REVIEW

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On the thesis of Prof. Jordan Atanassov Doumanov, PhD

"Organization and surface features of hBest1 protein in models of biological membranes" presented for the awarding of the scientific degree " Doctor of Sciences" in the Area of Higher Education 4. "Natural sciences, Mathematics and Informatics", Professional direction 4.3 Biological Sciences (Molecular Biology)

Brief biographical data

Prof. Jordan Atanassov Doumanov was born in 1973 in Bansko. In 1999 he graduated from Sofia University "St. Kliment Ohridski " MS in Biology with specializations - Cell Biology and Biology of Reproduction as well as Teacher of Biology. Since 2001 he was a PhD student at the University of Hohenheim, Stuttgart, Germany, where he successfully defended his PhD thesis on "Identification of the basolateral sorting signal in the cytoplasmic domain of the interleukin-6 signal transporter gp130" in 2006. After defending his PhD thesis he started his professional career in the Department of Biochemistry at SU "St. Kliment Ohridski ". Since 2015 he has been an associate professor and full professor since 2021 in the same department. Prof. Doumanov has been abroad several times on specializations in leading laboratories in the field of biochemistry and molecular biology in Germany, France and Spain, which has had a positive impact on his scientific qualification.

Currently Prof. Doumanov is a full professor in the Department of Biochemistry. He lectures basic courses at the Faculty of Biology and at the Faculty of Physics in the same University. He is a member of the Management Bord of the Scientific Research Sector at SU "St. Kliment Ohridski". Prof. Doumanov has published 64 scientific papers with a total IF 157.06, which have been cited 183 times. He was a Principal investigator of 6 research projects and participant in 14 others. He has supervised 7 MS as well 3 successfully defended PhD thesis. Some of the data in these PhD thesis are related to the topic of his thesis presented for the Doctor of Sciences.

Overview of the documentation related to the thesis and the author summary

All the documents related to the defense procedure, as well as the thesis itself and the author's summary have been prepared in the accordance of the requirements of the Low for the Development of the Academic Staff of the Republic of Bulgaria and the relevant regulation for its implementation of Sofia University. The thesis covers 196 pages and 294 references. The obtained results are illustrated with 3 tables and 54 figures and 30 appendices. The thesis is structured in a classic style: Introduction, Review of the Literature, Aims and Tasks, Materials and Methods, Results and Discussion, Conclusions, Contributions and References. The results of the thesis were reported at 18 articles and have been cited

44 times so far. Prof. Doumanov has participated with his results in several scientific forums. The Summary of the thesis covers 63 pages and fully corresponds to the structure and content of the thesis.

Actuality of the problems

The vision is one of the main senses through which people perceive the surrounding world. Light is received by the retina of the eye, which is made up of different types of cells, process it and transmit it to the brain so that the body can adequately respond to the changes that have occurred. One of the clinical pathologies in vision disorders is associated with mutations of the BEST1 gene called "bestrophinopathies". These changes lead to pathological degenerative conditions of the macula which have a very large social effect and the clarification of the reasons for the degenerative conditions of the macula are very relevant and of great social importance. The presented thesis is distinguished by the originality of the topic, the methods with which the research was conducted and its relevance. The thesis is based on a large number of genetic, biochemical, biophysical, physicochemical and molecular biological studies to clarify the relationship between the structure and function of the hBest1 channel protein and their pathophysiological significance.

Main results of the thesis

1. Several types of cell lines were investigated to demonstrate the protein expression and sorting of hBest1. In these studies, the MDCK II cell line was found to be the most successful model. In this cell line, the apical and basolateral domains can be easily distinguished, which is essential for the future studies, and it has been shown that hBest1 is preferentially localized to the basolateral membrane of the cells.

   To determine the reasons for localization of the protein to the basolateral membrane, hBest1 mutants were generated by site-specific mutagenesis at the basolateral tyrosine and dileucine sorting motifs, as such mutations have been observed in patients with macular degenerative conditions. The resulting mutant cells show a change in the localization of the protein in the direction of the apical domains. Through these studies has been proven the most important motif for the correct localization of the protein important for its proper functioning. Phosphorylation processes are essential for the correct localization of the protein, as the non-phosphorylated tyrosine in the investigated motifs is one of the main factors.

   To continue the studies, a MDCK II cell line stably transfected with hBest1 was established. The resulting cell line is characterized by a strong signal for hBest1, a correctly localized protein in the cell membrane, which does not change the growth profile and morphology of the cells as compared with the original cell line, as well as the time of polarization. When tracking changes in transepithelial resistance (TER) during polarization processes, close values were observed with the original cell line, which decreased after the fourth day of cultivation. After the seventh day of cultivation transepithelial resistance increased compared to the baseline cell line. These changes are due to the changes of ion flows in the extracellular space due to the presence of a large amount of hBest1 protein in the plasma membrane. Increased levels of the protein also explain changes in cell resistance, as tight junctions are altered by altered levels of ionic conductance. Due to the influence of Glu, GABA and ATP on the functional activity of hBest1, TER was investigated in MDCK II-hBest1 in the presence of these compounds. When treating the new cell

line with Glu and GABA, higher TER values were found due to a decrease in hBest1 activity, while ATP activates the protein and this leads to a decrease in TER.

2. To follow the influence of membrane bilayer structure on the role of hBest1 for transepithelial resistance, the untransfected and transfected cell lines were treated with $sPLA_2$. The results of this study showed a strong effect of phospholipase on untransfected cells, leading to a decrease in TER, while very weak changes were observed in transfected cells. $sPLA_2$ induces changes in the phase state of cell membranes, which in turn affects the structure and function of hBest1 in the direction of inhibiting its channel activity and increasing TER.

3. Since it is known that the function of transmembrane proteins depends on the lipid environment, the lipid composition of the cell membranes of both types of cells was studied. Increased amounts of phosphatidylinositol, phosphatidic acid, cardiolipin, lysophosphatidylcholine and lysophosphatidylethanolamine were found compared to non-transfected cells. An elevation of neutral lipids, phosphatidylcholine, and phosphatidylserine were observed in untransfected cells. These data elucidate the greater resistance of transfected cells to the action of $sPLA_2$. It can be hypothesized that the expression of hBest1 is associated with an increased content of non-lamellar lipids in the membranes and the possible role of the mutant forms for membrane remodeling in the pathological conditions. Examination of the structure of the cell membranes of both cell types demonstrated that the level of Ld domains in the transfected cells was increased compared to the non-transfected ones, which is evidence by the greater fluidity of the membranes in the transfected cells. This correlates with the change in established lipid composition in the transfected cells. By means of immunofluorescence it was proved that the localization of the protein is mainly in the Ld domains and that the activation of the protein takes place predominantly in the more disordered phase of the membranes.

4. The expression and localization of different mutant forms of hBest1 in MDCK II were investigated. Their localization was shown to be impaired compared to the normal protein. In the apoptosis assay, cells transfected with the wild-type protein showed higher survival, while early and late apoptosis were increased in the mutant forms. A change in the localization of the mutant forms in the cell membrane was also found, which leads to a change in ion transport and cell homeostasis. It has been suggested that that is due to a change in the lipid composition of the membranes.

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The expression and localization of different mutant forms of hBest1 which cause BVMD in MDCK II were investigated. Their localization was shown to be impaired compared to the normal protein. In the apoptosis assay, cells transfected with the wild-type protein showed higher survival, while early and late apoptosis were increased in the mutant forms. A change in the localization of the mutant forms in the cell membrane was also found, which leads to a change in ion transport and cell homeostasis. It has been suggested that this is due to a change in the lipid composition of the membranes.

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5. In order to investigate the behavior of hBest1 in model membranes, the protein was isolated and purified from the newly established MDCK II-hBest1 cell line. Gel filtration in combination with affinity chromatography was found to be the most successful method. Since no data exist in the literature on the secondary structure elements of the protein the secondary structure elements of human hBest1 protein were determined by Fourier-transform infrared spectroscopy. These studies prove that the secondary structure composition of a protein includes mainly helical structural elements - large and short α-helices, as well as 3_{10} -helices. After the addition of Ca^{2+}, conformational changes were observed in the direction of an increase in α-helical structures, which determines the functional activity of the protein.

6. One of the models of biological membranes are Langmuir monolayers and Lagmuir-Blodgett films. In order to determine the surface properties of hBest1, as well as the influence of Ca^{2+}, Glu and GABA on the structural and surface properties of the protein, the behavior of the protein in Langmuir monolayers under physiological conditions was investigated. The addition of Ca^{2+}, Glu and GABA was found to induce changes in the average molecular area. Determination of the lowest possible area of the molecule has shown that the area does not change in the presence of Glu and GABA, while the presence of Ca^{2+} results in a significant decrease in the area. This can be explained by a change in molecular electrostatic interactions, molecular packing density, conformation and orientation of protein molecules at the buffer/air interface. The observed differences in the molecular surface area are evidences that the protein changes its organization, as well as the packing density, which leads to changes in its physiological activity. In order to study the molecular interactions in more detail, the hysteresis of the compression-decompression of the π/A isotherms was also followed. These studies prove that the pushing of the protein in the subphase is a reversible process and is due to reorientation and reorganization of the protein molecule on the surface of the monolayer. BAM imaging of pure protein monolayers as well as in the presence of Ca^{2+}, Glu, and GABA demonstrate differences in density and packing due to changes in protein conformation, protein–protein interactions, and self-organization at the air/buffer interface. Atomic force microscopy was used to determine the nanometric changes in the size, structure and organization of the protein molecule after treatment with Ca^{2+}, Glu and GABA. The oval shape of the protein molecule, the lateral dimensions and the height of the molecule were determined. The addition of Ca^{2+} leads to a change in the secondary structure and the appearance of stable pentameric crystals. When Glu and GABA are added, no aggregation of the protein molecules is observed and they retain their original shape. For the first time in the literature such kind of results on the structure and behavior of hBest1were reported.

7. In order to investigate the influence of phospholipids on the interaction of the protein with Ca^{2+}, Glu and GABA, studies were carried out on binary monolayers. It has been shown that the protein does not mix with POPC at the air/buffer interface. When the monolayers are compressed, the protein molecules move to the water phase, while mainly the lipid molecules remain on the surface. A fluidizing effect of the protein molecules on the POPC monolayers and increased elasticity were also found. In order to avoid the extrusion of the protein into the aqueous subphase, the studies were carried out at certain values of the hysteresis of the two-component

monolayers. After the addition of Ca^{2+}, Glu and GABA under these conditions, no statistically significant changes were observed, which is an indication that POPC eliminates the effects of these components and all changes in pure protein monolayers are due only to conformational changes in the protein molecule. The study of the miscibility of the protein and the phospholipid, show that the protein does not mix with the POPC on the surface of the subphase. The surface morphology of the mixed monolayers was investigated by AFM. The shape of the protein was more irregular , with one or two lower formation around oval protein body. After the addition of Ca^{2+} , Glu and GABA, the monolayer became completely homogeneous, but uniformly distributed bright domains of different sizes were observed, probably due to tightly packed POPC molecules. Studies of pure POPC monolayers demonstrate that the presence of Ca^{2+} leads to the formation of domains, whereas such formations are not observed in pure monolayers of the protein. A change in the shape and size of the protein molecule in mixed monolayers was detected by AFM, suggesting the presence of boundary lipids that cover the hydrophobic surface of the protein. Addition of Ca^{2+}, Glu and GABA to pure protein monolayers changes its surface characteristics and dimensions, while phospholipid neutralizes their action. By BAM and AFM it has been shown that lipid-lipid and protein-protein interactions are much stronger than lipid-protein interactions. From these results, it can be concluded that Ca^{2+}, Glu, GABA and POPC affect not only the channel functions of hBest1, but also its interaction with other membrane components and thus participate in maintaining cellular homeostasis.

Since there are phospholipids in cell membranes, which affect the physicochemical properties of the membrane bilayer in a different direction, in contrast to POPC, studies were conducted on mixed monolayers protein /sphingomyelin. Mixed monolayers are in the same lipid:protein ratios as those with POPC. It has been shown that binary monolayers do not undergo a phase transition, and the values of π_{tr} do not change after addition of Ca^{2+}, Glu and GABA. The addition of protein caused fluidization of pure SM monolayer. The interactions between SM and the protein were shown to be much stronger than hBest1-hBest1 and SM-SM. In the presence of Ca^{2+}, the hBest1-SM intermolecular interactions were almost the same as those of hBest1-hBest1 and SM-SM, suggesting complete mixing or complete phase separation. Addition of GABA leads to separation of protein and lipid molecules, but this effect decreases with increasing surface pressure. It is suggested that Glu and GABA interact with the polar SM, head group, leading to repulsion between hBest1 and SM molecules or to the incorporation of Glu and GABA into the bilayers. It has been shown that the total free energy of mixing between the lipid and the protein is not affected by the addition of Ca^{2+}, Glu and GABA. The observed differences in the interaction of the protein with SM or with POPC are due to the different structures of the two phospholipids. The conclusion is that strong protein-lipid interactions exist in mixed protein/SM monolayers. The morphology of the binary monolayers proves compact homogeneous structures of the films. Addition of GABA did not change the morphology, whereas Ca^{2+} led to a partial separation of hBest1 and SM. The addition of Glu affects the monolayer morphology much less. Ca^{2+} induces much stronger protein-protein interactions and changes in protein conformation in mixed hBest1/POPC monolayers than in hBest1/SM monolayers. All these results are evidence of the spontaneous mixing of the protein and SM, which is due to the affinity between the protein and SM in cell membranes.

8. In order to verify the influence of the condensing effect of cholesterol on the behavior of the protein, experiments were carried out with monolayers of different composition. In the presence of Ca^{2+}, the condensing effect of cholesterol is more pronounced, and the effect of Ca^{2+} is stronger in the mixed monolayers of POPC/Chol. Through a combination of experimental and theoretical studies, it has been shown that the addition of Ca^{2+} does not change the shape of the isotherms, but shifts them in the direction of a lower average molecular area. Additional research has proven that the action of the ions on the mixed monolayers is mainly due to their action on the cholesterol molecules. The viscoelastic properties of the SM/Chol monolayers prove that they are in a liquid-condensed phase. The presence of Ca^{2+} slightly destabilizes the monolayer. The pure monolayer of SM undergoes a LE-LC phase transition, but upon addition of protein or Chol and protein, this phase transition disappears, which is evidence of the good mixing of the phases. The degree of miscibility and phase separation in the ternary systems was determined. Based on these studies, it has been shown that protein–lipid interactions are much stronger than protein–protein or lipid–lipid intermolecular interactions in the presence of POPC. When Ca^{2+} was added, separation of the individual phases was observed. By Goodrich's method, it has been shown that the mixing of protein and lipid molecules is spontaneous and thermodynamically advantageous. The presence of cholesterol has been shown to improve the miscibility and stability of mixed monolayers in the presence of POPC because it reduces the phase separation between the protein and POPC, while the presence of SM does not change the miscibility and stability of the monolayer. Stabilization of the protein miscibility/phase separation between the protein and the lipid phase in the presence of cholesterol has a direct effect on the association and localization of the protein in the lipid bilayer of cell membranes.

Conclusion

The thesis submitted for the acquisition of the scientific degree “Doctor of Science" by Prof. Jordan Doumanov and the related scientific publications which are proof of the originality of the presented results, meet the requirements of the Low for the Development of the Academic Staff of the Republic of Bulgaria and the relevant regulation for its implementation of Sofia University. This work is the result of almost ten years of research related to the study of the structure, interaction and localization of the hBest1 protein in the membrane bilayer thus clarifying the reasons for changing its function in pathological conditions. The research has an interdisciplinary nature, which has allowed the author to study his goals in details. The thesis is distinguished by the originality of the methods of molecular biology and physical chemistry. I think that one of the greatest achievements of the author is the obtaining and characterization of pure hBest1 protein, as well as the establishment of a stable cell line expressing the protein. Without these results, the following studies presented in the thesis would not have been possible. Based on part of the results 3 PhD thesis were defended. The articles published in international scientific journals have found a wide response among the international scientific community. The presented thesis is a complete scientific work with original results that are unique in nature and I strongly recommended to the members of the respected scientific jury to allow this thesis to be defended

and to award the scientific degree "Doctor of Sciences" to Prof. Dr. Jordan Doumanov in Higher Education Region 4, Professional Direction 4.3. Biological Sciences (Molecular Biology).

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Reviewer:

Prof. Diana Petkova