TPPP, A NEW PROTEIN FAMILY:
HOMOLOGUE SEQUENCES WITH DIFFERENT STRUCTURAL AND FUNCTIONAL PROPERTIES

PhD Thesis

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INTRODUCTION

Microtubules (MT) are one of the main components of the cytoskeleton. MTs are made up of tubulin heterodimers, and maintain multifarious functions in the cell. Beside being part of the structural network of the cell, they play role in the intracellular transport, cell motion and cell proliferation as well. All these functions could be achieved only because they are members of an accurately tuned system: MT interact with several different proteins. The most common MT-related proteins are the Microtubule-associated proteins (MAPs). They have been found to carry out a wide range of functions, which include the regulation of the stability and the dynamics of the microtubular system as well as the influence of the transport along the MTs. Soluble, cytoplasmic proteins, which establish dynamic contact with MTs and bind temporarily to MTs, are at least as important in the regulation of the microltubular system as the well-known structural- and motor-MAPs.

One of these cytoplasmic proteins is the recently identified Tubulin Polymerization Promoting Protein/p25 (TPPP/p25), which was identified on the basis of its function in Judit Ovádi's research group, in the Institute of Enzimology. This brain-specific protein main interacting partner is the tubulin/microtubular system, *in vitro* it induces aberrant MT-formation, binds to paclitaxel-stabilized microtubules and bundles them.

Different characteristics of TPPP/p25 (heat stability, high pI, very low α-helix content in contrast to the predicted values) suggested that this protein belongs to the group of intrinsically unstructured proteins.

Furthermore, the TPPP/p25 was found to be a member of a new protein family, because its primary sequence differs from that of other proteins identified so far; however, it shows high homology with p25-like hypothetical proteins.

Immunohistochemical and confocal microscopic studies demonstrated that TPPP/p25 is enriched in filamentous alpha-synuclein bearing Lewy bodies of Parkinson's (PD) and diffuse Lewy body (DLBD) diseases, as well as in glial inclusions of multiple system atrophy (MSA), which suggests the role of TPPP/p25 in the pathogenesis of synucleinopathies.

OBJECTIVES

The aims of my PhD thesis were to characterize the structure and function of TPPP family member proteins with different prediction and experimental methods. Consequently, the major objectives were

i) to compare the sequences of TPPP family member proteins, and to study, whether the sequential similarity reflects the structural and functional properties of TPPP/p25-like proteins;

ii) to identify interacting partners of TPPP/p25, and to further characterize the identified interactions *in vitro* and *in vivo* experiments, to elucidate the physiological and pathological role of TPPP/p25;

iii) to define the regions in TPPP/p25 responsible for the interaction with different known or newly recognized partner proteins, with special emphasis on tubulin, to find molecules, which may influence the emerging interactions selectively;

METHODS

Protein Purification

Tubulin was purified from bovine brain by the method of Na and Timasheff. Calmodulin (CaM) was also purified from bovine brain using the method of Gopalakrishna and Anderson. Recombinant human TPPP proteins and α-synuclein were expressed in E. coli BL21 cells, and isolated with His-Selected^{TM} cartidge in case of TPPP proteins, and with DE52 column in case of α-synuclein.

Circular Dichroism (CD) Measurements

CD spectra of TPPP proteins were acquired in the 190-280 nm wavelength range. The CD spectra of TPPP proteins as the function of temperature were also recorded, as well as the spectra in the presence of tubulin or trifluoroethanol. The α-helical content of proteins was calculated by the Chen-equation.

Affinity chromatography

Human recombinant TPPP/p25 was immobilized to CNBr-activated Sepharose affinity column. Cytosolic brain extracts were loaded to the column. The bound proteins were eluted,

separated by sodium dodecyl sulphate polyacrilamide gel eletroforesis (SDS-PAGE), and identified by Mass Spectrometry.

Limited proteolysis

To identify the proteolytic sensitivity of TPPP proteins (which gives information on the structural properties and compactness of proteins) limited proteolysis with trypsin IV and chimotrypsin was used. To specify the region of TPPP/p25 responsible for the interaction with tubulin, TPPP/p25 was digested with either subtilisin or chimotrypsin, followed by sedimentation experiments. To identify the region of tubulin responsible for interaction with TPPP/p25, the C-terminal region of tubulin was cleaved by subtilisin. Finally, to specify the region of α-synuclein which interacts with TPPP/p25, trypsin was used as a proteolytic enzyme.

Western-blot analysis

Proteins were transferred from SDS-PAGE onto Immobilon P^{SQ} membrane. After blocking, a suitable primary antibody was used. Antibody binding was revealed by using appropriate IgG coupled with peroxidase, ECL (enhanced chemiluminescence) Western Blotting Detection reagents and Kodak X-Omat AR film. To quantify the samples, series of known amount of protein and densitometry were used.

Turbidity Measurements

The assembly of 10 μM tubulin was assessed at 37 °C by addition of 20 μM paclitaxel, or that of 3-7 μM TPPP/p25. The turbidity was monitored at 350 nm by a Cary 100 spectrophotometer. At the final state of paclitaxel-induced polymerization, the solution was centrifuged at 30000g, and the pellet fraction was used as MTs.

Pelleting Experiments

The intact TPPP homologues or the digested fragments were incubated with tubulin or MT. The pellet and the supernatant fractions were separated by centrifugation, and both fractions were analyzed by a Tris-tricine SDS-PAGE.

Enzyme-linked immunosorbent assay (ELISA)

The 96-well microtiter plate was coated with TPPP/p25 solution. The wells were blocked with BSA containing buffer. Next, the plate was incubated with serial dilutions of native or digested α-synuclein. Then the plate was incubated with α-synuclein antibody and appropriate secondary IgG-peroxidase conjugate. O-phenylenediamine with 0,03% peroxide was used as substrate solution. The peroxidase catalysed reaction was stopped with H_2SO_4; absorbance was read at 490 nm with a Wallace Victor 2 multiplate reader (Perkin Elmer).

Pepscan analysis

TPPP/p25 decapeptides overlapping by 5 amino acid residues were synthesized on polyethylene pins and immunoscreening with an ELISA type of analysis were carried out in 96-well microtiter plates. To validate the method, the pins were incubated with anti-TPPP/p25 serum with known epitope (between amino acid 186 and 204) and then appropriate secondary IgG-peroxidase conjugate; the presence of antibodies was detected using o-phenylenediamine, peroxide and H_2SO_4, as described earlier. To identify the region of TPPP/p25 which interacts with α-synuclein, the experiment was carried out by adding α-synuclein and then anti α-synuclein antibody into the wells. The reaction of a pin-coupled peptide was scored positive (significance level) when the ELISA absorption was significantly higher than the twofold average absorption of the peptides.

RESULTS AND CONCLUSIONS

1. With BLAST analysis we have identified and then cloned 2 shorter protein homologues of TPPP/p25, namely TPPP/p20 and TPPP/p18. We experimentally proved the presence of TPPP/p20 in bovine brain at protein level: LC/MS/MS identified an approximately 20 kDa protein in a partially purified fraction of TPPP/p25 as the bovine homolog of TPPP/p20.

2. Sequence alignment demonstrated that the TPPP proteins show a high degree of homology. The sequence comparison of the three (hypothetical) proteins shows 53% identity (the pairwise comparisons of similarities are 81, 76, and 75% for p25-p20, p20-p18, and p25-p18, respectively) and they could be considered as a new protein family. However CD spectroscopy, limited proteolysis, differential scanning calorimetry and ^{1}H NMR experiments showed significant structural differences among the TPPP proteins: while TPPP/p25 is a flexible and inherently dynamic protein, TPPP/p18 rather than TPPP/p20 shows a stable structure.

3. These structural differences cannot be attributed exclusively to the presence of the disordered, 42 aa long N-terminal region of TPPP/p25. It is missing from both TPPP/p20 and TPPP/p18, and TPPP/p20, as well as TPPP/p18, is different from the truncated TPPP/p25-mutant (which lacks the N-terminal part of the protein), moreover, the major structural difference occurs between TPPP/p20 and TPPP/p18. It

could be concluded that the sequential alterations have an important role in the structural properties.

4. These structural differences manifest in functional alterations as well. By CD and surface plasmon resonance (SPR) experiments, we demonstrated that *in vitro* all three human TPPP proteins bind to tubulin, but they show significant differences in their binding affinities to tubulin. (The affinity constants of the interaction was 0.3 μM, 1 μM and 2,5 μM in the case of TPPP/p25, TPPP/p20 and TPPP/p18, respectively).

5. As the pelleting and transmission electron microscopic experiments revealed, the paclitaxel-stabilized MTs were extensively bundled in the presence of TPPP/p25 and TPPP/p20 proteins but not in the case of TPPP/p18. This effect was more explicit *in vivo*: in contrast to the co-localization of TPPP/p25 and TPPP/p20 with the MTs, TPPP/p18 apparently displayed homogeneous cytoplasmic distribution. These data indicate that the primary physiological interacting partner of TPPP/p18 is not the MT.

6. To identify possible physiological interacting partners of TPPP/p25, affinity chromatography and cell-free bovine brain extract was used. 23 proteins were identified:

   MAPK 1, SET protein, Phosphatase 2A inhibitor, Calmodulin, NCK associated protein, 14-3-3 protein (γ,ζ), Tubulin (β), Actin (α,β) GAPDH, Glutamate dehydrogenase, ATP citrate lyase, HSP 90 kDa, HSP 70 kDa, HSP 60 kDa, TER ATPase, Synapsin I a and b, α-synuclein, Myelin basic protein, Elongation factor α, Karyopherin β3 and β1, Nucleolin, p53 inducible protein 121 (CYFIP2), LanC-like protein 1.

   These proteins play role in signal transduction, translation, cellular metabolism; and structural, ribosomal- and (pre)synaptic proteins and lipoproteins were also identified. In some cases (GAPDH, CaM, MAPK1, α-synuclein, β-tubulin, HSP 90, Myelin basic protein) the interactions were corroborated by other, independent methods. Moreover, in 5 cases (GAPDH, MAPK1, CaM, α-syinuclein, β-tubulin) of functional significance the interactions were further characterized.

7. Affinity chromatography demonstrated that the amount of bound GAPDH was independent from the presence of microtubular proteins in the cell extract indicating

the direct interaction of the TPPP/p25 with GAPDH. However, the interaction was salt and NAD^+ sensitive.

8. *In vitro* experiments proved that the MAPK1 can phosphorylate TPPP/p25 at specific sites, and the phosphorylation affects the function of TPPP/p25: incorporation about 3 mol P/mol protein resulted in the loss of tubulin polymerization promoting activity of TPPP/p25. Our data suggest that MAPK1 is involved in the organization of microtubular system via the regulation of the tubulin polymerization promoting and MT bundling function of TPPP/p25.

9. CaM decreased the initial rate of tubulin polymerization induced by TPPP/p25 at a concentration dependent manner, suggesting that the calmodulin competes with tubulin to interact with TPPP/p25. The calmodulin-TPPP/p25 interaction was corroborated and further characterized by SPR.

10. Cerebrospinal fluid (CSF) samples from Multiple Sclerosis (MS) patients and non-MS patients were analyzed. The TPPP/p25 concentrations were found to be significantly higher in the samples of MS patients than in that of non-MS ones. Patients above the 50 μg/L TPPP/p25 level in their CSF suffer from MS or potentially compromised individuals. Thus the TPPP/p25 can be helpful diagnostic marker for MS.

11. Amino acid sequences in TPPP/p25 responsible for tubulin binding were identified by sequence alignment and binding experiments after limited proteolysis, as well as by the characterization of tubulin binding in the case of TPPP/p20 and N-terminal truncated version of TPPP/p25 (that lacks the first 43 amino acid residues TPPP/p25(Δ3-43)). These experiments showed that the region of TPPP/p25 between 167-187 amino acids plays an important role in its interaction with tubulin. This region is homologues to the so-called tau-MAP binding motif. Nevertheless, the basic and unstructured N-terminal tail of TPPP/p25 is also important for the interaction. Regarding the interaction of TPPP/p25 with tubulin/MT, our data suggested that the TPPP/p25 probably binds to the C-terminal region of tubulin, exposed on the outer surface of MT, in the same way as MAP2c or tau. The binding site includes not only the last, hypervariable region (about 20 amino acid) of tubulin, because TPPP/p25 was

able to bind to MT (even in less extent) after eliminating this region by subtilisin cleavage.

12. The investigation of α-synuclein – TPPP/p25 interaction by the means of „Pepscan" analysis showed that the region of TPPP/p25 between 147-166 amino acids is important for itse binding to α-synuclein. ELISA experiments following a limited proteolysis of α-synuclein suggested that the C-terminal region of α-synuclein (between 102-140 amino acids) binds to TPPP/p25, therefore the binding site could be assumed within this region. Information on the interacting surfaces could help to develop lead compounds influencing these interactions selectively, which is of great importance in the case of pathological processes.

PUBLICATIONS:

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