Quantitative live cell imaging of glucocorticoid receptor dynamics in the nucleus

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6 SUMMARY AND DISCUSSION

The glucocorticoid receptor is a well-studied transcription factor that serves as a model for transcription factor dynamics. In this thesis, we have developed a framework to advance our understanding of glucocorticoid receptor dynamics in the nucleus of live cells. To this end, we set out to gain biological insight as well as advance the analysis and technology used to investigate these processes. In this chapter, major findings are summarized and discussed and future directions are proposed.

The study carried out in **chapter 2** of this thesis has led to the discovery of several GR dynamics states and insight into how the receptor finds its target, which included repetitive switching between states. We also identified specific amino acids involved in long binding times of the GR. Quantification of single molecule data and FRAP experiments led us to develop a continuous time Markov chain model through which we could obtain information about switching even when using data obtained on shorter time scales. In **chapter 3**, through correction for the loss of fast molecules through a limiting depth of focus we obtained true fraction size for GR molecules in the nucleus of live cells. Through this, we could understand that switching of GR between states occurred at a larger time scale. In addition, **in chapter 4** GR clusters were revealed. Through quantification, we could describe these clusters and find characteristic that distinguished GR clusters from clusters of a closely related ER. Moreover, we could exclude that the distribution of the receptor within receptor specific foci was dependent on specific DNA binding. In particular, we have achieved progress in the analysis of single-molecule data. We have investigated alternative labeling methods for proteins in live cells in **chapter 5,** to observe the dynamics and localization of individual GRs on longer time scales. To this end, we have characterized the behavior of golden nanorods (GNRs) in both cytoplasm and nucleus. We showed that functionalization of GNR with NLS enables nuclear translocation. This indicates that GNRs can be functionalized and used as a label for GR tracking.

6.1 THE DYNAMICS OF THE GR (CHAPTER 2 AND 3)

In this thesis, we provide the most complete picture of GR dynamics to date. In **chapter 2**, GR dynamics in the nucleus of live cells was investigated by combining single-molecule microscopy and FRAP experiments. To this end, COS-1 cells were used that transiently expressed YFP labeled GR proteins. Four states with different dynamic properties were identified for the GR: two binding states and two diffusion states. Moreover, a mathematical framework was employed based on a continuous time Markov chain model to investigate the time spent in each of these states as well as the transition probabilities between states. In addition, DNA-binding domain mutants were exploited with a range of DNA-binding affinities to investigate the molecular mechanisms that underlie each of the states. When these results were combined, we concluded that GRs in the fast diffusion state is are freely diffusing through the nucleus, that GRs in the slow diffusion state are transiently interacting with DNA, that indirect binding to DNA leads to immobilizations with a short residence time and that direct DNA binding results in immobilization with a long residence time. Moreover, we show that the GR spends relatively long periods of time in the free diffusion state and once it leaves this state, it repeatedly alternates between brief nonspecific DNA interactions and longer immobilizations due to specific DNA binding ('repetitive switching'). Together these results provide a picture of how the GR finds its target on the DNA. This picture fits well within the theoretical description for target searching strategies of transcription factors, which propose a combination of 1D and 3D diffusion as the most effective way to reach a specific DNA target motif (1–3). Since the GR serves as a well-studied example of transcription factor functioning, it is likely that this mechanism is used by other transcription factors as well, but further studies should be carried out on other transcription factors to confirm this.

It is striking that the receptor has a high chance to switch from a free diffusion state to a slow diffusion state, but a low chance of switching from the free diffusion state to a direct or indirect binding state. This implies that the receptor has to switch to a different conformation or become part of a (different) multiprotein complex or that it reaches a different environment. This is supported by the long time that the receptor spends in the free

diffusion state. In this novel conformation, protein complex or nuclear environment, it is slowed down due to the increased number of (nonspecific) interactions with other (DNA) molecules. Another striking aspect of this research is alternating of the receptor between slow diffusion and binding. This indicates that once it reaches the slow diffusion state it is likely to remain within a limited region of the nucleus as it now only diffuses slowly. This allows for effective searching and binding within this region.

To better interpret the results obtained using single-molecule microscopy, in **chapter 3** we have developed novel analysis methods to faithfully determine the fraction of diffusing receptors and understand the timescales at which switching occurs. We can correct for the depletion of fast mobile fractions due to the axial limitation of the observation volume. Due to the limited detection volume, the fraction of fast diffusing molecules is depleted when longer time scales are used. We derived an analytical solution for a system with two populations based on the chance of a molecule to remain in the observation volume and integrated over the depth of the volume. We did single molecule microscopy on YFP-GR in COS-1 cells using increasing time between frames from 6.25 milliseconds up to 100 seconds. Through application of particle image correlation spectroscopy, we could correlate particles between consecutive frames as well as every other frame (4). When we corrected the obtained fraction size of the two populations, the fraction of GR molecules remained stable over time scales that we have observed (up to 150 milliseconds). This indicates that switching must occur on a much time scales larger than 150 milliseconds. This is congruent with the findings from chapter 2, in which we employed continuous time Markov chain models and find that switching must occur on the time scale of 600 milliseconds to tens of seconds.

6.2 THE LOCALIZATION OF THE GR (CHAPTER 4)

In the light of the results obtained in chapter 2, we expected to find regions in the nucleus of live cells to which the receptor is confined due to the slow diffusion and binding. Indeed, the occurrence of GR clusters has been observed previously, but thus far they had not been characterized extensively in a quantitative manner (5,6). In **chapter 4,** we carefully

characterized these clusters, or foci, in a quantitative manner. To this end, GFP-GR was stably expressed in U2OS cells, and spinning disk microscopy was used to obtain high-resolution images. An analysis pipeline was created in which individual nuclei were identified, a triangle threshold was applied and individual foci were identified. A Gaussian curve was fitted to the individual foci to obtain their width and intensity. We characterized the number of foci, their size and the relative number of molecules inside foci.

The molecular mechanism underlying the occurrence of these foci remains unclear. To obtain further insight into how foci differ between transcription factors, we compared GR foci with estrogen receptor (ER) foci. The ER was localized in significantly more, but smaller, foci than the GR. Together these findings imply that ER and GR foci are distinct entities. It has been hypothesized that foci correspond with specific DNA binding and subsequent transcriptional regulation. The GR and ER each recognize a different DNA binding motif (glucocorticoid or estrogen response elements (GREs or EREs)). Within the DNA-binding-domain of these receptors, the p-box dictates binding of the receptor to either GREs or GREs. Previous studies have shown that exchanging three amino acids within the p-box of the ER to those corresponding with the GR led to the recognition of GREs by the ER (7,8). This mutant was termed ERpGR. We exchanged the same amino acids in the GR, thereby generating GRpER. We have demonstrated that the foci's characteristics (like width and number) of these mutant receptors do not change compared to their WT receptors. This suggests that clustering of EREs or GREs in the nucleus, either pre-existing or formed upon ER or GR binding, does not explain the presence of foci.

Seemingly in contrast, previous studies have shown that the GR DNA-binding domain is required for the formation of focal domains inside the nucleus (5). In addition, the degree of inhomogeneity is dependent on the specific ligand that is bound to the GR (5,6). These results suggest that the receptor-specificity of the formation of foci depends largely on the ligand-binding-domain, but that specific DNA binding to EREs or GREs through the DBD is a prerequisite for the formation of foci in general. Binding of cofactors might play an important role in this as well as dimerization. Indeed, the latter was

previously shown to be of importance in the number of foci (9). To understand whether GR and ER foci are separate entities as their number and size suggest, colocalization experiments are essential. As the width of the foci show a wide range of distributions for both receptors, it is possible that ER and GR foci with an identical size colocalize. Furthermore, colocalization studies of the mutant receptors described earlier, with their original receptors might provide further clues as to which processes are dependent on direct DNA binding. To understand the role of response elements in the formation of transcription factor foci, future experiments should also aim at combining live cell imaging techniques of transcription factors and methods such as HiC and ChIP-seq.

It would be interesting to quantify the relative position of foci in the nucleus to understand their possible interactions with structural components of the nucleus, such as nucleoli, PML bodies or Cajal bodies. Additionally, it has been noted that the N-terminal-domain of the GR is quite disordered. Disordered proteins can undergo liquid-liquid phase separation under the right conditions, which may result in the formation of nuclear foci (10–12).

In order to study the functional role of the foci, we could study colocalization with markers for active chromatin in live cells, for example by using mintbodies (13). Colocalization with markers for active transcription such as RNA poll II could also be used. It has been shown that RNA poll II also exists in foci (14). However, van Steensel et al. (15) showed no correlation of RNA poll II. with GR foci. Colocalization with known interaction partners based on the GR interactome could also provide useful to disentangle the molecular mechanisms behind each state. These include many other transcription factors.

6.3 Coupling localization and dynamics

To obtain a full understanding of how the GR functions as a transcription factor we need to combine methods that capture dynamics (as described in **chapter 2**) with methods that provide information on the spatial distribution (as described in **chapter 4**). We would like to understand if DNA-bound receptors are the ones present in foci, whether these are different from

indirectly bound molecules and whether the slow diffusion occurs inside or close to the foci. Experimentally, this could be done using combined expression of receptors labeled with two different fluorescent molecules: one at a high concentration for the visualization of the distribution (in particular the localization of the foci), and one at a low concentration of imaging and tracking of individual molecules. This could be performed using two different fusion proteins, or by using a Halo-tagged receptor and labeling with a combination of two Halo ligands.

During the life-cycle of the receptor heat-shock proteins and subunits of the proteasome are involved in multiple steps. In the absence of ligand, the GR is confined to the cytoplasm (16). It is present in a complex that includes heat shock proteins as well as immunophilins (17,18). Once activated by the ligand, the GR is translocated to the nucleus and will find and bind to response elements in the DNA. Where heat shock proteins might stabilize GR interactions with the chromatin, the proteasome promotes GR removal from the chromatin (19,20). Whether the receptor is then targeted directly for degradation or can be recycled remains unclear. It is likely that both processes occur. This is supported by studies that show that the receptor is more stable in the absence of a DBD, but that in the presence of a DBD activation of the receptor by a ligand leads to increased degradation (Schaaf and Cidlowski, unpublished data). Moreover, this is ligand-specific. It is possible that the foci that were identified in chapter 4 represent clustering of the receptor with components of the proteasome in the process of, or after unloading from the DNA and could even represent degradation sites.

Furthermore, it was striking to find that the long immobilizations were dependent on the presence of two specific amino acids in the DNA-binding-domain, whereas the short immobilizations were completely independent of the integrity of the DNA-binding-domain. This suggests that these are really two different processes. We suggest that short immobilization corresponds with tethering to other transcription factors. Alternatively, it could represent other processes such as degradation of the receptor. In this light, it would be interesting to investigate colocalization of the GR with well-known tethering

partners such as AP-1 or block degradation using drugs such as geldanamycin.

6.4 Long-term visualization and tracking of individual GRs (chapter 5)

It would be interesting to be able to assess whether receptors alternate between long and short immobilization times, and whether this occurs with intermittent slow diffusion. To obtain this kind of information tracking of individual receptors over longer periods of time is necessary. Ideally, we would like to be able to track a single receptor through multiple cycles of DNA binding and unbinding. This requires imaging on the time scale of minutes, with a high framerate (~200 Hz). Fluorophores currently available are prone to blinking and bleaching and hence these types of measurements are not feasible with these tools. Thus, there is a need to develop methods that allow for long-term visualization. In **chapter 5**, we explored the use of a material that could serve as a label for proteins and allows for tracking of arbitrarily long times. Once this method has been established, the work provided in chapter 2 will serve as a benchmark to control for the effect of a different label on the behavior of transcription factors.

To this end, we have investigated a new method that allows prolonged tracking of the GR in the nucleus of live cells in chapter 5. Gold nanorods are unaffected by bleaching and easily functionalized. To assess their feasibility as a label for the GR, we have characterized the behavior of gold nanorods in live cells. We developed a delivery method using microinjection of the gold nanorods into the nucleus or the cytoplasm and subsequently assessed the diffusion properties and confinement radius of these particles in both the nucleus and the cytoplasm. The diffusion coefficient for the particles used in this study was much smaller than theoretically expected and much smaller than that of the GR (21,22), although it was consistent with previous reports or particles this size (23). When the GNR would be coupled to the GR this might influence the diffusion of the receptor and therefore its function. In addition, the number of freely diffusing and translocating particles was found to be limited. In the nucleus as well as the cytoplasm only ~45% of the particles were freely diffusing.

The gold particles we used were coated with polyethylene glycol (PEG) molecules of 5000 kDa. These are large proteins that likely form brush-like structures around the GNR with which other proteins or structures inside the cell can easily interact. In addition, the percentage of nuclear translocation of gold nanospheres was previously found to be size-dependent (24). These findings lay bare the current limitations of gold nanorods as labeling probes for the GR. By decreasing the size and interactions with the cellular environment these particles might still be promising as a probe for the GR in the future. To reduce interactions with the cellular environment the coating of GNR proteins could be altered. Alternatives could be a smaller polymer or a silica shell.

To function as a label for the GR it is essential that these particles translocate to the nucleus only upon active translocation of the GR after induction with the ligand. We showed successful functionalization of these particles with a nuclear localization signal is possible (NLS). However, only 8% of the particles translated to the nucleus after functionalization with this NLS. It is likely that this limitation is related to the low number of freely diffusing particles, since free diffusion seems like a prerequisite for translocation to the nucleus. Together these findings indicate that the GNR could be used as a label for the GR, but that the efficiency would be very low.

6.5 Future perspective

Together, the chapters of this thesis provide an outlook for the investigation of transcription factors such as the GR. In particular, it seems essential to carefully quantify the processes involved. To this end, we must keep on developing novel analysis techniques. In addition, we need to couple localization in foci with binding and diffusion of the receptor. Looking at colocalization of the receptor with other nuclear factors will uncover the underlying biological mechanisms that dictate the distribution and dynamics of the GR. To this end microscopy tools, in particular single molecule approaches, remain a powerful tool. These tools should be combined with information on the conformation of DNA through tools such as HiC. Finally, the most information would be obtained through following a single receptor through multiple DNA binding and unbinding cycles. Therefore, increased

efforts to develop methods for high frame rate acquisition (milliseconds) over long time periods (hours) must be are required.

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