MICROTUBULAR ULTRASTRUCTURES AND FUNCTIONS

effects of phophofructokinase, a glycolytic enzyme and TPPP/p25, an unstructured protein

PhD Thesis

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INTRODUCTION

The structure of eukaryotic cells is a beautiful example of the functional organization, in which spatiotemporal regulation of different processes manifests itself through different mechanisms. The cell's internal structure, in which the microtubule system is an important filament system, is not static; it dynamically changes depending on its tasks and environment. The microtubule system is formed from tubulin subunits by polymerization, a dynamic assembling process. The microtubule network is involved in cytoskeleton organization, transport processes, cell motility and the cellular process development beside a number of other functions. The diverse functions of the microtubular system built up from conservative tubulin subunits are regulated on one hand by microtubule associated proteins of high-affinity binding, MAPs, on the other hand by dynamically associated cytoplasmic proteins. The glycolytic enzymes can be classified to the latter group, they play a key role in glucose metabolism, the fundamental source of energy metabolism. The glycolytic enzymes in big compartments of the cells, where the protein concentration is high, form complexes with each other, bind to the cytoskeleton, membranes, but they can also interact with other metabolic enzymes or proteins. All these effects are of significant functional consequences, which are involved in energy balance of both physiological and pathological tissues.

The phosphofructokinase (PFK), which is a key enzyme regulating glycolysis, is only active in tetrameric form. The isoform (PFK-M) occurring in muscles and other tissues, like brain, dissociates into inactive forms on dilution or by specific metabolites, nucleotides. Consequently, any influence that affects the equilibrium between oligomer forms, substantially affects also the cytoplasmic energy production through the activity of PFK, namely, the intracellular ATP level. Our research group’s and other literary data show that the cytoskeleton is a potential target for the associations of glycolytic enzymes. Glucose as energy source is particularly significant in the brain, and the tubulin is an important constituent of the cytoskeleton.

Tubulin Polymerization Promoting Protein (TPPP/p25), has been identified in our research group as a brain-specific, intrinsically disordered protein, its principal partner is the microtubule system. After the discovery of the human proteome it has become clear that about one third of the hypothetical proteins do not have a well-defined tertiary structure. The lack of well-defined 3D structure, the unfolded state renders it possible for proteins to fulfill multiple functions with diverse interacting partners, or to manifest itself multiple features. The disordered proteins play an important role in those diseases where the consequence of conformational freedom results in the generation of structures which are toxic or deteriorative for the cells or tissues. The mechanisms responsible for

this are the aberrant protein-protein interactions leading to aggregation, inclusion formation (conformational diseases). Such typical unstructured or disordered proteins are β-amyloid, α-synuclein or mutant huntingtin.

TPPP/p25 occurs in normal brain tissue mostly in oligodendrocytes, the main task of which is the synthesis and generation of myelin sheath for axonal processes; the process of myelinization is indispensable for the normal physiological brain function. Their characteristic bipolar progenitor cells search the functional axons to establish myelin sheath. A number of proteins are expressed during the myelinization in coordinated manner with concomitant transformation of the cytoskeleton of oligodendrocytes. Microtubules play a key role in the rearrangement of the oligodendroglial cytoskeleton.

OBJECTIVES

My PhD thesis aims to characterize those microtubule ultrastuctures, which are generated by cytoplasmic, dynamically associated proteins interacting with microtubules; on the other hand to examine the function of these ultrastructures in physiological and pathological processes, to analyze them at molecular and cellular levels. Consequently, the major objectives were to characterize

i) the interaction of the microtubule system and an important glycolytic enzyme, the PFK *in vitro*, the consequences of this interaction on kinase function and on the structure of the microtubule system, the effect of the specific modulators on the interaction;

ii) the molecular and cellular effects of the expression of TPPP/p25, an unstructured protein, on the energy metabolism;

iii) the interaction of TPPP/p25 with the microtubule system and its structural and functional consequences in single cell experiments;

iv) the role of endogenous TPPP/p25 in formation of projections of rearranging microtubular network during the oligodendrocyte differentiation.

METHODS

Preparation of tubulin from bovine brain.
Fluorescence anisotropy measurements.
Measurement of phosphofructokinase enzymatic activity.
Follow-up of tubulin polymerization by turbidimetry.
Binding studies by pelleting.
Antiserum production and characterization.

Cell culture and differentiation.
Transient transfection of human cells with fluorescent fusion protein.
Stable cell line production, clonal selection.
Characterization of mitochondrial membrane polarization.
Primary production of oligodendrocyte culture.
Immunofluorescence studies.
Microscopic image analysis.

RESULTS

1. Tubulin and its polymerized form, microtubules (MT) was identified as potential interacting partner of phosphofructokinase. Interaction with the inactive dimers results in an extra enhancement in the turbidity indicating ultrastructural alteration. Electron microscopy identified specific microtubular ultrastructure, cross-bridged MTs formed by the association of PFK dimers attached to the taxol-stabilized MTs,. The consequence of the PFK interaction with MT results in decreased enzyme activity. On the basis of structural and functional studies with isolated proteins the affinity constant of MT-PKF (K_d) was determined which was in micromolar range indicating that the complex formation could be significant at physiological concentrations. PFK modulator compounds, such as ATP or fructose-2,6-bisphosphate, inhibited the binding of PFK to tubulin/MT, resulting changes in the stability of microtubule structures and the kinase activity.
2. The effect of the unstructured TPPP/p25 on glucose metabolism was studied in live human cells. It was found that in the presence of the unstructured protein the mitochondrial membrane polarization increased expressing positive effect on cell energy status. In concern with this issue, significant increase in the rate of glycolysis and the ATP level by the presence of TPPP/p25 was demonstrated in which PFK activation played an important role.
3. It was demonstrated that in HeLa cells TPPP/p25 targeted the microtubule network, it was aligned the microtubular network. TPPP/p25 cross-binded MTs causing their stabilization and resistance against depolymerizing effects. The inhibition of ubiquitin-proteosome system (UPS) resulted in increased TPPP/p25 level indicating the USP involvement in the regulation of the intracellular TPPP/p25 level .
4. The overexpression of EGFP-TPPP/p25 results in the development of novel microtubular ultrastructures: the aggresome, in the region of the centrosome, and the cage, which is formed from bundled microtubules around the nucleus. Novel, specific antibodies were developed for immunostaining TPPP/p25 and used for studies of human pathological brain tissues by immunofluorescence microscopy and immunohistochemistry. TPPP/p25 was identified in inclusions characteristic for synocleinopathies, like Lewy-bodies of Parkinson's disease or oligodendroglial cytoplasmic inclusions of multiple system athrophy (MSA). However, in oligodendrogliomas, TPPP/p25 could not be detected, while its occurrence in a normal oligodendrocytes is essential.
5. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an important glycolytic enzyme was found to be enriched in inclusions of Parkinson's disease samples. In agreement with this,

localization of GAPDH was identified in TPPP/p25-based aggresomes in HeLa cells, while in control cells GAPDH showed a homogeneous distribution in the cytosol.

6. It was proved that the level of TPPP/p25 increases significantly during oligodendroglial differentiation, TPPP/p25 showed co-localization with microtubule system in the projections, indicating its essential role in the microtubule system-derived projections stabilization. The enhancement of TPPP/p25 expression was shown to be linked to the expression of other proteins such as myelin basic protein or the class IV β-tubulin which are established markers of the oligodendrocyte differentiation,. Single cell experiments with oligodendrocytes proved that the inhibition of the expression of TPPP/25 with conservative specific microRNS, mir-206, arrested the differentiation process.

CONCLUSIONS

Dynamic associations of cytoplasmic proteins, in addition to the effects of statically linked, well-known MAPs, play an important role in the decoration of intracellular microtubule system, development of its ultrastructures and the diversity of its physiological functions. The microtubule system is a dynamic one which requires energy, which is primarily originated from glucose metabolism through glycolysis in the cytoplasmic compartment. Both the structural and functional characteristics of the glycolytic enzymes, including cell-specific isoforms, are well known at molecular level. However, their characteristics are less obvious at cellular and system level. Our research group and other laboratories over the past decade have provided evidence that the intracellular associations of glycolytic enzymes involving their cytoskeletal associations are major factors in the regulation of energy metabolism, in the determination of the ATP level..

My research on a glycolytic enzyme, PFK and the microtubule system was connected to the idea of compartmentation of the glycolytic enzymes, a hot topic in bioenergetics. The catalytic activity of PFK depending on its oligomerization state and its association to tubulin/microtubule system was characterized and showed its potential in the complex regulatory mechanisms of PFK. This mechanism is capable of ensuring a microtubule-driven system-level regulatory mechanism for the glycolysis by modulating PFK activity. Important function of PFK is also shown from my systemic cellular experiments, namely that an unstructured protein, the TPPP/p25 expression in neuroblastoma cells stimulates the energy metabolism, increases ATP level through the activation of the glycolytic flux.

My results also demonstrated that the tubulin/microtubule-PFK interaction has consequences for both participants: PFK is not only influenced in its catalytic activity, but the heterologous interaction results in stable microtubular ultrastructures. These stable structures resistant against

depolymerizing effects are formed by microtubule-bounded dimeric PFK molecules creating periodic cross-bridges due to dimeric-dimeric PFK interactions. The PFK-microtubule interaction may provide a new regulatiory mechanism for the glycolysis. At system level, however, further interactions amongst glycolytic enzymes, with the microtubule system and other cytoplasmic structures should also be taken into account. Our team's recently published results point out that heteroassociations of glycolytic enzymes are capable to create microcompartment in Huntington's disease transgenic mouse brain, by this way overcompensate the loss of GAPDH activity in the affected area by the increase of other glycolytic enzymes activity.

My research on live and fixed HeLa and SK-N-MC cells expressing EGFP-TPPP/p25 can answer exciting questions such as what happens with an unstructured protein within the cells. My results shed light on the fact that the TPPP/p25 targeted the microtubule network otherwise it was degraded by the proteosome system. The "bundling" of MTs by TPPP/p25 stabilized microtubules, which displayed resistance to depolymerizing effects. This effect does not hinder the rearrangement of the microtubule system, because the interaction is dynamic as demonstrated by Fluorescence Recovery After Photobleaching (FRAP) experiments showing very fast exchange rate of TPPP/p25 on microtubule network.

In normal brain tissue TPPP/p25 is expressed primarily in oligodendrocytes which play crucial role in the oligodendroglial differentiation. This microtubule-based physiological process could be inhibited by specific microRNS; miR-206 was identified as a powerful inhibitor of the differentiation of oligodendrocytes. Therefore, TPPP/p25 is a key player of the neuronal myelinization which could be regulated by its proteolitic degradation and posttranscritionally.

Involvement of TPPP/p25 in certain pathological brain processes was demonstrated which could be connected with the aggresome formation observed in living cell during over-expression of TPPP/p25. The accumulation of TPPP/p25 in pathological inclusions of neurons and glial cells together with α-synuclein was detected. The TPPP/p25 was found to be enriched in inclusions specific for Parkinson's disease and other synucleinopathias , while the phenomenon was not typical in Alzheimer's disease and other tauopathias. Thus, TPPP/p25 is not only a new MAP-like protein affecting microtubule ultrastructure but it is a potential biomarker of synucleinopathies. In the light of these results, the established experimental systems or a similar one could be useful as disease models to understand pathomechanism of certain neurodegenerative diseases at molecular and cellular level.

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