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Tau inhibits tubulin oligomerization induced by prion protein

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ABSTRACT

In previous studies we have demonstrated that prion protein (PrP) interacts with tubulin and disrupts microtubular cytoskeleton by inducing tubulin oligomerization. These observations may explain the molecular mechanism of toxicity of cytoplasmic PrP in transmissible spongiform encephalopathies (TSEs). Here, we check whether microtubule associated proteins (MAPs) that regulate microtubule stability, influence the PrP-induced oligomerization of tubulin. We show that tubulin preparations depleted of MAPs are more prone to oligomerization by PrP than those containing traces of MAPs. Tau protein, a major neuronal member of the MAPs family, reduces the effect of PrP. Importantly, phosphorylation of Tau abolishes its ability to affect the PrP-induced oligomerization of tubulin. We propose that the binding of Tau stabilizes tubulin in a conformation less susceptible to oligomerization by PrP. Since elevated phosphorylation of Tau leading to a loss of its function is observed in Alzheimer disease and related tauopathies, our results point at a possible molecular link between these neurodegenerative disorders and TSEs.

1. Introduction

Cellular prion protein (PrP^C), particularly in a misfolded form (PrP^{Sc}), is widely considered a key player in the pathogenesis of neurodegenerative diseases called TSEs [1,2]. Nonetheless, the molecular mechanisms of the pathogenic misfolding as well as the PrP-mediated neurodegeneration remain unresolved. PrP^C is a mostly extracellular glycoprotein anchored in the plasma membrane [3]. In pathology, however, its intracellular concentration significantly raises. PrP point mutations linked to some TSEs increase the fraction of a transmembrane form of PrP (^{Ctm}PrP) with the C-terminal domain localized in endoplasmic reticulum (ER) lumen and the N-terminal domain exposed to the cytosol [4,5]. C-terminally truncated stop mutants of PrP are detected in the cytosol and nucleus [6,7]. Furthermore, accumulation of cytosolic PrP (cytoPrP) may result from the ER stress common in TSEs, affected translocation of nascent PrP, and direct inhibition of the proteasome by PrP^{Sc} [8–10]. Importantly, intracellular PrP has been demonstrated to be neurotoxic [4,11,12]. It is postulated that mislocalized PrP may exert toxic effects through interactions with intracellular proteins leading to loss or modification of their physiological functions [10,13]. In line with this hypothesis co-aggregation of several cytosolic proteins with cytoPrP has been reported. For example, cytoPrP co-aggregated the anti-apoptotic protein Bcl-2 and the ubiquitin ligase mahogunin [14,15]. Moreover, we have demonstrated that PrP binds to tubulin and disrupts microtubular cytoskeleton by inducing tubulin oligomerization and formation of aggregates [16–18]. Subsequently, it was also found that PrP^{Sc} co-immunoprecipitated with tubulin in brain homogenates of TSE-infected animals [19]. Interestingly, loss of dendritic microtubules has been observed in ultrastructural studies of TSE brain sections [20].

Tubulin is the major building block of microtubules – dynamic cytoskeletal structures involved in crucial cellular functions [21]. The assembly and stability of microtubules are regulated by microtubule associated proteins (MAPs) [22]. One of the major neuronal MAPs is Tau protein whose dysfunction leads to neurodegeneration observed in Alzheimer disease (AD) and other tauopathies [23]. Phosphorylation of Tau reduces its binding to tubulin and thereby regulates microtubule dynamics. Hyperphosphorylation of Tau, a hallmark of AD, leads to its aggregation into paired helical filaments, disassembly of microtubular cytoskeleton, and neurodegeneration. Interestingly, hyperphosphorylation of Tau has also been reported in human TSEs cases [24–27]. This may result from induction of Tau hyperphosphorylation by misfolded PrP [28,29] indicating a molecular link between these protein misfolding diseases (PMDs). Furthermore, a direct interaction between PrP and Tau has been demonstrated [30].

Here, we checked whether MAPs/Tau protein may influence the PrP-induced oligomerization of tubulin. We have also verified an intriguing scenario where phosphorylation of Tau enhances the

Abbreviations: AD, Alzheimer disease; CBB, Coomassie brilliant blue; ^{Ctm}PrP, transmembrane form of prion protein with the C-terminus residing in the lumen of endoplasmic reticulum; cytoPrP, cytosolic prion protein; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; GSK3, glycogen synthase kinase 3; mAb, monoclonal antibody; MAPs, microtubule-associated proteins; pep1-30, peptide corresponding to PrP sequence 1–30; PKA, protein kinase A; PMDs, protein misfolding diseases; PrP, prion protein; PrP^C, cellular form of prion protein; PrP^{Sc}, TSE (scrapie) form of prion protein; rTau, recombinant Tau; SNHS, *N*-hydroxysulfosuccinimide; TSEs, transmissible spongiform encephalopathies

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deleterious effect of PrP exerted at the level of the microtubular cytoskeleton.

2. Materials and methods

2.1. Protein purification

Human prion protein (residues 23–231) was expressed in *E. coli* and purified as described previously [31,32]. As it was demonstrated by Zahn and colleagues [32], the applied purification procedure gives natively folded PrP. To ensure that PrP was not aggregated the preparations were ultracentrifuged (30 min at 200,000 *g*, 30 °C) before each experiment.

Tubulin containing traces of MAPs (crude tubulin) was purified from porcine brain by two cycles of polymerization/depolymerization according to the modified method of Mandelkow and colleagues [33], described in details in [16]. The last step of microtubule depolymerization was performed either for 2 or 12 h. Tubulin preparations were stored at −70 °C in PM buffer composed of 2 mM EGTA, 0.1 mM EDTA, 1 mM ATP, 2 mM DTT and 100 mM PIPES pH 6.9. Just before experiments the preparations were thawed and centrifuged for 20 min at 22,000 *g*, 4 °C. Obtained supernatants were used as crude tubulin.

Crude tubulin was depleted of MAPs by the modified method of Castoldi and Popov [34]. Tubulin supernatant, obtained as described above, was incubated with equal volume of 20% DMSO, 2 mM $MgCl_2$, 2 mM GTP and 1 M PIPES pH 6.9 for 1 h at 37 °C. The mixture was then layered (1:2, v/v) over a cushion composed of 60% glycerol, 1 mM EGTA, 1 mM $MgCl_2$, 80 mM PIPES pH 6.9 (warmed to 37 °C), and centrifuged for 1 h at 200,000 *g*, 37 °C. The obtained pellet of pure microtubules was briefly rinsed and homogenized in PM buffer on ice. The resuspended pellet was used at the day of preparation as pure tubulin. The supernatant, was dialyzed against 0.3 M KCl, 1 mM EDTA, 0.1 mM DTT, 0.5 mM PMSF and 10 mM MOPS pH 6.8 and subsequently used for preparation of MAPs fraction. MAPs were separated from remnants of tubulin on DEAE Sephacel according to Murphy and colleagues [35]. The fractions containing MAPs were concentrated on Centriprep (Millipore, Billerica, MA, USA) and dialyzed against 50 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 0.5 mM PMSF and 10 mM MOPS pH 6.8, and stored at −70 °C.

Brain Tau was purified directly from crude tubulin or from supernatants obtained upon centrifugation of crude tubulin over the glycerol cushion. Tau preparations of different electrophoretic mobility were obtained from tubulin depolymerized by incubation for different lengths of time (see above). Generally, preparations of higher electrophoretic mobility (H) were obtained from tubulin depolymerized for 2 h whereas those of lower mobility (L) from tubulin depolymerized for 12 h. Initial preparations were dialyzed against 0.75 M NaCl, 1 mM $MgCl_2$, 2 mM EGTA, 2% β-ME, 0.5 mM PMSF and 20 mM MES pH 6.8. Then, according to the method of Fellous and colleagues [36], the preparations were boiled for 5 min in a water bath and after cooling centrifuged for 30 min at 29,000 *g*, 4 °C. Obtained supernatants were treated with 2.5% perchloric acid as described by Lindwall and Cole [37], and immediately centrifuged for 15 min at 14,000 *g*, 25 °C. Subsequently, Tau was salted out from supernatants by ammonium sulfate at 50% saturation. The preparation was centrifuged for 30 min at 42,000 *g*, 4 °C. Tau pellet was resuspended, dialyzed against 50 mM NaCl, 0.5 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 50 mM MES pH 6.8, and stored at −70 °C.

Recombinant Tau (rTau, human isoform 2N4R) was purchased from Sigma (St. Louis, MO, USA). If not indicated otherwise, rTau at 28 μg/ml (0.6 μM) was used.

2.2. Phosphorylation of Tau

rTau at 0.85 mg/ml was incubated with catalytic subunit of PKA (Sigma) added at the proportion of 60 μg/mg of Tau for 2 h at 37 °C in 28 mM NaCl, 10 mM $MgCl_2$, 0.28 mM EGTA, 1.2 mM DTT and 28 mM MES pH 6.8. The phosphorylation was initiated by addition of 1 mM ATP. After 2 h, the buffer was supplemented with 0.01% Triton X-100 and the concentrations of NaCl, DTT and ATP were increased to 76 mM, 1.8 mM and 2 mM, respectively, whereas pH was increased to 7.4 with 16 mM Tris–HCl. The mixture was then incubated with GSK3β (GenScript, Piscataway, NJ, USA) at the proportion of 26 μg/mg of Tau for 3 h at 37 °C. In some experiments Tau was phosphorylated solely by PKA for 5 h. Solubility of phosphorylated Tau was confirmed in sedimentation experiments by centrifugation for 10 min at 200,000 *g*, 4 °C. The extent of phosphorylation was assessed by shift in electrophoretical mobility upon SDS-PAGE.

2.3. Dephosphorylation of Tau

Brain Tau at 0.4 mg/ml was incubated with phosphatase PP2A (Millipore) at the proportion of 17 μg/mg of Tau for 2 h at 30 °C in 240 mM NaCl, 0.4 mM $MgCl_2$, 0.04 mM $MnCl_2$, 0.7 mM EGTA, 25 mM β-ME, 1 mM DTT, 0.3 mM PMSF and 30 mM MES pH 6.8. The extent of dephosphorylation was assessed by SDS-PAGE and Western blotting.

2.4. Light scattering

Oligomerization of tubulin was assessed by turbidity measurements as previously described [17] at conditions not allowing microtubule formation. Tubulin at 0.2 mg/ml (4 μM, assuming molecular weight ≈ 50,000) was incubated at 25 °C in a buffer containing 10% (w/v) glycerol, 1 mM GTP, 16 mM $MgCl_2$ and 10 mM sodium phosphate buffer pH 7.0. In some experiments the buffer was supplemented with 50 μM $CaCl_2$. Monitoring of turbidity was initiated immediately after addition of PrP (final concentration of 10 or 15 μg/ml, corresponding to 0.4 or 0.6 μM) to the cuvette. All measurements were performed against tubulin or tubulin with MAPs/Tau as a reference.

2.5. Cross-linking experiments

Covalent cross-linking was performed at the same conditions and protein concentrations as the turbidity measurements. The reaction was initiated by addition of 1 mM SNHS and 1 mM EDC. After 1 h of incubation at 25 °C, cross-linking was stopped with 50 mM Tris. The cross-linking products were analyzed by SDS-PAGE and Western blotting.

2.6. Transmission electron microscopy

The samples analyzed by light scattering were also subjected to electron microscopic studies. After 20 min of incubation at 25 °C, 10-μl samples were placed on copper grids (400 mesh, Sigma) covered with collodion (SPI Supplies, West Chester, PA, USA) and carbon. Negative staining was performed with 2% (w/v) uranyl acetate (SPI Supplies) for 25 s. The grids were examined in a JEM 1400 electron microscope (JEOL Co., Japan) equipped with a digital camera (CCD MORADA, SiS-Olympus, Germany).

2.7. Electrophoretic separations

SDS-PAGE was performed on 10% separating gels according to Laemmli [38]. To improve separation of Tau preparations, the electrophoresis was continued until the 50 kDa marker reached the edge of the gel. The gels were stained with Coomassie brilliant blue R-250.

2.8. Western blotting

After SDS-PAGE proteins were electrotransferred onto nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) in a standard