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Cryptic glucocorticoid receptor-binding sites pervade genomic NF-κB response elements

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Glucocorticoids (GCs) are potent repressors of NF-κB activity, making them a preferred choice for treatment of inflammation-driven conditions. Despite the widespread use of GCs in the clinic, current models are inadequate to explain the role of the glucocorticoid receptor (GR) within this critical signaling pathway. GR binding directly to NF-κB itself—tethering in a DNA binding-independent manner—represents the standing model of how GCs inhibit NF-κB-driven transcription. We demonstrate that direct binding of GR to genomic NF-κB response elements (κBREs) mediates GR-driven repression of inflammatory gene expression. We report five crystal structures and solution NMR data of GR DBD-κBRE complexes, which reveal that GR recognizes a cryptic response element between the binding footprints of NF-κB subunits within κBREs. These cryptic sequences exhibit high sequence and functional conservation, suggesting that GR binding to κBREs is an evolutionarily conserved mechanism of controlling the inflammatory response.
Glucocorticoids (GCs) are a class of steroid hormones that are widely prescribed for inflammation-driven conditions such as asthma and arthritis. GCs exert their effects by binding to the GC receptor (GR), a ubiquitously expressed nuclear receptor that drives both the activation and repression of its target genes. Ligand-bound GR is able to antagonize the activity of immunogenic transcription factors such as nuclear factor-κB (NF-κB), AP-1, and T-bet, resulting in a potent attenuation of inflammation. Indeed, repression of pro-inflammatory genes such as interferon-γ by the GR is required to dampen immune responses that would otherwise be lethal.

Unfortunately, the therapeutic anti-inflammatory actions of GR are concomitant with a host of undesirable side effects that include skin atrophy, glaucoma, osteoporosis, adipogenesis, insulin resistance, and hypertension. These opposing actions by GR have led to an intense—and largely unsuccessful—search for dissociated ligands that would separate its anti-inflammatory properties from its more malicious side effects at pharmacological doses.

GR is normally sequestered in the cytoplasm and binding of GCs to its ligand-binding domain causes GR to translocate to the nucleus, where its DNA-binding domain (DBD) binds canonical activating GC response elements, or (+)GREs, which are palindromic hexameric sequences containing two AGAACCA (or similar) half-sites separated by 3 bp. The agonist-bound conformation of the GR ligand-binding domain enables the recruitment of transcriptional coregulators. An additional level of transcriptional regulation may occur through small variations in the canonical (+)GRE sequence, which slightly alters the conformation of DNA-bound GR. In contrast, the repressive effects of GR on pro-inflammatory transcription factors are generally thought to be DNA independent. In line with this hypothesis, GR has been shown in many circumstances to interact directly with NF-κB subunits and is thus believed to tether to NF-κB response elements (κBREs) without the use of its own DNA-binding capabilities.

Recently, some GR-mediated transcriptional repression has been attributed to direct interactions of the receptor with DNA. In 2011, the discovery of inverted-repeat negative GC response elements (nGREs) was found to mediate GC-induced repression of hundreds of genes. Our subsequent crystallographic analyses demonstrated that the GR DBD binds these nGREs in a distinct orientation from (+)GREs, which are pseudo-palindromic hexameric sequences containing two AGAACCA (or similar) half-sites separated by 3 bp. The agonist-bound conformation of the GR ligand-binding domain enables the recruitment of transcriptional coregulators. An additional level of transcriptional regulation may occur through small variations in the canonical (+)GRE sequence, which slightly alters the conformation of DNA-bound GR. In contrast, the repressive effects of GR on pro-inflammatory transcription factors are generally thought to be DNA independent.

In line with this hypothesis, GR has been shown in many circumstances to interact directly with NF-κB subunits and is thus believed to tether to NF-κB response elements (κBREs) without the use of its own DNA-binding capabilities.

To test the ability of GR to repress transcription of pro-inflammatory genes hosting κBREs within their promoter, we conducted RNA sequencing (RNA-seq) on A549 cells in the presence and absence of dexamethasone. Many NF-κB target genes were downregulated by dexamethasone treatment (Supplementary Figure 2a-b) and geneset enrichment analysis showed that dexamethasone-regulated genes had remarkable overlap with genes regulated by tumor necrosis factor (TNF)-α via NF-κB (p = 2 × 10^-45). However, measuring transcriptional changes of NF-κB target genes in response to dexamethasone is not ideal due to the low abundance of many of these transcripts in the absence of pro-inflammatory signaling. Unfortunately, their induction by TNF-α would introduce the confounding factor of NF-κB activation, including the nuclear translocation of potential tethering factors and/or chromatin remodeling. To remove these confounding factors, we tested the ability of the GR double mutant K442A R447A to repress constitutively active reporters harboring ~150 bp of κBRE-containing promoters. The K442A R447A mutant lacks two key side chains critical for sequence-specific DNA recognition by GR at multiple response elements (refs. 13, 19, and Supplementary Figure 2c). Although WT GR was able to repress several of these reporters, including IL8, CCL2, RELB, PLA2, and ICAM1, the K442A R447A mutant was generally unable to repress more than transfection with an empty vector (Supplementary Figure 2d-j). These results indicated that the DNA-binding ability of GR is critical for its ability to repress transcription of κBRE-containing promoters.

To understand how GR associates with native NF-κB target genes, we used a tacrycine-inducible system in HEK293T cells to express WT GR or κBRE mutants, including the S425G and K442A R447A mutants. As these cells express GR endogenously, the exogenous receptors were detected using their N-terminal hemagglutinin (HA) epitope tag (Fig. 1a). Recruitment of GR functional interaction with κBREs independent of TNF-α.

**Results**

**GR mutations preventing NF-κB repression dissociate (+)GRE and nGRE binding.** The GR is one of five paralogous steroid receptors in humans, along with the estrogen, androgen, progesterone, and mineralocorticoid receptors. Recently, we demonstrated that the GR is the only steroid receptor capable of binding to nGRE sequences and mediating transcriptional repression from these elements. A single amino acid substitution unique to the GR evolutionary lineage, G425S, altered the receptor’s backbone conformation, enhancing its ability to bind DNA as a monomer. The reverse mutation, S425G, renders the human GR incapable of binding nGREs. Previously, the S425G mutation had been reported to ablate the ability of GR to repress NF-κB-driven transcription. In line with these results, we found that the S425G mutation hindered the ability of full-length GR to repress a constitutively active reporter gene preceded by 400 bp of the IL8 promoter, which contains a κBRE, but not a (+) GRE (Supplementary Figure 1a). We hypothesized this effect could be due to direct binding of GR to DNA, given the similar effect seen at DNA-dependent nGREs.
together, these results suggest that GR associates with native steroid-deprived and treated with or without 1 GR and NF-κB subunit of NF-κB K442A R447A and GR S425G associated with the p65/RelA also interfere with this interaction. Similar to the WT receptor, interaction with NF-κB potentially in a sequence-specific manner—resulting in a sequence-specific effect on GR’s DNA-binding ability and recruitment to target genes (Fig. 1b). Critically, the K442A R447A mutation was not recruited to DNA-binding ability and recruitment to target genes (Fig. 1b). As GR could be tethered to exogenous GR to the native κBREs of NF-κB target genes (IL6, IL8, and ICAM1) and canonical (+)GREs of GC-induced genes (FKBP5, SGK1, and TSC22D3/GILZ; Supplementary Figure 3a) was then examined by ChIP assay using anti-HA antibody. Compared with WT GR, which was detected at every site examined, the S425G mutation reduced GR occupancy at specific NF-κB target genes (IL6 and ICAM1), but not IL8) and one of the three GC-induced genes (FKBP5 but not GILZ or SGK1), confirming that this mutation has gene-specific effects on GR’s DNA-binding ability and recruitment to target genes (Fig. 1b). Critically, the K442A R447A mutation was not recruited to canonical (+)GREs such as FKBP5 or κBREs in the inflammatory genes (Fig. 1c), demonstrating that recognition of DNA—potentially in a sequence-specific manner—is required at both classes of sites.

As GR could be tethered to κBREs via protein–protein interaction with NF-κB, we tested whether these DBD mutations also interfere with this interaction. Similar to the WT receptor, GR K442A R447A and GR S425G associated with the p65/RelA subunit of NF-κB when co-expressed (Fig. 1d and Supplementary Figure 3b), indicating that protein–protein interaction between GR and NF-κB is not disrupted by the mutations and is not sufficient for GR-mediated transcriptional repression. Taken together, these results suggest that GR associates with native NF-κB target genes via direct DNA binding, a mechanism that is completely abolished by the GR K442 R447A mutation and modulated by the GR S425G substitution.

GR binds directly to κBREs. Multiple ChIP-seq studies have been performed to determine DNA sequence motifs bound by GR in cells. In many of these, κBREs are highly enriched at genomic GR-binding sites. A recent study performed ChIP-seq on GR and the NF-κB subunit p65 in the presence of their respective ligands (or a combination), as well as with simultaneous lentiviral short hairpin RNA knockdown of p65. Our reanalysis of that study’s data reveals that upon treatment with triamcinolone acetonide (TA), a synthetic GC, ~ 476 GR ChIP-seq peaks contain κBRE motifs, and the vast majority of these (83%) remain even upon p65 knockdown (Supplementary Figure 4). Genes nearby these peaks appear to have important roles in the inflammatory response, with moderate overrepresentation of terms such as regulation of apoptosis, response to lipopolysaccharide (LPS), and response to cytokine stimulus in Gene Ontology analysis (Supplementary Figure 4). Further, GR is recruited to many of these motifs, such as that in the IL8 promoter, without corresponding occupancy by the NF-κB subunit p65 (Fig. 2a–c). Given the receptor’s localization to κBREs in multiple ChIP-seq studies and the requirement of its DNA-binding residues shown here, we...
postulated that GR may be binding directly to κBREs, to repress pro-inflammatory transcription in a sequence-specific manner.

To test this hypothesis, we used fluorescence polarization to measure the ability of soluble, full-length GR to bind both the IL8 κBRE and the SGK1 (+)GRE (Fig. 2d). Remarkably, the affinity of full-length GR for the two elements was very similar, with $K_d$s of 34 and 51 nM for the SGK1 (+)GRE and the IL8 κBRE, respectively. We were unable to purify enough full-length GR to test additional DNA elements so we tested the ability of the isolated GR DBD to bind the IL8, CCL2, and PLAU κBREs in vitro. The isolated GR DBD was able to bind to all three of these κBREs, with affinity similar to nGRE binding19 (Fig. 2e). The mineralocorticoid receptor DBD, which recognizes canonical GR binding sites31, is unable to bind any of the κBREs tested (Fig. 2f), suggesting that steroid receptor–κBRE interactions may be unique to GR, much similar to nGRE binding21.

Given the direct interaction between the GR DBD and κBREs, we sought to uncover the structural mechanism by which GR bound these elements. Using X-ray crystallography, we solved five crystal structures of the GR DBD bound to κBREs from the CCL2, ICAM1, IL8, PLAU, and RELB promoters, at resolutions ranging from 1.85 to 2.30 Å (Table 1, Fig. 3a-e and Supplementary Figure 5). In all of these crystal structures, the GR DBD formed a dimer within the asymmetric unit (Fig. 3a-e). However, in all structures, one DBD monomer was consistently located above the end-stacking junction of the pseudo-continuous DNA helix formed by crystal packing. Therefore, it is likely that sequence-specific contacts to κBREs are made with only one GR monomer.
GR recognizes kBREs in a sequence-specific manner. An interesting feature of the five crystal structures in complex with kBREs is the common binding footprint for the central GR DBD monomer (Fig. 3f). In each structure, GR DBD recognizes an AATTY sequence, with Y representing a pyrimidine base. Although each of the five structures were solved with a 16 bp oligonucleotide, the DNA packs in three distinct conformations driven by GR speciﬁcity for the AATTY sequence (Fig. 3f). PISA analysis indicates that the free-energy gain on formation of the BRE–GR DBD dimerization interface remains capable of repressing NF-κB activity33, as well as our previous observations

To test whether recognition of the AATTY motif is required for GR-kBRE binding in solution, we performed nuclear magnetic resonance (NMR) footprinting analysis to map the interaction between GR DBD and the IL8 kBRE (Fig. 5a-e). Two-dimensional (2D) homonuclear [1H,1H]- nuclear Overhauser effect spectroscopy (NOESY) NMR analysis reveals that the in-solution GR DBD-binding footprint on the IL8 kBRE is consistent with the crystal structure (Fig. 5a-c). Furthermore, the NMR data suggest the nucleotides near the AATTY sequence are most perturbed (Fig. 5c). The largest chemical shift perturbation occurred at guanine-21, which is directly adjacent to the first adenine of the AATTY motif. This is in strong agreement with the crystal structure of the GR DBD–IL8 kBRE structure, as the GR DBD makes two close interactions (2.7 Å) with the DNA backbone at this position (Fig. 5f). The next two largest chemical shift perturbations upon GR DBD binding occurred within the AATTY motif, including adenine-22, which is directly contacted by Lys442 of the GR DBD (Fig. 5g). Crucially, thymine-24, which is contacted directly by Val443 of the GR DBD (Fig. 5h), also had a significant chemical shift perturbation upon GR binding (Fig. 5b, c).

On the protein, 2D [1H,15N]-heteronuclear single quantum coherence (HSQC) NMR analysis reveals that binding of IL8 kBRE to 15N-labeled GR DBD causes large chemical shift perturbations for residues that contact DNA, such as Cys441 and Gly45914 (Fig. 5e). However, when bound to the IL8 kBRE, these “D-loop” residues were not affected (Fig. 5d), confirming that when GR DBD binds to this DNA sequence it does bind as a “D-loop” engaged dimer. This is consistent with recent reports that a monomeric full-length GR protein carrying mutations at both the DBD and LBD dimerization interfaces remains capable of repressing NF-kB activity33, as well as our previous observations.
NF-kB over-representation has been provided by structural analyses of six NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-03780-1 | www.nature.com/naturecommunications

“by NMR that monomeric nGRE interactions do not perturb the D-loop”21.

Cryptic GR-binding sites within kBREs are highly conserved. NF-kB binds as a homo- or heterodimer of Rel homology domain-containing proteins to kBREs by specifically recognizing two binding footprints surrounding a central spacer in which the AATTY site is found. In a p50/p65 heterodimer, the central base pairs of the kBRE are not specifically bound by NF-kB itself (Fig. 6a)34. Despite this lack of sequence discrimination by NF-kB, an AATTY motif is overrepresented in the spacer region in its genomic response elements15. No satisfactory explanation for this over-representation has been provided by structural analyses of NF-kB binding alone15, and a recent SELEX study to determine the optimal NF-kB-binding motif revealed little sequence preference by the protein at this spacer sequence36. Given our findings that GR recognizes a cryptic AATTY motif present within kBREs, we propose that this motif is widely prevalent to ensure that these elements can be bound and repressed by GR. Supporting this hypothesis, the AATTY motif is present in many NF-kB-responsive genes that are regulated by dexamethasone (Supplementary Table 1).

Furthermore, as GR, but not NF-kB, recognizes the AATTY motif within the central spacer sequence, we expected this motif to be highly conserved despite a lack of evolutionary pressure from NF-kB DNA-binding requirements. Indeed, we found that the AATTY motif is extremely well conserved—often more so than the bases that contact NF-kB (Supplementary Table 2). In a remarkable example, the AATTT motif or its reverse complement at the IL8 kBRE is perfectly conserved from mammals to reptiles (Fig. 6b). This element also exhibits strong functional conservation, as human GR DBD retains the ability to bind the IL8 kBRE from multiple species (Fig. 6c). Finally, mutation of one or more of these conserved bases from the human IL8 kBRE reduced or ablated the ability of dexamethasone to repress the human IL8 promoter (Fig. 6d), supporting both a sequence-specific recognition of the element by GR as well as a functional role for these highly conserved spacer bases at the IL8 kBRE. Taken together, our results demonstrate that GR binds a conserved, cryptic sequence within a subset of kBREs to repress pro-inflammatory transcription.

GR requires multiple coregulators to suppress cytokine genes. In MCF-7 cells, dexamethasone repressed TNF-α-induced expression of pro-inflammatory genes to different extents (Fig. 7a). To determine whether these differences reflect the ability of GR to compete against NF-kB for distinct nGREs/kBREs, we examined the recruitment of endogenous GR and the NF-kB subunit, p65, to the IL6, IL8, and ICAM1 promoters in MCF-7 cells stimulated for 1 h with 10 ng/ml TNF-α, 100 nM dexamethasone, or both. Compared with vehicle-stimulated cells, TNF-α and dexamethasone increased recruitment of p65 and GR, respectively (Fig. 7b). In dexamethasone-treated cells, TNF-α stimulation reduced GR recruitment to some of these sites, demonstrating that NF-kB signaling was not required for GR binding. Dexamethasone also blocked NF-kB recruitment in a gene-selective manner. In TNF-stimulated cells, dexamethasone abolished recruitment of p65 to IL6, without affecting p65 recruitment at IL8 or ICAM1 (Fig. 7b). These results indicate that other factors, in addition to sequence-specific kBRE binding by GR, are required to explain dexamethasone-dependent repression of NF-kB target genes.

To gain further insight into the molecular requirements of GR-mediated suppression of NF-kB activity, we used a small-scale small interfering RNA (siRNA) screen targeting 22 coregulator genes, to determine which are required for suppression of the IL6 gene in MCF-7 cells. These siRNAs have been previously shown to knockdown expression of their targets37, which include nuclear receptor corepressors, histone deacetylases, and the nuclear receptor coactivators, SRC1–3 (also known as NCOA1, NCOA2, and NCOA3), which have intrinsic lysine acetyltransferase (KAT) activity, and are primary scaffolds for recruitment of other KATs such as CBP, p300, and PCAF, and assorted coregulators.

We identified a number of coregulators whose knockdown partially or fully prevented dexamethasone from suppressing TNF-α induction (Fig. 7c). These include corepressors, NCoR, HDAC1, and HDAC3, but also the coactivator CBP, SIRT1 and SIRT2, NAD+−dependent corepressors that deacetyl- late p65 Lys32038,39, were both required for dexamethasone-mediated suppression of IL6 (Fig. 7c). We also identified three coregulators required for TNF-α-induced activation of IL6, including p300 and pCAF (Fig. 7d). SRC2 has been previously reported as required for GR-mediated anti-inflammatory effects40, but we observed that it had a dual role. SRC2 knockdown both reduced TNF-α-dependent activation and abolished dexamethasone-dependent suppression of IL6, demonstrating that it has roles in both activation and suppression at this locus.

There were also a set of coregulators whose knockdown led to global increases in IL6 activity with both vehicle and TNF-α treatment. Deleted in breast cancer-1 (DBC1/CCAR2) associates with SIRT1 and inhibits its deacetylase activity41,42. DBC1 was not required for GR-mediated suppression of IL6, but knockdown of DBC1 led to a TNF-α- and dexamethasone-
independent increase in IL6 expression, similar to effects of knockdown of HDAC2 and CoREST corepressors (Fig. 7c). Knockdown of SRC1 and SRC3 also globally increased IL6, but SRC3 displayed an additional role in dexamethasone-mediated suppression. Thus SRC1-3 family members displayed some distinct and some overlapping roles in integrating inflammatory and GC responses.

In contrast, silencing mediator of retinoid and thyroid hormone receptor (SMRT) or ligand dependent nuclear receptor corepressor (LCoR) siRNA did not increase IL6 expression or relieve the inhibitory effect of dexamethasone (Supplementary Figure 6), suggesting that SMRT and LCoR did not contribute to repression of IL6. Further, siRNAs against the lysine demethylase LSD1/KDM1A, the lysine methyltransferase GLP/KMT1D, and the corepressor CTBP1, which are found in complex with HDAC1 and CoREST, also did not have an effect on IL6 expression (Supplementary Figure 6). Taken together, these results suggest that GR requires a specific subset of coregulators, including SRC2, SRC3, CBP, HDAC1, and SIRT2 to mediate ligand-dependent suppression of NF-κB-mediated transcription at the IL6 gene.

**GR modulates the recruitment of coregulators at kBREs.** We examined recruitment of HDAC1 and SRC2 to the promoter regions of the IL6, IL8, and ICAM1 genes in MCF-7 cells by ChIP assay. All of the treatments induced recruitment of HDAC1 and SRC2 to the IL6 promoter, but dexamethasone induced the greatest recruitment (Fig. 7f), consistent with direct binding by GR in the absence of NF-κB signaling. Dexamethasone also
induced recruitment of HDAC1 to IL8, but did not induce recruitment of SRC2 to IL8 or ICAM1 (Fig. 7f), demonstrating that GR controls coregulator recruitment at native κBREs in a gene-specific manner. To test the role of monomeric GR binding, we compared the recruitment of endogenous SRC2 in HEK293T cells transfected with tetracycline-inducible expression plasmids for HA-tagged GR-WT or the mutant with reduced affinity for nGREs, GR-S425G. SRC2 was recruited to the IL6, IL8, and ICAM1 promoters, and this recruitment was completely abolished by the GR-S425G mutation that displays reduced affinity for nGREs (Fig. 7g). Taken together, these results are consistent with a model where GR controls recruitment of coregulators required for GC-dependent suppression of NF-κB target genes by binding directly to nGREs/κBREs via DBD-mediated, sequence-specific recognition of DNA.

**Discussion**

A defining feature of the inflammatory response is the integration of signals from a variety of pathways at the promoters of cytokine and chemokine genes, including direct binding of NF-κB, AP-1, IRF, STAT, and Cred family members. Opposing signaling through these pathways by GCs is strictly required to avoid lethal immune overactivation in response to infection or other inflammatory stimuli. Despite the importance of GR signaling, the mechanism of inflammatory gene repression by GCs has remained controversial. Here we show that GR binds directly to a
highly conserved, cryptic sequence within κBREs at these promoters, such as those of IL8, CCL2, and ICAM1.

Through reanalysis of ChIP-seq data, we find that the κBRE motif exists at 5%–10% of GR-bound sites after GC treatment alone, in line with other studies20,29,30. As GR activation does not alter the subcellular localization of NF-κB45-47, this data suggested the possibility of direct interaction between GR and NF-κB-driven promoters. Following NF-κB activation, the κBRE is present at ~25% of GR-occupied sites30. In contrast to the tethering hypothesis, we show here that GR’s DNA-binding ability is required for recruitment to these genes. This is in line with a previous study that demonstrated that some GR-binding sites, which did not co-occupy with NF-κB or AP-1, are only accessible and bound upon LPS treatment30.

The model we propose—that GR binds κBREs directly—represents a shift in the current models of GR action. Thus, it is worthwhile to re-examine previous studies in light of the hypothesis we propose here. Hundreds of studies have been performed with GR mutant proteins and their conclusions are quite complex24. At least four studies have demonstrated that GR activation affects the DNA binding of NF-κB3,46,-48, although this was not observed in at least three other reports45,49,50, whereas our data demonstrates that these effects are gene selective. Our model predicts that GR and NF-κB compete directly for the same binding site at some loci. However, experiments using GR variants demonstrate that two proteins binding the same response element can undergo dynamic exchange with short protein–DNA residency times and, in fact, do not compete51. Many studies, including ours, have shown that GR and the p65 subunit of NF-κB interact3,27,47,50,52. However, the existence of GR mutants,
such as the K442A R447A mutant described here, able to bind NF-κB yet deficient in its repression demonstration that a GR-NF-κB interaction is not sufficient for GC-mediated repression of NF-κB. In line with our results, the DNA-binding domain of GR has been previously shown to be crucial for repression of NF-κB.

Although we propose that direct GR binding to kBREs is an important mechanism of GC action, it is likely to be that multiple mechanisms govern GR-mediated suppression of inflammation and possible that multiple mechanisms act on any given gene. Some GR ligands affect multiple functions of the protein. For example, selective GR modulators have been reported to differentially affect repression of AP-1 and NF-κB. Newly discovered DNA-binding motifs mediate some of the transcriptional repression by GR, as nGREs may represent a genome-wide class of GR binding sites. Importantly, ChIP-seq studies have validated the widespread role of monomeric GR in GC function. Finally, ChIP-seq studies have also found similar GR-bound DNA sequences near genes both repressed and activated by GC treatment. This finding indicates that DNA sequence and/or oligomerization state may not be sufficient to predict GR action at a particular site, it also confirms that the current tethering model of protein–protein interactions between GR and NF-κB is insufficient to explain all GC-mediated transcriptional responses. Here we propose that direct, sequence-specific interactions between GR and some kBREs are critical for transcriptional repression previously explained exclusively by tethering.

Current models of transcriptional regulation suggest a signaling pathway controlled by ordered, cyclical patterns of protein recruitment. However, this occurs in a highly dynamic, stochastic manner, with rapid and transient assembly of different complexes, a subset of which facilitates active transcription. This suggests a model whereby GR samples the IL6 promoter in several structurally distinct modes that involve both direct binding to the kBRE and tethered binding via protein–protein interaction with DNA-bound transcription factors such as p65, and various coregulator complexes to disrupt NF-κB-mediated transcription.

**Methods**

**Analysis of ChIP-seq data.** Previously reported ChIP-seq reads were downloaded from the NCBI Sequence Read Archive (SRA): samples GSM604648, GSM604649, GSM604651, GSM604656, GSM604657, GSM604662, and GSM604663. These were aligned to the human GRCh38 genome using Bowtie2. ChIP-seq peaks were called using MACS 1.4. Peaks adjacent to each other were merged using bedtools intersect. The analysis was performed using the SICER tool. These were used as negative controls, bedtools intersect was used to determine shared peaks among samples with a minimum overlap of 1 bp.

**ARTICLE**

**Coregulators are essential for GR-mediated repression of NF-κB.**

**Fig. 7** Coregulators are essential for GR-mediated repression of NF-κB. a, b Effects of dexamethasone (Dex) on NF-κB target genes. Steroid-deprived MCF-7 cells were stimulated with vehicle, 10 ng/ml TNF-α, 100 nM dexamethasone, or both. a After 4 h, total RNA was isolated and analyzed by qPCR. b The cells were fixed after 1 h and promoter occupancies at IL6, IL8, and ICAM1 were compared by ChIP assay using anti-p65 or anti-GR antibody (mean ± SEM; n = 3). * Dunnett’s multiple comparisons test, p adj < 0.05 relative to vehicle-treated cells. c–e MCF-7 cells transfected with control or the indicated target siRNAs were stimulated with 10 ng/ml TNF-α alone or in combination with 10 nM dexamethasone for 2 h. IL6 mRNA levels (mean ± SEM; n = 3) determined by qPCR are shown relative to levels in control siRNA transfectants stimulated with the vehicle. d Genes required for TNF-α-induced activity. e Genes that globally suppress TNF-α-induced activity. f GR coregulators are recruited to native nGREs/kBREs. Steroid-deprived MCF-7 cells were stimulated with vehicle, 10 ng/ml TNF, 100 nM dexamethasone, or a combination of TNF and dexamethasone, and fixed after 1 h. Promoter occupancies at IL6, IL8, and ICAM1 were compared by ChIP assay using anti-HDAC1 or anti-SRC2 antibody (mean ± SEM; n = 3). * Dunnett’s multiple comparisons test, p adj < 0.05 relative to vehicle-treated cells. g GR modulates coregulator recruitment at native nGREs/kBREs. HEK293T cells were transfected with the indicated expression plasmids. The next day, the cells were steroid-deprived and treated with or without 1 μg/ml doxycycline (Dox) for 24 h. The cells were then stimulated for 1 h with 100 nM dexamethasone alone or in combination with 10 ng/ml TNF and analyzed by ChIP assay using an anti-HDAC1 or anti-SRC2 antibody. Shown as % input (mean ± SEM, n = 3). * Sidak’s multiple comparisons test, p adj < 0.05 relative to GR-WT transfectants.
Table Browser was used to obtain genomic sequences, and were considered to contain an xBRE if their genomic sequences contained at least one site matching the NFκB (Accession number: AHF2589-1) JASPAR (Ver. 2014) NRKEF1, with a score of 90% or greater, as determined using the Biostrings package in Bioconductor39. For Gene Ontology analyses, the nearest transcription start site to each peak was determined with the ChiPpeakAnno package39 and results input to the DAVID ontology server30.

RNA-sequencing. Approximately 10⁶ A549 cells cultured in complete Dulbecco’s modified Eagle’s medium (DMEM) were transfected with 2 μg pDNA3.1 with FuGene HD according to manufacturer’s protocols. Twenty-four hours after transfection, cells were treated with 100 mM dexamethasone or vehicle control (ethanol). Twenty-four hours after treatment, RNA was isolated with the Qiagen RNeasy kit, according to manufacturer’s protocols. Library preparation and sequencing were performed by the Baylor University Genomic and RNA Profiling Core.

Protein expression and purification. DBDs were expressed and purified as previously described: residues 417–506 of the human GR (GenBank ADP192525) and residues 345–431 of the human MR (GenBank NR_055971.1) were expressed as an N-terminal 6-×His fusion followed by a TEV protease cleavage site. Escherichia coli BL21(DE3)pLysS cells were induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside for 4 h at 30 °C after reaching an OD₆₀₀ of ~0.6. Proteins were purified via affinity chromatography (HisTrap) followed by gel filtration in 100 mM NaCl, 20 mM Tris-HCl pH 7.4, and 5% glycerol. Protein was concentrated to 4 mg/ml, flash frozen in liquid N₂, and stored at −80 °C until use. 15N-GR DBD was expressed in E. coli BL21(DE3)pLysS cells with 15N-Chi3 as the sole nitrogen source, purified as described above. The 6-×His tag was cleaved with TEV protease overnight at 4 °C, passed through a NiNTA column, and the flow through containing BSA was collected and verified to be >99% pure by SDS-polyacrylamide gel electrophoresis (PAGE). Residues 1–777 of human GR were expressed as an N-terminal 6-×His fusion in baculovirus-infected Sf9 cells64. Cells containing puriﬁed ERα were transfected into HEK293 cells with FuGene HD (Promega) according to the manufacturer’s protocol. Twenty-four hours after transfection, cells were treated with 10 μg/ml tamoxifen (10% DMSO in 4% FBS) and 24 h following treatment ﬁrefly luciferase activities were read using a luciferase assay system (Promega). WT and mutant GR were expressed in HEK293T cells treated with 1 μg/ml doxycycline for 16 h. Twenty-four hours following treatment, cells were treated with 1 μg/ml doxycycline for 16 h, and 24 h following treatment ﬁrely and Renilla luciferase activities were read using a Biotek Synergy 2 plate reader and the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer’s protocol. In Fig. 5d, 50 ng of the IL8 promoter (or the indicated mutant) in the pGL3 basic vector and 10 ng of a constitutively active Renilla luciferase under control of the pRL-TK promoter were transfected into HEK cells with FuGene HD (Promega) according to the manufacturer’s protocol. Twenty-four hours after transfection, cells were treated with indicated amounts of dexamethasone, TNF-α, and/or vehicle. Twenty-four hours following treatment, ﬁrely luciferase levels were measured as above. For reporter gene assays, firely luciferase divided by Renilla activity is shown, normalized to the control condition.

In vitro binding assays. Ten nM of double-stranded, 6-FAM-labeled DNA (Integrated DNA Technologies) was incubated with indicated amounts of protein in 100 mM NaCl, 20 mM Tris-HCl pH 7.4, and 5% glycerol. Formation of GR–DNA complexes was monitored viaﬂuorescence polarization on a Biotek Synergy plate reader at an excitation and emission wavelength of 485 and 528 nm, respectively. Data were graphed and analyzed in Prism 6 (Graphpad Software). For GR DBD binding to xBREs, a two-site binding model was used.

Sequences of DNA constructs used for ﬂuorescence polarization assays were: PLAU-5’- (FAM) CCCTGGGAAATTCCTGATA-3’ and 5’-TACGGGAAATTCG CAGG-3’; CCL2-5’-GAGTTGGAATTTTCACACTA-3’ and 5’-TGAGGGAATTTCCCA CTC-3’; ILB-5’-(FAM) AATCCGGAATTTCCCTGTA-3’ and 5’-CAGAGGAAATTC AGGAT-3’. In all cases, (FAM) indicates the position of 6-FAM (ﬂuorescein).

Cystatin and structure analysis. GR DBD was concentrated to 3.0 mg/ml with 0.125 M glycine for 10 min, and rinsed with cold 1 × PBS. 2D 15N−1H (1H,15N) NMR data were collected on a Bruker 700 MHz (1H frequency) NMR instrument equipped with a QCI cryoprobe. For DNA NMR experiments, the 19 nt IL8 xBRE DNA duplex was reconstituted in 20 mM phosphate (pH 6.7), 100 mM NaCl, 1 mM TCEP, 10% D₂O buffer to a ﬁnal concentration of 437 µM, subsequently annealed by denaturing at 95 °C for 3 min and equilibrated to room temperature (20–23 °C) overnight. A 2D 1H-detected NOESY was collected at 10 °C using 300 ms mixing time for 15N-GR DBD DNA before and after adding 0.441 or 2.313 of 15N-GR DBD. For protein NMR experiments, 2D [1H,15N]-HSCQ spectra were collected at 25 °C for free 15N-GR DBD protein or protein complexed with 0.441 or 2.313 of IL8 xBRE DNA duplex; or 1.5 × GBS consensus DNA sequence. Chemical shift perturbations were calculated using previously published GR DBD NMR chemical shifts35 and calculated using the minimum chemical shift perturbation procedure36 in the NMR analysis program NMRView (OneMoon Scientiﬁc, Inc.). Sequence alignments. Sequences were retrieved from the Ensembl database. Human sequences are from the GRCh38 genome build. Geneious version 6.1.6 (Biomatters Limited) was used for sequence alignment and visualization.

Co-immunoprecipitation. WT and mutant GR were expressed in HEK293T cells (ATCC, CRL-3216) using the Tet-On’ inducible gene expression system (Takara Bio USA, Inc., Mountain View, CA). To this end, 10⁶ cells were seeded in each 6 cm dish, co-transfected the next day with the TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI) and 2 µg/dish each of pTet-On Advanced reverse tetracycline-controlled transactivator (rTA), pT'ight-FRT-Hygro2-HA-GR WT-5425G-A442-R447A, and pCR3.1-p65 WT expression plasmids. Control cells were co-transfected with empty pTight vector instead of the GR expression plasmid. After 24 h, the media were replaced with phenol red-free DMEM + 10% CSFBS, with or without 1 µg/ml Doxycycline. The next day, the media were treated with 10 ng/ml TNF-α for 1 h and lysed in 600 µl RIPA buffer + protease inhibitor cocktail (P8340, Sigma-Aldrich Co. LLC, St. Louis, MO). Two hundred and ﬁfty microliter aliquots of lysate were transferred with 1 µg/ml of anti-GR (Y-11) antibody (Santa Cruz Biotechnology Inc., Dallas, TX) (Supplementary Table 3), 215 µl RIPA buffer, and 25 µl Dynabeads protein G (Invitrogen, thermo Fisher Scientiﬁc Inc., Waltham, MA) and rotated overnight at 4°C. The following day, the beads were washed 3 × with cold RIPA buffer and twice with cold phosphate-buffered saline (PBS). The beads were then incubated in 25 µl 2 x Laemmli sample buffer (1610737, Bio-rad Laboratories, Inc., Hercules, CA) for 5 min at 95 °C, and the supernatants were analyzed by western blotting using the anti-p65 (F-6) antibody (Supplementary Table 3). ChiP assay. Quantitative ChiP assay was performed as previously described with some modification. HEK293T transfectants in six well plates and MCF-7 cells (ATCC, CRL-11059) were grown on 12 well plates and 11% formaldehyde were fixed for 15 min, quenched with 0.125 M glycine for 10 min, and rinsed with cold 1 × PBS. The cells were disrupted in lysis buffer70, incubated at 4 °C for 1 h and sonicated. The lysates were washed 3 × with cold RIPA buffer and twice with cold phosphate-buffered saline (PBS). The beads were then incubated in 25 µl 2 x Laemmli sample buffer (1610737, Bio-rad Laboratories, Inc., Hercules, CA) for 5 min at 95 °C, and the supernatants were analyzed by western blotting using the anti-p65 (F-6) antibody (Supplementary Table 3).
were then incubated with 100 μl Dynabeads protein G (Invitrogen, Thermo Fisher Scientific, Inc.) and 10 μl pre-immune rabbit IgG for 1 h at 4 °C, and centrifuged at 12,000 rpm for 15 min at 4 °C. One hundred microliters of the pre-cleared supernatant was mixed with an antibody (Supplementary Table 3) (Santa Cruz Biotechnology, Inc.), 25 μl Dynabeads protein G (Invitrogen, Thermo Fisher Scientific Inc.), and lysis buffer to make a 200 μl MP mixture that were rotated overnight at 4 °C. The precipitates were sequentially washed in low-salt, high-salt, and LiCl buffers15, and twice in TE buffer. The crosslinks were then reversed at 65 °C for 3 h. DNA fragments were isolated using QIAquick PCR purification kit (QIAGEN, Hilden, Germany), and analyzed by qPCR using TaqMan® primers and analyzed by qPCR using TaqMan® bioproject/?term www.rcsb.org/structure/5E6C]. RNA-seq data have been deposited in the SRA under accession codes 5E69 [https://www.rcsb.org/structure/5E69], 5E6D[https://www.rcsb.org/structure/5E6D], 5E6A [https://www.rcsb.org/Data availability Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 5E69 [https://www.rcsb.org/structure/5E69], 5E6D[https://www.rcsb.org/structure/5E6D], 5E6A [https://www.rcsb.org/structure/5E6A], 5E6B[https://www.rcsb.org/structure/5E6B], and 5E6C [https://www.rcsb.org/structure/5E6C]. RNA-seq data have been deposited in the SRA under accession codes 5E69[https://www.ncbi.nlm.nih.gov/sra?term=PRJNA314815]. Other data are available from the corresponding author upon reasonable request.

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Additional information

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