

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

**DETERMINATION OF DYNAMIC PROPERTIES
OF NUCLEAR RECEPTORS**

by

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The Examination takes place at the Department of Biochemistry and Molecular Biology,
Faculty of Medicine, University of Debrecen;
at 11 AM; 13th of February 2014.

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The PhD Defense takes place at the Lecture Hall of the Building-A of the Department of
Internal Medicine, Faculty of Medicine, University of Debrecen
at 2 PM, 13th of February, 2014.

INTRODUCTION AND THEORETICAL BACKGROUND

The nuclear receptors

Regulatory signal that activates eukaryotic transcription factors can originate from a very distant source in the body. For instance, hormones released into the circulatory system by an organ that is part of the endocrine system travel through the circulation to essentially all parts of the body. The endocrine system can thus serve as a regulator to coordinate changes in transcription in cells of many different tissues. Some hormones are small molecules that, because of their lipid-solubility properties, can directly pass through the plasma membrane of the cell –like steroid hormones, such as glucocorticoid, testosterone, and estrogen. In the cell, steroid hormones bind to and regulate specific transcription factors in the nucleus. In metazoans they are called nuclear receptors (NRs).

Peroxisome Proliferation Activated Receptors (PPARs)

The unorthodox name of these receptors stands as a reminder of the initial cloning of one isoform as a target of various xenobiotic compounds that were observed to induce proliferation of peroxisomes in the liver. That isoform was the PPAR α . Soon the discovery of PPAR δ and PPAR γ came. Many cell types express more than one PPAR isoform, which raises the question of how isoform-specific targets are regulated. Most likely this occurs through a combination of subtle cis-sequence differences flanking the core RE, the presence of specific or selective coactivator proteins, and regulation of endogenous ligands.

A variety of fatty acids and their derivatives have been found to bind to PPAR γ with relatively low affinity. Eicosanoids, such as 13-HODE and 15-HETE, have also been suggested to act as PPAR γ ligands. Several high affinity synthetic PPAR γ ligands have been generated. These include the thiazolidinedione (TZD) class of drugs, which are used clinically as insulin sensitizers in patients with type-two diabetes.

They bind to DNA with the obligate heterodimer partner, the RXR. PPARs recognize the consensus PPRE half-site of a DR1 motif.

Retinoic Acid Receptors (RARs)

The nuclear protein called RAR binds retinoic acid, the biologically active form of vitamin A. Unlike members of the other protein superfamily, the steroid receptors, retinoid receptors are constantly localized in the nucleus. RARs are reported to be bound to their RE

and act as repressors in the absence of agonist ligand. They show high affinity for the RXR that is their obligate dimeric partner, just like for the PPAR. Their endogenous ligands are the all-trans retinoic acid (ATRA) and the 9-cis retinoic acid. Interestingly, the latter one can bind to the RXR as well. This phenomenon also shows the intimate and unique relationship between these two receptors.

Retinoid X Receptors (RXRs)

After the identification of the receptors for all-trans retinoic acid another receptor was discovered that was capable of mediating retinoid-signalling pathways. Parallely, a new cofactor was reported that appeared to be necessary for the RAR to bind to its RE. These reports were pointing at a new nuclear receptor, the RXR. The strong homology of the three isoforms (α, β, γ) indicates that they regulate common targets by binding similar ligands and recognizing similar sites. The difference is in their topological pattern of expression. RXR $_{\alpha}$ and RXR $_{\beta}$ are expressed in a wide range of tissues like kidney, spleen, placenta and epidermis. RXR $_{\gamma}$ is, in contrast mainly expressed in muscle and brain tissues.

Several molecules have been described as potential RXR ligands like 9-cis RA, docosahexaenoic acid or synthesized as selective ligands like LG100268. Still, the endogenous ligand for RXR has not been found yet.

RXRs are unparalleled in a sense that they can form heterodimers with at least twenty other nuclear receptors. ATRA, the ligand for RAR does not bind to or activate RXR. At the same time, 9-cis retinoic acid does. To distinguish the group of molecules that attribute their biological activities to interaction with RXR from the ones that do with RAR, rexinoids and retinoids are distinguished, respectively.

Based on their activation pattern in the mammalian two-hybrid studies RXR-heterodimers are divided into two groups. In a non-permissive heterodimer the partner (like RAR) actively interferes with the ability of RXR to activate transcription in response to RXR-specific ligands. The dimer cannot be activated selectively from the RXR side. Activation from both sides of the dimer has a synergistic effect on transactivation. In contrast, permissive heterodimers allow RXR signalling and act as bi-functional transcription factors. RXR forms permissive heterodimer with PPAR.

Nuclear receptors in action: a model

According to 'molecular switch model', in the absence of ligand corepressors and further members of the repressor complex, including HDACs are bound to the NR. This favours for the formation of condensed nucleosomal structure. The latter restricts

transcription factors access to the chromatin, resulting in a repressed state of transcription in that genomic region. As the agonist ligand appears and binds to the pocket of the LBD, conformation changes take place. Great amount of mutational, activity and structural studies were carried out with retinoic acid receptors, and others, revealing the working mechanism of the switch. The agonist-dependent repositioning of helix-12 (holo-form) causes a shift in the affinities between different coregulators and the NR. The holo-form has decreased ability to bind to the corepressors, but an increased affinity for the coactivators, such as ACTR (ACTivator for Thyroid hormone and Retinoid receptors) or DRIP/TRAP. In a cellular environment these sum up in the exchange of coregulators bound to the LBDs of the dimer. As a result of the coactivator binding, new sets of proteins are being recruited as members of the activator complex, including HATs. By creating an acetylated milieu in that genomic region via the histone-tail modifications, the change of coregulators ends up in transcriptional activation.

The temporal resolution of recent models of NR action

Chromatin immunoprecipitation (ChIP) revealed a new feature of transcription factors. The alteration of unproductive cycles marked by rapid DNA binding and ligand-dependent productive cycles with reduced mobility and longer binding-times seems to be the essence of estrogen receptor (ER) action. Based on the cyclic binding of ER, HDACs and RNA Polimerase II, the new model has pointed towards a highly integrated transcriptional ratchet that ensures dynamic and controlled response to stimuli, but requires elements being highly mobile.

Fluorescence Recovery After Photobleaching (FRAP)

The fluorescence techniques discussed here are based on the so-called fluctuation-dissipation theorem of statistical physics, which states that the fluctuation properties of a system and its response to an external perturbation are closely related. This characteristic of molecular systems gave rise to several techniques used to study equilibrium statistics by investigating relaxation to equilibrium after a small perturbation. One popular technique relying on external perturbation is FRAP, which can be applied to study binding kinetics and diffusion of fluorescent molecules in solution.

From a typical FRAP experiment the fraction of recovery and the half recovery time ($t_{1/2}$) can be determined. The first relates to the size of the mobile fraction.

FRAP was among the first methods allowing the study of transcription dynamics by detecting mobility in the sub-second range. Such studies led to the first challenge of the ‘rigid’ model with a “hit-and-run” model that introduced variable immobile fractions and half-recovery times of the bleached fluorescence signals of fluorophore-tagged NRs

Fluorescence Correlation Spectroscopy (FCS)

The discovery of fluorescent proteins and the possibility to detect molecules with single molecule sensitivity have revolutionized the way molecular interactions are measured. The sensitivity is allowed by confocal microscopy, which narrows the detection volume to a fraction of a femtoliter by applying a pinhole, and the power of fluorescence fluctuation analysis. FCS is similar to FRAP in the sense that it is based on the above mentioned fluctuation-dissipation theorem. However, FCS does not perturb the studied system because it does not cause any deviation from the equilibrium state. In fact this technique harnesses the deviations that occur spontaneously around the equilibrium. On the molecular level, equilibrium states are highly dynamic and the smaller the observed system, the higher are the relative fluctuations. FCS utilizes the fluctuations of fluorescence intensity resulted by the diffusion of fluorescently tagged molecules in and out of the confocal volume, which, in contrast to many other sources of noise, are correlated in time. The primary data detected in an FCS measurement is the time-dependent fluorescence intensity, which is proportional to the number of particles in the observed volume. From the recorded fluctuation of the fluorescence signal, the autocorrelation function is extracted, which reflects the photophysical and diffusion properties of the molecules.

Adding spatial to temporal: selective plane illumination microscopy – FCS (SPIM-FCS)

Despite its late second blooming, FCS has become a powerful tool for measuring the dynamics of fluorescently labelled molecules in solution and importantly also in live cells. With classical FCS setups it is usually possible to measure at several selected positions in a cell. Recently FCS has been extended to an imaging method by spinning-disk microscopy and selective plane illumination microscopy (SPIM).

In a SPIM microscope, in contrast to conventional wide-field and confocal fluorescence microscopes, a light sheet illuminates only the focal plane of the detection objective lens from the side, thus the detection of fluorescence light is at right angle to the illumination axis. Excitation is restricted to the fluorophores in the volume near the focal plane, so only those fluorophores that are actually observed are also illuminated. This provides optical sectioning and reduces photobleaching, phototoxicity and out-of-focus

background noise. The advantage of single plane illumination with detection in the entire image plane is that not only one but a whole set (up to 40x20) of FCS autocorrelation functions can be determined simultaneously in a single experiment. By fitting these curves, a nuclear mobility map can be created.

Going global: chromatin immunoprecipitation followed by sequencing (ChIP-Seq)

Much is known about the transcription factors binding to certain genomic locations, but recent sequencing technologies and the sequencing of more and more large genomes gave a chance to investigate an unseen face of transcription factors and the transcription machinery. In chromatin immunoprecipitation (ChIP) experiments, an antibody specific for a DNA binding factor is used to enrich target DNA sites to which the factor was bound to in a certain state of the living cell. The enriched DNA sites are identified, aligned to the reference genome and quantified. A sequence read distribution is one outcome of this process that is done by a peak locator algorithm. At this step the local concentration of sequence hits is determined and within these clusters a peak is called. These peaks are scattered along the whole reference genome showing the site and sequence preference of the factor in question. The information lies within the distribution and relative heights of these peaks, their changes and their correlations with that of peaks of other factors. Unlike the classical way of promoter analysis that could be like finding a needle in a haystack, ChIP-Seq provides a global view.

AIMS

Our studies concentrated on the dynamic nature of transcriptional regulation. The retinoic x receptor, as a central molecule of nuclear receptor action, was in the focus of our work. The general concept was to apply methods with different temporal (and spatial) resolution and gain insight to various sides of the involved mechanisms. In contrast with the majority of earlier investigations of the field, the used microscopy methods could be operated in live cells.

We started out with the use of a set of well-established methods for promoter analysis and then moved to the ones based on live cell confocal microscopy. The latter included FRAP and FCS. Various ligands and mutant forms of the receptors were utilized. As a completion of these studies SPIM-measurements for increasing spatial resolution and ChIP-Seq experiments for the review of effects on a whole genome scale were done.

The main questions we wished to answer were:

- What are the general mobility parameters of RXR and RAR?
- What is the effect of RXR and RAR activation on their intranuclear mobility?
- What events can be related to the changes in mobility?
- How the functional differences between RXR and RAR are reflected on their mobility?

After answering these questions we aimed to complete the models that describe nuclear receptor action with the description of highly dynamic elements.

MATERIALS AND METHODS

Cell culture and transfection

HeLa cells were maintained in phenol-red-free RPMI, supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin. Cells were plated 48 hours prior to measurement into Nunc 8-well chambered coverglass plates. 24 hours later, at 70% confluency, transfection was performed using 40 ng DNA mixed with 0.16 μ l FuGene (Roche) per well.

Plasmid constructs

cDNAs encoding hRXR α , hRAR α , hRXR α -LBD hRAR α -LBD, hRAR α -H12 cofactor interaction domains with nuclear localization signal NLS-SMRT-ID and NLS-ACTR-ID were subcloned after PCR amplification into pEGFP-C3 (Clontech) and pmCherry-C3 (created from pEGFP-C3 by replacing GFP with mCherry) using BglII/HindIII for RXR constructs, XhoI/HindIII for SMRT and NheI/SacI for ACTR constructs. GFP-RAR mutants were created using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturers' instructions. Integrity of all plasmids was confirmed by DNA sequencing.

Expression vectors for transient transfection assays, Gal-SMRT-ID1+2, VP-hRXR α -LBD, VP-hRAR α -LBD CMX-hRAR α , pMH100-TK-luc, bRARE-luc, pCMX- β -galactosidase, Gal-ACTR-ID1+2, Gal-DRIP-ID1+2 were described previously and were kindly provided by Drs. *R. Evans* and *Sz. Benkő*. Constructs of monomeric, dimeric, trimeric and tetrameric eGFP were kindly provided by Dr. *J. Langowski*.

Transient transfection assay

Functional characterization of proteins was performed by cotransfecting 500 ng of the cDNA with 120 ng of reporter retinoic acid response element (RARE) and 90 ng of the β -galactosidase plasmid into AD293T cells in 48-well plates. Luciferase activity was determined in the lysates using the Luciferase Assay Kit (Promega). Measurements were made with a Wallac Victor2 multilabel counter.

Pulsed ligand treatment

Transfected cells were incubated with 100 nM LG268 or AM580 ligand in serum-free medium for 10 minutes prior to FCS measurements. FCS measurements were carried out for 40 minutes afterwards. After washing out the ligand with pre-warmed (37°C) HBSS-buffer

cells were kept in serum-free medium in a CO₂ incubator at 37°C for 20 minutes. FCS measurements were then carried out in the absence of ligand for 40 minutes.

ChIP (Chromatin immunoprecipitation)

Cross-linking was carried out by disuccinimidyl glutarate for 30 minutes and by formaldehyde (Sigma) treatment for 10 minutes and was followed by RXR immunoprecipitation. After fixation chromatin was sonicated with Diagenode Bioruptor to generate 200-1000 bp fragments. Chromatin was immunoprecipitated with antibodies against pre-immune IgG (12-370, Millipore) and RXR (sc-774, Santa Cruz Biotechnologies, Inc.). Chromatin antibody complexes were precipitated with protein-A coated paramagnetic beads (Life technologies). After 6 washing steps complexes were eluted and reverse crosslinked. DNA fragments were column purified (Qiagen, MinElute). The amount of immunoprecipitated DNA was quantified with a Qubit fluorometer (Invitrogen). DNA was submitted to QPCR analysis or library preparation.

Fluorescence correlation spectroscopy (FCS) instrumentation and measurements

The 2-channel FCS extension (prototype designed by Dr. Jörg Langowski, DKFZ, Heidelberg, Germany) is attached to the 4th detection channel of the confocal scanning unit. FCS measurements on live HeLa cells were performed in 8-well chambered coverglass plates described above. Fluorescence of EGFP was excited by the 488-nm line of an Ar ion laser, and emission was detected through a 500-550 nm band-pass filter by a Perkin-Elmer avalanche photodiode (Perkin-Elmer, Wellesley, MA, USA). Fluorescence autocorrelation curves were calculated online by an ALV-5000E correlator card (ALV-Laser Vertriebgesellschaft m-b.H., Langen, Germany). Measurements of 10×8 s runs were taken at three selected points in the nucleus of each selected cell.

Fluorescence recovery after photobleaching (FRAP)

FRAP measurements were performed on an Olympus FluoView 1000 confocal microscope based on an inverted IX-81 stand with an UPlanAPO 60× NA 1.2 water immersion objective. The 488-nm line of an Ar ion laser excited EGFP, and emission was detected through a 500-550 nm band-pass filter. For quantitative analysis a 256×256 pixel area was selected, and scanned with an open pinhole and 10× zoom (pixel size 82 nm). Before each measurement 10 pre-bleach images were taken with 1% laser intensity followed by a 1500-ms bleach period with 100% laser intensity within the bleach area of 256×10 pixels that covered less than 30% of the whole nucleus. Fluorescence pixel intensities of background (outside the cell), bleach-

ROI (the strip) and whole-nucleus (the nucleus, including the strip, but excluding the nucleoli) were determined for each frame with NIH ImageJ ver. 1.45s. Recovery curves were created, normalized and evaluated with the IGOR software using Phair's Double Exponential model in the FrapCalc-EMBL module.

RESULTS AND DISCUSSION

The analysis of PPAR γ binding on the ABCG2-promoter (a ‘classical’ promoter analysis)

The previously described and published PPAR γ promoter includes a 1647 bp long region (-1285/ +362). Sequence analysis of this did not reveal any canonical PPAR γ response elements in that part. Next, a larger region (5000bp) including conserved sequences between the human, dog and bovine genome was taken into consideration. A 150bp, well-conserved region (-3946/ -3796) was identified by a bioinformatics approach. This sequence contains three potential PPAR γ response elements as they all include direct repeats of AGGTCA (DR1). The newly identified ABCG2-enhancer element was cloned upstream the minimal TK-luciferase promoter (enhancer TK-luciferase). This construct was cotransfected into COS1 and 293T cells along with the constitutively active form of PPAR γ (VP- PPAR γ) alone or with RXR. The increased luciferase activity indicates specific binding of the NRs to the enhancer element. This could be detected when the luciferase activity levels of the enhancer TK-luciferase (including the enhancer element) construct was compared to that of the TK-luciferase (control) element. Heterodimer formation has an enhanced effect as compared to the monomeric form. The appearance of further induced the PPAR-related induction of the reporter. These experiments revealed the binding of PPAR/RXR heterodimers to the newly identified enhancer element of the human ABCG2 gene.

Nuclear receptors at the single-cell level

RXR dynamics in live cells, as detected by FRAP

The intracellular mobility of RXR was studied by FRAP, which allows analysis of RXR dynamics on the scale of seconds. We wished to answer whether an immobile fraction appears after ligand treatment and how the agonist-dependent activation affects the mobility of RXR. Geometry of the bleached area largely determines the usable diffusion model, thus a strip was chosen as ROI. Recovery curves were double normalized and fitted to Phair’s double exponential recovery model. The size of immobile fraction and the half-recovery times could be determined and compared.

In the absence of ligand, the fluorescence signal showed a rapid recovery after bleaching with a half-recovery time, $t_{1/2} = 2.5 \pm 0.4$ s, and no immobile fraction ($3 \pm 3\%$). Ten minutes after the addition of 100 nM LG268, the $t_{1/2}$ of the recovery increased to 7.3 ± 0.7 s, but still no significant immobile fraction was detected ($7 \pm 3\%$). Agonist treatment also

caused an increase, though to a smaller extent, in the $t_{1/2}$ of the GFP-RXR-LBD construct that lacks direct DNA-binding ability. In these FRAP experiments, slowing-down of RXR was detected during activation, but unlike several other NRs, RXR did not form an immobile fraction that would indicate a longer DNA residence time.

Dynamics of RXR and RAR at the sub-second timescale as detected by live-cell FCS

To achieve a characterization with higher time resolution we moved to FCS, quantifying the mobility parameters of RXR in the millisecond range. The two-component, normal diffusion model was chosen. The concept of this part of the dissertation is built up as to highlight the analogies and more importantly the differences between RAR and RXR dynamics as detected by FCS. The regimes of the diffusion times were between $\tau_1 = 1.5\text{-}10\text{ ms}$ for the fast component and $\tau_2 = 60\text{-}240\text{ ms}$ for the second, slower component (with corresponding diffusion coefficients of $D_1 = 3\text{-}12\text{ }\mu\text{m}^2/\text{s}$ and $D_2 = 0.07\text{-}0.5\text{ }\mu\text{m}^2/\text{s}$).

Large fraction of RAR and RXR moves around in the nucleus relatively freely

The distributions for the two receptors are similar, but two main differences appear: the faster overall diffusion and the smaller size of the slower population of the RXR. $r_2 = 29\%$ of GFP-RAR $r_2 = 16\%$ of GFP-RXR molecules belong to the slower population, and the large majority to the faster one.

Activation shifts the receptors towards a slower state

A central concept of nuclear receptor action is the molecular switch model describing ligand-dependent coregulator exchange as the main event that makes activation possible. Our question was how ligand-dependent activation and coregulator-exchange is reflected in the mobility of the receptors. Treatment with a saturating concentration of a selective agonist (100 nM AM580 or LG268 for the RAR and RXR, respectively) caused only a slight change in diffusion times. Surprisingly, the redistribution of the populations appeared already five minutes after the addition of the agonist. The ratio of the slower (second) population increased to 43%, and the distribution of the fast diffusion time shifted to larger values. The mean of the slow diffusion time increased slightly, and its distribution broadened. As a starting point, the size of the slower population of RAR was larger than that of the RXR before the activation (29%). AM580 resulted a similarly immediate increase to 43%.

The ligand dependent shift in receptor mobility is transient in RXR, unlike in RAR

The level of small molecule metabolites and other potential NR ligands is not consistent in time in the circulating blood. Seeing the immediate response of receptor mobility to ligand treatment, we were curious about its durability. ‘Wash-out’ experiments were carried out. The very same well of cells was measured without and with agonist, then (after a short (20 minute) medium wash) the agonist-containing medium was replaced by ligand-free medium. Cells were incubated for 15 minutes and FCS measurement was carried out again in the same well. These experiments were carried out with the GFP-RXR (with LG268), and the GFP-RAR (with AM580) cell lines as well.

A surprising difference between these retinoid receptors appeared. The redistribution of the two RXR-populations was reverted completely when the agonist was removed from the medium. In contrast, the agonist-dependent redistribution of RAR was not reverted when the ligand was removed from the medium, implying a low off-rate of ligand binding.

Coactivator binding is needed for the ligand-dependent shift in RAR and RXR mobility

According to the molecular switch model of nuclear receptors, the main feature of the mechanism is coregulator exchange, which could also affect receptor dynamics. The process of receptor activation consists of corepressor release and subsequent coactivator binding, accompanied by a conformational change of the receptor. We created a series of (GFP-fused) point mutants modified at the surface residues of the fourth and eleventh helix of the RAR ligand-binding domain.

In the case of the ‘activator-binding’ mutant the diffusion properties of the untreated receptor were similar to those of the wild type. On average, $r_2=30\%$ of the population showed slow diffusion before ligand treatment. Apparently, the loss of repressor binding did not have a dramatic effect on the dynamics of the receptor. Contrarily, the increased SMRT-binding affinity of the ‘repressor-binding’ mutant caused a slight increase in τ_2 and a slight decrease of r_2 ($r_2=25\%$) for the untreated sample.

The mutants showed a disparate behavior after AM580-treatment. A clearly significant increase of r_2 ($\sim 12\%$) could only be detected in the case of the ‘activator-binding’ mutant, which behaved similarly to the wild type.

As there are no well-described point mutations of the RXR that would reportedly modify its coregulator binding, we applied a different strategy here to test the effect of coactivator binding on its mobility. The LG1208 is a synthetic (RXR α -specific) ligand that acts as a competitive antagonist. In a dual ligand treatment LG1208 diminished the LG268-

effect when it was applied in ten-fold excess. We presume that LG1208 occupies the ligand-binding pocket of the receptor, which induces a conformation of RXR incompatible with coactivator binding. These findings were corroborated with FCS measurements. The antagonist alone did not change r_2 , but in combination with the agonist ligand it prevented redistribution. This suggests that, just like in the case of RAR, coactivator binding is a prerequisite for the mobility shift of RXR.

DNA-binding determines the steady state of the receptors but has limited effect on the activation-dependent changes in mobility

Being transcription factors, one of their key characteristics of NRs is the ability to directly bind DNA. Therefore, we expected that reducing or abolishing the DNA binding affinity of the receptors would have major effect on their diffusion. Truncated forms of the receptors that contain only the LBD (thus lacking the DBD) were fused to GFP (GFP-RXR-LBD, GFP-RAR-LBD). Our FRAP measurements already showed that the truncated form of RXR had a somewhat higher mobility than the full-length receptor, but it responded to activation with an increased half-recovery time. This mutant is still capable of ligand- and coregulator binding and dimer formation, but unable to bind to DNA directly. It is important to emphasize, though, that DNA binding of the LBD construct via the (full-length, endogenous) dimer partners cannot be excluded. The FCS measurements in the nucleus showed slightly shorter diffusion times in the fast population as compared to the FL forms. This can be related to the smaller molecular weight of the truncated receptor. The apparent diffusion time of the second component did not differ significantly from that of FL, but its fraction was lower than that of the full-length form. This result implies that there is some DNA binding or chromatin association in the unliganded state of the full-length RXR and RAR.

Interestingly, as the agonist was added to the transfected cells, the fraction size of the slower population of LBDs changed. But at this point again, a difference between RXR and RAR was detected; in case of RXR the size of the slower fraction that was just detectable before the ligand treatment increased to 40%, that is close to the level of the activated FL form. The r_2 of RAR-LBD construct also showed agonist-dependent increase but at a level that is far below that of the RXR; it just reached the inactivated level of the FL form.

The mobility map of RXR

Visualization of the intracellular spatial distribution of different RXR fractions can give us an unprecedented view of transcription factors. We applied single plane illumination microscopy FCS (SPIM-FCS). The advantage of single plane illumination with detection in the entire image plane is that not only one, but a whole set (up to 40x20) of FCS autocorrelation functions can be determined simultaneously in a single experiment. By fitting these curves, a nuclear mobility map of RXR can be constructed, which shows the nuclear distribution of r_2 . The redistribution detected by FCS was thus confirmed and refined by SPIM-FCS. In addition, it was revealed that the distribution of populations was rather homogenous, as no nuclear architecture related pattern was recognized either before or after activation.

A global view on the DNA binding of RXR

As seen, NRs recognise certain DNA elements and partner proteins and interactions are formed between them. These interactions have dramatic effects on each other. Many of these receptors diffuse freely along the nucleus. As the right stimulus arrives, the picture changes; they slow down. This is probably due to their chromatin binding; an interaction that largely depends on the coregulator and dimer binding ability. But what happens between the transcription factor and the chromatin? Are there more sites appearing as potential binding sites during activation? Is there an increase in the rate of both unspecific and specific chromatin binding? Are these interactions getting stronger? These questions can only be addressed when the chromatin binding of a NR is investigated at the whole genome level.

We carried out ChIP-Seq experiments on HeLa cells to have a glimpse on the distribution on RXR-occupied regions over the whole chromatin before and after the activation. Chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-seq) was performed using the HeLa cell line, which was treated with RXR-agonist (100 nM LG268) for one hour. Samples were cross-linked and immunoprecipitated by a pan-RXR antibody. After sequencing, the genomic locations that showed RXR binding were detected as genomic binding sites. These are typically referred to as peaks, because a stack of short sequences shows up as peaks on genome browsers.

We determined the number of binding sites that RXR occupies and the impact of ligand on the cistrome in our HeLa cell line-based model system. 6636 genomic regions were determined as binding-regions in the vehicle treated samples. This number increased in the LG268 activated samples, where 8302 binding sites were detected. 5138 (more than 50%) of

all peaks were identical before and after agonist treatment, these are sites that are permanently occupied by RXR. 1498 sites disappeared and 3164 new sites appeared upon activation.

The activation of RXR by its agonist ligand does not induce RXR binding to a significantly larger set of sites specific for the active state. At the same time, chromatin occupancy, expressed as the mean of tag counts of the peaks, increased from 9.3 to 12 (with 28 and 39 maximum values, respectively), as shown by metagene analysis. Thus, within one hour, ligand activation increases the probability of RXR binding to DNA or chromatin.

SUMMARY

We characterized and compared the nuclear dynamics of RXR and RAR during activation in single cells on the sub-second scale using live-cell imaging methods. By applying FRAP and fluorescence correlation spectroscopy (FCS), techniques with different temporal resolution, a highly dynamic behaviour could be shown, which is best described by a two-state model of receptor mobility. In the unliganded state most NRs belonged to the fast population. Upon agonist treatment, the ratio of the slow population increased to as a result of an immediate redistribution. Coactivator binding appears to be indispensable for redistribution and has a major contribution to chromatin association.

A comparison of the FCS results gained from the RXR and from the RAR studies revealed differences on the behaviour of these two molecules: RXR appeared to be more dynamic. It changed its mobility at a larger scale upon ligand activation. The redistribution detected by FCS was confirmed and refined by single plane illumination microscopy (SPIM-FCS). In addition, it was revealed that the distribution of populations was rather homogenous, as no nuclear architecture related pattern was recognized either before or after activation.

Investigation of activation dependent changes showed that the occupancy of RXR's genomic binding regions increased, but no significant change in the number of sites was revealed by ChIP-Seq.

The relationship of the RXR and other nuclear receptors during activation is an interesting question of the field. How the available binding sites, the ligands and the dimer partners determine and regulate the formation of different RXR heterodimers might now be answered. Another interesting topic is the relationship of the chromatin, the transcription factors and the actual active sites of transcriptions. Newer applications partially related to these methods are already available; such as SPIM-FCCS (SPIM - Fluorescence Cross- Correlation Spectroscopy) for the nuclear map of protein-protein interactions, or the GROseq (Global Run-On sequencing) for the detection of active RNA-production on a global scale. When the aim is to describe a mechanism, the key might still be the combined use of methods with different ways of targeting.

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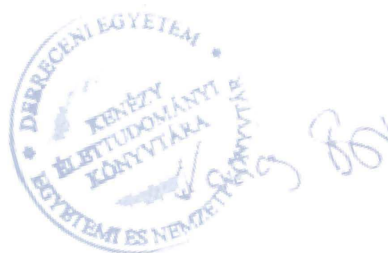
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Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **Brázda, P.**, Krieger, J., Dániel, B., Jonas, D., Szekeres, T., Langowski, J., Tóth, K., Nagy, L., Vámosi, G.: Ligand binding shifts highly mobile RXR to chromatin-bound state in a coactivator-dependent manner as revealed by single cell imaging.
Mol. Cell. Biol. "accepted by publisher", 2014.
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SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)



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List of other publications

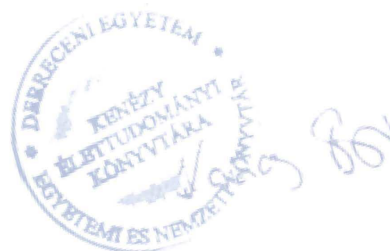
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Total IF of journals (all publications): 30.823

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The Candidate's publication data submitted to the Publication Database of the University of
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LIST OF PRESENTATIONS

P., Brazda, K., Toth, L. Nagy, Gy., Vamosi (2011) “Dissecting the role of coregulator exchange and chromatin binding in retinoic acid receptor (RAR) mobility by live cell FCS” (presentation)

– 8th European Biophysics Congress (EBSA), Budapest

P., Brazda, B., Reho, J., Krieger, K., Toth, Gy., Vamosi (2013) “Activation enhances DNA-binding of RXR but not the number of binding sites, as shown by FCS and ChIP-Seq” (poster)

– 9th European Biophysics Congress (EBSA), Lisbon

Gy., Vamosi, **P., Brazda, B., Reho, J., Krieger, K., Toth** (2013) “Liganded RXR displays highly dynamic behavior governed by coactivator binding as revealed by single cell imaging” (presentation)

– 23rd Wilhelm Bernhard Workshop, Debrecen

FURTHER PUBLICATIONS

Brazda P, et al. (2007) A transzkripció szabályozás dinamikus arca.

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