to express an N-terminally His₆-tagged human 14-3-3 ζ , 14-3-3 σ or 14-3-3 $\sigma\Delta C$. An overnight 20 mL pre-culture in *Luria-Bertani* (LB) medium was used to inoculate 1 L of culture medium. For the expression of proteins with isotopic natural abundance, cells were inoculated in 1 L of M9 minimal medium supplemented with 4 g/L D-Glucose, 1 g/L ammonium chloride, 5% (v/v) LB medium and 100 μ g/mL ampicillin. For $^{15}\text{N}^{13}\text{C}^2\text{H}$ isotopic labeling, the cells were inoculated in 1 L of deuterated M9 minimal medium supplemented with 2 g/L $^{13}\text{C}_6^2\text{H}_7$ D-Glucose, 1 g/L ^{15}N ammonium chloride, 0.4 g/L Isogro $^{15}\text{N}^{13}\text{C}^2\text{H}$ Powder – Growth Medium (Sigma Aldrich) and 100 μ g/mL ampicillin. Cells were grown at 37 °C to an OD₆₀₀ of 0.9 and induced with 0.5 mM IPTG. Incubation was continued for 12 h at 25 °C for the natural abundance proteins and for 15 h at 28 °C for the $^{15}\text{N}^{13}\text{C}^2\text{H}$ labelled protein. Cells were harvested by centrifugation, lysed with a homogenizer and the proteins were purified using a Ni-NTA column (GE Healthcare). The selected fractions were then mixed with the TEV protease in NMR buffer (100 mM sodium phosphate, pH 6.8, 50 mM NaCl) during 2 hours at 20 °C and 12 hours at 4 °C for cleavage of the N-terminal His₆-tag. The cleavage was performed during dialysis and the molar ratio between 14-3-3 and the TEV protease was 50:1. The cleaved protein was separated from the uncleaved fraction and from the His₆-tagged TEV protease by flowing the proteins in NMR buffer through the same Ni-NTA column. The cleaved proteins were then concentrated, aliquoted, flash frozen and stored at -80 °C. Typical yields were in the range of 40 to 80 mg of protein per liter of culture.

Tau proteins expression and purification

E.coli BL21 (DE3) cells were transformed with the pET15b vector carrying the cDNA encoding wild-type Tau (2N4R, 441 residues – for NMR experiments) or His₆-tagged Tau Y394W (2N4R, 441 residues - for AUC and SPR experiments). A 20 mL pre-culture in LB medium containing 100 µg/mL ampicillin was grown overnight at 37 °C and was used to inoculate a 1 L culture in M9 minimal medium supplemented with 4 g/L D-Glucose, 1 g/L NH₄Cl, 5% (v/v) LB medium and 100 µg/mL ampicillin. For ¹⁵N labeling, cells were inoculated in 1L of M9 minimal medium supplemented with 4 g/L D-Glucose, 1 g/L ¹⁵NH₄Cl, 500 mg Isogro ¹⁵N¹²C¹H Powder – Growth Medium (Sigma Aldrich) and 100 µg/mL ampicillin. The culture was grown at 37 °C to an OD₆₀₀ of 0.8 and induced with 0.5 mM IPTG. Incubation was continued for 3 h at 37 °C and the culture was then harvested by centrifugation. The lysate was obtained by homogenization followed by centrifugation at 30 000 g for 30 minutes to recover the supernatant. A first purification step was achieved by heating the sample at 75 °C for 15 min, followed by centrifugation at 15 000 g for 20 minutes to eliminate the precipitate. The recombinant Tau protein, dissolved in buffer containing 50 mM sodium phosphate pH 6.6 and 2mM EDTA (buffer A) was then loaded on a cation-exchange chromatography column (GE Healthcare). Elution was performed with a gradient from 0% to 50% using a buffer containing 50 mM sodium phosphate pH 6.6 and 2 mM EDTA, 1 M NaCl (buffer B). The selected fractions were further buffer-exchanged with 50 mM ammonium bicarbonate using a desalting column (GE Healthcare) before lyophilization. Protein concentration was estimated by absorption at 280 nm. A more detailed protocol can be found in [43]. Typical yields were in the range of 5-15 mg of recombinant Tau protein per liter of culture.

Protein Kinase A catalytic subunit production and purification

E.coli BL21 (DE3) cells were transformed with the pet15b Vector carrying the cDNA coding the N-terminally His₆-tagged PKA catalytic subunit α from *Mus musculus* (PKAc) (Addgene plasmid # 14921). A 20 mL pre-culture in LB medium containing 100 μ g/mL ampicillin was grown overnight at 37 °C and was used to inoculate a 1 L culture in LB medium. The culture was grown at 37 °C to an OD₆₀₀ of 0.8 and production was induced with 0.5 mM IPTG. Incubation was continued for 20 h at 20 °C and the culture was then harvested by centrifugation. After being suspended in a buffer containing 50 mM Tris, pH 8.0, 500 mM NaCl, 2 mM β -mercaptoethanol and 10 mM imidazole, the pellet was lysed using an homogeneizer, followed by centrifugation at 30,000 g for 30 minutes. The supernatant containing the His₆-tagged PKAc was then loaded on a Ni-NTA column (GE Healthcare) and eluted with a buffer containing 50 mM Tris, pH 8.0, 500 mM NaCl, 2 mM BME and 250 mM imidazole. The purest fractions were then buffer-exchanged with 250 mM potassium phosphate pH 6.5, 0.1 mM DTT on a desalting column (GE Healthcare). The protein was aliquoted, flash frozen in liquid nitrogen and stored at -80 °C. Typical yields were in the range of 10-15 mg per liter of culture.

In vitro phosphorylation of Tau

Recombinant Tau or TauY394W proteins (100 μ M) were incubated with 1.5 μ M recombinant PKAc at 30 °C for 3 h, in a buffer consisting of 50 mM Hepes pH 8.0, 5 mM ATP, 12.5 mM MgCl₂, 50 mM NaCl, 5 mM DTT, 1 mM EDTA. The reaction was heat-inactivated at 75 °C for 15 min and the solution was centrifuged in order to eliminate the precipitated PKAc. Buffer exchange with 50 mM ammonium bicarbonate on a desalting column (GE Healthcare) was performed before lyophilization. Tau phosphorylation by PKAc was accessed by ¹H NMR [41,43].

¹H-¹⁵N TROSY-HSQC spectroscopy

¹H-¹⁵N TROSY-HSQC (Transverse Relaxation Optimized Spectroscopy- Heteronuclear Single Quantum Coherence Spectroscopy) spectra were acquired in 3 mm tubes (sample volume 200 μ L) using a 900 MHz Bruker Avance NEO spectrometer, equipped with a cryoprobe. The spectra were recorded with 3426 complex data points in the direct dimension and 128 complex data points in the indirect dimension, with 300 scans per increment, at 32 °C, in a buffer containing 100 mM sodium phosphate, 50 mM NaCl, pH 6.8, 1 mM DTT, EDTA-free protease inhibitor cocktail (Roche) and 10% (v/v) D₂O. Experiments on 125 μ M ¹⁵N¹³C²H labeled 14-3-3 σ (full-length, comprising residues 1-248) were acquired in the presence and absence of 80 μ M Tau-PKA. Experiments on 125 μ M ¹⁵N¹³C²H labeled 14-3-3 σ Δ C (comprising residues 1-231 out of 248) were acquired in the presence or in the absence of 500 μ M wt-Tau, 500 μ M Tau-PKA, 500 μ M Tau peptide surrounding pS214 (R₂₁₁TPpSLPTP₂₁₈) (bought from Genecust) and 500 μ M Tau peptide including pS214 and pS324 (5,6-FAM (fluorescein)-R₂₁₁TPpSLPT₂₁₇GGSGGGSGGGSS₃₂₀KCGpSLGNIHHK₃₃₁) (bought from Caslo). The reference for the ¹H chemical shift was relative to trimethylsilyl propionate. ¹⁵N chemical shifts were referenced indirectly. Spectra were collected and processed with Topspin 4.0 (Bruker Biospin) and analyzed with Sparky 3.12 (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco). The backbone resonance assignments of 14-3-3 σ Δ C have previously been reported [44].

Backbone chemical shift assignment of the C-terminal stretch (A232-S245) of 14-3-3 σ

Backbone assignment experiments were recorded using a 900 MHz Bruker Avance NEO spectrometer, equipped with a cryoprobe. Samples, in a buffer containing 100 mM sodium phosphate, pH 6.8, 50 mM NaCl, 1 mM DTT (Dithiothreitol), EDTA-free Protease Inhibitor

Cocktail (Roche, Switzerland) and 10% (v/v) D₂O, were transferred to Shigemi tubes (sample volume of 330 μ L) and experiments were acquired at 32 °C. The concentration of ¹⁵N¹³C²H labeled protein for assignment was 1.0 mM. Backbone assignments were obtained from TROSY-HNCACB, TROSY-HNCA, TROSY-HN(CO)CACB and TROSY-HNCO spectra on ¹⁵N¹³C²H labeled 14-3-3 σ (full-length, comprising residues 1-245). Non-Uniform Sampling was employed for the acquisition of all 3D spectra. The reference for the ¹H chemical shift was relative to Trimethyl silyl propionate. The ¹⁵N and ¹³C chemical shifts were referenced indirectly. All spectra were collected and processed with Topspin 3.5 (Bruker Biospin) and analyzed with Sparky 3.12 (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco).

Analytical Ultracentrifugation

Analytical ultracentrifugation (AUC)-sedimentation velocity (SV) experiments were conducted in a ProteomeLabTM XL-I analytical ultracentrifuge (Beckman Coulter), as described previously [45]. Prior to measurement, proteins were dialyzed against a buffer containing 50 mM Tris-HCl pH 6.7, 30 mM NaCl, 2.5 mM EDTA and 1 mM Tris(2-carboxyethyl)phosphine (TCEP). Samples were loaded in charcoal-filled Epon centerpieces with 12 mm optical path length and centrifuged at 20 °C, during 24 hours at 45 000 rpm/ 163 004 g rotor speed (An-50 Ti rotor, Beckman Coulter). All sedimentation profiles were recorded using both interference and absorption optics at 280 nm. The diffusion-deconvoluted sedimentation coefficients distributions *c(s)* were calculated using the SEDFIT software [46]. A Tau construct containing a tryptophan residue (TauY394W) was used to improve its detection by UV absorption optics. In order to study the effect of the phosphorylation of Tau on the formation of a complex with 14-3-3 σ , solutions containing 24 μ M 14-3-3 σ in the presence and absence of 12 μ M wt-Tau or 12 μ M

Tau-PKA were analyzed. In order to study the stoichiometry of the 14-3-3 σ /Tau-PKA complex, mixtures containing 14-3-3 σ /Tau-PKA in the molar ratios of 1:1 (20 μ M 14-3-3 σ : 20 μ M Tau-PKA) and 2:1 (24 μ M 14-3-3 σ :12 μ M Tau-PKA) were analyzed. In order to determine the apparent K_d of the 14-3-3 σ /Tau-PKA complex, sedimentation velocity analysis of mixtures containing 8.4 μ M 14-3-3 σ and different concentrations (0.42 μ M, 1.4 μ M, 4.2 μ M, 12.6 μ M and 42 μ M) of Tau-PKA were performed. The apparent K_d was estimated using the global Lamm equation modeling with a 1:1 Langmuir binding model implemented in the SEDPHAT software [47] considering the interaction of one molecule of Tau-PKA with one 14-3-3 dimer. Figures of normalized continuous sedimentation coefficient distributions were prepared using GUSSE 1.4.2 [48].

Surface Plasmon Resonance

SPR experiments were conducted with a Biacore 3000 optical biosensor instrument (Biacore, Inc) equipped with a research-grade BDM500M sensor chip (XanTec Bioanalytics). For the determination of the dissociation constant between phosphorylated Tau and 14-3-3 σ or 14-3-3 ζ , the Tau mutant Y394W was used for more accurate determination of protein concentration. Biotinylation reactions of 14-3-3 σ /14-3-3 ζ were conducted by mixing the proteins at 350 μ M with the EZ-Link NHS-PEG12-Biotin reagent (Thermo Scientific) at 385 μ M for 2 h at room temperature, at pH 7.4. The biotinylated 14-3-3 σ or 14-3-3 ζ proteins were mixed with Switchavidin before immobilization (1:1 or 2:1 ratio, ~ 2500 RUs). Measurements were carried out at 20 °C, at a flow rate of 5 μ L/min in HBSP+ buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.05% surfactant Tween 20). Binding of Tau-PKA was assessed by injecting 12 different concentrations of Tau-PKA, as 3-fold serial dilutions, for 5 minutes in two channels of the sensor chip. One channel was used as a blank surface and the other contained immobilized 14-3-3 σ or

14-3-3 ζ . After each cycle, the surface was regenerated with a 5 M GUSCN solution acidified with 5 mM TCEP. Dissociation constants (K_d) were calculated with GraphPad Prism 8.1.2 Software using a nonlinear regression analysis.

Results and Discussion

Phosphorylation of Tau by PKA enhances its interaction with 14-3-3

We first confirmed that we could detect the stimulation of Tau binding to 14-3-3 by specific phosphorylation in our experimental system. It has been reported that Tau can bind to 14-3-3 even without being phosphorylated [31] and that phosphorylation by PKA increases the affinity of Tau to 14-3-3 [38]. The increase in the affinity upon phosphorylation was shown to be related to the pS214 [36,38] and pS324 epitopes [36].

The ^1H - ^{15}N TROSY-HSQC spectra of 125 μM ^{15}N / ^{13}C labeled 14-3-3 $\sigma\Delta\text{C}$ in the presence of either 500 μM wt-Tau (**Figure 1 A**) or 500 μM Tau-PKA (**Figure 1 B**) were recorded. We used 14-3-3 $\sigma\Delta\text{C}$ (containing residues 1-231 out of 248), lacking the 17-residue disordered C-terminal tail since it significantly improves the quality of NMR spectra. The spectrum of the 14-3-3 $\sigma\Delta\text{C}$ /wt-Tau complex does not show a remarkable difference compared to the spectra of 14-3-3 $\sigma\Delta\text{C}$ alone (**Figure 1 A, Figure S1**), except a loss of intensity for a small number of resonances. On the other hand, it is possible to detect major changes in the spectrum of 14-3-3 $\sigma\Delta\text{C}$ in the presence of Tau-PKA (**Figure 1 B**) corresponding to intensity decrease of the resonances to the point that some peaks cannot even be detected. In spite of not being a quantitative measure of interaction affinities, this shows that Tau interacts more tightly with 14-3-3 $\sigma\Delta\text{C}$ when phosphorylated by PKA. For further characterization, we performed AUC-SV experiments in order to study the formation of 14-3-3 σ /wt-Tau and 14-3-3 σ /Tau-PKA complexes. The normalized continuous sedimentation coefficient distributions $c(s)$ calculated

from the absorbance data (A_{280}) are shown in **Figure 1 C**. Each of the sedimentation profiles of 14-3-3 σ , wt-Tau or Tau-PKA displays a single peak suggesting homogeneity and a unique monomeric or multimeric state for each of the isolated proteins (orange, violet and blue curves).

The sedimentation profile of a mixture containing both wt-Tau and 14-3-3 σ (**Figure 1 C, light blue curve**) corresponds to the combined profiles of 14-3-3 σ and wt-Tau isolated in solution, as it contains only peaks at exactly the same positions as observed for proteins alone. This indicates that the interaction between wt-Tau and 14-3-3 σ is too weak to form a stable complex in solution. On the other hand, for Tau-PKA and 14-3-3 σ centrifuged together, peaks corresponding to each of the isolated proteins were not observed whereas a new peak shifted towards higher values of sedimentation coefficients was detected, confirming the formation of a stable complex between Tau-PKA and 14-3-3 σ (**Figure 1 C, green curve**).

Therefore, both NMR and AUC-SV data clearly showed that the phosphorylation of Tau by PKA enhanced its interaction with 14-3-3 σ , to the point of being essential for the formation of a stable complex in solution.

Tau interacts with 14-3-3 exclusively via the amphipathic binding groove

Having demonstrated that our experimental system was able to detect the PKA phosphorylation-dependent formation of the 14-3-3:Tau complex, we next investigated the interaction between full-length Tau-PKA and 14-3-3 $\sigma\Delta C$. The details of this protein-protein interface are of interest in regards to the efforts underway to modulate the interaction of these proteins[7]. In this context, it is of importance to understand whether the interaction is only mediated by the 14-3-3 phospho-binding motifs of phosphorylated Tau or requires additional contacts of Tau with the 14-3-3 surface, as has been reported for some of the 14-3-3 complexes

whose structures have been investigated using full-length proteins [49–54]. The X-ray crystal structures of 14-3-3 σ in complex with Tau peptides show that the pS214 and pS324 residues of Tau bind to the phospho-accepting pocket of the amphipathic binding groove of 14-3-3 [36]. In addition, by the analysis of the ^1H - ^{15}N HSQC of Tau-PKA in the presence of 14-3-3 σ , CSP are detected mainly for resonances corresponding to residues in close proximity to these phosphorylation sites. In order to further explore the interface, we recorded ^1H - ^{15}N TROSY-HSQC of $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ in the presence of unlabeled Tau-PKA (**Figure 1 B**). We next compared the effect caused by the full-length protein to the one caused by peptides containing only the pS214 epitope (**Figure S2**) or both pS214 and pS324 epitopes (**Figure S3**). The previously reported backbone assignment of 14-3-3 σ [44] allows the link of a resonance to a specific residue of 14-3-3 σ . Addition of the full-length Tau-PKA or of Tau peptides containing pS214 or both pS214 and pS324 to $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ induced, as expected, broadening of a number of resonances in the spectrum of 14-3-3 $\sigma\Delta\text{C}$. The broadening of a resonance causes a drop of its intensity and consequently of the I/I_0 ratio - I being the intensity of a resonance in the analyzed spectrum and I_0 the intensity of the corresponding resonance in the control spectrum (125 μM $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ alone). Note that this analysis does not detect residues in direct contact with Tau but rather residues affected by the binding, allowing to map a region of interaction. By analyzing the intensity plots along the 14-3-3 σ sequence corresponding to the formation of the three complexes, a similar profile is observed (**Figure 2 A**). The magnitude of the intensity decrease is gradual from the peptide surrounding pS214 (**Figure 2 A, red plot**) that showed the smallest decrease, followed by the pS214+pS324 double-phosphorylated peptide (**Figure 2 A, green plot**) and finally the full-length PKAc phosphorylated Tau protein (**Figure 2 A, blue plot**). This data suggests that, as expected, the

affinity of the monophosphorylated pS214 peptide for 14-3-3 σ ($K_d = 102 \pm 31 \mu\text{M}$ determined by [36] on the homologous 14-3-3 ζ) is lower than that of the diphosphorylated peptide ($K_d = 2 \pm 0.2 \mu\text{M}$ determined by [36] on the homologous 14-3-3 ζ). In the case of the full-length protein, the magnitude of the I/I0 ratio decrease is even stronger. However, we cannot conclude on this basis that the affinity of Tau-PKA for 14-3-3 σ is higher than either of the peptides, because the formation of a high MW complex (14-3-3/Tau-PKA) by itself leads to a general decrease of the resonance intensities due to the longer correlation time, and thus shorter relaxation time of the complex. The analysis of the residues corresponding to the resonances of the spectrum of $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ most affected by the presence of Tau-PKA indicates that it binds similarly to the peptides (**Figure 2 B**). Indeed, the I/I0 plots show a similar profile for the three spectra, indicating that all three molecules share the same binding surface on 14-3-3 σ . Keeping in mind that the peptides only bind to the amphipathic binding groove [36], our study suggests that the contacts of Tau-PKA with 14-3-3 were with the residues that compose the amphipathic binding groove, as was the case for the much shorter Tau-derived phosphopeptides.

The C-terminal stretch of 14-3-3 σ does not participate in the interaction with Tau-PKA

Our data obtained from the spectra of 14-3-3 $\sigma\Delta\text{C}$ suggested that the binding of Tau-PKA occurs exclusively through the amphipathic binding groove. Nevertheless, the data acquired with this C-truncated form (comprising residues 1-231 out of 248), which is normally used for crystallographic and NMR studies, did not allow us to exclude a possible participation of the disordered C-terminal stretch (residues A232-S248). Due to better quality NMR spectra, the reported backbone chemical shift assignments of 14-3-3 σ were performed on the C-truncated form of 14-3-3 σ [44], which means that the chemical shift assignments of this C-terminal stretch are not available. Therefore, in order to study the effect of this C-terminal stretch on the

interaction of 14-3-3 σ with Tau-PKA, the first step was to obtain the backbone chemical shift assignments of these residues. As previously reported [44], the NMR signals corresponding to this C-terminal stretch are very intense (**Figure S4**), which allowed the straightforward sequential assignment of the corresponding backbone chemical shifts using standard 3D NMR experiments (**Table S1**). A ^1H - ^{15}N TROSY HSQC of $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled full-length 14-3-3 σ in the presence and absence of Tau-PKA was recorded (**Figure S5**) and the corresponding I/I0 ratios were plotted (**Figure 3**). The I/I0 plot shows that the intensities of the resonances corresponding to residues of the C-terminal stretch were not affected by the presence of Tau-PKA, indicating that these residues are not involved in the interaction.

Tau-PKA occupies both binding grooves of the 14-3-3 dimer

Various configurations of 14-3-3 complexes are observed in crystal structures of 14-3-3 with full-length protein partners. 14-3-3 proteins can form complexes constituted of one 14-3-3 dimer for one protein partner by binding two distinct phosphorylation sites of the partner protein, each occupying one groove of the dimer [49]. Alternatively, one 14-3-3 dimer can accommodate two copies of a protein partner with a single phosphorylation, each occupying one groove of the dimer [50]. In the specific case of 14-3-3 interaction with Tau, it is suggested that the binding grooves of a 14-3-3 σ dimer could be simultaneously occupied by pS214 and pS324 sites, because the affinity of a doubly phosphorylated Tau peptide is higher than either of the corresponding singly phosphorylated peptide, suggesting cooperativity[36]. In addition, using cross-linking of 14-3-3 ζ /Tau-PKA complexes, and gel electrophoresis, complexes with an apparent molecular weight (MW) of 80 kDa (suggesting a 1:1 14-3-3 dimer/Tau stoichiometry), 120 kDa (suggesting 1:2 14-3-3 dimer/Tau stoichiometry) and 150 kDa (suggesting 2:2 14-3-3 dimer/Tau stoichiometry) were observed [55]. However, the complex formed by the full-length

Tau-PKA with 14-3-3 has not yet been directly investigated in solution, making a conclusion on the subunit composition 14-3-3/Tau complex difficult. We therefore decided to get further insights into the Tau-PKA/14-3-3 σ complex using AUC-SV. When Tau-PKA and 14-3-3 σ were mixed in an equimolar ratio, a complex was formed as indicated by the presence of a peak corresponding to higher molecular weight compared to that of the separate components. Nevertheless, in this 1:1 ratio of the partner proteins, some free, unbound Tau-PKA remained in solution (**Figure 4 A, light blue curve**). On the other hand, when Tau-PKA and 14-3-3 σ were mixed in a 1:2 molar ratio, a complex with the same value of sedimentation coefficient was formed and there were no traces of unbound Tau-PKA detected in solution (**Figure 4 A, green curve**). In addition, the observed value of weight averaged sedimentation coefficient $s_{w(20,w)}$ of 4.2 S for the peak identified as 14-3-3/Tau-PKA complex corresponded approximately to a MW of 100 kDa. Given that the theoretical mass for a complex containing one molecule of Tau-PKA (considering 5 phosphorylation sites) and two 14-3-3 molecules is 102.6 kDa, this experiment confirmed that in solution Tau-PKA occupies simultaneously both amphipathic binding grooves of the subunits of a 14-3-3 σ dimer (Figure 4 B). These experiments are compatible with a 14-3-3/Tau-PKA PPI in which Tau-PKA binds in each monomer of 14-3-3, with the pS214 and pS324 Tau phosphorylation sites. Indeed, these Ser residues are the preferential sites for phosphorylation by PKA[41] and the only ones that can be completely modified into pSer in *in vitro* experiments[7,41]. Therefore, despite the presence of a few minor phosphorylation sites (only a small fraction of the sample is modified at these positions) in the C-terminal part of PKA-Tau used in our experiments, these two Tau phosphorylation sites (pS214 and pS324) are the ones mostly binding in the amphipathic binding grooves of the 14-3-3 dimer (**Figure 4 B**).

The affinity of the 14-3-3/Tau-PKA interaction is in the low μM range

K_d value determination for the interaction between Tau-PKA and 14-3-3 is of importance when trying to modulate the interaction by small molecules. We determined K_d for this interaction in solution using AUC-SV by titrating 14-3-3 σ with increasing concentrations of Tau and recording the sedimentation profiles of these solutions. Using AUC-SV, a K_d of $1.3 \pm 0.2 \mu\text{M}$ was obtained (**Figure 5 A**), a value compatible with a previously reported K_d of $6.5 \pm 1.9 \mu\text{M}$ estimated by NMR [36], another method of affinity determination in solution. In addition, we used SPR to estimate the K_d because SPR is often used in drug discovery screening and a reference value is of interest in this context. Increasing concentrations of Tau-PKA were thus injected over surface-immobilized biotinylated 14-3-3 σ (**Figure 5 B**), and a K_d of $12.7 \pm 0.5 \mu\text{M}$ was obtained (**Figure 5 C**). Further SPR experiments were performed in order to evaluate whether there was a difference between the σ and ζ isoforms regarding the affinity for Tau-PKA. A K_d of $1.4 \pm 0.5 \mu\text{M}$ was derived for the 14-3-3 ζ /Tau-PKA interaction (**Table S2**). Note that saturation was not reached in these experiments, given the limitation to inject concentrated full-length Tau-PKA, and the risk of unspecific binding at the surface.

In contrast to these results, the affinity of 14-3-3 ζ for Tau-PKA was reported to be $27.4 \pm 1.3 \text{ nM}$ [38] using SPR experiments with PKA-Tau attached to the chips. This discrepancy illustrates the technical difficulty to obtain K_d for interactions involving long disordered phosphorylated proteins, especially when surface effects can interfere with the measurements.

Conclusions

Here we elucidate, using a range of biophysical techniques, several important aspects of the interaction of 14-3-3 and Tau-PKA. We first showed that, similarly to the peptides surrounding pSer214 or pSer324 phosphorylation sites of Tau, the interaction of the full-length PKA-

phosphorylated Tau protein is only mediated by the binding of the phosphorylation sites in the amphipathic binding groove of 14-3-3 σ . Despite the 441 amino acid residues of Tau protein, its interaction with 14-3-3 is thus only mediated by short peptides of about 6 residues, surrounding the phosphorylation sites. We also demonstrated that in solution, Tau phosphorylated by PKA occupied the amphipathic binding grooves of both monomers of the 14-3-3 dimer, leading to a 1-10 μ M affinity interaction. These aspects are important considerations for the pharmacological modulation of this PPI: the binding interface being small and the interaction of moderate affinity make the interface likely druggable for inhibition. It reinforces therefore the strategy followed in previous work where this PPI was targeted by competitive inhibitors binding to the amphipathic binding groove of 14-3-3 [7,8]. In addition, given the challenge represented by providing specific inhibitors for 14-3-3 that has many protein partners, it is essential to obtain detail on the molecular basis of complex formations. In summary, our work provides valuable structural insights into the 14-3-3/Tau interaction that could be of use for future drug discovery projects aiming at the pharmacological modulation of the 14-3-3/Tau interaction.

Author Contributions

JFN performed all the experiments and treated the data, OP performed the AUC-SV experiments and treated the data, FB performed the SPR experiments and treated the data, F-XC designed and recorded the NMR spectra, HM prepared recombinant proteins, G.O'M, TO and XH supervised the experiments, IL initiated and coordinated the research project. JFN and IL wrote the initial version of the manuscript. All authors have contributed to, revised and approved the final version of the manuscript.

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Supporting Information

Plot of the I/I₀ values of ¹H-¹⁵N correlation peak intensities in the spectrum of ¹⁵N¹³C²H labeled 14-3-3 $\sigma\Delta$ C in the presence of wt-Tau and Tau-PKA; ¹H-¹⁵N TROSY-HSQC spectra of ¹⁵N¹³C²H labeled 14-3-3 $\sigma\Delta$ C in the presence and absence of Tau peptides; ¹H-¹⁵N TROSY-HSQC spectra of ¹⁵N¹³C²H labeled full-length 14-3-3 σ in the presence and absence of Tau-PKA; Backbone chemical shift assignments of the C-terminal stretch of 14-3-3 σ and SPR derived dissociation constants for the Tau-PKA/14-3-3 interaction.

Accession codes: Human microtubule associated protein Tau (UNIPROT: P10636); Human 14-3-3 protein sigma (UNIPROT: P31947); Human 14-3-3 protein zeta/delta (UNIPROT: P63104); Mus musculus cAMP-dependent protein kinase catalytic subunit alpha (UNIPROT: P05132).

Figure legends

Figure 1. Phosphorylation of Tau by PKA increases the affinity of Tau for 14-3-3. (A) ^1H - ^{15}N TROSY-HSQC spectra of 125 μM $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ alone (in black), or in the presence of 500 μM wt-Tau (superimposed in red). (B) ^1H - ^{15}N TROSY-HSQC spectra of 125 μM $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ alone (in black), or in the presence of 500 μM Tau-PKA (superimposed in blue). Note that the chemical shift perturbations (CSP) on the spectrum of 14-3-3 $\sigma\Delta\text{C}$ are only detected in the presence of Tau-PKA, and only minor changes are observed in the presence of wt-Tau. (C) The normalized continuous sedimentation coefficient distributions for wt-Tau (purple), Tau-PKA (dark blue), 14-3-3 σ (orange), wt-Tau mixed with 14-3-3 σ (ratio 1:2, light blue) and Tau-PKA mixed with 14-3-3 σ (ratio 1:2, green).

Figure 2. Tau-PKA interacts with 14-3-3 σ through the amphipathic binding groove of 14-3-3. (A) Plot of the I/I₀ values of ^1H - ^{15}N correlation peak intensities in the spectrum of 125 μM $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ in the presence of 500 μM Tau peptide surrounding pS214 (red plot), 500 μM Tau peptide surrounding pS214 and pS324 (green plot) and 500 μM full-length Tau-PKA (blue plot) (I) compared to corresponding resonances in the reference spectrum of 125 μM 14-3-3 $\sigma\Delta\text{C}$ (I₀) (y axis) versus 14-3-3 $\sigma\Delta\text{C}$ amino acid sequence (x axis). A total of 135 correlation peak intensity ratios are shown. The x axis is not proportional to the sequence length. The helices of 14-3-3 σ are identified below the x axis as blue cartoons, while disordered regions are represented by red lines. (c) Mapping on the crystal structure of 14-3-3 σ (PDB ID: 1YZ5, represented as a grey cartoon) of the amino acid residues corresponding to the 20 most affected resonances by the presence of 500 μM Tau-PKA. The residues corresponding to the 10 most affected resonances are colored in red and the following 10 are colored in orange. Note that these

mostly affected residues of helices αC , αG - αH - αI mapping the interaction to the amphipathic binding groove.

Figure 3. The C-terminal disordered tail of 14-3-3 σ is not involved in the interaction with Tau-PKA. Plot of the I/I0 values of 1H - ^{15}N correlation peak intensities in the spectrum of 125 μM ^{15}N ^{13}C 2H labeled 14-3-3 σ in the presence of 80 μM Tau-PKA (I) compared to corresponding resonances in the reference spectrum of 125 μM 14-3-3 σ (I0) (y axis) versus 14-3-3 σ amino acid sequence (x axis). A total of 148 correlation peak intensity ratios are shown. The x axis is not proportional to the sequence length. The helices of 14-3-3 σ are identified below the x axis as blue cartoons, while disordered regions are represented by red lines. A diagram of the location of the C-terminal stretch in the sequence of 14-3-3 σ is presented above the plot.

Figure 4. Tau-PKA occupies both binding grooves of the 14-3-3 dimer. (A) Normalized continuous sedimentation coefficient distributions of 36 μM Tau-PKA alone (purple), 30 μM 14-3-3 σ alone (dark blue), and mixtures of Tau-PKA with 14-3-3 at ratio 1:1 (light blue) and at ratio 1:2 (green). Note that in the mixtures containing both proteins there is the presence of a complex with $s_{w(20,w)}$ of 4.2 S (estimated MW of 100 kDa), confirming that one molecule of Tau-PKA can bind simultaneously both monomers of 14-3-3 σ . (B) Proposed model for the 14-3-3/Tau-PKA PPI in which Tau-PKA binds in each monomer of 14-3-3 with the pS214 and pS324 Tau phosphorylation sites.

Figure 5. The dissociation constant of the 14-3-3 σ /Tau PPI is in the low μM range. (A) Area normalized continuous sedimentation coefficient distributions of mixtures of 14-3-3 σ with Tau-PKA at different ratios: 20:1 (purple), 6:1 (dark blue), 2:1 (light blue), 1:6 (green) and 1:20 (orange). (B) Example of an SPR sensorgram in which increasing concentrations of Tau-PKA

were allowed to flow over a chip functionalized with biotinylated 14-3-3 σ . The measured Response Units (RUs) (y axis) are plotted as a function of the elapsed time since the start of the flow, in seconds (x axis). For each curve, the values at equilibrium response (i.e. binding coverage) were extrapolated and fitted in a dose-response curve, shown in (C), against the log of the molar concentration of Tau-PKA.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supporting Information for

Phosphorylated full-length Tau interacts with 14-3-3 proteins via two short phosphorylated sequences, each occupying a binding groove of 14-3-3 dimer

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Figure S1. Plot of the I/I_0 values of ^1H - ^{15}N correlation peak intensities in the spectrum of 125 μM ^{15}N - ^{13}C labeled 14-3-3 $\sigma\Delta\text{C}$ in the presence of 500 μM wt-Tau (red plot) and 500 μM full-length Tau-PKA (blue plot) (I) compared to corresponding resonances in the reference spectrum of 125 μM 14-3-3 $\sigma\Delta\text{C}$ (I_0) (y axis) versus 14-3-3 $\sigma\Delta\text{C}$ amino acid sequence (x axis). A total of 135 correlation peak intensity ratios are shown. The x axis is not proportional to the sequence length. The helices of 14-3-3 σ are identified below the x axis as blue cartoons, while disordered regions are represented by red lines.

Figure S2. ^1H - ^{15}N TROSY-HSQC spectra of $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ 125 μM alone (in black), or in the presence of 500 μM Tau peptide surrounding pS214 (superimposed in red).

Figure S3. ^1H - ^{15}N TROSY-HSQC spectra of $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled 14-3-3 $\sigma\Delta\text{C}$ 125 μM alone (in black), or in the presence of 500 μM Tau peptide surrounding pS214 and pS324 (superimposed in green).

Figure S4. ^1H - ^{15}N TROSY-HSQC spectra of $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled full-length 14-3-3 σ 125 μM (purple). The assignments of the ^1H - ^{15}N resonances corresponding to the backbone amide function of the amino acid residues constituting the C-terminal stretch of 14-3-3 σ (residues 232-248) are presented. Note that all these resonances are more intense than the average resonances of the protein's spectrum.

Table S1. Backbone chemical shift assignments of the C-terminal stretch (residues A232-S248) of 14-3-3 σ .

Residue number	$\delta(^{15}\text{N})$ [ppm]	$\delta(^1\text{H})$ [ppm]	$\delta(^{13}\text{C})$ [ppm]		
	N^{H}	H^{N}	C_α	C_β	CO
A232	125.7	7.410	53.09	18.06	175.3
D233	119.2	8.184	54.27	40.53	173.6
N234	119.4	8.141	52.94	38.50	172.4
A235	124.5	8.150	52.73	18.31	175.7
G236	108.2	8.251	44.85	-	171.6
E237	120.9	8.147	56.00	29.62	174.2
E238	122.5	8.461	56.41	29.50	174.5
G239	110.5	8.369	44.99	-	172.1
G240	109.1	8.124	44.71	-	171.3
E241	120.9	8.147	55.68	29.69	173.4
A242	127.1	8.287	50.03	17.23	-
P243	-	-	-	-	174.3
Q244	121.2	8.369	55.10	29.07	173.2
E245	124.0	8.337	53.96	28.87	-
P246	-	-	-	-	174.3
Q247	121.5	8.425	55.32	29.06	172.7
S248	123.1	7.908	59.54	64.30	-

Figure S5. ^1H - ^{15}N TROSY-HSQC spectra of $^{15}\text{N}^{13}\text{C}^2\text{H}$ labeled full-length 14-3-3 σ 125 μM alone (in black), or in the presence of 80 μM Tau-PKA (superimposed in red).

Table S2. Determination of dissociation constants (K_d) by SPR for the interactions between Tau-PKA with 14-3-3 σ and 14-3-3 ζ .

	14-3-3σ/Tau-PKA	14-3-3ζ/Tau-PKA
First experiment	$K_d = 13.1 \mu\text{M}$	$K_d = 0.9 \mu\text{M}$
Second experiment	$K_d = 12.2 \mu\text{M}$	$K_d = 1.8 \mu\text{M}$
Average	$K_d = 12.7 \pm 0.5 \mu\text{M}$	$K_d = 1.4 \pm 0.5 \mu\text{M}$