Single cell biochemistry to visualize antigen presentation and drug resistance

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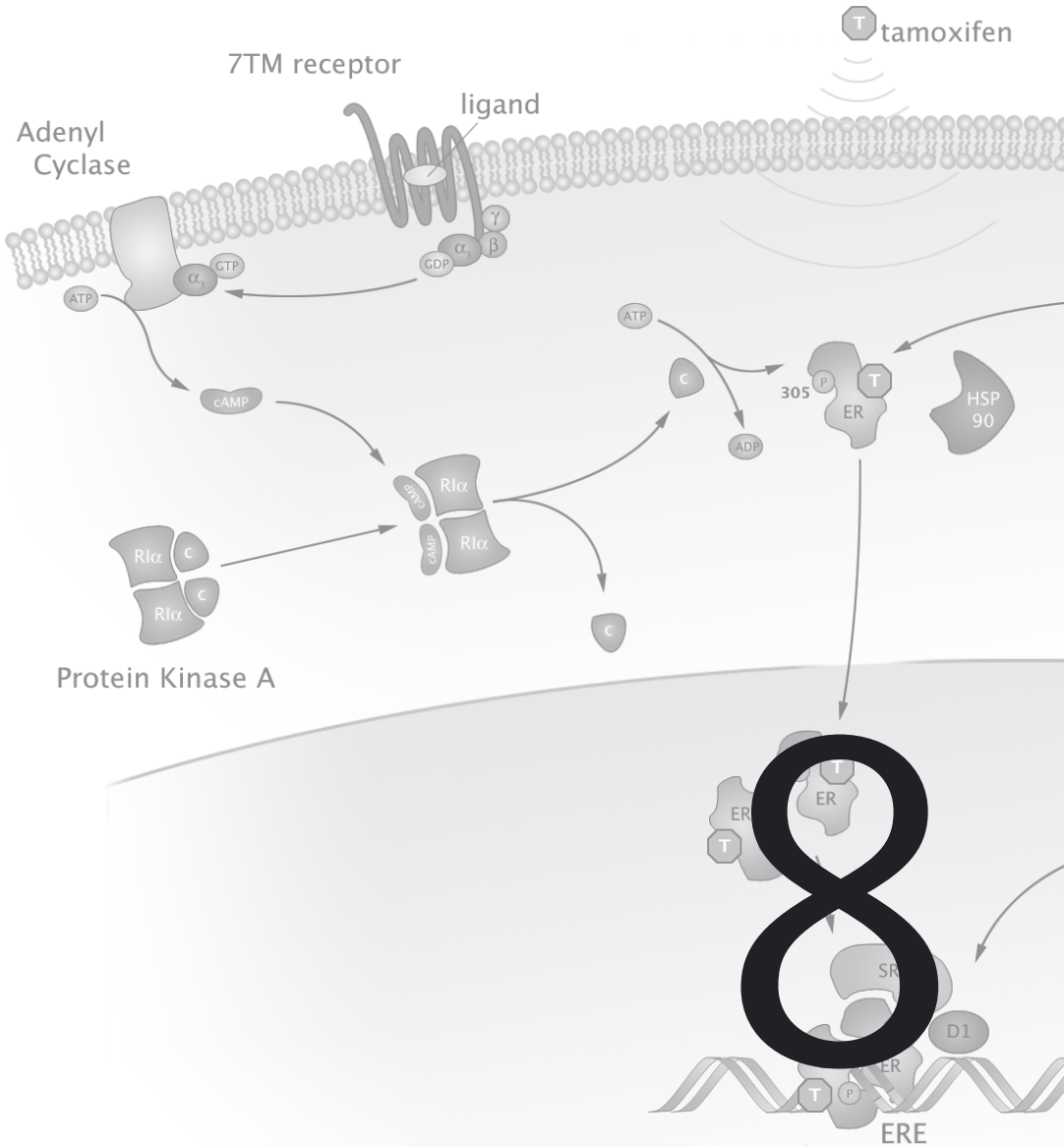
Part 2
Drug Resistance

Visualizing the action of steroid hormone receptors in living cells

Submitted



Visualizing the action of steroid hormone receptors in living cells

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Transcription controlled by Steroid Hormone Receptors (SHRs) plays a key role in many important physiological processes like organ development, metabolite homeostasis, and response to external stimuli. Evidently, the members of this family have drawn a lot of attention from the scientific community since their discovery, four decades ago. Still, after many years of research we are only beginning to unravel the complex nature of these receptors. The pace at which we do has improved significantly in recent years with the discovery of genetically encoded fluorescent probes, and the accompanying revival of biophysical approaches that allow more detailed study of SHRs. Here, we will look into the different aspects of SHR signalling, and discuss how biophysical techniques have contributed to visualize their functioning in their native context, the living cell.

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Steroid Hormone Receptors

Already in 1896 Thomas Beatson described that removal of the ovaries in advanced breast cancer patients often resulted in remarkable improvement (1). With that he had revealed the stimulating effect of the female ovarian hormone estrogen on breast cancer, even before the hormone itself was discovered. His work provided a foundation for the modern use of hormone therapy in treatment and prevention of breast cancer. Only much later the cellular counterpart that mediated the described effects was revealed, the estrogen receptor (ER) (2, 3). As it turned out, this receptor plays a key role in the development and maintenance of the sexual reproductive tissues, and therefore, as Beatson had discovered, in breast cancer as well. We now know that the estrogen receptor is part of the nuclear receptor superfamily, and comes in two forms, ERα [NR3A1] and ERβ [NR3A2]. More specifically, both receptors are members of the subgroup of Steroid Hormone Receptors (SHRs), to which also the cortisol binding glucocorticoid receptor (GR) [NR3C1], the aldosterone binding mineralocorticoid receptor (MR) [NR3C2], the progesterone receptor (PR) [NR3C3], and the dihydrotestosterone (DHT) binding androgen receptor (AR) [NR3C4] belong (**Figure 1A**). In addition, the SHR subgroup contains three orphan receptors closely related to ER: the estrogen related receptors α (ERRα) [NR3B1], β (ERRβ) [NR3B2], and γ (ERRγ) [NR3B3], for which a natural ligand remains to be identified. All SHRs function as nuclear transcription factors whose activity is regulated by small lipid-soluble ligands, and each member plays an important role in key physiological processes like reproduction, glucose metabolism, salt balance, and stress response.

Structural Overview

The members of the Steroid Hormone Receptor family share a similar, modular architecture, consisting of a number of independent functional domains (**Figure**

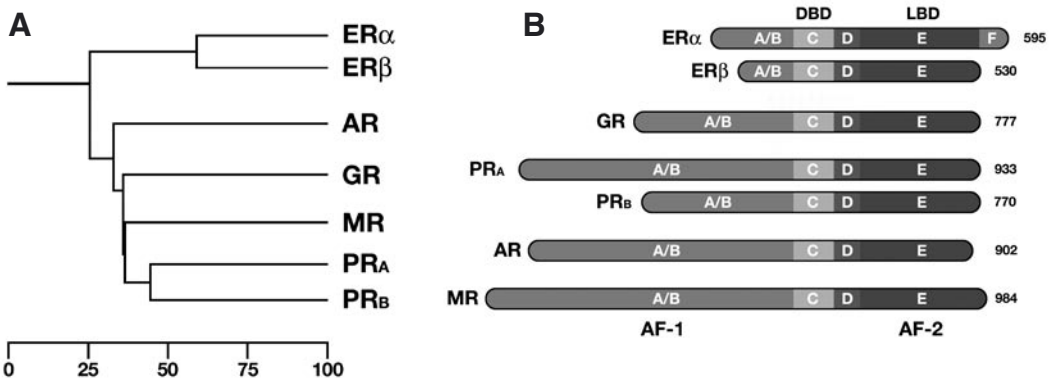

Figure 1. Overview of the Steroid Hormone Receptor family. (**A**) Phylogenetic tree of the Steroid Hormone Receptor (SHR) family showing the evolutionary interrelationships and distance between the various receptors. Based on alignments available at The NucleaRDB (56). (**B**) All steroid receptors are composed of a variable N-terminal domain (A/B) containing the AF-1 transactivation region, a highly conserved DNA Binding Domain (DBD), a flexible hinge region (D), and a C-terminal Ligand Binding Domain (LBD, E) containing the AF-2 transactivation region. The estrogen receptor α is unique in that it contains an additional C-terminal F domain. Numbers represent the length of the receptor in aminoacids. Abbreviations: ER - estrogen receptor, GR – glucocorticoid receptor, PR - progesterone receptor, AR - androgen receptor, MR - mineralocorticoid receptor.

1B). Most conserved is the centrally located DNA binding domain (DBD) containing the characteristic zinc-finger motifs. The DBD is followed by a flexible hinge region and a moderately conserved Ligand Binding Domain (LBD), located at the carboxy-terminal end of the receptor. The estrogen receptor α is unique in that it contains an additional F domain of which the exact function is unclear. The LBD is composed of twelve α-helices (H1-H12) that together fold into a canonical α-helical sandwich. Besides its ligand binding capability, the LBD also plays an important role in nuclear translocation, chaperone binding, receptor dimerization, and coregulator recruitment through its potent ligand-dependent transactivation domain, referred to as AF-2. A second, ligand independent, transactivation domain is located in the more variable N-terminal part of the receptor, designated as AF-1. To date, no crystal structure of a full-length SHR exists, though structures of the DBD and LBD regions of most SHRs are available. These have helped significantly in understanding the molecular aspects of DNA and ligand binding, but have to some extent also led to biased attention to these parts of the receptor only. For example, many co-regulator interaction studies are still performed with the LBD only, while numerous studies have demonstrated that the AF-2 domain often tells only part of the story. With the help of biophysical techniques it is however feasible to study the full-length receptor in its native environment (**Figure 2**).

Ligand Binding

Steroid Hormones (SHs) reach their target cells via the blood, where they are bound to carrier proteins. Because of their lipophilic nature it is thought that they pass the cell membrane by simple diffusion, although some evidence exist that they can also be actively taken up by endocytosis of carrier protein bound hormones (4). For a long time it has been assumed that binding of the ligand resulted in a simple on/off switch of the receptor (**Figure 2**, step 1). While this is likely the case for typical agonists like estrogen and progesterone, this is not always correct for receptor antagonists. These antagonists come in two kinds, so-called partial antagonists (for the estrogen receptor known as SERMs for Selective Estrogen Receptor Modulators) and full antagonists. The partial antagonist can, depending on cell type, act as SHR agonist or antagonist. In contrast, full antagonists (for ER known as SERDs for Selective Estrogen Receptor Downregulators) always inhibit the receptor, independent of cell type, in part by targeting the receptor for degradation. Binding of either type of antagonist results in major conformational changes within the LBD and in release from heat shock proteins that thus far had protected the unliganded receptor from unfolding and aggregation (**Figure 2**, step 2). This process was nicely visualized for the estrogen receptor by Devin-Leclerc *et al*. who showed using fluorescence microscopy that the nuclear ER-

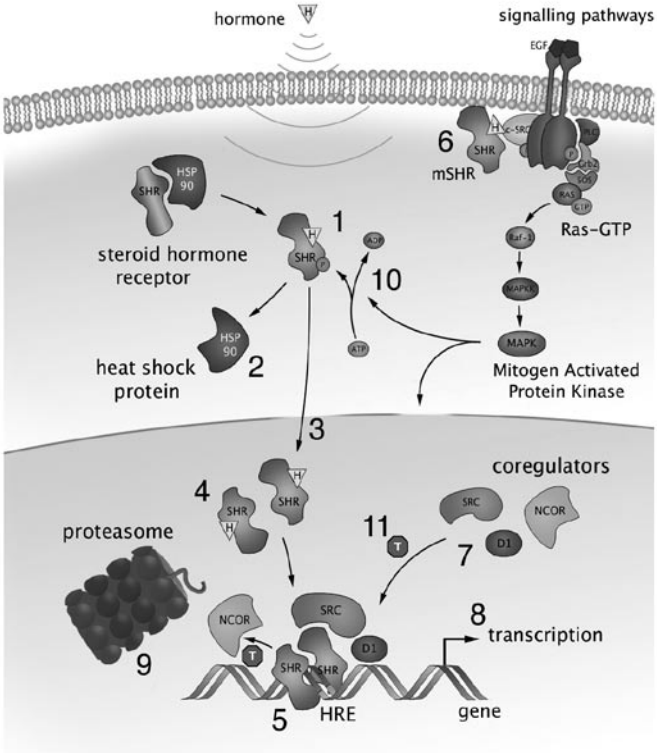

Figure 2. Steroid Hormone Receptor signalling. Steroid Hormone Receptors (SHR) act as a hormone dependent nuclear transcription factor. Upon entering the cell by passive diffusion, the hormone (H) binds the receptor, which is subsequently released from heat shock proteins, and translocates to the nucleus. There, the receptor dimerizes, binds specific sequences in the DNA, called Hormone Responsive Elements or HREs, and recruits a number of coregulators that facilitate gene transcription. This latter step can be modulated by receptor antagonists like tamoxifen (T), and cellular signalling pathways.
Examples of processes studied using biophysical techniques and discussed in this review include: hormone binding (1), chaperone interaction (2), nuclear translocation (3), receptor dimerization (4), DNA binding (5), putative membrane-bound receptors (6), coregulator recruitment (7), transcription (8), proteasomal degradation (9), modulation by cellular signalling pathways (10), and antagonist resistance (11).

HSP90 complex dissociated after addition of either agonist or antagonist, followed by rapid relocation of the heat shock protein to the cytoplasm (5).

Nuclear Translocation

The constitutive nuclear localisation of ER is a unique feature of this SHR only. Although family members, SHRs are located differently in cells. The subcellular localisation of SHRs in living cells has been extensively studied using fusion constructs of green fluorescent protein (GFP). This showed that SHRs can be divided in three groups based on their unliganded distribution: ERα and ERβ are found predominantly in the nucleus (6), GR and AR are found primarily in the cytoplasm (7, 8), while MR and PR have a mixed distribution over both cytoplasm and nucleus (9, 10). The progesterone receptor is of particular interest as it exists in two forms with different ratios of nuclear versus cytoplasmic localization of the unliganded receptor. In most cell contexts, the PRA isoform is a repressor of the shorter PRB isoform, and without hormone induction it is mostly located in the nucleus, whereas PRB distributes both in the nucleus and in the cytoplasm. PRB accumulates in the nucleus after progesterone binding, a process that directly correlates with PR mediated transcription (10, 11).

Rapid and almost complete nuclear translocation following ligand addition is a common behavior observed for almost all SHRs (**Figure 2**, step 3). This translocation coincides with a striking alteration in receptor distribution within the nucleus, most apparent in case of the already nuclear ER. Htun *et al.* observed that GFP-ER's uniform distribution changes into a punctuated pattern upon the addition of either agonistic or antagonistic ligands (6). A few years earlier the same group had already made similar observations for GFP-GR (8). Other groups confirmed similar behavior for the other SHRs, some directly by tagging two receptors with different variants of GFP and following both at the same time (12). Fejes-Toth and colleagues demonstrated that hormone-activated MRs accumulated in dynamic discrete clusters in the cell nucleus, a phenomenon that only concurred with agonistic mineralocorticoids and not with full antagonists

(9). Further work on MR and AR showed that the accumulation of these receptors in about 250-400 foci requires both the DBD and LBD regions, and is possibly influenced by AF-1 function (13-17). The exact nature of these foci is still unclear and multiple roles have been proposed, including storage depots and sites of transcription, splicing, aggregation or degradation. What is clear however is that nuclear and subnuclear translocation of SHRs is ligand and concentration dependent. Martinez *et al.* recently made use of this finding to devise a molecular screen for ER ligands based on a fluorescent GR-ER chimera (18). Instead of a constitutive nuclear localization, this chimeric receptor adapted the cytoplasmic localization of unliganded GR, and translocates to the nucleus upon ER ligand addition. Interestingly, the GR-ER chimera retained the (anti-)estrogen binding properties, and could thus be used to screen for new ER ligands.

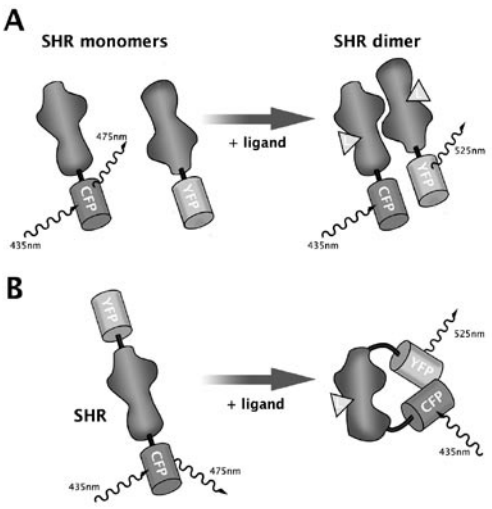

Figure 3. Fluorescence Resonance Energy Transfer.
(**A**) Principle of FRET to measure intermolecular interaction. Exciting the cyan variant of GFP (CFP) linked to one Steroid Hormone Receptor (SHR) monomer at 435nm results in emission at 475nm, unless energy is transferred to a SHR monomer coupled to the yellow variant of GFP (YFP). This phenomenon only occurs when both monomers physically interact as a dimer, and results in increased YFP emission at 525nm at the cost of CFP emission at 475nm.
(**B**) A similar protocol is followed to measure intramolecular FRET. A single SHR monomer is tagged with two variants of GFP. Ligand binding induces conformational changes within the receptor and alters the relative orientation and distance between the two fluorophores, leading to changes in FRET efficiency.

Dimerization

Nuclear receptors that bind steroid hormones typically form homodimers (**Figure 2**, step 4). Dimer formation is facilitated mainly through interactions between the LBDs of both receptors, and is essential for their function, as mutations in the dimerization domain typically render the receptor inactive. ER has been reported to exist as a dimer even in the absence of ligand, although it is important to note that these studies have been performed with the LBD domain only (19). Biophysical *in vitro* studies, again with the LBD only, have confirmed these data and show slow dissociation of unliganded dimers, which is further retarded by ligand binding (20). Recent *in vivo* studies suggested that this might not hold for full-length receptors though, at least not for AR. Schaufele *et al.* used Fluorescence Resonance Energy Transfer (FRET) to study AR dimer formation (21). FRET is the radiationless transfer of energy from an excited donor fluorophore to a suitable acceptor fluorophore (22). Importantly, FRET is extremely sensitive to the distance between the fluorophores (its efficiency decays with the distance to the sixth power), and will therefore only occur when two proteins are on average no more than one molecule in distance apart, but usually they interact directly (**Figure 3A**). Schaufele and colleagues measured FRET between CFP and YFP labeled AR receptors, and their results suggest that dimerization only takes place after ligand binding, and predominantly in the nucleus. It should be noted that absence of FRET does not imply absence of protein-protein interaction since the relative orientation of two fluorophores is also critical for FRET to occur (22). Further study is required to confirm these findings and to determine whether this behavior is unique for AR or also applies to other SHRs.

Dimers of SHRs are only efficiently formed between closely related receptors. In this light the previously mentioned two isoforms of the progesterone receptor and the two estrogen receptors are of particular interest. In both cases one of the two seems to exhibit a repressive function on the other. ERβ efficiently dimerizes with ERα and mixed dimers show identical subnuclear distribution as homodimers (23). However, binding of ERβ suppresses ERα mediated gene transcription, accordingly the mouse knockouts of either receptor show completely opposing phenotypes (24).

DNA Binding

Upon binding of ligand and translocation to the nucleus, SHRs bind to specific regions in the DNA called Hormone Responsive Elements (HREs) through zinc-finger motifs present in the DBD (**Figure 2**, step 5). The exact mode of binding has been characterised in detail with help of available crystal structures and extensive biophysical *in vitro* measurements. Consensus nucleotide binding sequences have been determined for all SHRs, but these show a significant amount of ambiguity, making it hard to pinpoint true target HREs in the genome. A HRE is made up of two so-called half-sites that each binds one monomer of the SHR dimer. Interestingly, also single half-sites have been found in genes that clearly respond to hormone, hinting at a possible role for receptors in their monomeric configuration.

Immobilization of SHRs on DNA and other nuclear structures has been studied with photobleaching techniques like Fluorescence Recovery After Photobleaching (FRAP). By bleaching fluorescent molecules in a region of interest in a living cell and measuring recurrence of fluorescence levels in the bleached area, the mobility of the tagged molecules can be determined (**Figure 4**). Using this technique several groups could demonstrate a clear correlation between receptor immobilization in the nucleus and the appearance of the typical punctuated receptor distribution, which was most convincingly demonstrated by Schaaf and colleagues who compared 13 GR ligands (25). FRAP measurements show that fluorescently tagged SHRs such as ER, GR, and AR are highly mobile and dynamic in the unliganded state, whereas ligand-bound forms are less mobile (26-28). Stenoien et al. further showed that in the case of ERα, FRAP could discriminate between ligands with potential agonistic properties and full antagonists on the basis of receptor immobilization in the nucleus (28). The nature of the substrate on which the receptor immobilizes remains uncertain, but almost certainly includes DNA. Carefully controlled FRAP measurements from Sprague *et al.* show that in free form GR is bound to a single type of substrate, most probably DNA, with each molecule binding on average 65 sites per second (27). This rapid sampling of GR is likely to be important in finding a specific HRE. Upon ligand binding, the residence time on DNA is significantly increased. According to Farla *et al.* on average one out of five ARs is immobilized in the presence of ligand, each individual AR being immobile for 1-2 min. This immobilization is depended on DNA binding since GFP-ARs mutated in the DNA-binding domain were not immobilized (26). Likewise, FRAP analysis by Kino *et al.* on several GR mutant receptors showed a

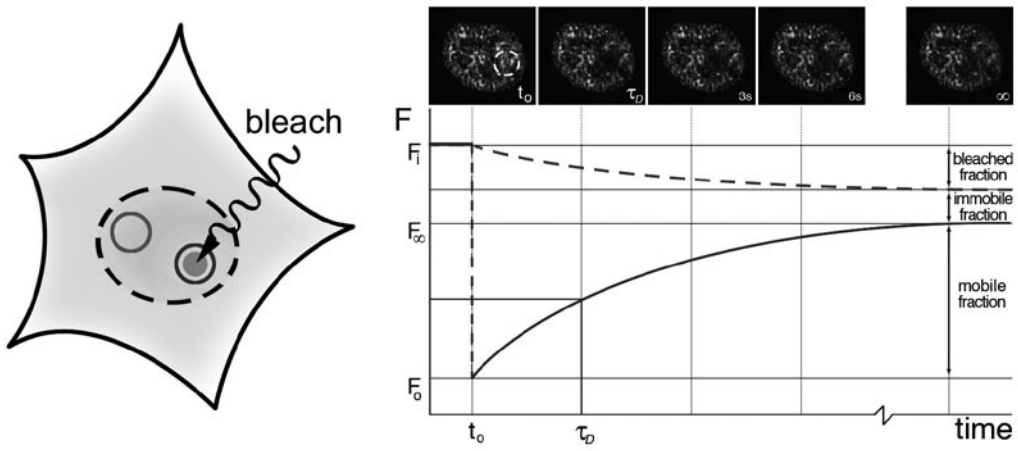

Figure 4. Fluorescence Recovery After Photobleaching (FRAP). By bleaching the indicated region in the fluorescent area (here the nucleus of a cell expressing ERα fused to GFP) is bleached at time t_0. Fluorescence decreases from the initial fluorescence F_i to F_0. The fluorescence recovers over time by diffusion. The characteristic diffusion time τ_D indicates the time at which half of the fluorescence has recovered. The mobile fraction can be calculated by comparing the fluorescence in the bleached region after full recovery (F_∞) with the fluorescence in a distant region in the nucleus (dashed line).

significantly increased nuclear motility and decreased chromatin retention, which correlated with impaired transcriptional activity (29).

DNA binding and transcription has been visualized directly by using cells that have stably integrated a tandem array of HREs. Pioneering work in this area has been performed by the Hager lab, which used this approach to study the interaction of GR with a natural promoter (30). The promoter array allows significant amounts of GFP-GR to accumulate for direct detection under the microscope. The recruitment of GFP-GR leads to gross alterations in chromatin structure of the array that correlate with gene transcription (31). Interestingly, FRAP analysis on the array again shows a rapid exchange of receptors between chromatin and the nucleoplasmic compartment. Further analysis demonstrated that following binding of GR to the promoter, the receptor is actively displaced from the template during a chromatin remodeling reaction facilitated by the hSWI/SNF complex (32). Further evidence comes from work on PR by the same group, which showed that the exchange of PR-GFP on the array was slowed down (but still in the order of seconds) upon agonist addition, and even further slowed down after addition of a partial antagonist (33). Strikingly, addition of a full-antagonist showed the opposite effect, with ongoing exchange at a rate faster than for an agonist bound receptor. In contrast to an agonist or partial antagonist bound receptor, addition of a full-antagonist does not lead to recruitment of the SWI/SNF chromatin remodeling complex, which may partly explain the above results. Together, these findings have led to the so-called hit-and-run model. In contrast to static binding of the receptor to a HRE and the subsequent build up of the transcription complex, this model suggests a receptor to continuously probe the DNA for potential binding sites. Transcriptional activation reflects the probability that all components required for activation will meet at a certain chromatin site.

Besides binding to Hormone Responsive Elements, SHRs can also exert their effects by binding directly to other transcription factors. For example, ERα is able to bind to fos/jun, and thus regulate AP-1 mediated transcription of genes like cyclin D1. Similarly, ERα can bind Sp1 proteins and regulate transcription of genes that contain an Sp1/ER binding site. Interestingly, antagonists often have agonistic effects in this setting, which may be important when it comes to resistance to antagonistic compounds. This is illustrated by work from Kim *et al*. who used FRET to visualize the interaction between ERα and Sp1 (34). Addition of the full anti-estrogen ICI 182,780 inhibits normal ERα mediated transcription, yet like agonist estradiol induced a FRET signal between ERα and Sp1 that correlated with Sp1 mediated transcription of a reporter construct.

Recently a number of groups have claimed a role for SHRs in non-genomic, extranuclear signalling events (**Figure 2**, step 6). Targeting ERα artificially to the plasma membrane has marked influence on ERK1/2 signalling, which was not affected by full anti-estrogens (35). Similar effects on the Mitogen Activated Protein Kinase (MAPK) and Protein Kinase A (PKA) pathways have also been attributed to the wildtype receptor (36, 37). However, most studies are based on biochemical approaches where post-lysis artefacts are hard to exclude. Moreover, convincing microscopic pictures of SHR membrane localization are still lacking. Nevertheless, accumulating evidence seems to point to possible functions for SHRs other than mediated by DNA binding.

Co-regulator Recruitment

The classical mode of action of SHRs involves ligand and DNA binding. For transcription to occur the subsequent recruitment of co-regulator proteins is absolutely required (**Figure 2**, step 7). These regulatory proteins come in two types, co-activators and co-repressors that respectively enhance or diminish transactivation activity through various enzymatic activities, including acetylating, deactylating, methylating, ubiquitinating, and kinase activity. Ligand dependent recruitment of coregulators occurs through a hydrophobic cleft formed by helices 3, 4 and 12 in the AF-2 domain of the receptor (38). In free receptors this pocket is shielded by a short amphipathic α-helix (H12) located at the carboxy-terminal end of the receptor that prevents AF-2 mediated coregulator binding in the absence of ligand. Upon hormone binding, this helix is repositioned, which opens a functional interface for coregulator recruitment through conserved LXXLL motifs in the co-factor. Antagonists exert their function by inducing a different conformational change of H12 that blocks or modulates the recruitment of these essential coregulators. However, not all coregulator binding occurs through the AF-2 region. Also other conformational changes within the receptor and events like dimerization are likely to be involved in coregulator recruitment. Moreover,

the AF-1 region of SHRs plays an important role in ligand independent binding of coregulators. The exact coregulator requirements for transcription are dependent on cell type, and probably also on ligand and promoter context. This explains why partial antagonists can have antagonistic properties in one tissue, while exhibiting agonistic properties in another.

The most well studied coactivators are of the Steroid Receptor Coactivator (SRC) family, which includes SRC-1 (or NcoA-1), SRC-2 (also known as TIF-2 or GRIP1, NcoA-2) and SRC-3 (also known as RAC3, ACTR, AIB1, P/CIP and TRAM). Llopis *et al*. were the first to directly visualize the interaction between SRC-1 and a SHR in living cells using FRET (39). They showed that the ERα LBD exhibited some basal interaction with coactivators in unstimulated cells that was increased upon agonist addition and abolished by receptor antagonists. A large number of publications have since confirmed these findings, also with full-length ER constructs. Interestingly, these studies clearly show that the receptor adopts a slightly different conformation for various ligands, and this conformation significantly influences the binding of specific coregulatory proteins (40-42). Likewise, the small structural differences between the LBD of ERα and ERβ can result in profound differences in SRC-1 recruitment with the same ligand (43). We have visualized these conformational changes by fusing full-length ERα with YFP to its N- and CFP to its C-terminus (44). This allowed us to monitor conformational alterations of the receptor after ligand binding in the form of a change in FRET between CFP and YFP (**Figure 3B**). Indeed, subtle FRET differences between the various anti-estrogens tested were observed, showing that the receptor had adopted ligand specific conformations.

The stoichiometry of interaction between ERα and SRC-1 was studied using fluorescence correlation spectroscopy (FCS). By measuring the fluorescence signal from a very small excitation volume only, this technique allows precise determination of the diffusion coefficient of fluorescently labeled proteins, which is in part dependent on protein complex formation (**Figure 5**). In this way Margeat *et al*. could show that the ERα dimer binds a single SRC-1 coactivator molecule (45).

One caveat of the above experiments is the use of only the AF-2 binding part of SRC-1. This may give false impressions when conclusions are extrapolated to the full-length receptor. Work from others and unpublished data from our lab have demonstrated that full-length SRC-1 binds the receptor through its AF-1 domain in a ligand independent manner (46) (Zwart *et al*., manuscript in preparation). AF-2 binding still functions as a switch to invoke full transactivation activity, but in contrast to what the above studies suggest, SRC-1 is already bound to unliganded

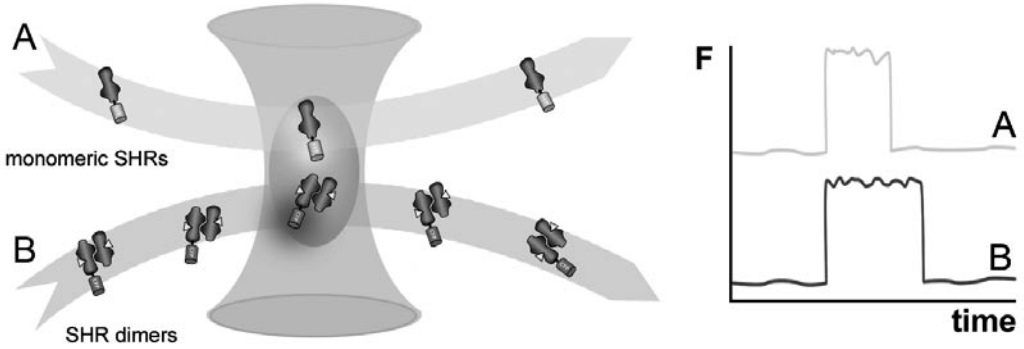

Figure 5. Fluorescence Correlation Spectroscopy (FCS). By using the microscope objective lens to focus the laser beam, a diffraction limited excitation volume is created. The emitted fluorescence signal (F) from this observation volume fluctuates as labeled molecules diffuse in and out, and the duration of the fluctuations are related to the average time individual models reside within the volume. One such an event is depicted in the graph. The residence time can be used to determine the diffusion coefficient for the fluorescent molecules, which is dependent on their size and interaction with other proteins. For example, it can be used to discriminate monomeric Steroid Hormone Receptors (SHRs, A) from dimeric complexes (B).

receptors. This was also demonstrated by Stenoien *et al.*, who used a fusion construct of CFP-ERα with a lac repressor domain to artificially target the receptor to an integrated lac operator array (47). Even in the absence of ligand, significant levels of SRC-1 were already present on the array, which further increased upon addition of agonist, and decreased after addition of antagonist. Further work by this group suggested that agonist binding predominantly stabilized SRC-1 binding, which translated in identical mobility of both proteins (28). Similar results were reported for other SHRs like GR and AR, and for other coregulators including CREB binding protein (CBP), glucocorticoid receptor (GR) interacting protein 1 (GRIP-1), and RIP140 (48-50). Interestingly, in all cases dynamic DNA binding of SHRs was observed even in the presence of agonist and coregulators.

Transcription

Our view on SHR mediated transcription has more and more shifted from one in which a static holoenzyme of transcription factors is steadily build up after initial binding of the receptor to a response element, into a highly dynamic picture where different factors rapidly move in and out to perform temporary and local functions (**Figure 2**, step 8). In this so-called hit-and-run model, transcription only takes place when all factors coincidentally meet at the same time at the same location. Factors like DNA binding on specific HREs, and ligand dependent co-regulator recruitment simply increase the odds that a successful transcriptional unit is formed and a gene is transcribed. FRAP analysis shows that indeed the majority of nuclear proteins is highly mobile (51), except for Polymerase II, which once recruited has a residence time on the DNA in the order of minutes.

Whichever model is closer to reality, it is clear that transcription is a complex process, requiring dozens of proteins. Large-scale Chromatin IP assays have shown the recruitment of at least 46 factors to an empty promoter before continued transcription can take place (52). Interestingly, these experiments revealed a striking ATP-dependent periodicity in the recruitment of these factors, which was confirmed using FRAP analysis (52, 53). The observed cycling time was in the order of 1h, much slower than the rapid exchange of SHRs on the template described earlier. Receptor degradation by the proteasome plays an important role in this process, as a block of proteasomal function halts the cycle after one round of transcription (**Figure 2**, step 9). This corresponds to previous observations that GR and ERα completely immobilize in the nucleus upon proteasome inhibition (28, 54). However, since proteasome inhibition rapidly de-ubiquitinates histones, these effects may also be indirect, and the result of chromatin alterations (55). An important role to maintain proper cycling has also been suggested for heat shock proteins like HSP-90 (54).

The exact role of this cyclic recruitment of transcription factors to the promoter is still unclear. It is also difficult to interpret how this slower cycle relates to the rapid exchange of SHRs that forms the basis of the hit-and-run model. Interestingly, the cyclic pattern of transcription factor presence on the promoter does suggest that some form of order in the build up of a functional transcriptional unit must exist, resulting in a so-called transcription factory. This may very well represent the summation of all rapid exchange events over a longer period of time, which suggests that both models may not necessarily be mutually exclusive, and might act in subsequent steps of transcription initiation.

Note

The literature on steroid hormone receptors is extensive and only selected studies were cited in this review due to space limitation. We sincerely apologize to all investigators whose work was not mentioned.

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