Physiological and pathological signaling complexes in the plasma membrane and the nucleus studied with single molecule sensitivity (K103965)

Final report

PI: György Vámosi, PhD

SUMMARY

Our research focused on the following main areas:

1. Protein-protein interactions along the interlukin-2/15 receptor signaling pathway
2. Protein-protein and protein-DNA interactions of nuclear receptors
3. Development of techniques to quantitate protein-protein interactions

Interleukin-2 and -15 receptors are important regulators of T cell activation, apoptosis and immunological memory. We have shown that the expression of MHC glycoproteins forming supramolecular clusters with IL-2 and -15 receptors regulates co-clustering and mobility of these receptors and may thereby influence receptor function. We have proven that these receptors start to preassemble during trafficking, but this process is only completed in the plasma membrane. This may have importance in understanding the resistance of certain lymphomas to blocking antibody therapies. We found that the c-Fos transcription factor, which is a member of the IL-2/15 signaling pathway, can form homodimers when overexpressed, which may thus act as an autonomous oncogene. We have refined the molecular switch model of nuclear receptor function based on the study of mobility and interactions of RXR and RAR: binding of the receptors to the chromatin is enhanced by binding agonist ligand, and DNA binding also enhances dimerization of the receptors. We have shown that RXR can also form homodimers in live cells. We have worked out several methods to quantitate protein-protein interactions in live cells. We participated in the testing of biocompatible nanoparticles that target MR contrast agents to cells expressing their target, folic acid receptor.

1. PROTEIN-PROTEIN INTERACTIONS ALONG THE INTERLEUKIN-2 AND -15 RECEPTOR PATHWAY

Interleukin-2 and -15 (IL-2, IL-15) are cytokines critically involved in controlling T cell homeostasis and function. Their multisubunit receptors share the β and γ_c chains responsible for one set of ligand-induced signal transduction events, but both have their "private" α chains ensuring the high-affinity binding of the appropriate cytokine and specificity of the immune response. Due to the shared subunits, IL-2 and IL-15 activate similar signaling (JAK/STAT, lck, Bcl-2, Ras/Raf/MAPK, PI-3K/Akt) pathways. Besides similar cellular responses (e.g. proliferation of T and NK cells), IL-2 and IL-15 can also provide contrasting contributions to T cell mediated immunity: whereas IL-2, through its pivotal role in activation induced cell death (AICD), inhibits survival of CD8+ memory T cells and is critically involved in peripheral self-tolerance, IL-15 counteracts AICD and promotes long-term immunological memory. mechanism of rece Understanding the distinct interactions of the two receptors may be key to deciphering the bifurcation of the signaling pathways.

1.1 MHC I expression regulates co-clustering and mobility of interleukin-2 and -15 receptors in T cells

An important feature of cell membranes is their intrinsic lateral heterogeneity, which must be a key to functionality. Plasma membrane lipids and proteins are compartmentalized in specific domains and clusters, whose size and composition vary.

MHC glycoproteins form supramolecular clusters with interleukin-2 and -15 receptors in lipid rafts of T cells. The role of highly expressed MHC I in maintaining these clusters is unknown. We knocked down MHC I in FT7.10 human T cells, and studied protein clustering at two hierarchic levels: molecular aggregations and mobility by Förster resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FCS), and segregation into larger domains or superclusters by superresolution STED microscopy. FCS based molecular brightness analysis revealed that the studied molecules diffused as tight aggregates or nanoclusters of several proteins of a kind. Knockdown reduced the number of MHC I containing molecular aggregates and their average MHC I content as revealed by FCS, and decreased the heteroassociation of MHC I with IL-2Rα/IL-15Rα as evidenced by FRET. The mobility of not only MHC I but also that of IL-2Rα/IL-15Rα increased, corroborating the general size decrease of tight aggregates. We analyzed the diameter of nanometer to micrometer scale aggregates by three methods: direct measurement of the width of intensity peaks, determining the characteristic radius of the spatial correlation function of the intensity distribution, and by a cluster segmentation method assessing the size of contiguous high-intensity areas. This multifaceted analysis of STED images revealed that the diameter of MHC I superclusters diminished from 400-600 to 200-300 nm, whereas those of IL-2Rα/IL-15Rα hardly changed. MHC I and IL-2Rα/IL-15Rα colocalized with GM1 ganglioside-rich lipid rafts, but MHC I clusters retracted to smaller subsets of GM1- and IL-2Rα/IL-15Rα-rich areas upon knockdown. Our results prove that changes in expression level may significantly alter the organization and mobility of interacting membrane proteins.

We published our results in ***Biophysical Journal (1)***. Our paper was highlighted in the "New and Notable" section of the journal (Jacobson and Liu, Biophys J 111 (1), 1–2, 2016): "This study will undoubtedly have general implications for how diverse species of nano- and superclusters are both assembled and maintained. ... Mocsár et al. both raise significant scientific questions and provide a paradigm, including the attendant interpretational limitations, for further investigation of lateral heterogeneity in the plane of the plasma membrane." This publication with two other papers **formed the basis of the PhD dissertation of Gábor Mocsár, who defended his thesis in 2017**.

1.2. Preassembly of interleukin-2 and -15 receptors during trafficking

The preassembly of IL-2 and 15 receptor subunits in the plasma membrane, even in the absence of ligand, has been shown previously in our lab. It has not been studied whether the subunits are associated directly after their synthesis in the ER or later in the Golgi. We have expressed IL-2R and IL-15R subunits tagged with EGFP and mCherry, and assessed their association state by measuring the FRET efficiency between the fluorophores in these organelles. The ER and the Golgi were identified by BFP-tagged (blue) marker proteins, and FRET efficiencies between the receptor subunits were evaluated in an organelle specific manner in regions expressing these markers. In spite of the different lengths of the intracellular C-terminal tails of the receptor subunits, we could measure low but positive FRET efficiencies between the receptor subunits (γ_c + IL-2Rα, γ_c + IL-15Rα, and IL-2/15Rβ + γ_c). To bring the fluorescent protein labels at the C-termini in register, we truncated the longest, IL-2/15Rβ subunit. By using this construct, the FRET efficiency between IL-2/15Rβ + IL-2Rα or IL-2/15Rβ + IL-15Rα slightly increased in the Golgi from 2 to 5% (see figure below). On the other hand, FRET efficiency between these subunits in the plasma membrane was ~15%. These data indicate that preassembly of the IL-2/15R subunits in the ER and the Golgi is only partial.

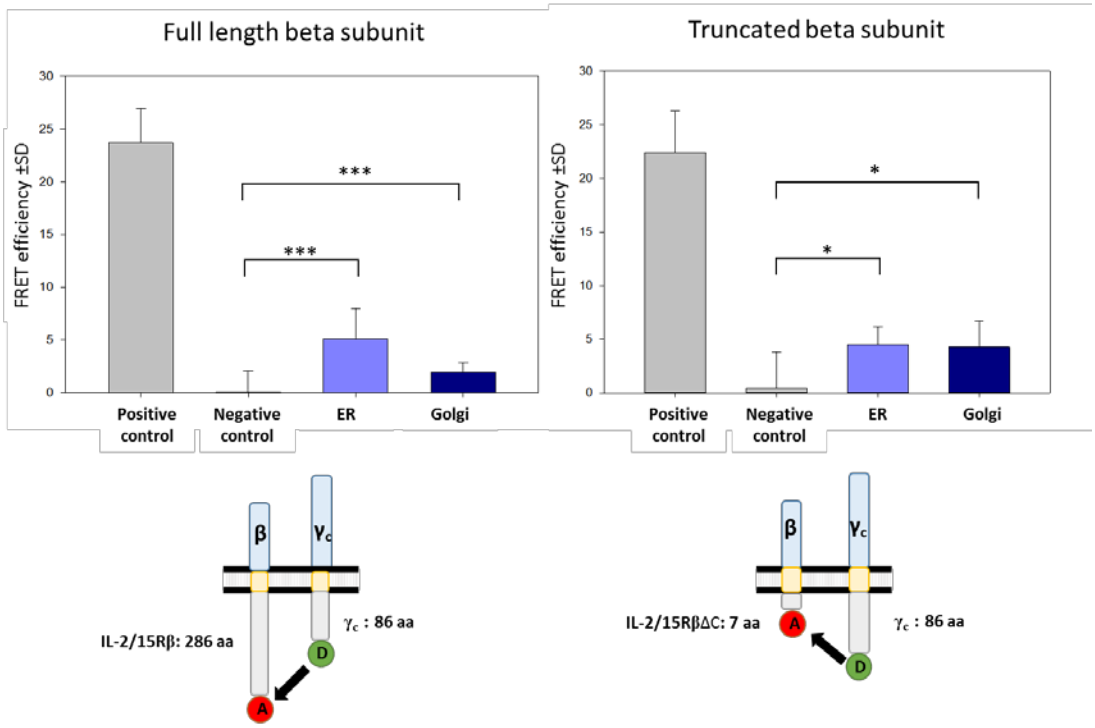

Fig. 1 FRET efficiencies measured between IL-2R β and γ_c subunits

Both IL-2R and IL-15R are heterotrimeric receptors, and the stability of pairwise interactions between any two subunits may be affected by the presence of the third subunit. Therefore, we created cell lines stably expressing one of the subunits (with no fluorescent marker), and measured the association between the other two, fluorescently labeled, coexpressed subunits by FRET. Transfection of the γ_c subunit did not significantly influence the interaction between the α and β subunits for either IL-2R or IL-15R.

Our results show that a fraction of receptor subunits is already preassembled in the ER and the Golgi, but assembly becomes complete only in the plasma membrane. This may suggest the possibility that activated T cells expressing both IL-2R and the IL-2 cytokine could signal even before the receptors actually reach the plasma membrane. This could explain the inefficiency of IL-2 receptor-targeted antibody therapies in certain T leukemia/lymphoma types. Our results have been presented on posters at several conferences, and a manuscript is in preparation.

1.3. Measurement of MHC I – IL-2R interaction and IL-2-induced signaling in peripehral $CD4^+$ T cells from ankylosing spondylitis (AS) patients

Ankylosing spondylitis is linked with the HLA B27 allele of the MHC I glycoprotein, which can form nonphysiological heavy chain dimers via disulfide bridges. Earlier we have shown that IL-2R, IL-15R and MHC I form common clusters in T cells. We hypothesized that the alternate conformation of MHC I, via its interaction with IL-2R, may modify the assembly and function of the receptors, and thereby modulate the immune response. In our experiments we measured whether homoassociation of MHC I or its free heavy chain (MHC I FHC), hetero-association of IL-2Rα with MHC I or the MHC I FHC, and consequently, the efficiency of IL-2 signaling are changed in AS patients. On peripheral T cells of control (n=4) and AS patients (n=17) we measured the expression of IL-2Rα, MHC I and MHC I FHC and assessed the above mentioned protein associations via FRET. Neither the expression levels, nor the FRET efficiencies measured on control and AS patients differed significantly. We did not find any significant

change in the IL-2 induced STAT5 phosphorylation levels either. Therefore, we did not pursue this line of investigation further.

1.4. Distinct spatial relationship of the interleukin-9 receptor with interleukin-2 receptor and major histocompatibility complex glycoproteins in human T lymphoma cells

The interleukin-9 receptor (IL-9R) consists of an α subunit and the γ_c chain that is shared with other cytokine receptors, including interleukin-2 and -15 receptors. We previously showed that IL-2R and IL-15R are expressed in common clusters with major histocompatibility complex glycoproteins in lipid rafts of human T lymphoma cells, which raised the question what the relationship between clusters of IL-2R/MHC and IL-9R was. Confocal microscopy colocalization and FRET experiments showed the nonrandom association of IL-9R with IL-2R/MHC clusters at the surface of human T lymphoma cells. Accommodation of IL-9Rα in membrane areas segregated from the IL-2R/MHC domains was also detected. The bipartite nature of IL-9R distribution was mirrored by signal transducer and activator of transcription (STAT) activation results. Our data indicate that co-compartmentalization with MHC glycoproteins is a general property of γ_c receptors. Distribution of receptor chains between different membrane domains may regulate their function. Our results were published in ***ChemPhysChem (2)***.

1.5. Homoassociation of c-Fos in HeLa cells: a new form of the AP-1 complex

The AP-1 complex composed of the c-Fos and c-Jun transcription factors is an important element of the IL-2/IL-15 signaling pathway controlling early response genes. c-Fos and c-Jun form heterodimers and bind to DNA via a basic leucine zipper, and regulate the cell cycle, apoptosis, differentiation, etc. Purified c-Jun leucine zipper fragments could also form stable homodimers, whereas c-Fos leucine zipper homodimers were found to be much less stable in earlier in vitro studies. The importance of c-Fos overexpression in tumors and the controversy in the literature concerning c-Fos homodimerization prompted us to investigate c-Fos homodimerization. FRET and molecular brightness analysis of fluorescence correlation spectroscopy (FCS) data from live HeLa cells transfected with fluorescent protein-tagged c-Fos indicated that c-Fos formed homodimers. We developed a method to determine the absolute concentrations of transfected and endogenous c-Fos and c-Jun, which allowed us to determine dissociation constants of c-Fos homodimers (Kd=6.7±1.7 μM) and c-Fos-c-Jun heterodimers (on the order of 10-100 nM) from FRET titrations. Imaging fluorescence cross-correlation spectroscopy and molecular modeling simulations confirmed that c-Fos homodimers were stably associated and could bind to the chromatin. Our results establish c-Fos homodimers as a novel form of the AP-1 complex, which may be an autonomous transcription factor in c-Fos overexpressing tissues, and could contribute to tumor development. We intend to identify genomic binding sites of Fos homodimers, and prove their presence in Fos-overexpressing tumor tissues.

Our technique combining FCS and immunofluorescence to determine the absolute concentration of endogenous proteins, and the FRET titration method to measure Kd values between proteins in live cells also can be applied to other molecules such as nuclear receptors.
Our work has been published in ***Molecular and Cellular Biology (3)***. Together with two other papers, it also formed the basis of **Nikoletta Szalóki's PhD thesis (defended in 2016)**.

2. PROTEIN-PROTEIN AND PROTEIN-DNA INTERACTIONS OF NUCLEAR RECEPTORS

Nuclear receptors (NRs) are transcription factors regulating the transcription of their target genes in a ligand dependent fashion. Their ligands are lipid soluble molecules that can pass the membrane and bind to their receptors in the nucleus or the cytoplasm. They regulate many cellular processes including

growth, differentiation, metabolism, death and immune responses. The current model of nuclear receptor function, the “molecular switch model”, assumes that DNA-bound receptor dimers bind corepressors, which, through their histone deacetylase activity and via binding further histone modifying enzymes, actively repress transcription. Ligand binding induces a conformational change in the activation function-2 domain of the receptor, which results in the dissociation of the corepressor and formation of a molecular surface that binds a coactivator protein, leading in several steps to the transcription of the target gene. In our experiments we studied the chromatin binding and dimerization of nuclear receptors during activation, and the dependence of these processes on the presence of ligands and cofactors by using advanced fluorescence microscopy and genomic tools. This work is carried out in collaboration with the groups of Prof. László Nagy (Dept. of Biochemistry and Molecular Biology, U. Debrecen) and Prof. Jörg Langowski and Dr. Katalin Tóth (DKFZ Heidelberg).

2.1. Ligand binding shifts highly mobile retinoid X receptor to the chromatin-bound state in a coactivator-dependent manner, as revealed by single-cell imaging.

Retinoid X receptor (RXR) acts as a heterodimer interacting with other nuclear receptors: retinoic acid receptor (RAR), peroxisome proliferator-activated receptor gamma (PPARg), vitamine D receptor (VDR), liver X receptor (LXR), etc. Earlier we have shown that the majority of RAR molecules (70%) diffuses freely in the absence of ligand (fast component), whereas the minor fraction (30%) is bound to the chromatin (slow component). Upon activation by RAR agonist, the bound fraction increases by ~15%. We were interested in how the mobility of the promiscuous RXR reacts to agonist treatment. We carried out mobility measurements at three different temporal and spatial scales with sensitive fluorescence microscopy techniques. We studied long-range diffusion (at a distance scale of a few micrometers and a time scale of a few tens of seconds) by FRAP, and showed that long-range receptor mobility decreased significantly upon ligand treatment. We detected no significant completely immobile fraction either in the presence or absence of ligand, suggesting that receptor binding to the chromatin is transient, lasting shorter than a few tens of seconds. We studied local diffusion in the submicrometer range and at a time scale of tens of milliseconds by using fluorescence correlation spectroscopy (FCS). We proved that RXR is even more mobile than RAR in the absence of ligand: 85% of RXR molecules diffuses freely, and a mere 15% is chromatin-bound. Upon agonist treatment the slow fraction increases significantly, to ~43%. When binding of full length (endogenous) coactivator to RXR was impaired by a peptide competing with the coactivator, ligand induced transition to the slow state did not take place. The condition for this transition is binding full length coactivator. We also carried out our experiments by mutants lacking the DNA-binding domain; this molecules possesses only the ligand binding domain (GFP-RXR-LBD), which is also responsible for ligand binding. For this mutant the slow complex constituted only 5% of the population. However, the slow fraction also increased by 30% upon RXR agonist treatment, suggesting that RXR-LBD, via interacting with its dimeric partner (e.g. RAR), can indirectly bind to the DNA. We also repeated our experiments by using the selected plane illumination microscope-FCS (SPIM-FCS) allowing 2D mapping of the mobility in the whole cell nucleus. SPIM-FCS measurements showed that RXR binding occurs homogenously throughout the entire nucleus, not only at a few selected binding sites. These measurements also confirmed the applicability of the two-component model. Our results support a model in which RXR has a distinct, highly dynamic nuclear behavior and follows hit-and-run kinetics upon activation. We mapped the genomic binding sites of RXR by using the ChIP-seq technique. We found that the number of binding sites in the absence of ligand is ~6600, which increases to ~8300 upon agonist treatment, and about 54% of all binding sites contains the consensus sequence of RXR response elements. The occupancy of binding sites significantly increases after ligand treatment, in accordance with the results of the biophysical techniques. Our results were published in ***Molecular and Cellular biology (4).***

2.2. Dimerization of nuclear receptors studied by FRET and SPIM-FCCS

As a continuation of our studies on the mobility of RAR and RXR, we examined the dimerization of nuclear receptors by using FRET, FCS and 2D-imaging SPIM-FCCS (single plane illumination fluorescence cross-correlation spectroscopy). Our hypothesis is that dimeric partner selection of RXR (from among RAR, PPARg, VDR, LXR, etc.) depends on the presence of specific agonists of the partner receptors, which increase the stability of a given dimer. By FRET measurements between GFP- and mCherry-tagged receptors we have shown that RXR and RAR dimerized even in the absence of ligand, and there was only a slight increase in the extent of dimerization upon RXR or RAR agonist binding. We got similar results for the PPARg-RXR interaction. FCCS measurements require lower protein concentrations than FRET studies. We carried out SPIM-FCCS measurements (at DKFZ) at lower expression levels closer to the endogenous concentrations in order to study the dimerization/co-diffusion of RXR and RAR, and also their binding to chromatin. Our results showed that RAR and RXR dimerized both in the presence and absence of ligand even at these lower concentrations, and a significant fraction of the dimer was bound to DNA. We also performed SPIM-FCCS measurements with the ligand binding domains (LBDs) of the receptors lacking DNA binding capacity, and found that the fraction of heterodimeric RAR-LBD + RXR-LBD was only ~5-10% in the absence of ligand, which increased to ~80% when RAR or RXR agonist was added. From our FRET and SPIM-FCCS experiment we concluded that **i)** ligand binding increases the affinity of receptor dimerization and **ii)** DNA-binding also enhances receptor dimerization. The latter effect can be explained by the occurrence of nuclear receptor response elements as repeats (direct, inverted, etc.) in the genome, which can dock two receptors and promote their interaction by bringing them in close proximity. This model can be considered as a refinement of the molecular switch model.

We created plasmids coding for further nuclear receptors that are heterodimeric partners of RXR: LXRa, PPARg, and VDR labeled with blue, green and red fluorescent protein tags (TagBFP, EGFP, mCherry). We started the study of the ligand dependence of heterodimer/homodimer formation of RXR by using tripartite systems: RXR and two partner receptors competing for dimerization in the presence of specific ligands. With these experiments we want to generalize the hypothesis that the presence of a specific ligand increases the stability of the corresponding heterodimer. In these experiments the cellular expression levels of all three receptors (e.g. BFP-PPARg + GFP-RAR + mCherry-RXR) should be approximately equal. However, it was not possible to achieve equal expression of all three proteins in cells by using transient cotransfection of three receptors. To reduce variability of expression, we made a lentiviral transfer plasmid to create a cell line stably expressing FP-tagged RXR. Experiments using this cell line are under way. As a continuation of the present project, we would also like to measure the dissociation constants of purified, fluorescently tagged receptors in FRET titration experiments *in vitro* by single molecule microscopy. We designed vectors to express FP-tagged receptors with Halo-tags enabling protein purification.

We also examined **RXR homoassociation** to answer a long-debated question whether RXR could also act as a homodimer. By using FRET and SPIM-FCCS we have proven that RXR forms homodimers in live HeLa or 293T cells. We wanted to identify the genomic binding sites of the homodimers by using the ReChIP-seq technique. In this experiment, RXR molecules were tagged with one of two different tags (GFP or FLAG), which were coexpressed and immunoprecipitated sequentially, thus we could fish out dimers of RXR molecules. Before the ReChIP experiment, we successfully validated the DNA-binding of GFP-RXR and FLAG-RXR separately and also as a complex by using ChIP-qPCR at a number of known PPARg-RXR or RAR-RXR binding sites. By ChIP-seq of GFP-RXR (using anti-GFP-antibody) we could also identify peaks at enhancers of known nuclear receptor-regulated genes. Unfortunately, neither ChIP-seq, nor ReChIP-seq gave specific peaks with FLAG-RXR. The anti-FLAG antibody may not have the

sufficient specificity needed for ChIP-seq. We designed a Halo-tag-RXR vector, which binds covalently to its resin during purification, allowing more stringent washing and a better selection of specific binders. This is work is in progress.

We created **FRET-based ligand sensors** from the ligand binding domains of RAR and RXR. These constructs are labeled at the N and C termini with GFP and mCherry. When agonist ligand is bound, the C-terminal helix-12 undergoes a conformational change, and the distance between the two dyes changes, resulting in a change of FRET efficiency between them. For RAR-LBD the FRET efficiency increased from 7% to 8%, whereas for RXR-LBD from 12% to 13%. We intend to optimize the constructs spatially and spectroscopically to get a larger change in FRET efficiency: we modify the linker length connecting the LBD and the dyes, and replace the mCherry with mScarlet to increase the overlap integral with the GFP spectrum.

3. DEVELOPMENT OF TECHNIQUES TO QUANTITATE PROTEIN-PROTEIN INTERACTIONS

3.1. High throughput FRET measurement for studying protein-protein interactions in large populations of adherent cells

The application of FRET (Förster resonance energy transfer) sensors for monitoring protein-protein interactions under vital conditions is attracting increasing attention in molecular and cell biology. Laser-scanning cytometry (LSC), a slide-based sister procedure to flow cytometry, provides an opportunity to analyze large populations of adherent cells or 2-D solid tissues in their undisturbed physiological settings. We worked out an LSC-based three-laser protocol for high-throughput ratiometric FRET measurements utilizing cyan and yellow fluorescent proteins as a FRET pair. Membrane labeling with Cy5 dye was used for cell identification and contouring. Pixel-by-pixel and single-cell FRET efficiencies were calculated to estimate the extent of the molecular interactions and their distribution in the cell populations examined. We also presented a non-high-throughput donor photobleaching FRET application for obtaining the required instrument parameters (alpha factor) for ratiometric FRET imaging. In the biological model presented, HeLa cells were transfected with the ECFP- or EYFP-tagged Fos and Jun nuclear proteins, which heterodimerize to form active AP1 transcription factor. The method, due to its high throughput, could be applied for screening the effect of drugs on protein-protein interactions. We published the procedure in ***Current protocols in Cytometry (5).***

3.2. EGFP oligomers as natural fluorescence and hydrodynamic standards

We are studying protein complexes (e.g. nuclear and interleukin receptors) by observing fluorescent protein (FP) tags. These proteins can be present in various oligomerization states, which differ in their mobility and brightness. These parameters are influenced by the photophysical behavior of the FP, which depends on the measurement conditions (excitation light intensity, pH, etc.). Exact knowledge of e.g. the brightness of oligomers is important for accurate interpretation of data derived from fluorescence fluctuation methods such as FCS or Number and brightness analysis. We characterized the fluorescence and hydrodynamic properties of recombinant, covalently linked EGFP oligomers composed of 1 to 4 EGFP subunits in solution in order to validate them as natural standards for higher protein complexes.

Diffusion coefficients (D) of EGFP1-4 were determined by analytical ultracentrifugation with fluorescence detection and by FCS. A “barrels standing in a row” model agreed best with the sedimentation data. Fluorescence anisotropy decreased, indicating homoFRET between the subunits.

Fluorescence lifetime decreased only slightly (4%) indicating no significant quenching by FRET to subunits in dark states. FCS-measured D, particle number and molecular brightness depended on dark states and light-induced processes in distinct subunits, resulting in a dependence on illumination power different for monomers and oligomers. Since subunits may be in "on" (bright) or "off" (dark) states, FCS-determined apparent brightness is not proportional to that of the monomer. From the dependence of molecular brightness on the number of subunits, the probability of the "on" state for a subunit was determined to be 96% at pH8 and 77% at pH6.38, i.e., protonation increases the dark state fraction. Our results with EGFP oligomeric standards can help to interpret results for oligomerized EGFP fusion proteins of biological interest. Our method to determine the fraction of fluorophores in a dark state can be used for red FPs as well, which are prone to have high (sometimes >50%) dark state fractions due to slow maturation and various photophysical processes. Our collaboration partners at DKFZ have prepared red FP oligomers from the often used mCherry fluorophore and the newly developed mScarlet (lab of Prof. T. J. Gadella, Amsterdam), which has improved photophysical properties; we will characterize and compare these dyes using the above described procedures. Since red FPs are often used as FRET acceptors, these studies will help the quantitative interpretation of FRET data. Our results were published in ***Scientific Reports (6)***.

3.3. Triple FRET measurement between 3 proteins

We worked out the measurement of FRET between 3 proteins labeled with spectrally distinct dyes, which allows for the identification of protein trimers. This method can be used for the study of multisubunit receptors or multiprotein complexes such as the IL-2/IL-15R or nuclear receptor/cofactor complexes. Our results were published in **Cytometry A** (7).

4. COLLABORATIVE PROJECT: BIOCOMPATIBLE NANOPARTICLE AS MR CONTRAST MATERIAL

In our research the efficient transfection of lymphocytes often causes problems. Presently we use either electroporation, which reduces cell viability, or lentiviral transduction, which is a more time consuming procedure. Both procedures are expensive. We participate in a collaborative project aiming at developing nanoparticles targeting various molecules such as MR contrast agents, nucleic acids, chemotherapeutic agents to selected cells. The research group at BBS Nanotechnology Ltd. consists of biocompatible polymers: chitosan and poly-gamma-glutamic acid, which are held together by electrostatic interaction. Nanoparticles were conjugated with folic acid, which binds to cells expressing folic acid receptors. We have shown by fluorescence microscopy and flow cytometry that human cervix carcinoma (HeLa) and rat epithelial (HeDe) cells overexpressing folic acid receptors specifically take up the nanoparticles. When receptors were previously saturated with folic acid, the uptake of the nanoparticles was almost completely abolished suggesting the role of folic acid receptors in the internalization of the nanoparticles. Cellular proliferation and viability assays proved the nanoparticles were not toxic. Gd^{3+} ions were bound to the nanoparticles via electrostatic interaction. MR studies indicated that Gd^{3+} loaded nanoparticles were taken up by HeDe cells, and change the T2 relaxation time. HeDe cells injected into rats and forming tumors also showed nanoparticle accumulation and a shift of T2. Our data were published in ***International Journal of Pharmaceutics*** and ***Anticancer Research (8, 9)***, and served as the basis of **István Hajdú's PhD thesis (defended in 2014).**

The publications arising from further collaborations are listed on page 10.

EXPLANATION OF CHANGES:

We planned to probe IL-2R – MHC I interactions and IL-2 signaling efficiency in Ankylosing Spondylitis (AS) patients having the HLA B27 MHC I allele. Neither the expression levels of IL-2R, MHC I and MHC I free heavy chains, nor the FRET efficiencies measured between MHC I molecules or between the MHC I – IL-2R alpha pair differed significantly on control vs. AS patients. We did not find any significant change in the IL-2 induced STAT5 phosphorylation levels either (see 1.3.). Therefore, we did not pursue this line of investigation further.

Instead of this line of research, we studied the preassembly of IL-2R and IL-15R subunits in the ER and the Golgi, which may have clinical significance in understanding resistance of lymphomas to antibody therapies as described above (see 1.2.). In addition, we proved that c-Fos oncogene, an important element of the IL-2/15 signaling pathway, forms homodimers in Fos-overexpressing cells, which may contribute to cancer development in a series of tumors overexpressing this protein (see 1.5.). We developed a technique to determine the dissociation constant of interacting proteins in live cells via FRET.

We also studied the hydrodynamic and fluorescence properties of EGFP oligomers, which will be useful in interpreting fluorescence brightness data of protein dimers and higher oligomers (see 3.2.).

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