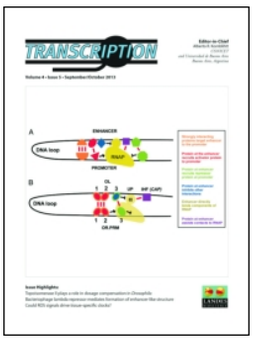

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POINT-OF-VIEW

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More than meets the dimer: What is the quaternary structure of the glucocorticoid receptor?

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ABSTRACT

It is widely accepted that the glucocorticoid receptor (GR), a ligand-regulated transcription factor that triggers anti-inflammatory responses, binds specific response elements as a homodimer. Here, we will discuss the original primary data that established this model and contrast it with a recent report characterizing the GR–DNA complex as a tetramer.

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DNA binding; dimer; glucocorticoid receptor; monomer; steroid receptors; tetramer

The glucocorticoid receptor (GR) is a modular transcription factor, organized in three distinct structural and functional domains: the N-terminal domain (NTD), a central DNA-binding (DBD) domain, and a C-terminal Ligand-binding domain (LBD).[1] The GR is expressed in most cell tissues, and is involved in critical biological processes including homeostasis and metabolism. Moreover, pharmacological activation of the GR triggers powerful anti-inflammatory and immunosuppressive actions, making the receptor one of the most targeted proteins for therapy. GR oligomeric manipulation is considered a key aspect in the search of synthetic ligands that would eliminate the side-effects associated with chronic glucocorticoid treatment.[2]

In current dogma, GR is considered to bind to specific glucocorticoid response elements (GREs) as a homodimer. However, primary data references for this model are largely absent in current review articles, or in research articles. This is expected for long-standing, widely accepted models. We recently reported that DNA binding at response elements induces tetramerization of the GR,[3] suggesting that tetramers maybe the final, active chromatin bound form of the receptor. The report[3] presents the first *in vivo* experiments that directly address the oligomeric state of GR when bound to chromatin. In this brief comment, we will argue that the dimerization model exclusively relies on *in vitro* data. Furthermore, the recent observations in living cells are, to some extent, consistent with early *in vitro* data, indicating that new efforts are needed to resolve the long-standing issue regarding the active form of the DNA bound GR.

GR as a dimer: In vitro studies

We refer here to *in vitro* studies as any experiment that has been performed in a cell-free environment. The first description of GR's oligomeric status was presented in 1983 by the Yamamoto lab. The authors purified GR from rat liver and analyzed by electron microscopy GR "particles" either bound or unbound to a DNA fragment containing a GRE sequence from the mouse mammary tumor virus (MMTV) promoter region.[4] Based on the size of the particles, these investigators concluded: "…the 94 k_D receptor subunits seem to form homotetramers in a DNA-independent manner under these conditions (…) but this has not been confirmed by independent methods." Later, the Gustafsson group evaluated the relative stoichiometry between GR and DNA.[5] Purified receptor bound to ^{3}H-triamcinolone acetonide (a GR agonist) is incubated with ^{32}P-labed DNA. After glycerol centrifugation, each fraction is analyzed and the relative

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radioactive signals are obtained. The authors concluded that only one GR molecule binds a single GRE, suggesting that GR binds to DNA as a monomer. However, the same technique applied on a "stronger" GRE sequence indicated that the activated GR exists as a homodimer when unbound as well as when bound to DNA.[6]

Another commonly used technique to study DNA binding is the electro-mobility shift assay (EMSA). In this case, *in vitro* translated, GST-purified (usually only the DBD fragment) or endogenous purified receptor is mixed with labeled DNA and the binding products analyzed by polyacrylamide gel. Early efforts identified two distinct shifted bands. These were assigned as monomeric and dimeric complexes, a conclusion perhaps also biased from the dyad symmetry of the GRE sequence.[7] Therefore, results from EMSA experiments suggested that the functional entity that binds to a GRE is a dimer.[7,8] Although some studies reported that the monomer binds first to DNA,[7,9,10] other reports provided evidence for DNA-independent dimer formation.[6,8,11–14] In general, groups that worked with the entire receptor argued for a DNA-independent pathway,[8,11,12,14] whereas those working with the DBD fragment found that it was the monomer that first binds DNA, which in turn favors the binding of the second monomer, a concept known as *positive cooperative effect*.[7,9,10] The use of other *in vitro* approaches did not help to solve the controversy. Although DNA-independent positive interactions between *in vitro* translated GR and cell-extracted immunoprecipitated GR have been observed in solution,[15] other investigators argue that GR exists almost exclusively in a monomeric state.[16] Finally, several early papers suggested that either adjacent GREs[17–19] or unusual GREs[20] could lead to the formation of homo-tetramers. However, when the solution and crystal structures of GR's DBD were elucidated,[21,22] the community rapidly adopted the idea that the activated GR was in fact a homodimer.[23]

From the structural perspective, only the DBD[22] and LBD[24] domains have been crystalized, although separately. The NTD has eluded crystallization and high-resolution structure, most likely due to its intrinsically disordered domain.[25] The first crystal structure of GR's DBD bound to DNA revealed a clear dimerization region between the two receptor monomers.[22] Although several studies in the 90s suggested a region outside the DBD could be involved in GR dimerization,[10,11,26,27] the predominant view portrayed the DBD as the exclusive domain responsible for dimer formation. This dogma developed largely because a point mutation in that region (known as the GRdim mutant) was allegedly sufficient to generate a monomeric GR,[28,29] although no direct evidence was provided at the time.[30,31] When the crystal structure of the LBD was reported and a second dimerization region discovered,[24] concerns about the physiological relevance and functional contribution of both domains arose.[32] Some investigators still argue that the LBD dimers are an artifact of crystallization, and that the LBD dimerization of GR is "unlikely."[33]

Taken together, the evidence from *in vitro* data appears to indicate that GR is a dimer, although no clear consensus exists as to whether GR dimerizes before or after DNA binding, nor which domains are involved. The clearest results come, however, from studies performed on the DBD fragment alone and not the entire GR protein.

The in vivo perspective on GR dimerization

We refer here to *in vivo* studies as any experiment that has been performed inside living cells, or experiments wherein the biological parameter measured occurred in intact cells but was revealed with an *in vitro* technique. Inside the cell, in the absence of ligand, the receptor is mostly retained in the cytoplasmic compartment, as a monomer,[34] by being part of a heterocomplex with Hsp90, Hsp70, p23, and immunophilins, among others.[35] However, GR overexpression has been reported to induce ligand-independent cytoplasmic dimerization.[36] Since the heterocomplex is necessary for proper folding that allows GR to bind hormone,[37] it is not clear how the GR dimers can still remain associated with the heterocomplex, as they are able to bind ligand and translocate into the nucleus.[36]

Once GR is activated by ligand, it translocates almost completely to the nuclear compartment. Using a nuclear-import deficient receptor mutant in the context of its wild-type counterpart, the Hache lab demonstrated that GR can interact with itself before and/or during retrograde transport.[15] This experiment constitutes the first demonstration that the GR can actually interact with itself *in vivo* (i.e., inside living cells), many years after the community had already adopted the dimerization paradigm. The Hache group noted at the time: "At present we cannot exclude the potential formation of higher-order

complexes of GR." Co-immunoprecipitations experiments from cell extracts also confirmed the presence of GR–GR interactions inside the cell,[30,38] however, the interaction was always detected both in the presence and absence of hormone. Finally, using Förster resonance energy transfer (FRET) *in vivo*,[39] GR–GR interactions have also been detected,[40] although the assay cannot discriminate between different oligomerization states or accurately quantify the proportion of monomers against dimers in the population.

The first *in vivo* quantification of the quaternary structure of GR came from the use of the Number and Brightness assay (N&B), a microscopy technique that measures the oligomerization state of fluorescent proteins with high spatial resolution.[41] Unexpectedly, results revealed that most of the ligand–GR complexes were dimeric in the nucleus,[34] although a small proportion of monomers or higher oligomerization states could not been ruled out as well. Because the fraction of GR specifically bound to DNA at any given time *in vivo* is very low (3–5%),[42–44] the virtually complete population of dimers observed in the nucleoplasmic N&B assay must arise from mostly unbound receptors, strongly suggesting a DNA-independent model for GR dimerization.[31]

Collectively, from the *in vivo* perspective, the activated GR appears to be mostly dimeric in the nucleoplasm. However, until recently, no *in vivo* experiment has measured directly the quaternary structure of the GR–chromatin complex. Theoretical models based on *in vivo* experimental kinetic studies predict that it is the GR monomer the entity that first binds to DNA.[45] Alternatively, chromatin immunoprecipitation coupled with exonuclease digestion (ChIP-exo) "footprints" have been interpreted as *in vivo* evidence for dimeric binding of GR,[46,47] based on the protection signature of the exonuclease from the DBD–DNA contacts. Since ChIP assays neither measure directly nor indirectly oligomerization states, the conclusion that GR is a dimer was drawn by assuming that (i) one-half GRE footprint always represents a monomeric GR event and (ii) GR dimerization exclusively relies on DBD–DBD contacts. However, direct measurement of the quaternary structure by N&B demonstrates that LBD–LBD contacts are more relevant in stabilizing the dimers than DBD–DBD contacts.[3,31] Hence, it is conceivable that GR dimers can still bind a half-GRE wherein LBD–LBD contacts held the dimer together. In fact, when directly measured at a tandem array of GR-binding sites, the receptor appears tetrameric at full GREs.[3]

In vitro versus in vivo data: Any room for reconciliation?

In a recent report,[3] we concluded that GR is mostly a tetramer when bound to DNA. This conclusion is based on four independent findings: (i) N&B measurements at an array of response elements show the presence of tetramers; (ii) A mutation that mimics the DNA-bound conformation of GR (P493R) triggers tetramerization in the whole nucleoplasm; (iii) Homo-FRET studies show higher oligomerization states in both nucleoplasm and the array; and (iv) Single-molecule photobleaching experiments detect the presence of greater-than-two-subunit oligomers randomly in the nucleus. Using several mutations, we also reported that tetramer formation depends on the presence of the LBD, is independent of dimerization surfaces, and requires DNA-induced conformational changes in the DBD.

How can these results be reconciled with the previous literature? One of the strongest evidence for GR dimerization is the crystallographic X-ray structure.[22] However, lack of tetramerization of the DBD fragment is not surprising since tetramer formation depends on the LBD. Moreover, the monomer-to-dimer transition observed in EMSA assays[7,9,10] is completely consistent with the N&B data once the LBD domain is removed. The NTD–DBD fragment behaves *in vivo* as the DBD behaves *in vitro*: fully monomeric in the nucleoplasm and fully dimeric at GREs.[3] It is worth mention one study, by the Miguel Beato's group,[8] where they mixed full-length and DBD fragments to form whole-GR/DBD-only heterodimers, strongly suggesting the presence of dimeric forms of the receptor. The *in vivo* evidence, on the contrary, only points to tetramerization[3] as there are no other direct measurements reported.

If GR binds to DNA as a dimer *in vitro* and as a tetramer *in vivo*, then something must be missing in the *in vitro* system. They are several key elements present *in vivo* that are absent in the controlled *in vitro* environment: post-translational modifications, cofactor interactions, nucleosomes, the interphase chromatin landscape, to name a few. Some of these elements could be proven essential for this "next regulatory step" GR seems to have within live cells.

Toward a new model for GR oligomerization

After review of more than 30 years of research, we suggest that the evidence for GR dimerization is not as solid as originally thought. Based on the new N&B

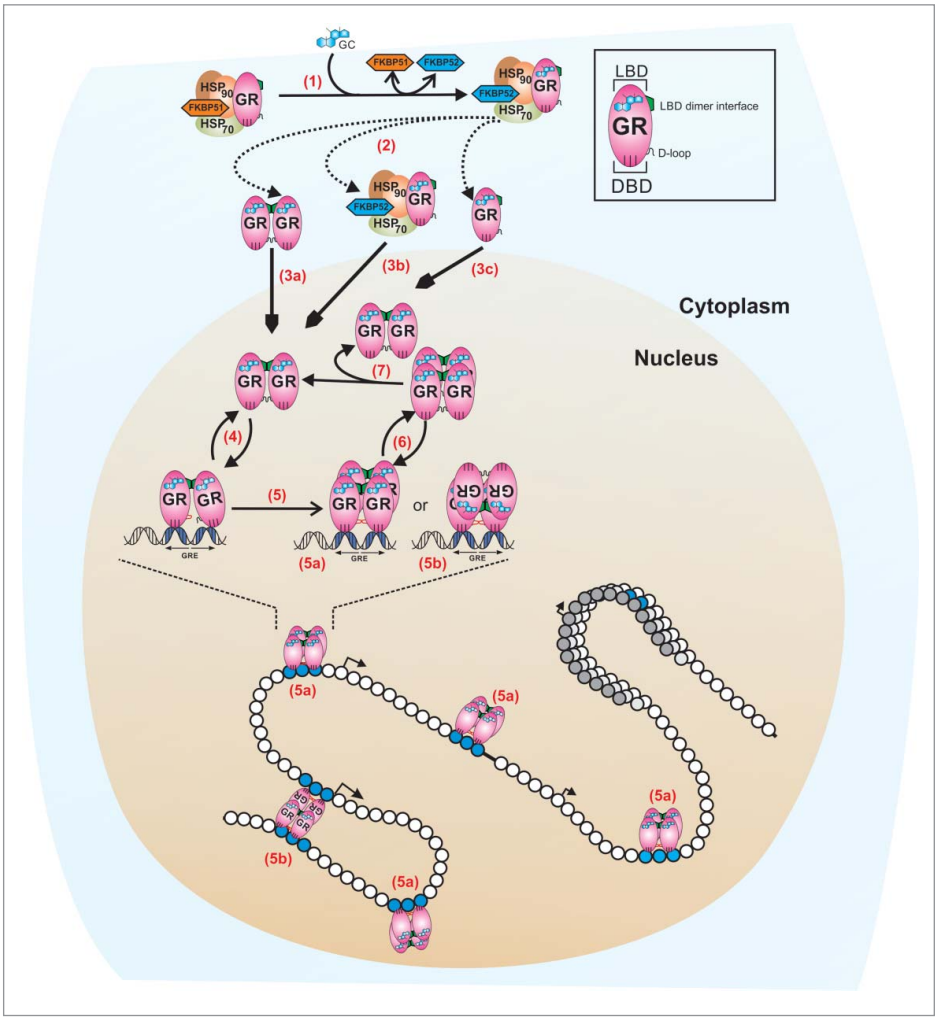

Figure 1. *A revised model for GR quaternary structure dynamics*. In the absence of ligand, the monomeric cytoplasmic GR forms a heterocomplex with Hsp90, Hsp70, FKBP51, and other proteins (not shown). (1) Ligand binding (GC) induces a conformational change in the GR that leads to either FKBP51-FKBP52 exchange within the heterocomplex, or the complete dissociation from the complex (2). At least two mechanisms appear to regulate the influx of GR molecules to the nuclear compartment: a microtubule/Hsp90 dependent pathway (3b, microtubule not shown) and a microtubule/Hsp90 independent pathway (3a, 3c).[35] Early *in vivo* studies suggests that GR is a dimer before translocation[15] (3a) but a monomeric population cannot be rule out (3c). Nucleoplasmic GR appears to be mostly dimeric,[3,31,34] with at least two contact surfaces localized in the LBD (shown in green) and the DBD (D-loop, shown in black).[31] The interaction with specify response elements (GREs) is very dynamic (4), in the order of seconds.[42] DNA induces an allosteric change in the receptor's DBD domain[51] (D-loop shown in red) which triggers a conformational change in the LBD, allowing the formation of tetramers, either in a head-to-head (5a) or head-to-tail (5b) configuration.[3] GR tetramers also exchange dynamically with DNA (6) and, if it is the tetramer the quaternary structure that detaches from DNA, then the dissociation into dimers should occur at a much faster temporal scale (7), to account for the mostly complete population of dimers observed in the nucleoplasm.[3] This highly dynamic regulation occurs in the context of a chromatin landscape. We speculate that a head-to-tail configuration (5b) may assist in bridging different points in the genome, thus favoring a looping mechanism between distant regulatory sites.

findings, we propose a new model that attempts to resolve all available data (Fig. 1).

In the absence of ligand, the inactivated GR appears to be fully monomeric inside the nuclear compartment,[31, 34] while their behavior in the cytoplasm could depend on its concentration: At endogenous levels, the GR is most likely a monomer but overexpression of the receptor could lead to ligand-independent dimerization.[30,36,38,40]

When the GR is activated by dexamethasone or its natural ligand corticosterone, dimer formation is initiated before or during the nuclear translocation process.[15] The reported inability of the non-steroid GR ligand Compound A to efficiently promote GR nuclear translocation[48] may be explained by its incapacity to induce GR dimer complexes.[31] Once in the nucleus, virtually all agonist-bound GR molecules are

in the dimeric form[31,34] through LBD–LBD[24] and DBD–DBD[22] interactions, although mutational analyses indicate that these dimeric surfaces are not functionally equivalent. In fact, dimerization through the DBD is dependent upon the presence of the LBD.[3] It has also been documented that after specific DNA binding, the DBD changes conformation,[49] potentially favoring DBD–DBD interactions.[50] Hence, the positive cooperative binding between monomers observed *in vitro* when only the DBD fragment is used[10,50] may not be a key factor *in vivo*, and reflects further stabilization of the pre-formed dimers after engaging chromatin. Since GR molecules are already dimeric before binding to DNA, it is the dimer and not the monomer that is the hormone-activated GR entity (Fig. 1).

The GR is allosterically modulated not only by ligand binding, but also by DNA itself.[51] This suggests that GREs do not merely serve as GR docking points, but may also modulate GR activity by altering its conformation.[52] In fact, thermodynamic studies using GR's DBD have shown an induce-fit binding mode to DNA,[53] therefore suggesting at least a two-step, and possibly a multi-step mechanism. We propose that this new conformation triggers a structural rearrangement in the LBD, promoting the formation of higher order oligomers, predominantly tetramers, through LBD surfaces that are yet to be identified.[3] This phenomenon may be more common than previously thought, as STAT3 has been recently described as transitioning from dimer to tetramers in a DNA-dependent manner.[54] A deeper understanding on the intricacies of GR quaternary structure may help find new strategies in the search for safer glucocorticoids, or at least finally close some roads taken in the past[2] that have led us nowhere. Finally, as combinatorial long-range interactions between regulatory elements play an important role in gene regulation,[55] we speculate the tetrameric nature of some transcription factors such as GR or STAT3 can serve as a platform to bridge different points in the genome.

Abbreviations

ChIP	chromatin immunoprecipitation
DBD	DNA-binding domain
EMSA	electro-mobility shift assay
GC	glucocorticoids
GR	glucocorticoid receptor
GRE	glucocorticoid response element
LBD	ligand-binding domain
MMTV	mouse mammary tumor virus
N&B	number and brightness
NTD	N-terminal domain

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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