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EFR3A protein as a potential partner of flotillin 2 and a possible organizer of resting rafts

The subject of this thesis is an attempt to define the mechanism of membrane rafts formation and regulation in animal cells plasma membrane. According to the concept proposed by Simons and Ikonen, living cell membranes are not laterally homogeneous and contain highly dynamic structures such as protein-lipid complexes (raft precursors < 10 nm) and nanodomains (resting state rafts > 10 nm). Raft precursors can link with each other and create membrane rafts called also „resting rafts", which can in turn create signalling platforms. Rafts are made up of lo-phase lipids, primarily cholesterol, and a set of characteristic proteins. Both resting rafts and microdomains/signaling platforms, by affecting changes in mobility and conformational changes resulting from association/dissociation of proteins with these domains, regulate their activity, playing a key role in the regulation of many important biological processes, including signal transmission from growth factor receptors related to proliferation and control of cell motility. They also participate in the transport of lipids and proteins. All these processes make membrane rafts important in processes related to immunity, pathogen-host interactions as well as in neoplasia.

Previous studies by the team of the Department of Cytobiochemistry indicate that specific interactions of proteins from the flotillin family and the MPP1 protein seem to be responsible for the organization and regulation of the membrane raft domain in erythroid cells. Therefore, in this work, it was assumed that the formation of a flotillin-based protein network, which interacts with specific lipids, underlies the mechanism of raft domain formation and regulation in cells that do not express *MPP1* at a high level. Flotillins are highly conservative peripheral membrane proteins. These proteins are ubiquitous in eukaryotic organisms and they belong to the proteins family which is characterised by the presence of SPFH domain (stomatin/ prohibitin/ flotillin/ HflK/C). Flotilins are one of the main membrane raft proteins that are permanently associated with nanodomains and remain closely associated with them, despite, for example, removal of cholesterol from the membrane.

In this work an affinity approach based on immobilized recombinant flotillin 2 was used to isolate and identify the protein partner using the MS/MS technique. The protein identified

by this technique may be responsible for the organization and regulation of the raft domain. Use of affinity chromatography allowed to identify EFR3A as a potential protein which interacts with flotillin 2. Interaction between flotillin 2 and EFR3A was confirmed using the same approach, but the identification was performed by Western Blot and anti EFR3A antibodies as well as by co- immunoprecipitation. The interaction between flotillin 2 and EFR3A was also confirmed by an overlay assay using recombinant EFR3A protein and recombinant flotillin 2. Moreover, it was found that EFR3A is a stable component of the HeLa DRM fraction and that its presence was found to be sensitive to removal of cholesterol from the membrane. Silencing of EFR3A gene expression may support the hypothesis that this protein is a stable component of the raft domain and may be involved in the organization and regulation of this domain. In cells with a reduced level of EFR3A, so-called KnD cells, a reduction in membrane order of living cells was observed. This was also the case for giant plasma membrane vesicles (GPMV) derived from KnD *EFR3A* cells. In addition, data obtained by fluorescence correlation spectrometry (svFCS) indicated increased mobility of the raft probe (a fluorescent sphingomyelin analog) in *EFR3A* KnD cells. The consequences of silencing the EFR3A gene are not a random effect, but are specific and closely related to EFR3A. As shown, after transfection of KnD *EFR3A* cells with the „rescue" vector encoding *EFR3A*, membranes of living cells in FLIM and svFCS analysis showed a regressive trend of physicochemical parameters in plasma membrane. In the analysed second cell line, MCF7, less impact of silencing the *EFR3A* gene on membrane fluidity was observed.

Silencing of EFR3A expression has also been shown to affect EGF-dependent intracellular Ca^{2+} concentration. In conclusion, the obtained results indicate an interaction of flotillin 2 and the EFR3A protein, about which there is no information in the literature and databases so far. Moreover, according to several criteria, the interaction between flotillin 2 and the EFR3A protein appears to be responsible for the organization and regulation of membrane rafts. The results obtained from the cytometric analysis also indicate the participation of this interaction in the regulation of at least several cellular processes, including the ones related to the control of the cell cycle, namely observed an increase in the duration of the G0/G1 cycle and S phase compared to control cells.

This interaction has not been observed so far. According to the criteria approved for MPP1, the EFR3A protein may turn out to be the „organizer" of the raft domain because:

- interacts with flotillin 2,

- is a membrane protein located in DRM fractions and its localization is sensitive to removal of cholesterol from the membrane,
- reduction of *EFR3A* gene expression causes reduction of cell membrane order.