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Study of the interactions of IL-2 and -15 receptors on human T lymphoma cells

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UNIVERSITY OF DEBRECEN

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The Examination took place at the Discussion room of the Department of Immunology, Faculty of Medicine, University of Debrecen, at 12:00 PM, December 15, 2015.

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at 1 PM, September 4, 2017.

1. Introduction

1.1. Membrane models, lateral organization of membrane proteins

The main function of the cell membrane is the isolation of the cell from its environment, and to control communication and material transport. In the last decades several findings indicated that the improvement of Singer & Nicolson membrane model is necessary. Based on the experiments of Simons & Ikonen in 1997 when they found detergent resistance membrane fractions (DRM), the lipid raft hypothesis became widespread. Lipid rafts are mainly composed of cholesterol, sphingomyelin, glycosphingolipids (GSL) and GPI-anchored proteins. Lipid rafts are described as structural and functional protein platforms with heterogenic size and lifetime distribution.

Beyond the modulation of the movement of the membrane proteins by the viscous environment, the diffusion of membrane proteins can also be restricted by the cell cytoskeleton. The inhomogeneous protein distributions may be caused by the combination of the following factors:

1. Forces acting in the plane of the membrane: lipid domain structure of the membrane, viscous membrane domains, protein–protein interactions.
2. Intracellular factors: the cytoskeleton can directly or indirectly restrict the motion of proteins, interaction with different cytosolic signaling elements, clusterization induced by vesicular transport mechanisms.
3. Extracellular factors: electrostatic forces, cross-linking ligands and the extracellular matrix.
4. Other factors: changes in the membrane potential or other perturbing factors acting indirectly either through inducing conformational changes or via modification of the factors listed above.

These factors together control the lateral distribution of membrane elements, mobility and aggregation states of membrane proteins in a mutually interdependent manner.

1.2. Interleukin-2 and -15 receptors

The cytokines IL-2 and -15 play important roles in the activation, proliferation, death and survival of immune cells. The cytokine binding affinity of their receptors is determined by the composition of receptor complexes. The high affinity receptors are built of three subunits, two of which, the β and γ_c chains are shared by the two cytokines, while the α subunits are cytokine specific. The IL-2 and IL-15 receptors are preassembled on lymphoma/leukemia cells and form a heterotetrameric complex composed of IL-2Rα, IL-15Rα and the β and γc chains; they are

enriched in lipid rafts. Since they share the same signaling receptor chains, IL-2 and IL-15 also have several functions in common: they promote the proliferation of CD4+ T lymphocytes and augment the cytotoxicity of T and NK cells in vitro. However, they may also play opposing roles in T cells: IL-2 induces apoptosis in activation induced cell death, whereas IL-15 preferentially promotes the survival of CD8+ memory T cells and inhibits apoptosis.
The importance of the receptors is shown by the gene mutation of the γ_c subunit, which results in the formation of X-linked combined immunodeficiency syndrome (X-linked severe combined immunodeficiency, X-SCID).

1.3. Major histocompatibility complex I, II

There are two main classes of major histocompatibility complex (MHC) molecules, MHC I and II. MHC I is a heterodimer consisting of a transmembrane heavy chain and an extracellular light chain, the beta-2 microglobulin (β2m), which has no transmembrane domain. MHC II molecules are also heterodimers, they are built of two homogenous chains.
MHC I is expressed in most nucleated cells, while MHC II molecules are expressed only professional antigen presenting cells: B cells, macrophages and dendritic cells, but they can also be induced on tumor cells. MHC I presents self or viral antigens to T cell receptors expressed by CD8+ T cells initiating T cell activation upon antigen recognition. MHC II molecules present exogenous peptides to CD4+ T cells. The main function of activated CD4+ cells is controlling the development of immune cells (B cells, macrophages, CD4+ T cells), and maintaining the immune response by secretion of cytokines (interferon-γ, IL-2, -4, -5, -6, -9, -10, -13).

In the plasma membrane, MHC I can form oligomers and is enriched in lipid rafts. In addition to IL-2Rα and IL-15Rα, it can also associate with a series of other membrane proteins including receptors in various cell types: MHC II, EGF receptors, ICAM-1, insulin receptors, tetraspan molecules (CD53, CD81, CD82) and transferrin receptors also.
The first pieces of evidence for the close molecular proximity of MHC I and IL-2Rα were obtained from FRET experiments on a T lymphoma cell line and later on activated peripheral T cells. FRAP showed the slowing down of IL-2Rα diffusion after ligating MHC I with an antibody. TEM analysis indicated that the two molecules were partially co-clustered in Kit 225 K6 T lymphoma cells.

The polymorphism of MHC genes is associated with several diseases, e.g. sclerosis multiplex, type I diabetes, systemic lupus erythematosus, ulcerative colitis, Crohn's disease and

rheumatoid arthritis. Thus, in the last decades a multifaceted view of MHC I is emerging, which goes beyond its classical antigen presenting function.

1.4. c-Jun and c-Fos transcription factors

A transcription factor is a DNA binding protein that controls the rate of transcription of genetic information. Transcription factors bind to the enhancer or promoter regions of DNA of the specific genes that they regulate. The c-Jun and c-Fos transcription factors can form homo- or heterodimers by their leucine zipper domains. The heterodimer binds the elements of AP-1 (Activator protein 1), and controls a number of cellular processes including differentiation, proliferation and apoptosis in a cell type dependent manner. Their induction can be brought about by several stimuli and signaling cascades: G protein-linked receptors, growth factors (TGFβ, EGFR), neurotransmitters, UV radiation, oncoproteins, mitogen activated kinase, cyclic AMP or Ca^{2+} dependent signaling pathways. The induction of proliferation by Jun-Fos complex is achieved via growth receptors and mitogen activated protein kinases (MAPK) pathways.

In several pathological disease condition the overexpression of c-Fos was shown: in breast tumors, oral cancer, in the carcinogenesis of liver; the high expression levels can be influenced via the thyroid hormone receptor. In some cell types, c-Fos overexpression resulted in apoptosis.

c-Jun plays a role in the regulation of the cell cycle: Jun activates cyclin D transcription factors, and the lack of activation results in the G1 phase block. The overexpression of c-Jun decreases (p53, p21) the expression of certain tumor suppressors, thereby its high expression may accelerate cell division.

1.5. multiple-τ algorithm, field-programmable gate array (FPGA)

Fluorescence correlation spectroscopy (FCS) is a powerful method for determining the diffusion coefficients, photophysical properties and concentrations of fluorescent molecules in solution and living cells. For resolving photophysical and diffusion processes, the fluctuations of fluorescence are analyzed with varied time resolution. In a typical FCS measurement, the correlation time of photophysical processes is $\sim 10^{-9} - 10^{-6}$ s, the mean dwell time of diffusing molecules in the focus falls between $10^{-3} - 1$ s; therefore, the determination of the correlation function requires to store and access several data spanning a time range of several orders of magnitude, which would be difficult by using equidistant time intervals on a linear scale. Instead, the multiple-τ algorithm, a widely used method in fluorescence correlation

spectroscopy, estimates the correlation function with quasi-logarithmic timescale increments of lag time.

FPGAs are semiconductor devices that are based on a matrix of programmable and configurable logic blocks. They can be reprogrammed for a desired application, resulting in a hardware performing specific functions.

FPGA devices combine the application-specificity of integrated circuits and the flexibility of processor-based systems.

Taking into account the memory and computing capacity of FPGA chips as well as the characteristics of the multiple-τ algorithm, even a multi-channel (hardware) correlator may be implemented on a single modern FPGA chip.

2. Objectives

The main topic of my dissertation is the analysis of the structure and dynamics of small and large scale clusters of membrane proteins. The associations of MHC I and II glycoproteins and interleukin-2 and -15 receptors have been described earlier, but the role of the highly expressed MHC glycoproteins in these complexes has not been elucidated. We were interested in finding the role of MHC I in the clustering and mobility of MHC I/IL-2Rα/IL-15Rα complexes. To this end, we knocked down MHC I expression at the cell surface by using anti-β2m siRNA.

A validation of an FPGA-based dual channel multiple-τ hardware correlator is presented in my dissertation, which can be used to measure the mobility and co-mobility of fluorescently tagged proteins.

In the remaining part of my dissertation, the DNA binding, dynamics and conformation of heterodimer-forming c-Jun and c-Fos transcription factors, which are part of the signaling pathway of IL-2, were analyzed.

In our work we were interested in the following questions:

- What is the role of MHC I molecules in the clustering and dynamics of IL-2R/IL-15R?
- How does the change of the expression level of MHC I alter the mobility and clustering of MHC I and IL-2R/IL-15R?
- What is the conformation of c-Jun and c-Fos transcription factors in live cells?

Our results may help to understand the dynamics and clustering of membrane proteins and transcription factors. The presented approaches demonstrate the usefulness of combination of different modern microscopic techniques.

3. Materials and methods

3.1. Cell lines, cell culture

For analyzing the cell surface patterns of IL-2 and -15 receptors the FT7.10 CD4+ cell line was used. The cells originated from Kit 225 cells, which is an IL-2-dependent human adult T lymphoma cell line. It expresses all three subunits of IL-2R constitutively, and is stably transfected with IL-15Rα carrying an N-terminal FLAG-tag. The expression level of MHC I was knocked down using anti-β2m siRNA. Membrane proteins were targeted by fluorescently tagged antibodies, gangliosides were labeled with Cholera toxin B.

Experiments for analyzing the dynamics of c-Fos and c-Jun transcription factors were carried out using HeLa human cervical cancer cells. Proteins were tagged either with EGFP or mRFP1. Since the Fos C-terminal domain is 164 AAs longer than that of Jun, when counted from the end of the dimerization domain, we also constructed a truncated Fos moiety (Fos^{215} -EGFP) to gain distance information between another pair of points of the C-terminal domains. Fos^{215} is a truncated version of Fos whereby the last 164 amino acids have been removed.

3.2. Förster resonance energy transfer (FRET)

Measuring Förster resonance energy transfer (FRET) efficiency is a widely used approach in cellular biology to quantify conformational changes and associations of proteins. The rate of energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor making FRET a sensitive and suitable molecular ruler, it is became frequently and widely used method for monitoring the molecular distances. In the acceptor photobleaching FRET measurements, the acceptor molecule of the FRET pair is bleached with high laser power, therefore they will be unable to quench the donor dyes. Thus, measuring the increase of donor fluorescence due to the loss of acceptors allows the determination of FRET efficiency. Considering the low expression level of IL-2Rβ in the FT 10.7 cell line, the FRET efficiency between IL-2Rβ and IL-2Rα was determined with the acceptor photobleaching approach by using the FluoView 1000 Olympus confocal microscope.

To gain information about the conformation of c-Jun and c-Fos transcription factors, an intensity based FRET approach was used. The measurements were performed using a Zeiss LSM 510 confocal microscope.

3.3. Super-resolution STED microscopy, image analyses methods

Using super-resolution techniques allows resolving structures smaller than Abbé's diffraction limit. In the dissertation the clusters of MHC I molecules and IL-2/15 receptors were analyzed by stimulated emission depletion (STED) microscopy.

The sizes of IL-2/15R and MHC I protein clusters in control and MHC I knockdown cells were determined by using three different image analysis methods. The three methods are based on three different approaches, so instead of a direct comparison of the results, the trends of the cluster size changes are more meaningful for proper interpretation. In the first procedure the full widths at half maximum of the intensity peaks detected on the cell surface were measured. In the second procedure the spatial correlation curves of super-resolution images of MHC I, IL-2Rα and IL-15Rα were calculated. Empirical curves were fitted with a model function, which allowed estimation of the average cluster diameter. The third procedure, the cluster segmentation method is based on the classification of pixels of STED images of cell surface protein distributions.

Fractions of IL-2Rα and IL-15Rα in common clusters with MHC I were calculated by dividing the total intensity from receptor subunits in common clusters with MHC I by the total intensity of receptor subunits in the whole region of interest. Molecular and domain level colocalization were also assessed by calculating the Pearson's correlation coefficients between the pixel intensities of the two detection channels.

3.4. Fluorescence correlation and cross-correlation spectroscopy (FCS, FCCS)

FCS is a powerful method for determining the diffusion coefficients, photophysical properties and concentrations of fluorescent molecules in solution and living cells. To measure diffusion properties of membrane proteins, we used fluorescence correlation spectroscopy. The measurements were performed on a modified Olympus FluoView 1000 Olympus confocal microscope, which is extended with a 2-channel FCS detector. The TTL signal of the individually detected photons was fed into an NI 7833 field programmable gate array (FPGA) card. The autocorrelation function of the signal was calculated offline by a home-written software written in C. Bleaching fractions, mean number of diffusing molecules, molecular brightness and mobilities were estimated.

FCCS is a widely used technology for measuring molecular interactions between two differentially colored molecules. The mobilities of c-Jun and c-Fos transcription factors were analyzed with a two-color custom-built confocal FCCS setup in the laboratory of our collaboration partner. Auto- and cross-correlation curves were fitted by using the QuickFit 3.0

software. Autocorrelation curves were fitted with assuming triplet state formation and two diffusion components. The percentage of DNA-bound dimer was estimated from the fraction of the slow component of the crosscorrelation curve of the co-diffusing species.

4. Results

4.1. Effects of MHC I knockdown on the dynamics of IL-2/15 cytokine receptors

4.1.1. Expression of IL-2/15R and MHC glycoproteins before and after MHC I KD

To unravel the role of MHC I in clustering of IL-2/IL-15 receptors, we knocked down MHC I expression by using anti-β2m siRNA. KD was specific: expression levels of IL-2Rα, IL-15Rα or MHC II did not change. MHC I expression at the cell surface decreased with increasing siRNA concentration reaching a maximal effect at 100 μg/ml where expression was reduced to 5-10%.

Fluorescence intensity distributions of MHC I, IL-2Rα and IL-15Rα expressed at the cell surface were measured by using flow cytometric Qifikit beads. The total numbers of proteins at the cell surface were estimated from the number of proteins in the confocal detection volume also. The cells picked for FCS measurements had similar protein expression distributions as the original cell population.

4.1.2. Estimation of aggregate size of IL-2Rα, IL-15Rα and MHC I with molecular brightness analysis

The molecular brightness, which is the fluorescence intensity per particle (*F/N,* measured in photon counts per second per particle), is proportional to the number of labeled proteins forming an aggregate. This parameter gives an estimate of the number of fluorescent molecules in a jointly moving complex, therefore the degree homoaggregation of membrane proteins can be determined. Before direct comparison of *F/N* data, the irreversible photodestruction of dyes was estimated by calculating the bleached fractions. The fractions were estimated from intensity traces of all FCS experiments. After 20 s of illumination, the fraction of bleached proteins was in the range of 50-70%, therefore, molecular brightnesses were analyzed from the first 15 s of measurements.

We found a strong positive correlation between the expression density of MHC I and the number of aggregates. The dependence of the specific brightness had an increasing tendency with increasing local expression density, therefore, the homoaggregation state of MHC I molecules was strongly affected by their expression level. The homoaggregation of IL-2Rα, IL-15Rα and MHC II showed only a slight decrease, which was statistically not significant.

4.1.3. Increased mobility of IL-2Rα, IL-15Rα and MHC I upon MHC I KD

If a protein is in a jointly moving cluster/aggregate, its mobility in the membrane is inversely related to the size of the aggregate, therefore, we expected increased mobilities upon MHC I KD. The value of D was not dependent on the run number; therefore, D values for all good runs were included in the statistics. Upon MHC I KD, ACFs shifted toward shorter diffusion times indicating an increase of mobility. D decreased with increasing expression density (increasing local F) of MHC I, we concluded that the mobility of MHC I molecules depended on the expression level. Diffusion coefficients (D) increased significantly for IL-2Rα, IL-15Rα and MHC I in the MHC I KD cells. Altogether, brightness and mobility data suggested an overall decrease of the size of IL-2Rα/IL-15Rα/MHC I aggregates. We separately analyzed diffusion coefficients of MHC I and IL-2Rα residing in large patches and outside patches. The mobility of these proteins was lower inside patches than outside them. These data were in line with the previous findings: the mobility and the number of molecular entities in a cluster are dependent on the level of membrane protein expression.

4.1.4. Analysis of STED images shows a reduction of MHC I protein supercluster size upon KD

Our FCS data suggested that the size of small-scale protein aggregates diminished upon MHC I KD. We used super-resolution STED microscopy to get a direct insight into the effect of MHC I KD on the higher order clustering of the studied proteins. STED images of the (co)distributions of the fluorescently labeled proteins were taken in KD and control cells. Cells were labeled with antibodies against either MHC I and IL-2Rα or MHC I and IL-15Rα. The optical resolution of the STED microscope was ~40 nm, and the pixel size 20 nm.

First, the full-width-at-half-maximum (FWHM) values of intensity peaks of STED images were calculated in control and MHC I KD cells. The mean diameter of MHC I clusters decreased significantly after KD, from 640±120 nm to 260±80 nm. In contrast, the average size of IL-2Rα clusters did not change significantly, and the FWHM of IL-15Rα peaks decreased only slightly from 360±60 nm to 300±60 nm.

Second, we applied spatial correlation analysis of intensity distributions to assess cluster sizes. The angle-averaged radial correlation functions of fluorescence images were calculated and fitted with model a function. This analysis confirmed the impact of KD on cluster sizes: the mean radius of MHC I clusters was reduced from 340±120 nm to 200±120 nm after MHC I KD. The average radii of IL-2Rα and IL-15Rα clusters were in the range of 280-320 nm, and did not change significantly.

Third, we measured the mean occupied areas of MHC I, IL-2Rα and IL-15Rα clusters by an image segmentation method. The previous methods estimate the average diameter of clusters, whereas this procedure assesses the area of contiguous high-intensity pixels. The mean area of MHC I clusters decreased significantly upon KD from $(7.2 \pm 0.9) \times 10^4$ nm^2 to $(1.8 \pm 0.2) \times 10^4$ nm^2. The areas occupied by interleukin receptors did not change significantly.

4.1.5. Analysis of occupied areas of clusters of MHC I and IL-2Rα / IL-15Rα

The previous analyses dealt with the spatial dimensions of large protein clusters, but not with their protein content, which also depends on the density of proteins in the clusters. To quantify the amount of receptors in clusters of different sizes, the summed fluorescence intensities of clusters of a given size were calculated. Intensities were normalized to the total intensity of all clusters. In line with the results of previous image analysis methods, the most populated cluster size of MHC I decreased significantly after KD. The most preferred cluster sizes of IL-2Rα and IL-15Rα receptors did not change significantly.

4.1.6. Estimation of molecular fractions of colocalized proteins

To assess changes of colocalization with a resolution of 40 nm, molecular fractions of colocalized proteins were calculated from STED images. Remarkably, the mutual vicinities of MHC I and interleukin receptors showed an asymmetric behavior after MHC I KD. The fraction of IL-2Rα found near MHC I in STED images was reduced from 75±3% (control) to 5.4±3% (KD). Similarly, the proportion of IL-15Rα near MHC I was significantly reduced, from 91±4% to 20±6%. Conversely, the fraction of MHC I colocalized with IL-2Rα decreased only slightly, from 61±8% to 52±7%, and its fraction near IL-15Rα even increased from 12±2% to 19±5% upon KD. MHC I molecules probably retracted to a smaller total area, but remained in the vicinity of interleukin receptors, suggesting that their association with IL-2Rα and IL-15Rα was stable.

4.1.7. Pearson colocalization analyses of cell surface distributions of MHC I and IL-2Rα/IL-15Rα upon KD

Colocalization between MHC I and IL-2Rα/IL-15Rα subunits was also analyzed by calculating Pearson's correlation coefficients (*C*) of their pixelwise intensity distributions. We calculated *C* values with three thresholding conditions: for pixels where the intensity in one, the other or both channels was above the threshold. *C* values were significantly reduced for both pairs (MHC I + IL-2Rα as well as MHC I + IL-15Rα) upon KD of MHC I. Our results on

Pearson's correlation coefficients are in line with the findings that after KD the majority of interleukin receptors were situated in clusters free of MHC I.

We applied the same analyses to confocal images acquired from the same cells, and found similar tendencies; however, numerical values of colocalized fractions and Pearson's coefficients were different due to the inability of confocal microscopy to resolve finer details.

4.1.8. Membrane microdomain localization of IL-2Rα and IL-15Rα subunits remained unchanged upon MHC I knockdown

Based on our FCS and STED results, we hypnotized that the enrichment of MHC I and IL-2Rα /-15Rα in lipid rafts were changed by the KD. Co-distributions of IL-2Rα, IL-15Rα or MHC I with the lipid raft marker GM_1 ganglioside were recorded by confocal microscopy followed by intensity correlation analysis. Correlation coefficients of the images of the receptor subunits and GM_1 did not change significantly upon gene silencing. These results implied that the enrichment of IL-2Rα and IL-15Rα in lipid rafts did not depend on the presence or absence of MHC I. On the other hand, the C values between MHC I and GM_1 decreased from 0.4±0.1 to 0.28±0.08. The experimentally observed decrease of C could partially be explained by the change of the S/N ratio upon KD, even though the extent of co-clustering was maintained.

4.1.9. Study of the interaction between IL-2Rβ and IL-2Rα by photobleaching FRET

In addition to the mobility and clustering analyses, we tested weather MHC I molecules played a critical role in the conformation or assembly of IL-2/15 receptors. FRET between IL-2Rβ and IL-2Rα was analyzed by the acceptor photobleaching technique on a pixel-by-pixel basis in control and KD cells. The heteroassociation of IL-2Rβ with IL-2Rα did not change significantly, suggesting that interleukin receptor assembly and conformation were not influenced by interactions with MHC I.

4.2. Study of the AP-1 complex

4.2.1. Analysis of heterodimerization of thec-Fos–c-Jun dimer by FRET measurements

To investigate the relative positions of the labeled molecular moieties of the transcriptional activator AP-1 in live cells, we performed FRET experiments. Even though the distribution of the individual transcription factor molecules was homogenous, their interaction could be influenced by the local environment. This raises an interesting question whether the dimerization of Jun and Fos occurred preferentially at specific nuclear compartments/gene segments, or their association state was independent of the exact localization within the

nucleus. We found that the distributions of FRET efficiency values were rather homogeneous in the nucleus for the Fos^{215} -Jun heterodimer, such as for the positive and negative controls. The homogeneity was also reflected in the narrow distributions of the FRET histograms calculated from the pixel values of each image. This suggests that the association state of Jun and Fos and the conformation of the dimer do not depend crucially on the local environment in the nucleus or on the presence of specifically located DNA sequences. The average FRET efficiencies for the full length Fos-Jun (3.0 ± 1%), Fos 215 -Jun (9.9 ± 0.5%), the EGFP-mRFP1 fusion proteins (22.1 ± 2.8%), and the separately coexpressed EGFP and mRFP1 dyes (0.3 ± 0.8%) were determined from; 30–80 cells. Assuming a Förster radius of 4.5 nm, for Fos^{215} -EGFP Jun-mRFP1 dimer, the dye-to-dye distance estimation was 6.8 ± 0.1 nm. This data was in good agreement with the prediction of MD modeling (6.7 ± 0.1 nm). The dye-to-dye distance for full length dimers was 8.4 ± 0.5 nm, thus, these data are in line with the truncation of the Fos protein at the C-terminal by 164 amino acids is resulting in equally long C-termini, therefore, the increase of FRET efficiency is expected.

4.2.2. Analysis of binding of c-Fos and c-Jun to DNA by FCCS measurements

We performed FCS and FCCS measurements on cells transfected with Fos^{215} and Jun to determine whether the truncation of the Fos protein at the C-terminal influences the dimerization, mobility, or DNA-binding of the proteins. The extent of dimerization was monitored by the cross-correlation amplitude; the mobility of the different species was inferred from the diffusion times, whereas the percentage of DNA bound dimer could be assessed from the fraction of the slow component. The cross-correlation amplitude calculated as a percentage of the autocorrelation amplitude of the green channel was 34 ± 6%. This value is close to that found earlier for the full-length Fos and Jun (31 ± 6%), and is significantly different from the negative control (separately expressed EGFP and mRFP1: 13 ± 3%, arising from the spectral crosstalk of the EGFP signal into the red detection channel. The percentage of cross-correlation amplitude for positive control cells relative to the autocorrelation amplitudes was 45±4%. There were two components present in the auto- and cross-correlation signals; the fast one had diffusion times ranging from 1.0 to 1.4 ms, whereas the slow one, 67–91 ms on average, was similar to those published earlier for the full-length constructs. The two components can be attributed to the free and DNA-bound proteins.

The long diffusion times may also mean average residence times of the Jun-Fos complex spent in a DNA-bound state. The fraction of the slow component in the cross-correlation curves was 56 ± 4% for the Fos 215 -Jun complex, whereas it was 77 ± 13% in the case of the full-length

Fos-Jun construct. The decrease in the fraction of the slower component may suggest that the C-terminal domain, which was deleted from the full length Fos-EGFP, might be involved in anchoring the dimer to other nuclear components. This is concordant with a possible transactivation role of this C-terminal domain observed in fibroblasts.

4.3. Validation of the FPGA hardware correlator

In my dissertation, I have demonstrated the validation of the software correlator and FPGA hardware correlator developed by our team by comparing the correlation functions of the test solutions. Autocorrelation functions of the doubly labeled antibodies were fitted to a model with triplet state and two diffusion components to account for dyes bound to antibodies (slow component) and free, dissociated dyes (fast component). The cross-correlation curves of the antibodies were fitted without triplet formation because triplet formation of the two dyes are uncorrelated events. A fast diffusion component is not necessary because dissociated Alexa Fluor 488 and Cy5 molecules move separately, yielding no cross-correlation.

For comparing the correlation data, it is important to note that the commercially available ALV 5000E correlator is not suitable for simultaneous measurement of auto- and cross-correlation; the functions can be determined only sequentially, from separate measurements. The raw correlation functions calculated by the ALV correlator and the FPGA correlator overlapped perfectly and yielded practically identical fit parameters. These results demonstrated the correctness of calculations performed by the FPGA correlator and its applicability to calculate two ACF and two CCF curves in real time.

5. Discussion

5.1. Effects of MHC I KD on the dynamics of IL-2/15 cytokine receptors

Cell surface structures from membrane proteins have varying spatial and temporal structure, they are organized on several hierarchical levels. The inhomogeneous protein distributions is caused by the combination and interaction of several factors. The size and lifetime of the structures can be influenced by both the intra- and extracellular interactions and factors.

MHC I and II glycoproteins and interleukin-2 and -15 receptors are all important players in immunological processes. We were interested in finding the effect of gene silencing of MHC I glycoproteins on the dynamics and clustering of MHC I and IL-2Rα/IL-15Rα.

Earlier results showed that MHC I glycoproteins homo- and heteroassociated on the molecular level with IL-2 and -15 receptors, and the molecular clusters were enriched in lipid rafts. Between large clusters of MHC I and IL-2R, the exchange of the components is dynamic process. Using cross-correlation spectroscopy technique, the co-mobility of MHC I and IL-2/15 receptors was demonstrated. The cytoskeleton may also play a role in stabilization of large MHC I clusters.

However, the role of the highly expressed MHC glycoproteins in these complexes has not been elucidated. We examined how the assembly, interactions, and mobility of IL-2 and -15 receptors are influenced by MHC I in T lymphoma cells. For describing the clustering of membrane proteins, all hierarchical levels of clustering were analyzed with a combination of different modern biophysical approaches.

Molecular brightness data indicated that the extent of aggregation was highest for the MHC I molecules in control cells. The homoaggregation of MHC I depended strongly on its expression level, their specific brightness decreased upon KD. The homo- and heteroaggregation of IL-2Rα and IL-15Rα were found to be independent from MHC I. Our data highlight that the properties of certain membrane proteins (i.e.: cluster size, lateral mobility, number of molecules in a cluster) can be dependent on the level of membrane protein expression, while the aggregation of other membrane proteins does not show such correlation.

Our measurements revealed the presence of small protein nanoclusters with a size similar to that of lipid nanodomains, and contributed to the understanding of the factors necessary for the formation of building blocks of multi-level organization. The mobility of MHC I molecules was found to be higher when reducing the size of their homoaggregates. The mobility of IL-2Rα and IL-15Rα subunits also increased by reducing the number of interacting

partners (MHC I). Our results reveal that the dynamics of cytokine receptors are sensitive to changes in their micro-environment.

We hypothesized that the increased mobility of the proteins may be caused by their reduced cluster sizes. Higher level organization of the membrane proteins was studied with super-resolution STED microscopy. The cluster sizes of proteins were estimated by three different algorithms. The results of all three methods confirmed that the cluster size of the MHC I molecules depends on its expression level. The cluster sizes of IL-2Rα and IL-15Rα subunits and the average distance between IL-2/15Rβ and IL-2Rα or IL-15Rα were not significantly influenced by the elimination of MHC I. We concluded that the reduction of size of the large MHC I clusters and nano-homoaggregates were consistent with the higher mobilities: the smaller nanoaggregates diffused faster.

By analyzing super-resolution images, we demonstrated that MHC I gene silencing also reduced the associated fractions and the Pearson correlation coefficients between MHC I and the receptors. Therefore, the elimination of MHC I contributed to the increasing the mobility of the receptors by reducing the number of their interaction of partners, while the mean geometry of receptors was unaffected by the KD. Based on data on the associated fractions, the remaining MHC I molecules stayed in the vicinity of IL-2Rα / IL-15Rα subunits, which suggests a stable associations between MHC I and IL-2 and -15 receptors.

We hypothesized that the enrichment of MHC I, IL-2Rα and IL-15Rα in lipid rafts are changed by the KD. Our data imply that the enrichment of IL-2Rα and IL-15Rα in lipid rafts does not depend on the presence or absence of MHC I. Based on literature data, the enrichment of receptors in lipid rafts could only be altered by cholesterol extraction and addition of their ligands.

In conclusion, the clustering of IL-2 and -15 cytokine receptors and MHC I glycoproteins was significantly influenced by reducing the amount of the most highly expressed component in the clusters. However, in my thesis I did not perform functional studies. In general, the protein nano- and microclusters can be considered essential platforms for cellular signaling, therefore, clustering of membrane proteins might alter several signaling pathways and cell functions.

The understanding of the hierarchical and dynamic clustering of membrane proteins requires a thorough exploration of intra/extracellular and intramembrane factors. In my dissertation, I examined how the permanent reduction of a highly expressed component of protein clusters affects the dynamics of the clusters at the nano- and micrometer-scale.

5.2. Analysis of the AP-1 complex

The c-Fos and c-Jun transcription factors are members of the activator protein-1 (AP-1) complex. In the second part of my dissertation I examined the conformations of the c-Jun and c-Fos heterodimer and its ability to bind to DNA. The heterodimer is linked together by a leucine zipper. Its activation plays an important role in controlling several genes, and is one of the possible endpoints of IL-2 signaling. According to the literature, the structure of their dimerization and DNA-binding domains are known, however, the conformation of their C-terminal domains remained unknown.

Using FRET data between GFP and mRFP1 tagged C-terminal ends of Jun and Fos, we were able to constrain the possible range of conformations allowed by MD modeling, at least for the majority of the population, to those in a less extended state (dye-to-dye distance between 6 and 10 nm). Based on this, we can conclude that the C-terminal ends of the two proteins are significantly closer than the maximal distance allowed by the linear length, indicating the possible vicinity of the transactivation domains. By fluorescence cross-correlation spectroscopic analysis, we found that the DNA-bound fraction decreases if the C-terminus of c-Fos is truncated; therefore, the C-terminal end plays an important role in DNA binding. This finding is consistent with the transactivation role of the C-terminal domain of c-Fos.

Our results demonstrated that the range of possible conformations calculated from molecular dynamics simulations can be significantly reduced by using distances from FRET measurements. Since heterodimerization of c-Fos and c-Jun is a known phenomenon, this model system can be used for optimizing and designing FRET measurements between GFP-mRFP1 in other biological systems.

The preassembly of the subunits of the interleukin 2 receptors in plasma membranes was demonstrated by fluorescence resonance energy transfer (FRET) measurements. In our workgroup, we are currently investigating the intra-cellular assembly of IL-2 and -15 receptor subunits in the endoplasmic reticulum and the Golgi. Thus, the demonstrated methods can be used in other model systems also, e.g.: GFP and mRFP1 dyes are linked to different subunits of interleukin receptors.

5.3. Validation of FPGA hardware correlator

In the third part of my dissertation, I presented the application of a multifunctional re-programmable hardware in FCS / FCCS measurements. In FCS measurements, correlation functions are typically calculated by a dedicated hardware, the curves are calculated and displayed in real time. The multi-τ algorithm produces correlation curves on a quasi-

logarithmic time scale, thus this method is an often used approach in FCS measurements. Thanks to the small memory consumption of the algorithm and to calculating the curves block by block, we were able to develop a multi-τ hardware correlator with two input channels on a single FPGA device.

Advantages of FPGAs include their re-programmability, flexibility and their lower cost relative to commercially available correlators. With their multiple analog and digital inputs, they can be used as a general data collection tools, not just as a single correlator element.
In our lab, an ALV 5000E correlator was only available, which can only calculate either the autocorrelation functions of two channels or their cross-correlation functions at a time. Since the cell membrane is an inhomogeneous planar membrane, the properties of aggregation and diffusion states of membrane protein molecules are also heterogeneous. Because of this and the fast dynamics of membrane components, auto- and cross-correlation measurements performed sequentially might describe different molecular populations, which is disadvantageous for determining the associated ratio of proteins by using the amplitudes of the autocorrelation and cross-correlation functions.

Field programmable gate arrays are capable of parallel calculations of ACFs and CCFs in real time. In my dissertation, I demonstrated the validation of the FPGA hardware correlator developed by our team by comparing the correlation functions of double labelled antibodies with those obtained from the same samples by our commercially available ALV 5000E correlator. The parameters derived by nonlinear fitting of the correlation functions obtained from the two devices were nearly identical.

By computing parallel auto- and cross-correlation functions, the associated fractions of molecular complexes can be estimated more accurately, and the stability of complexes can also be characterized precisely. We plan to use the device to determine the associated fractions of proteins in cell surface protein complexes. In addition, we will develop a three-channel correlator, which will measure the mobilities and associated ratios of three distinct proteins (e.g. IL-2Rα, IL-2Rβ, IL-2Rγ).

6. Summary

Membrane proteins form dynamic clusters at different hierarchical levels. MHC I and II glycoproteins and interleukin-2 and -15 receptors, which are all important players in immunological processes, form supramolecular clusters in lipid rafts of T cells. The role of the highly expressed MHC glycoproteins in these complexes has not been elucidated, we were interested in finding the effect of gene silencing of MHC I glycoproteins on the dynamics and clustering of MHC I and IL-2Rα/IL-15Rα. The expression level of MHC I was reduced using anti-β2m siRNA. Combination of superresolution microscopy with image analysis methods showed that the diameter of MHC I superclusters significantly reduced, whereas those of IL-2Rα/IL-15Rα hardly changed upon gene silencing. Fluorescence correlation spectroscopy-based brightness analysis revealed that the molecular level of homoaggregation of MHC I molecules was reduced by decreasing their expression level. The mobility of IL-2Rα, IL-15Rα and MHC I increased significantly upon knockdown of MHC I, corroborating the general size decrease of protein aggregates. MHC I molecules retracted to a smaller total area, but remained in the vicinity of interleukin receptors. The interaction between IL-2Rα and IL-2/15Rβ subunits did not change significantly, suggesting that the interactions holding these subunits together were not dependent on the presence of MHC I. Our colocalization analyses suggested that the enrichment of IL-2Rα/IL-15Rα in lipid rafts was not affected by removing MHC I molecules.

In the second part of the dissertation the dynamics and conformation of heterodimer-forming c-Jun and c-Fos transcription factors, which are part of the signaling pathway of IL-2, were analyzed. The conformation of the dimers was analyzed by FRET measurements between the fluorescently labeled C-termini of Jun and Fos proteins. Two-color cross-correlation analysis was used to assess co-mobility and DNA binding of the constructs. The data showed that the fraction of DNA-bound dimers decreased if we truncated the C-terminus of c-Fos, suggesting that the C-terminal end may play a role in the stabilization of the DNA-binding of the transcription factors. The average distances of the C-termini derived from our FRET measurements allowed us to narrow down the range of possible conformations of the Fos-Jun dimer predicted by molecular dynamic simulations.

Finally, an application of a reprogrammable hardware device (FPGA) in biophysical measurements, and its validation were presented in fluorescence (cross)correlation experiments. The correlation functions calculated by our commercial correlator and the FPGA correlator overlapped perfectly and yielded practically identical fit parameters. By using this hardware, the associated fraction of diffusing molecules and their co-mobility can be quantified, allowing the establishment of more precise biophysical models.

7. Publications

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Registry number: DEENK/284/2016.PL
Subject: PhD Publikációs Lista

Candidate: Gábor Mocsár
Neptun ID: W56ZNY
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10034348

List of publications related to the dissertation

1. **Mocsár, G.**, Volkó, J., Rönnlund, D., Widengren, J., Nagy, P., Szöllősi, J., Tóth, K., Goldman, C. K., Damjanovich, S., Waldmann, T. A., Dóczy-Bodnár, A., Vámosi, G.: MHC I expression regulates co-clustering and mobility of interleukin-2 and -15 receptors in T cells.
   Biophys. J. 111 (1), 100-112, 2016.
   DOI: http://dx.doi.org/10.1016/j.bpj.2016.05.044
   IF: 3.632 (2015)
2. **Mocsár, G.**, Kreith, B., Buchholz, J., Krieger, J. W., Langowski, J., Vámosi, G.: Multiplexed multiple-tau auto- and cross-correlators on a single field programmable gate array.
   Rev. Sci. Instrum. 83 (4), 046101, 2012.
   DOI: http://dx.doi.org/10.1063/1.3700810
   IF: 1.602
3. Vámosi, G., Baudendistel, N., von der Lieth, C. W., Szalóki, N., **Mocsár, G.**, Müller, G., Brázda, P., Waldeck, W., Damjanovich, S., Langowski, J., Tóth, K.: Conformation of the c-Fos/c-Jun complex in vivo: a combined FRET, FCCS, and MD-modeling study.
   Biophys. J. 94 (7), 2859-2868, 2008.
   DOI: http://dx.doi.org/10.1529/biophysj.107.120766
   IF: 4.683

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List of other publications

4. Buchholz, J., Krieger, J. W., **Mocsár, G.**, Kreith, B., Charbon, E., Vámosi, G., Kebschull, U., Langowski, J.: FPGA implementation of a 32x32 autocorrelator array for analysis of fast image series.
   Opt. Express. 20 (16), 17767-17782, 2012.
   DOI: http://dx.doi.org/10.1364/OE.20.017767
   IF: 3.546

5. Beck, Z., Balogh, A., Kis, A., Izsépi, E., Cervenak, L., László, G., Bíró, A., Liliom, K., **Mocsár, G.**, Vámosi, G., Füst, G., Matkó, J.: New cholesterol-specific antibodies remodel HIV-1 target cells' surface and inhibit their in vitro virus production.
   J. Lipid. Res. 51 (2), 286-296, 2010.
   DOI: http://dx.doi.org/10.1194/jlr.M000372
   IF: 6.115

6. Erdélyi, K., Bai, P., Kovács, I., Szabó, É., **Mocsár, G.**, Kakuk, A., Szabó, C., Gergely, P., Virág, L.: Dual role of poly(ADP-ribose) glycohydrolase in the regulation of cell death in oxidatively stressed A549 cells.
   FASEB J. 23 (10), 3553-3563, 2009.
   DOI: http://dx.doi.org/10.1096/fj.09-133264
   IF: 6.401

7. Dóczy-Bodnár, A., Nizsalóczki, E., **Mocsár, G.**, Szalóki, N., Waldmann, T. A., Damjanovich, S., Vámosi, G.: A biophysical approach to IL-2 and IL-15 receptor function: localization, conformation and interactions.
   Immun. Lett. 116 (2), 117-125, 2008.
   DOI: http://dx.doi.org/10.1016/j.imlet.2007.12.014
   IF: 2.858

Total IF of journals (all publications): 28,837
Total IF of journals (publications related to the dissertation): 9,917

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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