Interaction of TPPP/p25 protein with glyceraldehyde-3-phosphate dehydrogenase and their co-localization in Lewy bodies

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Abstract TPPP/p25, a flexible unstructured protein, binds to tubulin and induces aberrant microtubule assemblies. We identified hereby glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a new interacting partner of TPPP/p25. The immunoprecipitation and affinity chromatographic experiments with bovine brain cell-free extract revealed that the interaction was salt and NAD\textsuperscript{+} sensitive while ELISA showed resistant and firm association of the two isolated proteins. In transfected HeLa cells at low expression level of EGFP-TPPP/p25, while the green fusion protein aligned at the microtubular network, GAPDH distributed uniformly in the cytosol. However, at high expression level, GAPDH co-localized with TPPP/p25 in the aggresome-like aggregate. Immunohistochemistry showed enrichment of TPPP/p25 and GAPDH within the \(\alpha\)-synuclein positive Lewy body.

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1. Introduction

The search of protein–protein interactions becomes a more and more important issue for understanding the multiple functions of the gene products in the post-genomic era. Recently, we have isolated a new brain-specific protein from bovine brain tissue and denoted it to TPPP/p25 protein reflecting its function (Tubulin Polymerization Promoting Protein) as well as its molecular mass (25 kDa) \([1,2]\). This bovine protein showed high homology with p25\(\alpha\), a human gene transcript \([3]\). We have shown that TPPP/p25 is a heat stable cationic intrinsically unstructured protein \([1,4]\), which displays structural similarity to \(\alpha\)-synuclein \([4]\). TPPP/p25 interacts with tubulin, induces aberrant tubulin assemblies and bundling of microtubules (MTs) both in vitro and in vivo systems \([1,5]\). We have proved that TPPP/p25 extensively co-localizes with the microtubular system in transfected HeLa cells and at high expression level it causes the formation of aggresome-like body at the centrosome region \([5]\). Our data on pathological brain tissues have revealed that TPPP/p25 co-localizes with \(\alpha\)-synuclein in cytosolic inclusions in the case of Parkinson’s disease (PD) and other synucleinopathies \([6]\). We have suggested that the formation of protein aggregates in HeLa cells induced by the over-expression of TPPP/p25 could be related to a pathological process leading to neurodegeneration \([5]\).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was considered as a classical glycolytic housekeeping enzyme with its glycolytic activity. Nowadays, more and more evidence have been accumulated which suggest that GAPDH displays a number of additional activities due to its multiple interactions with different cytosolic and nuclear proteins, nucleic acids and subcellular particles \([7]\) and references therein). GAPDH has a general mediator role in the initiation of apoptotic cascades \([8]\), and interacts with different proteins involved in age-related neurodegenerative disorders such as Huntington’s disease, Alzheimer’s disease and ataxias \([9]\). It has been recently shown that the over-expression of both GAPDH and \(\alpha\)-synuclein in COS-7 cells induced Lewy body (LB)-like cytoplasmic inclusions \([8]\). In fact, it has been suggested that \(\alpha\)-synuclein itself is not sufficient to cause aggregation into LB-like inclusions, but additional factors such as oxidative stress, mitochondrial dysfunction and macromolecular interactions probably play role in the pathogenesis. For example, GAPDH was found to be a stimulator of \(\alpha\)-synuclein aggregation in PD. Recently, proteomic analysis suggested the co-occurrence of TPPP/p25 and GAPDH in rodent brain postsynaptic density \([10,11]\).

In this work we identified GAPDH as a potential interacting partner of TPPP/p25. We demonstrated that these two proteins interacted with each other in vitro, and co-localized in

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DHB, 2,5-dihydroxybenzoic acid; DTE, 1,4-dithioerythritol; DTT, 1,4-dithiothreitol; FCS, fetal calf serum; GAPDH (EC 1.2.1.12), glyceraldehyde-3-phosphate dehydrogenase; IAM, iodoacetamide; LB, Lewy body; MALDI-TOF, matrix-assisted laser-desorption ionization time-of-flight; MT, microtubule; PD, Parkinson’s disease; PSD, post source decay; TMRE, tetramethylrhodamine ethyl ester
HeLa cells overexpressing TPP/p25 as well as in LBs of pathological brain tissue.

2. Materials and methods

2.1. Materials

Dithiothreitol (DTT), iodoacetamide (IAM), ammonium bicarbonate (NH₄HCO₃) and 2.5-dihydroxybenzoic acid (DHB) were obtained from Sigma (Germany), the sequencing grade Modified Trypsin (modified by reductive methylation) was ordered from Promega. Millipore C18 ZipTip and prelubricated microcentrifuge tubes (washed with HPLC grade methanol) were used. Monoconal anti-α-tubulin antibody in body (DMA1) was purchased from Sigma.

Rabbit muscle GAPDH was Sigma product in ammonium sulphate. The salt was removed from GAPDH solution by extensive dialysis against PBS buffer containing 1 mM 1,4-dithioerythritol (DTE). The ratio of OD₅₆₂/OD₂₈₀ of the GAPDH solution was 1.35 ± 0.05, which corresponded to about 2 moles firmly bound NAD⁺ per mole tetrameric enzyme [12]. To obtain apo-GAPDH, the firmly bound NAD⁺ molecules were removed by using activated charcoal [13].

2.2. Antibody production

Two different anti-TPPP/p25 antisera were used in the experiments. One of them was raised against a fragment of TPP/p25 (186-200 residues) by describing strategy [6]; the other antiserum was raised against human recombinant His₆-tagged TPP/p25 [25]. Antiserum raised against rabbit muscle GAPDH was produced in rat. The protocols used for immunization and testing the sera were similar to that described earlier [6].

2.3. ELISA

The microtitre plate was coated with 5 µg/ml (100 µl/well) apo-GAPDH or GAPDH containing ~2 firmly bound NAD⁺ per tetrameric enzyme in PBS containing 1 mM DTE in the presence or in absence of NAD⁺ overnight at 4 °C. The wells were blocked with 0.5% BSA in PBS for 1 h at room temperature. Next, the plate was incubated with serial dilutions of pure TPP/p25 in the concentration range between 2 μm and 0.0195 μm, in the presence and in absence of NAD⁺ or 150 mM NaCl for 1 h at room temperature in PBS. Then the plate was sequentially incubated with the anti-TPPP/p25 serum (dilution 1:2000), and with the anti-α-tubulin IgG-peroxidase conjugate (dilution 1:5000). Both antibodies were in PBS buffer containing 0.5% BSA, and incubated for 1 h at room temperature. Between each incubation steps the wells were washed three times with PBS. When the TPP/p25 was incubated with a buffer containing 10 mM NaCl, then the wash was performed in the same condition (10 mM phosphate, 10 mM NaCl, pH 7.4). o-Phenylenediamine in the concentration of 3.7 mM with 0.03% peroxide was used as substrate solution. The peroxidase catalyzed reaction was stopped after 15 min with 1 M H₂SO₄; absorbance was measured at 495 nm with a Wallace Victor 2 multiplexer.

2.4. Preparation of cytosolic brain extract

Cytosolic extract was prepared from bovine brain tissue. The tissue was homogenized in PEM buffer (50 mM PIPES pH 6.6, containing 1 mM EGTA, 5 mM MgCl₂, 1 µg/ml leupeptin, 1 µg/ml pepstatin) at a 1:1.5 ratio of tissue and buffer. The homogenate was centrifuged at 30000 x g at 4 °C for 20 min, and then the supernatant was again centrifuged at 100000 x g at 4 °C for 30 min. This supernatant of a ~25 mg/ml total protein concentration was used as cytosolic bovine brain extract. In some case the microtubular proteins were removed by assembling the MTs with adding paclitaxel and GTP as described previously [14]. In some case the microtubular proteins were removed by assembling the MTs with adding paclitaxel and GTP as described previously [14]. In some case the microtubular proteins were removed by assembling the MTs with adding paclitaxel and GTP as described previously [14]. In some case the microtubular proteins were removed by assembling the MTs with adding paclitaxel and GTP as described previously [14]. In some case the microtubular proteins were removed by assembling the MTs with adding paclitaxel and GTP as described previously [14]. In some case the microtubular proteins were removed by assembling the MTs with adding paclitaxel and GTP as described previously [14]. In some case the microtubular proteins were removed by assembling the MTs with adding paclitaxel and GTP as described previously [14]. In some case the microtubular proteins were removed by assembling the MTs with adding paclitaxel and GTP as described previously [14].

2.5. Affinity chromatography

TPPP/p25 was immobilized to CNBr-activated Sepharose 4B (Amersham) according to the manufacturer’s instructions. The TPP/p25 bound to Sepharose was packed into columns. The binding capacity of a column was ~3 mg TPP/p25 per 1 ml Sepharose. The affinity column was equilibrated with phosphate buffer (10 mM phosphate buffer pH 7.0 containing 10 mM NaCl). The extracts (with or without microtubular proteins) were loaded to the column, and the column was washed with phosphate buffer. The bound proteins were eluted with phosphate buffer plus 0.5 M or 150 mM NaCl. Typically, 2 ml ~25 mg/ml extract was loaded to the column (1 ml) and the total amount of the eluted protein was ~500–800 µg. After each experiment the column was regenerated using 5 cycles of 0.1 M Na-acetate pH 4.0 buffer containing 0.5 M NaCl and 0.1 M Tris pH 8.0 buffer containing 0.5 M NaCl. The eluted proteins were concentrated using an Amicon ultrafiltration stirred-cell apparatus fitted with an YM-10 membrane, followed by SDS/PAGE. Proteins were electrotransferred onto Immobilon-P° transfers membranes. The filters were subjected to immunoblotting with an antiserum directed against rabbit muscle GAPDH in the dilution 1:10000 or with an antibody directed against α-tubulin in mouse (dilution 1:2500). Antibody binding was revealed by using anti-rat (dilution 1:2500) or anti-mouse (dilution 1:2500) IgG coupled with peroxidase, ECL® (enhanced chemiluminescence) Western Blotting Detection reagents (Amersham Biosciences) and Kodak X-Omat AR film.

2.6. Immunoprecipitation

The tubulin-free bovine brain extract (~25 mg/ml, 50 µl) was incubated with 4 µl serum raised against the human recombinant TPP/p25 protein in 50 mM Tris pH 8.0 buffer containing 25 mM NaCl and 0.05% SDS (total volume of each sample was 100 µl). After over-night incubation at 4 °C, the samples were centrifuged at 15000 x g at 4 °C for 10 min; the pellets were washed with 100 µl buffer, resuspended in 20 µl Laemmli sample buffer, and then loaded to SDS/PAGE. The same samples were used for immunoprecipitation with the anti-rat IgG coupled with G Affinity Gel (Sigma). The respective beads were collected and were washed three times with 500 µl buffer, the beads were resuspended in 20 µl Laemmli sample buffer, and then the samples were boiled. Immunoprecipitates were analysed by SDS/PAGE in each case.

2.7. Mass spectrometry

SDS-PAGE purified proteins were reduced with DTT (10 mM DTT, 25 mM NH₄HCO₃, 30 min, 56 °C) and alkylated with IAM (55 mM W33 mM NH₄HCO₃, 30 min, room temperature). This was followed by digestion with trypsin (4 h, 37 °C) and extraction of the peptides. The digests were purified on C18 ZipTip and unfractonated tryptic digests were analyzed on a Bruker Reflex III matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometer in reflectron mode. DHB was used as the matrix. In the MALDI-TOF mass measurements external calibration was used. Post source decay (PSD) spectra were collected in 12–14 steps, lowering reflector voltage by 25% in each step, and then stitching the data together. The peak lists were generated with X-Tof (version 5.1.5) software. Mascot database search software (http://www.matrixscience. com/) was used to identify the proteins in the full NCBI database (2007). Monoisotopic mass of precursor ions within ±20 ppm mass accuracy were considered in the peptide mass fingerprint search, while average mass fragment ions within ±1 kDa were considered for the PSD data. Only tryptic peptides, with two missed cleavages were permitted. Cys-carbamidomethylation was considered as fixed modification, while a series of variable modifications, such as pyroglutamic acid formation from N-terminal Gln residues, the oxidation of methionine and the acetylation of protein N-termini were also permitted. Protein scores greater than 78 were accepted as significant (P < 0.05).

2.8. DNA manipulations of TPP/p25 coding sequence, cell culture and transfection and live cell imaging

The coding region of human TPP/p25 was fused to the C terminus of EGFP by cloning TPP/p25 ORF into pEGFP-C1 (Clontech) using the Bgl II and the EcoRI restriction sites (pEGFP-TPPP/p25) as described previously [5]. HeLa (American Type Culture Collection, CCL-2) cells were grown and transfected as described earlier [5]. For immunofluorescence studies, cells were grown on 12 mm round coverslips and transfected with 50 ng DNA, otherwise they were grown on 60 mm dishes. To characterize the mitochondrial membrane polarization in live experiments, tetramethylrhodamine ethyl ester (TMR) excitation and fluorescence were monitored at excitation and emission wavelengths of 590 nm and 530 nm, respectively. The excitation was used at 550 nm. Transfected cells were incubated with 50 nM TMR in complete medium for 20 min. Cells on coverslips were washed with incubation medium (PBS supplemented with 50 mM TMR, 1 mM sodium pyruvate and 1 g/L glucose), then the coverslips were placed on slides with cells upside. The cells were covered in incu-
bation medium with an extra coverslip, and were sealed with molten agarose. Fluorescent images of live cells were recorded within 1 h on tempered microscopic stage.

2.9. Immunocytochemistry

Transfected HeLa cells were fixed with cold methanol for 12 min, were rehydrated in PBS, and next were blocked for 60 min in PBS containing 10% fetal calf serum (FCS) (FCS-PBS). Subsequently, cells were stained with anti-GAPDH antibody overnight at 4°C, followed by Texas-Red conjugated anti-rat antibody (Jackson Laboratories) for 1 h at 25°C, all diluted in FCS-PBS. After washing in PBS, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and coverslips were mounted on Gel Mount Aqueous Mounting Medium (Biomeda). Images were recorded on Leica DMLS microscope (Leica Microsystems, Germany) equipped with cooled CCD camera (Spot, Digital Instruments, USA), with C-PLAN 100x immersion objective. The signal of EGFP was investigated by narrow band GFP filter set (Chroma Technology Inc.). Texas-Red signal was detected with Leica filter set N2.1 (illumination of samples was kept minimally in live experiments). Spot 4.0.9 was used to acquire digital images. Analysis of digital images was done with ImageJ (PC-based version of NIH Image).

2.10. Immunohistochemistry

Formalin fixed paraffin-embedded tissue blocks from the mesencephalon including the substantia nigra of one neuropathologically confirmed PD case (76-year-old woman) was obtained. Rat polyclonal anti-GAPDH antibody (1:20), anti-TPPP/p25 (1:200), and anti-α-synuclein (mouse monoclonal, 1:10000, Signet) after a pretreatment of digital images was done with ImageJ (PC-based version of NIH Image).

3. Results and discussion

3.1. TPPP/p25 interacts with GAPDH in bovine brain extract

The major objective of this work was to identify new interacting protein(s) of TPPP/p25, a tubulin binding unstructured protein [1], the physiological and/or pathological functions of which are obscure. Two sets of experiments were performed: immunoprecipitation and affinity chromatography. Cell-free extract from bovine brain was prepared for these experiments. In some cases, microtubular proteins were removed from the extract to abolish the possible competition of the new target protein(s) and microtubular proteins for TPPP/p25 binding, or exogenous recombinant TPPP/p25 was added to the extract to enhance the amount of immunoprecipitate.

In these co-immunoprecipitation experiments, the brain extract was pre-incubated with anti-TPPP/p25 serum raised against human recombinant TPPP/p25 protein. Fig. 1A shows the SDS/PAGE images of control samples as well as those of the immunoprecipitates. No pellet (protein) was obtained if the extract or the antiserum was preincubated alone. However, immunoprecipitate was formed when both the extract and the specific antiserum were co-incubated. The SDS/PAGE analysis of this fraction revealed the presence of some protein in the precipitate. In addition to the protein bands corresponding to TPPP/p25 and the heavy and light chains of IgG (~60 and ~23 kDa), a characteristic band of a protein with ~35 kDa was visible on the SDS/PAGE image, the intensity of which was not enhanced by the addition of exogenous TPPP/p25 (Fig. 1A). This protein band was identified from a trypptic digest by mass spectrometry. This band corresponds to GADPH (NCBI LOCUS: AA102590, NP_001029206, P10096, MW 36 kDa). 64% of the detected masses matched predicted tryptic peptides of the protein and 51.6% of the protein sequence was covered by these fragments (Fig. 1B). This identification was confirmed by MS/MS (PSD) analysis of MH⁺ = 1032.51 and MH⁺ = 1763.62, that correspond to sequences VKGV-MMHGR [2–11] (Fig. 1C) and LISWYDNEFGYSNR [308–321] (data not shown).

In the other set of experiments, TPPP/p25 affinity column was prepared as described in Section 2, and bovine brain extracts were loaded onto the affinity column to isolate protein(s) interacting with TPPP/p25. Fig. 2A shows the SDS/PAGE
images of the loaded extracts with or without endogenous tubulin, the controls and the fractions retarded on the affinity column. In the bound fraction two intensive protein bands of 50 kDa and 35 kDa can be visualized. As a control, a mocked column (prepared using the same procedure, but without bound TPPP/p25) did not retard any protein from the brain extract. Western blotting using specific anti-tubulin antibody
or an antiserum raised against GAPDH provided evidence that the 35 kDa and 50 kDa bands corresponded to GAPDH and tubulin, respectively; and that the binding of GAPDH to the immobilized TPPP/p25 was rather independent of the presence of microtubular proteins in the loaded extract (Figs. 2B and 2C). The intensity of the ~50 kDa protein band in the retarded fraction significantly decreased when tubulin-free extract was loaded (cf. Fig. 2C). This faint band was identified as Bos taurus elongation factor 1 alpha (NCBI LOCUS: CAB88863 MW 50 kDa) by mass spectrometry. 17% of the detected masses matched predicted tryptic peptides of the protein and 17% of the protein sequence was covered by these fragments. This identification was confirmed by MS/MS analysis of MH⁺ = 1025.61 and MH⁺ = 1314.74, that correspond to sequences IGGIGTVPVGR [256–266] (Fig. 2D) and EHALLAYT-LGVK [135–146] (data not shown).

To test the nature of the interaction of TPPP/p25 with GAPDH, similar sets of experiments were carried out on TPPP/p25 affinity column as described above, except that NAD⁺ or salt was added to the extract. As shown in Fig. 2A, at the addition of 0.5 mM NAD⁺ the binding of GAPDH to the immobilized TPPP/p25 was reduced; at 5 mM NAD⁺ concentration virtually no GAPDH binding was detected. To test the effect of physiological salt concentration, the bound GAPDH was eluted with 150 mM NaCl-containing buffer. No significant binding of GAPDH to the immobilized TPPP/p25 from brain extract could be detected in these cases (data not shown). These data, therefore, suggest that the association of TPPP/p25 with GAPDH is NAD⁺ and ionic strength sensitive. It should be added that the binding of tubulin to the TPPP/p25 column was also salt sensitive, even if in less extent than that of GAPDH. About 150 mM salt concentration

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Fig. 4. Localization of endogenous GAPDH in HeLa cells expressing EGFP-TPPP/p25 at different expression levels. Note the homogeneous cytosolic distribution of GAPDH at low expression level (A–C) in contrast to its aggregation within the aggresome-like body at high expression stage (D–F). Pictures (G–K) represent different level of mitochondrial membrane polarization measured by TMRE uptake. Note the highly polarized mitochondrion at low expression level of EGFP-TPPP/p25 (G–I) similar to that of the untransfected cells (red only), in contrast to the membrane potential damage of the highly expressing cells (J–K).
significantly reduced the binding of tubulin to the TPPP/p25 affinity column (data not shown), although their association has been extensively demonstrated in various alive cells [2,5]. A plausible interpretation of findings could be the crowding effect in vivo, which is known to significantly increase the heteroassociations of proteins [16].

3.2. Interaction of TPPP/p25 and GAPDH is direct
ELISA was used to test whether the interaction between TPPP/p25 and GAPDH is direct, and whether it occurs at physiological conditions concerning the concentration of NAD⁺ and salt. For this purpose the following ELISA experiments were performed. Rabbit muscle GAPDH, with and without NAD⁺, in absence and in the presence of physiological salt concentrations was immobilized on the plate and various concentrations of TPPP/p25 were added to the wells. TPPP/p25 bound to GAPDH was detected by the addition of anti-TPPP/p25 antiserum as primary antibody (cf. Section 2). As shown in Fig. 3, the two isolated proteins interact with each other in a concentration dependent manner; however, the interaction is practically resistant against the effects of NAD⁺ and salt concentrations. (The experimental curve measured at low ionic strength does not reach saturation probably due to unspecific binding.) The results obtained with purified proteins are apparently in disagreement with the finding evaluated from experiments with brain extract. However, taking into account the significant difference in the complexity of the two systems, one can suggest that the nature of the interaction, the interacting forces are different. This difference could be due to indirect, piggy back binding of GAPDH, or GAPDH displays multiple interactions with TPPP/p25 and other targets.

3.3. Co-localization of GAPDH with TPPP/p25 in aggresome-like aggregate of HeLa cells
In order to get information whether the interaction of TPPP/p25 with GAPDH occurs under intracellular conditions, we studied the co-localization of these proteins. EGFP-TPPP/p25 was expressed in HeLa cells, and the localization of the green fluorescent fusion protein and the immunostained endogenous GAPDH were visualized by epifluorescence microscopy. The untransfected cells (no green signal) appeared as “red” ones due to the immunostaining of endogenous GAPDH with Texas Red-conjugated secondary antibody. As we demonstrated previously [5], while the empty EGFP-C1 vector expressed green fluorescent protein distributed homogeneously within the cell, EGFP-TPPP/p25, at low expression level, was aligned along the microtubular network in HeLa cells [5]. Under this condition GAPDH did not show co-localization with the TPPP/p25-decorated microtubule system, it distributed homogeneously within the cytosol similarly to that of untransfected cells (Fig. 4A–C). High expression of EGFP-TPPP/p25 resulted in significant ultrastructural alterations of the microtubular network with concomitant appearance of a protein aggregate (aggresome-like body) around the centrosome [5]. In these cells GAPDH did not display uniform distribution in the cytosol, but it co-localized with TPPP/p25, which are extensively enriched within the protein aggregates (see orange cell in the merged image, Fig. 4F). To obtain quantitative data for the overlap of TPPP/p25 and GAPDH we counted the transfected cells which contain aggresome-like bodies at high TPPP/p25 expression level. The GAPDH was enriched in 10–15% of the aggresomes.

To clarify if the cells forming aggresome-like structure by overexpression of TPPP/p25 execute apoptotic death, DAPI staining was performed (cf. Fig. 4). Rather interestingly, nei-

Fig. 5. Immunohistochemistry (A, B) and double immunolabelling for GAPDH (red) and α-synuclein (green) (C), and TPPP/p25 (green) and α-synuclein (red) (D) in the substantia nigra from a patient with Parkinson’s disease. All images show a neuronal intracytoplasmic Lewy-body. In addition, in the left corner of (D), fragments of Lewy neurites are also shown. Bar represents 10 μm in A and B, and 20 μm in C and D.
ther intranuclear localization of GAPDH nor DNA fragmentation was visualized, which suggested that TPPP/p25 overexpression did not induce apoptosis. The polarization state of the mitochondrial membrane related to the energy metabolism of living cell was investigated by TMRE, a fluorescent dye which stains exclusively the hyperpolarized mitochondrial membrane [17]. As shown in Fig. 4G–I in the case of the untransfected cells and of those expressing TPPP/p25 at low level, the mitochondrial membrane appeared to be hyperpolarized. However, at high expression level of EGFP-TPPP/p25, when aggresome-like body was formed, red fluorescence is virtually undetectable. This indicates that the mitochondrial membrane potential is collapsed resulting in the depletion of both cytosolic and mitochondrial NAD\(^+\) pools by opening permeability transition pore [18]. Therefore, non-physiological situation may favour the co-accumulation of TPPP/p25 and GAPDH within the aggresome-like body. This situation probably mimics pathological circumstances, when the accumulation of proteins occurs at specific microenvironment.

3.4. Co-localization of TPPP/p25 and GAPDH in human pathological brain

The co-localization of TPPP/p25 and GAPDH was tested under pathological conditions in human brain tissues. Previously we reported the extensive co-localization of TPPP/p25 with \(\alpha\)-synuclein and their enrichment in inclusions characteristic for \(\alpha\)-synucleinopathies including LBs of post-mortal PD brain [6]. Immunostaining for \(\alpha\)-synuclein as well as for TPPP/p25 revealed numerous typical intra- and extracellular LBs and Lewy neurites throughout the substantia nigra and less in the dorsal raphe (data not shown). Herein, we tested the localization of GAPDH within LBs in nigral sections of PD.

Fig. 5 shows the immunohistochemical studies by using antibodies against TPPP/p25, \(\alpha\)-synuclein and GAPDH. Almost complete co-localization of TPPP/p25 with \(\alpha\)-synuclein can be visualized within LBs in agreement with our previous data where 90% co-localization was quantified [6]. In contrast, GAPDH immunoreactivity can be seen only in the rim of typical compact LBs (Fig. 5A and B), thus its co-localization with \(\alpha\)-synuclein (Fig. 5C) is focal. This finding qualitatively corresponds to a recent data where about 20% co-localization of \(\alpha\)-synuclein with GAPDH was observed in inclusions of PD [8, 19].

3.5. Conclusion

Recent in vitro and in vivo data have shown that the primary target of TPPP/p25 is the tubulin/microtubule, and its physiological function is likely the stabilization of the microtubular system, which is fulfilled via its bundling activity [20]. The interaction of TPPP/p25 with \(\alpha\)-synuclein, a marker of PD and other synucleinopathies, was demonstrated as well [21]. Our present data suggest that TPPP/p25 interacts with GAPDH, however, the nature and strength of this interaction depend on the presence of other proteins, which can form protein complexes with GAPDH or just function as crowding agents, and on the microenvironment such as local ionic strength or NAD\(^+\) concentration. In fact, the effect of NAD\(^+\) on the multiple interactions of GAPDH has been reported suggesting the role of NAD\(^+\) binding site in its non-glycolytic function [9]. Extreme circumstances could occur within an aggresome-like body and LB. Our present data suggest that these conditions favour the assemblies of TPPP/p25, \(\alpha\)-synuclein and GAPDH (cf. Figs. 4 and 5). The state of our knowledge with respect to the intracellular situation is still in its infancy. It may be accurate to say that the post-translational modifications of proteins play a role in the neurodegenerative process. Thus, for example, GAPDH was found to be an excessively nitrated protein in AD hippocampus [22], or phosphorylated in postsynaptic density [23]. These modifications result in inactivation or different associative capability of GAPDH (for review see [9]). It is worth noting that apart from GAPDH, elongation factor 1 alpha and TPPP/p25 were found as well in postsynaptic densities [23, 24].

The knowledge of these molecular interactions through which cells regulate intracellular localization and function as well as formation of pathological entities is crucial, because such factors would clarify not only the genesis of LBs but could also provide clues about the therapeutic targets in PD and other synucleinopathies.

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