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Widespread Negative Response Elements Mediate Direct Repression by Agonist-Liganded Glucocorticoid Receptor

Milan Surjit,[1] Krishna Priya Ganti,[1,2] Atish Mukherji,[1,2] Tao Ye,[1] Guoqiang Hua,[1] Daniel Metzger,[1] Mei Li,[1] and Pierre Chambon[1,*]

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS UMR7104, Inserm U964, Université de Strasbourg, Collège de France, Illkirch, 67404, France

[2]These authors contributed equally to this work

*Correspondence: chambon@igbmc.fr

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SUMMARY

The glucocorticoid (GC) receptor (GR), when liganded to GC, activates transcription through direct binding to simple (+)GRE DNA binding sequences (DBS). GC-induced direct repression via GR binding to complex "negative" GREs (nGREs) has been reported. However, GR-mediated transrepression was generally ascribed to indirect "tethered" interaction with other DNA-bound factors. We report that GC-induces direct transrepression via the binding of GR to simple DBS (IR nGREs) unrelated to (+)GRE. These DBS act on agonist-liganded GR, promoting the assembly of *cis*-acting GR-SMRT/NCoR repressing complexes. IR nGREs are present in over 1000 mouse/human ortholog genes, which are repressed by GC in vivo. Thus variations in the levels of a single ligand can coordinately turn genes on or off depending in their response element DBS, allowing an additional level of regulation in GR signaling. This mechanism suits GR signaling remarkably well, given that adrenal secretion of GC fluctuates in a circadian and stress-related fashion.

INTRODUCTION

Glucocorticoids (GCs) are peripheral effectors of circadian and stress-related homeostatic functions fundamental for survival throughout vertebrate life span (Chrousos, 2009; Nader et al., 2010). They are widely used to combat inflammatory and allergic disorders and their therapeutic effects have been mainly ascribed to their capacity to suppress the production of proinflammatory cytokines (Rhen and Cidlowski, 2005). GCs act by binding to the GC receptor (GR), a member of the nuclear receptor (NR) superfamily. In absence of GCs, GR is maintained in the cytoplasm by molecular chaperones. Binding of GCs generates a conformational switch in the GR ligand binding domain (LBD) which affects GR interactions with chaperones and facilitates nuclear translocation (Ricketson et al., 2007). Once in the nucleus, GR binds to GC response elements (GREs) and regulates transcription of target genes. "Simple" GREs belong to a family of imperfect palindromes consisting of two inverted hexameric half-site motifs separated by three base pairs (bp) (Meijsing et al., 2009). Such "simple" (+)GRE confer transcriptional transactivation to agonist-liganded GR through association with coactivators (e.g., SRC1, TIF2/SRC2 and SRC3) (Lonard and O'Malley, 2007). "Composite" GREs consist of DNA binding sites (DBS) for GR which, in association with binding sites for other factors, can act synergistically to mediate transactivation or transrepression. In a few of cases, binding of GR to promoter regions has been implicated in GC-induced transrepression, but no consensus sequence for "repressing" negative GREs (nGREs) has emerged (Dostert and Heinzel, 2004). Remarkably, "tethering" GREs do not contain DBS for GR per se, but instead contain binding sites for other DNA-bound regulators, such as NFκB and AP1, that recruit GR (Karin, 1998; Kassel and Herrlich, 2007). Thus, tethering GREs confer "indirect" transrepression to agonist liganded GR.

Atopic dermatitis (AD) is an inflammatory skin disease that exhibits a high prevalence (Bieber, 2008). We recently developed mouse models which closely mimic human AD (Li et al., 2005, 2006), and revealed that induction of the Thymic Stromal Lymphopoietin (TSLP) cytokine in epidermal keratinocytes is necessary and sufficient to trigger a human AD-like syndrome. As topical GCs are important tools for AD treatment, we wondered whether their effect could result from TSLP repression. We report that GCs transcriptionally repress TSLP expression in AD mouse models, and demonstrate that this repression is mediated through direct binding of GR to a "simple" nGRE, which belongs to a novel family of evolutionary-conserved *cis*-acting negative response elements (IR nGREs) found in numerous GC-repressed genes.

RESULTS

Glucocorticoid-Induced GR-Mediated Transcriptional Repression of TSLP Expression

The GC agonist fluocinolone acetonide (FA) was applied to ears of mice concomitantly treated with the "low-calcemic" Vitamin D3 (VitD3) analog MC903 (Calcipotriol; hereafter called MC) to trigger TSLP expression (Li et al., 2006). In wild-type (WT) mice,

FA application inhibited basal TSLP RNA level by ~50%, which interestingly could be relieved by coapplication of the GC antagonist RU486 (mifepristone, hereafter named RU), while MC-induced increase of TSLP RNA, which was fully blocked by FA, was also restored by RU cotreatment (Figure 1A). Similarly, the retinoic acid (RA)-induced increase of TSLP transcripts (Li et al., 2006) was blocked by FA and restored by RU (Figure S1A available online). As expected, the expression of the GC-inducible GPX3 (glutathione peroxidase 3) gene which harbours a (+) GRE (Tuckermann et al., 1999) was enhanced by FA (Figure 1A) and inhibited by RU cotreatment, while FA or RU had no effect on MC-dependent expression of the CYP24A1 gene (a VitD3 target) (Figure 1A). Inhibition of TSLP expression by FA was not dependent on its induction by MC or RA, as a 3-day FA application to ears of RXR$\alpha\beta^{ep-/-}$ or VDR/RAR$\alpha\gamma^{ep-/-}$ mice (selectively lacking in epidermal keratinocytes both RXRα and β, or VDR and both RARα and RARγ, respectively) which express high levels of TSLP in epidermal keratinocytes in the absence of MC or RA treatment (Li et al., 2005 and unpublished data) reduced TSLP RNA by ~70% (Figure 1B, left panel, and data not shown).

The GR involvement in FA-induced inhibition of MC-induced TSLP expression in keratinocytes was demonstrated using adult mice in which GR was selectively ablated in keratinocytes ($GR^{ep-/-}$ mice). Although the basal TSLP level was similar in vehicle-treated WT and $GR^{ep-/-}$ mice, FA blocked MC-induced TSLP expression in WT, but not in $GR^{ep-/-}$ mice (Figure 1B, right panel). MC treatment was more efficient in $GR^{ep-/-}$ than in WT mice, indicating that endogenous GCs may partially inhibit MC-induced TSLP expression in WT epidermis. TSLP expression was similarly repressed by FA and restored by RU in mouse intestinal epithelium (Figure S1B) and in human lung epithelial cells A549 (Figure S1C), whereas expression of the (+)GRE-containing mouse GPX3 and human GILZ (Wang et al., 2004) GC-induced genes was enhanced by FA and inhibited by RU. Nuclear run-on assays demonstrated that GR-mediated FA inhibition of TSLP expression was transcriptional (Figure 1C).

A Putative Negative GRE Is Located in the TSLP Promoter Region

As neither NFκB nor AP1 are involved in TSLP induction by MC in epidermis (unpublished data), its repression was unlikely to be mediated by a tethering GRE. A bioinformatics analysis of 20 kb of DNA located upstream and downstream from the mouse and human TSLP translation startsite (+1) did not reveal any (+)GRE or known "composite" activating or repressing GRE, but unveiled the presence of a palindromic sequence consisting of two inverted repeated (IR) motifs separated by one bp (called hereafter IR1 nGRE), in the upstream promoter region of both mouse (m) and human (h) TSLP genes (Figure 1E). Recombinant human GR protein in electrophoretic mobility shift (EMSA) and supershift assays with GR antibody showed that this putative mTSLP IR1 nGRE and its human counterpart, as well as the TAT (+)GRE (Meijsing et al., 2009), bound to the GR protein (Figure 1D, left panel). These bindings were specific, as shown by lack of GR binding to a mutant (+)GRE and to three mTSLP IR1 nGRE mutants (Mut1, 2, and 3) (Figures 1E and 1D, middle panel). Complexes formed between the recombinant GR and either putative IR1 nGREs or (+)GRE similarly migrated. As GR binds (+)GREs as a dimer (Wrange et al., 1989), two GR monomers may bind these putative nGREs. Competition bindings between [32P]-labeled mTSLP IR1 nGRE probe and excess cold (+)GRE probe, and vice-versa, indicated that GR has a higher affinity for (+)GRE than for TSLP IR1 nGRE (Figure 1D, right panel).

Binding of Agonist-Liganded GR to the Putative TSLP IR1 nGRE Enables the Formation of a Repressing Complex

GC-induced binding of GR to TSLP IR1 nGRE, the generation of a repressing complex, and its effect on the organization of the TSLP promoter regions, were investigated in vivo by chromatin immunoprecipitation (ChIP) with WT epidermis and intestinal epithelium, as well as in vitro with cultured A549 cells. Four regions of the TSLP promoter were analyzed: the proximal promoter region (PP), the region containing the IR1 nGRE, and those containing the DR3d VitD3 (VDRE) and the DR2b Retinoic Acid (RARE) response elements (unpublished data) (Figure 1F and Figure S1D). ChIP assays of epidermis revealed weak bindings of GR, as well as of SMRT and NCoR corepressors (Lonard and O'Malley, 2007) to the nGRE region, which were strongly increased upon a 6hr topical FA treatment (Figure 1G). The concomitant disappearance of both GR and SMRT/NCoR bindings to the nGRE region in $GR^{ep-/-}$ mutant mice (Figure 1G) indicated that corepressor bindings were associated with that of GR, which was confirmed by colocalization of GR and corepressors, when shorter segments of the nGRE region were explored (Figure S1E). Upon FA treatment, in the same cells, GR as well as SRC2, SRC3 and Pol II but not SMRT and NCoR, were recruited to the GPX3 (+)GRE region (Figure S1F). Moreover, binding of GR to (+)GRE, was antagonized by RU, which on its own, did not allow the binding of GR to GPX3 (+)GRE (Figure S1F).

SMRT and NCoR are known to recruit histone deacetylase (HDACs) to repressing complexes. As for GR and corepressors, HDAC2 and HDAC3 were weakly bound to the nGRE in vehicle-treated epidermis, and FA strongly enhanced this recruitment (Figure S1G), further supporting that GC-induced binding of GR to TSLP IR1 nGRE generates a repressing complex. RU topical treatment precluded FA-induced generation of this repressing complex on nGRE, whereas application of MC (Figure 1G and Figure S1G) or retinoic acid (RA) (Figure S1H, upper panels) had no effect.

We also used mouse intestinal epithelium, which revealed, upon FA intraperitoneal injection, a strong binding of GR together with SMRT and NCoR corepressors to the TSLP IR1 nGRE region. RU addition precluded the generation of this FA-induced repressing complex, whereas VitD3 had no effect (Figure S1I, upper panel). Similarly, FA addition to A549 cells resulted in a stronger binding of GR together with SMRT and NCoR to the nGRE region (which was suppressed by RU, Figure S1J), whereas it induced binding of an activating complex to the GILZ gene (+)GRE (Figure S1K).

To demonstrate that SMRT and NCoR are instrumental in GC-induced IR1 nGRE-mediated TSLP repression, we knocked-down their expression in A549 cells by 60% and 80%, respectively, with selective siRNA (Figure 1H). Upon single siRNA treatment, ChIP assays showed a marked decrease in SMRT binding and complete disappearance of NCoR binding to TSLP nGRE, while

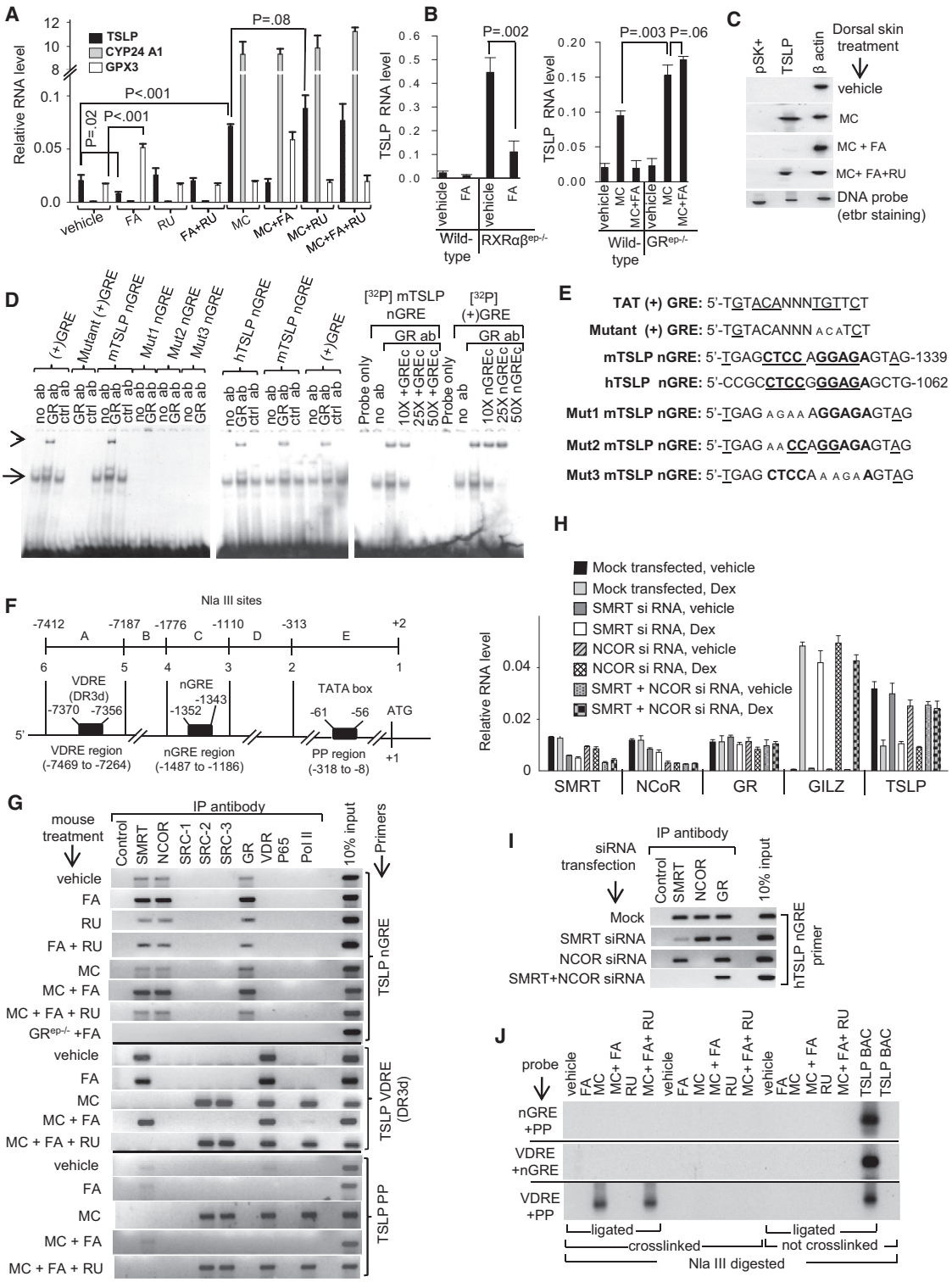

Figure 1. Glucocorticoid-Induced Inhibition of TSLP Transcription Is Mediated through GR and Corepressors Binding to a Negative GRE

(A) Q-RT-PCR (Quantitative-RT-PCR) of TSLP, Cyp24A1 and GPX3 RNA from WT ear epidermis topically treated for 6 hr.

(B) TSLP Q-RT-PCR from RXRαβ$^{ep-/-}$ and WT ear epidermis FA-treated twice a day for 3 days (left panel). TSLP Q-RT-PCR from GR$^{ep-/-}$ and WT ear epidermis treated for 6 hr (right panel).

(C) Nuclear run-on assays using WT dorsal epidermis treated as indicated. Upper panels: autoradiograms of labeled transcripts hybridized with TSLP, β actin or pSK+ vector DNA. Lower panel: ethidium bromide (etbr) staining.

no SMRT and NCoR binding could be detected upon siSMRT and siNCoR RNAs cotreatment (Figure 1I). Dex-induced TSLP repression was prevented by concomitant knockdowns of SMRT and NCoR, but not significantly affected by their single knockdown, thus demonstrating that SMRT and NCoR are instrumental in GC-induced IR1 nGRE-mediated repression, and that these two corepressors can be functionally redundant. Interestingly, the ChIP data suggest that GR is less efficiently bound to the IR1 nGRE in the absence of the two corepressors (Figure 1I). Note that SMRT and NCoR knockdowns (on their own or together) did not affect GR expression and Dex-induced transactivation of the (+)GRE GILZ gene (Figure 1H).

Generation of a Repressing Complex on the TSLP IR1 nGRE Precludes the Formation of an Activating Complex on VDRE, RARE, and Proximal Promoter Regions

In the absence of an agonist ligand, a repressing complex containing VDR and SMRT was bound to TSLP DR3d VDRE in epidermal keratinocytes, whereas it was replaced by a VDR-SRC2/SRC3-Pol II activating complex upon MC topical treatment (Figure 1G and unpublished results). Upon MC and FA cotreatment, VDR association with the VDRE was not inhibited, whereas those of SRC2, SRC3, and Pol II were drastically reduced, and an association of SMRT was observed (Figure 1G). No association of GR to DR3d VDRE was detected, but these latter changes are clearly related to binding of FA to GR, as RU cotreatment (MC+FA+RU) restored the activation binding pattern observed upon treatment with MC alone (Figure 1G). No GR binding to the PP region could be detected upon FA treatment. However, this treatment precluded VDR, SRC2, SRC3 and Pol II bindings induced by MC treatment, and a RU cotreatment (MC+FA+RU) reversed the effect of FA, indicating an involvement of FA-liganded GR in preventing the association of VDR, SRC2/SRC3 and Pol II with the PP region (Figure 1G, lower panels). Similarly, the generation of a repressing complex on IR1 nGRE precluded the formation of an activating complex on DR2b RARE and the PP regions (Figure S1H). Note that, in contrast to the DR3d VDRE complex, the DR2b RARE complex contains SRC2 only.

In keeping with the above data, in intestinal epithelium, the generation of a repressing complex on TSLP IR1 nGRE also precluded formation of a VitD3-induced activating complex on DR3d VDRE and the PP regions (Figure S1I, middle panels).

GC-Induced Formation of a Repressing Complex on the TSLP IR1 nGRE Precludes Interaction between VDRE and PP Regions

That, in presence of MC, the same activating complexes (VDR, SRC2/SRC3 and Pol II) were associated with DR3d VDRE and PP regions indicated that these two regions could be in close apposition through chromatin looping. We therefore performed Chromosome Conformation Capture (3C) assays on epidermal chromatin of mice topically treated with vehicle, FA, MC, MC+FA, RU, and MC+FA+RU. Cross-linked chromatin was digested with Nla III restriction enzyme to separate DR3d, nGRE and PP regions (Figure 1F), which were then ligated to reveal possible interactions between PP and DR3d VDRE or nGRE regions (Figure 1J). No interaction between nGRE and PP region, nor between nGRE and VDRE, could be detected upon FA or RU treatment, whereas an interaction was observed upon MC treatment between DR3d VDRE and PP regions, which was precluded by FA cotreatment (MC+FA), and restored upon RU addition (MC+FA+RU) (Figure 1J). Interactions between DR3d VDRE and PP regions were similarly revealed upon Alu I digestion (Figures S1L and S1M). Thus, the TSLP IR1 nGRE which is located ~1.3 kb upstream from the PP region could act as a silencer element precluding the formation of a chromatin loop between the PP and the VDRE enhancer region located ~7.3 kb upstream.

The Repressing Activity of the TSLP nGRE Tolerates Changes in Spacing and/or Sequence of Its Repeated Motifs

To investigate whether additional DNA elements could be required to generate a repressing activity, we inserted the TSLP IR1 nGRE upstream of an enhancerless SV40 early promoter located 5′ to the luciferase coding sequence of pGL3 vector (Figure 2A). A VDRE separated from the IR1 nGRE by a 314bp-long DNA segment devoid of any known transregulator binding site (not shown) was inserted to generate a luciferase-expressing reporter plasmid (pGL3 vector 1), which was transfected into A549 cells, followed by addition of VitD3

(D) Left panels: EMSA with recombinant human GR protein, and GR antibody (ab) supershift assays, using [32P] 5′-labeled probes (panel E). Arrow and arrowhead point to the position of shifted [32P]- GREs in absence and presence of GR ab, respectively. Right panel: Competition EMSA and GR ab supershift assays using either cold (+)GREc and [32P]- TSLP nGRE probes or cold TSLP nGREc and [32P]- (+)GRE probes, as indicated. 10×, 25×, and 50× refers to fold molar excess of cold probe.

(E) Comparison of mouse (m) and human (h) TSLP nGRE sequences with that of TAT (+) GRE. Palindromes are underlined. Conserved bases in nGREs are in bold letters. N, any base. Mutated residues are in smaller font size.

(F) mTSLP promoter region. +1 is the "A" base of the translation initiation codon (ATG). A to E are Nla III-digested DNA fragments. 1 to 6 denotes Nla III sites. Boxes represent promoter elements with their coordinates. Coordinates of DNA segments (regions) PCR-amplified in ChIP assays are indicated.

(G) ChIP assays using dorsal skin from WT and $GR^{ep-/-}$ mice treated as indicated. Amplified DNA regions are on the right side. IP antibody: immunoprecipitating antibodies. Control is rabbit IgG. 10% input indicates the signal obtained after PCR amplification of the relevant DNA region contained in 10% of chromatin used for each immunoprecipitation with a given antibody.

(H) Q-RT-PCR of SMRT, NCoR, GR, GILZ and TSLP transcripts from siSMRT and/or siNCoR RNA-transfected A549 cells, treated as indicated.

(I) ChIP assays on Dex-treated A549 cells transfected with siSMRT and/or siNCoR RNA, (as indicated), to detect the binding of GR and corepressors to the human TSLP IR1 nGRE region.

(J) Chromosome conformation capture (3C) assays using WT epidermis. Upper, middle and lower panels reveal interaction between nGRE and PP regions, VDRE and nGRE regions, and VDRE and PP regions, respectively.

Values are mean ± SEM.

See also Figure S1, Figure S7, Table S5, and Table S6.

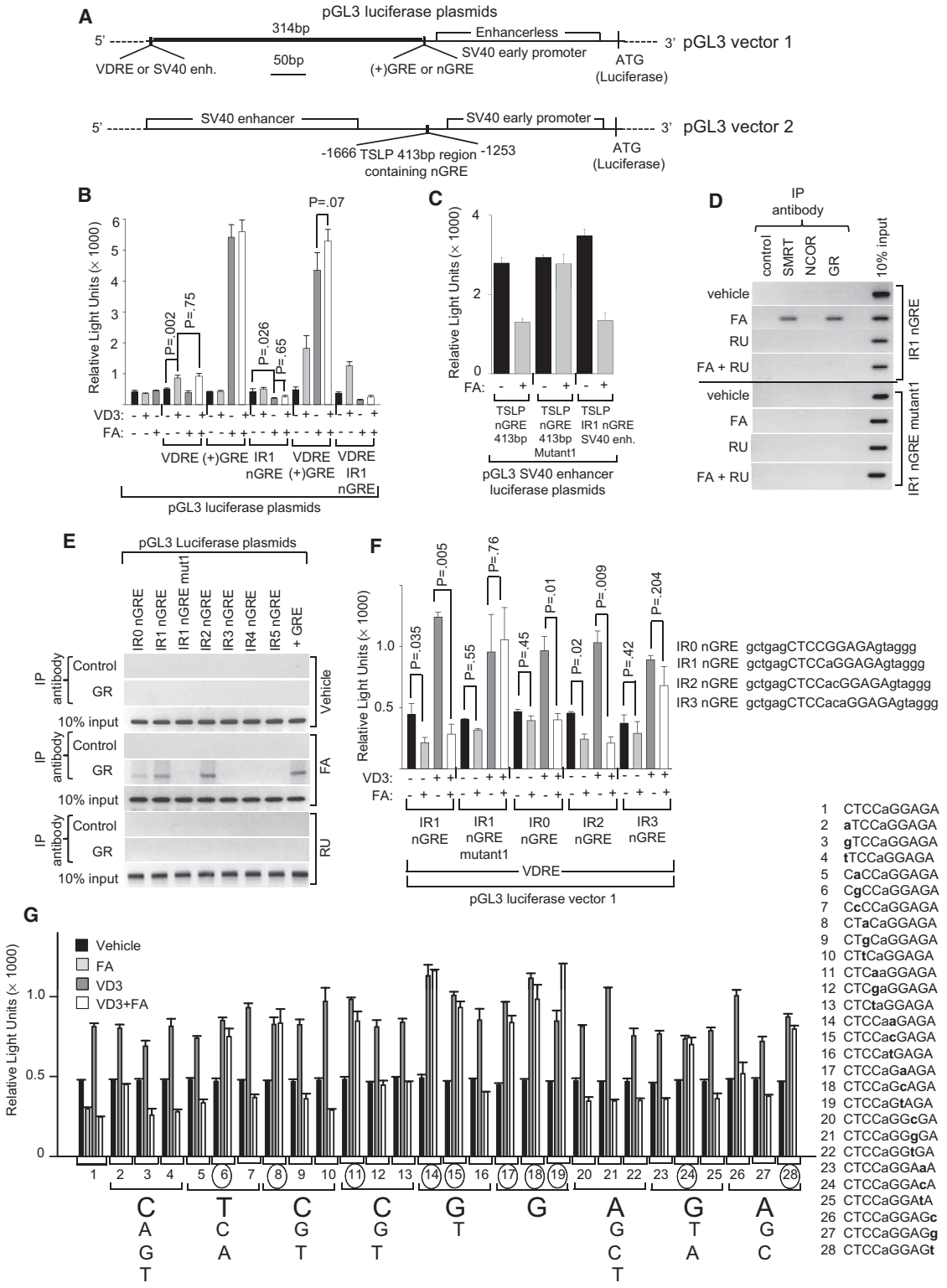

Figure 2. The TSLP nGRE Is Not a Composite Element, but Its Activity Is Affected by Changes in Spacing and/or Sequence of Its Inverted Repeated Motifs

(A) pGL3 luciferase reporter plasmids (see Extended Experimental Procedures).

(B) Luciferase assays of A549 cells transfected with pGL3 vector 1 derivatives, and treated with FA and/or vitD3 (VD3).

and/or FA. FA addition did not affect luciferase expression in absence of IR1 nGRE, whereas its presence resulted in decreased expression (which could be prevented by RU addition) of basal and VDRE-mediated VitD3-induced transcription (Figures 2A and 2B). As expected, FA-induced increase in luciferase expression was observed when IR1 nGRE was replaced by a (+)GRE. The TSLP IR1 nGRE exhibited a similar FA-inducible repressing activity when embedded in 413 bp of its natural environment within the reporter pGL3 vector 2 (Figures 2A and 2C). Thus, on its own, TSLP IR1 nGRE is sufficient to mediate a FA-inducible repressing activity, which resulted from the generation of a repressing complex containing the SMRT corepressor and GR, as shown by ChIP assay (Figures 2D). Replacing, in the reporter, IR1 nGRE by a mutant to which recombinant GR does not bind (IR1 nGRE mut1, Figures 1D and 1E) resulted in no GR binding (Figures 2D and 2E) and no FA-inducible repressing activity (Figure 2C and F), thus supporting the conclusion that GR binds directly to TSLP nGRE in cultured cells. In keeping with our above data in vivo, the addition of RU to FA prevented the formation of the repressing complex (Figure 2D), and RU on its own was unable to induce GR binding to TSLP IR1 nGRE inserted in the pGL3 vector 1 (Figures 2D and 2E).

To investigate whether the 1 bp spacer between the inverted repeated motifs of TSLP IR1 nGRE was critical for its repressing function, pGL3-based luciferase plasmids containing the VDRE and TSLP nGRE motifs spaced by 0 to 5 bases (IR0 to IR5 nGREs) were transfected into A549 cells (Figures 2E and 2F, and data not shown). ChIP assays showed that, upon FA addition, GR similarly bound IR1 and IR2 nGREs, whereas its binding was less efficient on IR0 nGRE, and not detectable on IR3, IR4, and IR5 nGREs (Figure 2E). Accordingly, upon FA addition, the decrease in basal and VitD3-induced luciferase activity was stronger for IR1 and IR2 than for IR0 nGRE, whereas no significant change could be detected for IR3, IR4 and IR5 nGREs (Figure 2F, and data not shown).

pGL3-based luciferase plasmids containing VDRE and TSLP IR1 nGREs (Figure 2A), in which individual bases had been changed one by one, were used to study whether non-canonical IR1 nGREs could function as efficient nGREs (Figure 2G). With one exception, at least a one base pair change was "tolerable" at any position of the TSLP IR1 nGRE and did not impair its activity in vitro, suggesting that GR bound to "degenerate" IR1 elements might also mediate GC-induced direct repression in vivo (see below). A similar analysis carried out with plasmids containing IR2 nGREs showed that a one base pair change was tolerable at any position of IR2 nGRE (Figure S2).

Mouse Genes that Contain IR0, IR1, and IR2 nGREs Conserved in Their Human Orthologs Are Repressed upon GC Agonist Treatment In Vivo

Bioinformatics analyses of mouse and human genomes revealed thousands of genes containing IR elements made up of inverted repeated motifs identical to those of TSLP (CTCC and GGAGA) with either no (IR0), 1 (IR1) or 2 (IR2) bp spacers (Table 1A). A comparison of mouse and human orthologs indicated conservations of such IR elements (51 IR0, 379 IR1, and 566 IR2; Table 1A and Table S1). Within these ortholog gene families, we randomly chose 35 IR0, 50 IR1, and 50 IR2 nGRE-containing mouse genes (Extended Experimental Procedures) to investigate whether (1) they were expressed in epidermis, intestinal epithelium and liver, (2) their expression was inhibited by the GC agonist Dexamethasone (Dex), (3) this inhibition could be relieved by RU486 coadministration, and also if it could be correlated with GR and corepressor binding to their putative IR nGREs. These genes were found to be expressed in one, two or all three tissues, and when expressed, were cell specifically repressed or not repressed (Table 1B).

For nine of the above "IR1" genes, we tested the repressing activity of their putative IR1 nGREs in vitro using the luciferase assay (Figure 2A and Figure 3D). In all cases, and irrespective of the tissue pattern of GC-induced repression, these putative IR1 nGREs "repressed" luciferase activity (Figure 3D), indicating that these IR1 nGREs elements are bona fide IR1 nGREs. Importantly, upon GC treatment in vivo, there was a tight correlation between repression of a given gene in a given tissue (and its relief by RU coadministration), and GR and corepressor association with the IR1 nGRE of that gene in that tissue (compare in Figures 3E–3G with Figures 3A–3C, respectively). In contrast there was no GR and corepressor association with the IR1 element of BMP3 gene which is not "repressed" in either tissue (Figures 3E–3G). These data indicate that, not only could the tissue-specific expression of genes, which potentially can be negatively controlled by GCs, be subjected to epigenetic control, but also that their GC-repression itself could also be epigenetically controlled, which suggests that additional mechanisms operate in the negative control of gene expression mediated by IR nGREs (e.g., USF1 in Figures 3A–3G). Note that depending on the gene and the time of exposure of the animal to FA or Dex (6 and 18 hr), the corepressor components of the repressing complex may change (Figures 3E and 3F).

The "IR0" genes (Figure S3A–S3C, and Table 1B) and "IR2" genes (Figures S4A–S4C, and Table 1B) were also cell specifically or noncell specifically expressed and repressed in the three tissues. We tested the GC-induced repressing potential of putative IR nGREs of some of the selected "IR0" and "IR2" genes using the luciferase assay, and found that they exhibited a repressing activity, irrespective of their activity in vivo (Figure S3D

(C) Luciferase assays of FA-treated A459 cells transfected with TSLP nGRE 413 bp pGL3 vector 2, TSLP nGRE mutant1 (Figure 1E) 413 bp pGL3 vector 2, or a pGL3 SV40 enhancer vector1 (see panel [A]) containing the TSLP IR1 nGRE (sequence in panel [F]).
(D) ChIP analysis of GR and SMRT recruitment to IR1 nGRE or IR1 nGRE mutant1 of pGL3 IR1 nGRE vector 1 transfected in A549 cells treated with FA and RU.
(E) ChIP analysis of GR binding to IR0, IR1, IR2, IR3, IR4, IR5 nGRE and (+) GRE present in pGL3 vector 1, following FA and RU treatment of transfected A459 cells.
(F) Luciferase assay showing the effect of IR nGRE spacer length (as indicated) on repressing activity.
(G) Functionality of various mutant IR1 nGRE elements bearing a 1 bp change. Right panel: sequences of the canonical IR1 nGRE and its variants are listed 1 to 28. Circled numbers: intolerable mutations. Bigger letters: sequence of canonical IR nGRE; smaller letters: tolerable base changes.
Values are mean ± SEM.
See also Figure S2 and Table S5.

Table 1.

(A) Distribution of Mouse and Human IR0, IR1, and IR2 nGREs (see also Table S1)

	IR0	IR1	IR2
Mouse genes containing a putative nGRE	1105	3303	3949
Human genes containing a putative nGRE	1169	4281	5443
Mouse and human orthologue genes containing identical IR nGREs	51	379	566

(B) Repression, upon Mouse Treatment with a GC Agonist, of Genes Containing IR0, IR1, and IR2 nGREs Conserved in Their Human Orthologues

IR0 nGRE Containing Genes	Epidermis			Intestinal Epithelium			Liver			IR1 nGRE Containing Genes	Epidermis			Intestinal Epithelium			Liver			IR2 nGRE Containing Genes	Epidermis			Intestinal Epithelium			Liver		
	repressed	not repressed	expressed	repressed	not repressed	expressed	repressed	not repressed	expressed		repressed	not repressed	expressed	repressed	not repressed	expressed	repressed	not repressed	expressed		repressed	not repressed	expressed	repressed	not repressed	expressed	repressed	not repressed	expressed
ADAMTS19	●		+	●		+				ADRA2A	●		+		♦	+		♦	+	CCR10				●		+		♦	+
AIP		♦	+		♦	+		♦	+	AGPAT2		♦	+		♦	+		♦	+	COL4A3BP		♦	+		♦	+		♦	+
ANO4					♦	+	●		+	AKAP6		♦	+	●		+	●		+	DPAGT1	●		+		♦	+		♦	+
CCND1	●		+	●		+	●		+	ARHGAP23		♦	+	●		+	●		+	ELMOD3		♦	+		♦	+		♦	+
CITED1					♦	+		♦	+	ATN1		♦	+		♦	+	●		+	ERP27					♦	+		♦	+
DDI1		♦	+				●		+	BCL2L1		♦	+		♦	+	●		+	FKBP2		♦	+	●		+	●		+
DLAT		♦	+		♦	+		♦	+	BEST2		♦	+							FLOT2	●		+	●		+	●		+
FBXL18		♦	+		♦	+		♦	+	BMP3		♦	+		♦	+		♦	+	FOXJ1				●		+		♦	+
FSD2		♦	+	●		+				BTG2				●		+	●		+	FOXQ1		♦	+	●		+		♦	+
GPIHBP1		♦	+		♦	+		♦	+	B4GALT2		♦	+	●		+	●		+	FSTL1	●		+		♦	+	●		+
HCST		♦	+	●		+	●		+	CACNA2D3		♦	+							GAS6	●		+	●		+			
INHBE								♦	+	CADM4		♦	+		♦	+		♦	+	GAS2L2				●		+			
KLF5		♦	+		♦	+	●		+	CDH11	●		+		♦	+		♦	+	GGT1		♦	+	●		+		♦	+
LAG3		♦	+		♦	+	●		+	CDK9		♦	+		♦	+		♦	+	GIT2		♦	+		♦	+	●		+
MAPK4					♦	+	●		+	CTSA		♦	+		♦	+		♦	+	GKN1	●		+						
MTG1		♦	+		♦	+		♦	+	CYP26A1	●		+				●		+	GLU1		♦	+		♦	+	●		+
N MYC	●		+	●		+		♦	+	DYNLT3		♦	+		♦	+		♦	+	GOT1		♦	+		♦	+		♦	+
NDUFA4L2	●		+		♦	+	●		+	FBLN2	●		+	●		+	●		+	GPR68					♦	+	●		+
NEU1		♦	+	●	♦	+				GBA2				●		+	●		+	GPR97		♦	+	●		+		♦	+
PPIL5	●		+		♦	+				GNG10		♦	+	●		+	●		+	GRASP		♦	+	●		+		♦	+
PTMS		♦	+		♦	+		♦	+	GPR108		♦	+		♦	+		♦	+	GUK1					♦	+	●		+
SLC25A5		♦	+		♦	+	●		+	GPR153		♦	+		♦	+		♦	+	HSPB2		♦	+		♦	+		♦	+
SP9					♦	+				HSD11B2	●		+					♦	+	HSPB3					♦	+		♦	+
SPARC	●		+		♦	+	●		+	JUND		♦	+	●		+	●		+	ISL2				●		+			
TCF25		♦	+		♦	+		♦	+	KLC3		♦	+		♦	+		♦	+	ITPKA	●		+	●	♦	+	●		+
TRPM7		♦	+		♦	+	●		+	KRT14	●		+							MUC20					♦	+		♦	+
ZBED4		♦	+		♦	+		♦	+	LRRC		♦	+		♦	+		♦	+	POMGNT1		♦	+	●		+		♦	+
ZFP113		♦	+		♦	+		♦	+	MT2		♦	+	●		+		♦	+	PRKRA		♦	+		♦	+		♦	+
ZFP467		♦	+		♦	+	●		+	MTHFR		♦	+		♦	+		♦	+	RDH5				●		+	●		+
ZFP784		♦	+		♦	+		♦	+	MXI1		♦	+		♦	+		♦	+	RORA		♦	+	●		+	●		+
										NKRF		♦	+		♦	+	●		+	SLC25A34					♦	+	●		+
										PRKCB	●		+	●		+	●		+	SLC2A8		♦	+		♦	+	●		+
										RDH11				●		+	●		+	SOCS1					♦	+		♦	+
										SCP2		♦	+		♦	+		♦	+	TGM6		♦	+		♦	+			
										SGK3								♦	+	TNFRSF25					♦	+		♦	+
										SOCS3					♦	+	●		+										
										SREBF1		♦	+		♦	+		♦	+										
										SSTR4		♦	+		♦	+		♦	+										
										STAB1		♦	+		♦	+		♦	+										
										STRA13	●		+	●		+	●		+										
										TNFRSF19	●		+	●		+	●		+										
										USF1		♦	+	●		+	●		+										

RNA isolated from 18 hr- Dex-treated WT mice epidermis, intestinal epithelium, and liver was analyzed by Q-RT-PCR. Genes, for which no signal could be detected in tissue samples from vehicle treated mice after 60 cycles of amplification, were considered to be not expressed in that tissue. (+) indicates that the gene was expressed in that tissue while "repressed" denotes statistically ($p < 0.05$) significant decrease in transcript levels after Dex treatment. For individual data, see Figures 3A, 3B, 3C, (IR1 nGRE), Figures S3A, S3B, S3C (IR0 nGRE), and Figures S4A, S4B, and S4C (IR2 nGRE).

and Figure S4D). Upon Dex treatment, a decrease in gene expression in a given tissue always correlated with GR and corepressor association with the IR0 or IR2 element in that tissue (Figures S3E and S3F and Figures S4E and S4F), and in all cases the GC-induced repression was relieved by coadministration of RU. We conclude that, as expected from our in vitro luciferase assays, not only IR1, but also IR0 and IR2 nGREs can efficiently mediate the *cis*-acting GC agonist-induced repressing activity of the GR, in vivo.

IR nGREs of Mouse and Human Orthologs May Differ by a Tolerable One-Base-Pair Mutation

As Keratin 5 (K5), known to be downregulated by GCs (Ramot et al., 2009, and Figure 3H showing that Dex treatment-induced

decrease in epidermal K5 transcript levels can be prevented by RU coadministration), was not present in the list of human/mouse orthologs that contain a canonical nGRE (Table S1), we looked whether this absence could reflect the presence of a canonical IR1 nGRE in human K5, while a tolerable change would exist in its mouse ortholog, or vice-versa. We found one canonical IR1 nGRE in human K5 gene and 3 putative nGREs in its mouse ortholog, each of them exhibiting one tolerable change in vitro (Figure S5A). Only one of them (mK5 IR1 nGRE2) allowed formation of a GR-SMRT repressing complex in epidermis of Dex-treated mice (Figure 3I), suggesting that not all in vitro tolerable IR1 nGRE variants are functional in a given tissue.

Similarly, we looked at insulin (ins) and insulin receptor (insr) genes, as their expression was reported to be downregulated by GCs (Delaunay et al., 1997; see also Figure 3H, showing that these downregulations - in pancreas for insulin, and in liver for insulin receptor - can be prevented by RU cotreatment). A canonical IR1 nGRE was found in the human insulin receptor gene (hinsr IR1 nGRE), and two IR1 nGREs, each bearing one tolerable change, were present in the mouse (Figure S5B). Upon Dex treatment, functional GR-SMRT/NCoR repressing complexes were assembled on both mouse insulin receptor IR1 nGRE variants (minsr IR1 nGRE1 and 2 in Figure 3I). Remarkably, in addition to a canonical IR2 nGRE in both human and mouse insulin genes, a tolerable IR1 nGRE variant was present in mouse insulin gene, while a canonical IR1 nGRE was present in the human gene (Figure S5B). Interestingly, in vitro studies suggested 20 years ago that this latter human element could act as a negative GRE (Boam et al., 1990; Goodman et al., 1996). Upon Dex treatment, repressing complexes were bound, in mouse pancreas, to both the IR1 nGRE variants and the IR2 nGRE of the insulin gene. However, in the absence of Dex treatment, this complex (whose formation could be prevented by RU administration; data not shown) could be detected (albeit fainter) on the IR1 nGRE variant, but not on the IR2 nGRE (Figure 3I), suggesting that the affinity of the latter for liganded GR could be lower.

We also looked at Reverbα, as its downregulation by GCs was previously reported (Duez and Staels, 2008, and Refs therein). A canonical IR1 nGRE was found in mouse, but not in human Reverbα gene, which contains 3 IR1 nGREs each bearing one tolerable variant (Figure S5C). Note that, in the mouse, this gene also contains an IR1 nGRE variant (mReverbα IR1 nGRE2), that is identical to one of the human nGRE variants (hReverbα IR1 nGRE2 in Figure S5C), and appears to be as functional as the mouse canonical IR1 nGRE1, as judged from ChIP assays with liver extracts (Figure 3I).

Genes Downregulated by Glucocorticoids in Human A549 Cells Contain Functional IR nGREs

DNA microarray and ChIP scanning (Wang et al., 2004), and RNA-seq (Reddy et al., 2009) searches for GC target genes have revealed the existence of a number of genes, the expression of which is downregulated by GCs in A549 cells. In the first study, three out of 21 genes have been ChIP-scanned for GR binding. Upon Dex-treatment of A549 cells, we found that two of these human genes [BHLHB2 which is the ortholog of the STRA13 mouse gene (Table 1B), and GEM] contain IR nGREs (Table S2), on which assembly of repressing complexes could be prevented by excess RU cotreatment (Figures S6A and S6B). In Reddy et al. (2009) RNA-seq study, out of 85 GC-downregulated genes, 31 contain IR nGREs (Table S2). We analyzed 15 of them by Q-RT-PCR and ChIP assay after treatment with Dex or Dex + RU. All of them exhibited a Dex-induced downregulation, which was reversed by excess RU (Figure S6A). For all of these 15 genes, a repressing complex, of which the formation upon Dex treatment of A549 cells was inhibited by excess RU, was associated with at least one of their nGREs (Figure S6B). Interestingly, it appears that SMRT or NCoR can be selectively bound to the IR nGRE of a given gene, whereas both SMRT and NCoR are bound to the TSLP IR1 nGRE (Figure S1J). Moreover, as previously seen for TSLP (Figure 1 H and I), siRNA knockdowns of SMRT and NCoR in A549 cells demonstrate that these corepressors were instrumental in Dex-induced IR nGRE-mediated transrepression (Figures S7A and S7B). Furthermore, these knockdowns reveal that, even though binding of these corepressors exhibit gene specificity, they can be redundant for their repression function when one of them is knocked-down. Note that in several cases, binding of GR to the IR nGRE was altered when the two corepressors were knocked-down (Figure S7B).

Reddy et al. (2009) also reported a whole-genome ChIP-seq analysis of GR DBS in Dex-treated A549 cells. Among all of these GR DBS (4392), we selected those containing IR1 or IR2 nGREs (allowing for 1 tolerable change) located within known genes and their promoter regions (see Extended Experimental Procedures; these 313 genes are listed in Table S4). Five of them were randomly chosen among those the transcription of which was decreased in A549 cells by Dex treatment for 18 hr, and rescued by RU cotreatment (Figure S6C). For all 5 genes, ChIP assays with vehicle-treated A549 cells showed the presence of Pol II on the promoter proximal (PP) region, its disappearance upon Dex treatment, and reappearance upon RU addition. Conversely, GR and NCoR were bound to IR nGREs in the presence of Dex, but not in vehicle or Dex+RU-treated cells, thus demonstrating that the expression of these genes is transcriptionally repressed through GC-induced IR nGRE-mediated direct transrepression (Figure S6D).

Differential Effects of the GRdim Mutation and RU486 Treatment on GC-Induced Tethered and IR nGRE-Mediated Transrepressions

The GRdim mutation does not affect GC-induced NFκB and AP1-mediated "tethered" transrepression, whereas it impairs (+)GRE transactivation (Tuckermann et al., 1999). In contrast, we found that Dex-induced IR nGRE-mediated gene repression was abolished by this mutation (Figure 4A, and data not shown). Expression of "IR0 nGRE" CCND1, "IR1 nGRE" PRKCB and "IR2 nGRE" FSTL1 genes, was repressed by Dex treatment in WT epidermis, but not in GRdim mutant. Note that epidermis was treated as indicated with TPA, to activate NFκB and AP1 factors. As expected, the repression of the Cox2 gene that contains NFκB and AP1 binding sites, was not impaired in mutant epidermis, while the (+)GRE-containing GPX3 gene was not GC-induced (Figure 4A). Accordingly, ChIP assays showed that Dex-induced repressing complexes

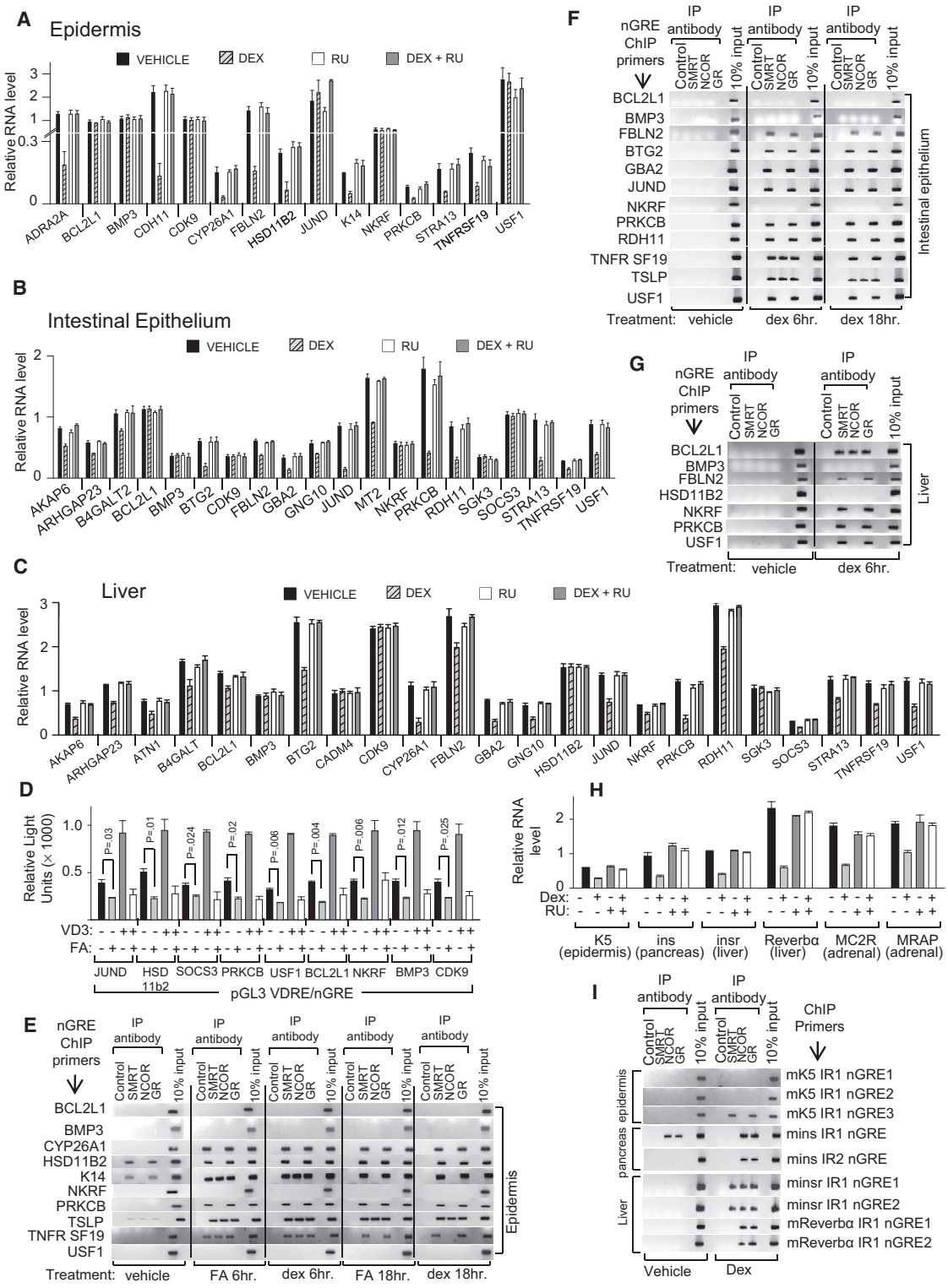

Figure 3. Glucocorticoid-Induced Repression of Mouse Genes that Contain IR1 nGREs Conserved in Mouse and Human Orthologs Is Relieved by RU486 Coadministration

(A) Q-RT-PCR for transcripts of IR1 nGRE-containing genes (Table 1B) in WT epidermis topically-treated with Dex and/or RU486 (RU) for 18 hr.

(B) As under (A), but using intestinal epithelium of intraperitoneally (IP)-injected WT mice.

(C) As under (B), but using WT liver.

were formed on IR nGREs of CCND1, PRKCB and FSTL1 genes of WT but not of GRdim mice (Figure 4B), while no activating complex was formed on the GPX3 (+)GRE gene in Dex-treated epidermis of GRdim mutants (Figure 4C), and repressing complexes were assembled, upon epidermis Dex treatment, on the Cox2 NFκB/AP1-containing region in both WT and dim mutants (Figure 4D).

Importantly, the effect of Dex treatment was reverted by excess RU cotreatment in the case of IR nGRE-mediated transrepression (Figures 4A and 4B), whereas RU had little effect on NFκB/AP1-mediated tethered transrepression (Figures 4A and 4D). Similar results concerning the differential RU effects were obtained when NFκB and AP1 factors were activated in MLE12 mouse cells by addition of IL-1β, instead of TPA treatment (Figures 4E–4G; in panels 4E and 4F, the USF1 gene was analyzed instead of the PRKCB gene in panels 4A and 4B, because the latter is not expressed in MLE12 cells). Note that no NFκB or AP1 are bound to the IR nGRE regions of CCND1, PRKCB, USF1, and FSTL1 genes (Figures 4B and 4F).

Failure of Dissociated GCs to Prevent Undesirable Side Effects of Corticoid Therapy Could Be Due to IR nGREs-Mediated Transrepression

The anti-inflammatory and immune-suppressant properties of GCs represent the central target of pharmacological GC therapy. It is thought that debilitating effects of GC treatment are due to (+)GRE-mediated gene transactivation, while GC beneficial anti-inflammatory effects have been mostly ascribed to tethered transrepression (Karin, 1998; Kassel and Herrlich, 2007). This led to a search for "dissociated" GR ligands which would preferentially induce tethered transrepression. Such a ligand, RU24858, was found to exhibit the expected dissociated profile in vitro (Vayssière et al., 1997). However, upon RU24858 administration in vivo, pathophysiological studies failed to confirm this dissociation (Belvisi et al., 2001). Our present results, led us to posit that GC-induced transcriptional repression of IR nGRE-containing genes could contribute to the GC undesirable effects.

IL-1β-"activated" A549 cells transfected with pGL4- and pGL3-based reporter plasmids were used to examine the activities exhibited in vitro by RU24858 for (1) tethered transrepression (Figure 5A; NFκBluc and AP1luc plasmids), (2) (+)GRE-mediated transactivation [Figure 2A; (+)GRE pGL3 luciferase plasmid], and (3) IR0, IR1, or IR2 nGRE-mediated transrepression (VDRE/IR0, IR1, or IR2 nGRE pGL3 luciferase plasmids, Figures 2A and 2F). As expected from its dissociated profile in vitro, RU24858 was almost as efficient as Dex at repressing IL-1β-induced activation of transcription by NFκB (Figure 5B, left panel) and AP1 (Figure 5B, right panel). Accordingly, RU24858 was as efficient as Dex at recruiting a repressing complex tethered to NFκB bound to its cognate element in the NFκBluc plasmid (Figure 5C). Most interestingly, RU24858 was also as efficient as Dex at inducing repression mediated by IR0, IR1 and IR2 nGREs in VDRE/IR0, IR1 and IR2 nGRE pGL3 luciferase plasmids (Figure 5D), as well as at recruiting GR and SMRT to form repressing complexes on nGRE-containing regions (Figure 5E). In contrast, RU24858 was much less efficient than Dex at inducing transactivation of (+)GRE pGL3 luciferase plasmid (Figure 5D).

We next investigated the "activity profile" of RU24858 in vivo. Unlike Dex, a topical RU24858 treatment did not activate GC-dependent expression of the (+)GRE-containing GPX3 gene in epidermis, nor of the GGT1 and ERP27 genes in liver (Figure 5F), and did not induce assembly of an activating complex on their (+)GRE (ChIP assays in Figure 5H, and data not shown). In contrast, RU24858 was as efficient as Dex at downregulating, through NFκB-mediated tethered transrepression, genes of which the skin expression was enhanced by topical TPA treatment (Figure 5G). Most interestingly, and as in vitro, RU24858 was also as efficient as Dex at inducing transrepression of IR0 (CCND1), IR1 (TSLP, CYP26A1, K14, PRKCB) and IR2 (DPAGT1) nGRE-containing genes, through recruitment of GR-SMRT/NCoR repressing complexes on nGRE regions (Figures 5F and 5H).

IR nGRE-Containing Genes Exert Physiological Homeostatic Functions Related to Debilitating Effects of Glucocorticoid Therapy

Long-term treatments with GCs generate numerous debilitating effects (Table 2A, and references therein). An ontology search revealed that the known or putative functions of almost 15% of IR nGRE-containing ortholog genes could possibly be implicated in physiological homeostatic processes leading to side-effects upon GC therapy (Table S3). Moreover, there is evidence that repression of expression of a number of these latter genes could actually be instrumental to the generation of defects subsequent to GC administration, either because their expression is known to be decreased upon GC treatment, and/or because their decreased expression is known to generate defects related to those produced by GC-therapy (Table 2A; see also references

(D) Luciferase assays on A549 cells transfected with pGL3 vector 1 derivative (Figure 2A) containing IR1 nGREs from genes (Table 1B) as indicated, were treated for 6 hr with vehicle, VD3 and FA, as indicated.
(E) ChIP analysis of FA- and Dex- induced binding of GR and corepressors to IR1 nGRE regions of genes analyzed in panel (A).
(F) ChIP analysis of Dex- induced binding to the IR1 nGRE regions of genes analyzed in panel (B).
(G) ChIP analysis of Dex- induced bindings to the IR1 nGRE regions of genes analyzed in panel (C).
(H) Q-RT-PCR of gene transcripts, as indicated (see also Table 2A and Table S3), in WT mice topically-treated (epidermis) or IP-injected (for other tissues) with Dex and/or RU, for 18 hr.
(I) ChIP analysis of epidermis, pancreas and liver showing binding of GR and corepressors to the IR nGRE regions of indicated genes. WT mice were topically-treated with vehicle or Dex (in the case of K5) or IP-injected with vehicle or Dex (in the case of ins, insr and Reverbα) for 18 hr.
Values are mean ± SEM.
See also Figure S3, Figure S4, Figure S5, Figure S6, Table S2, Table S4, and Table S5.

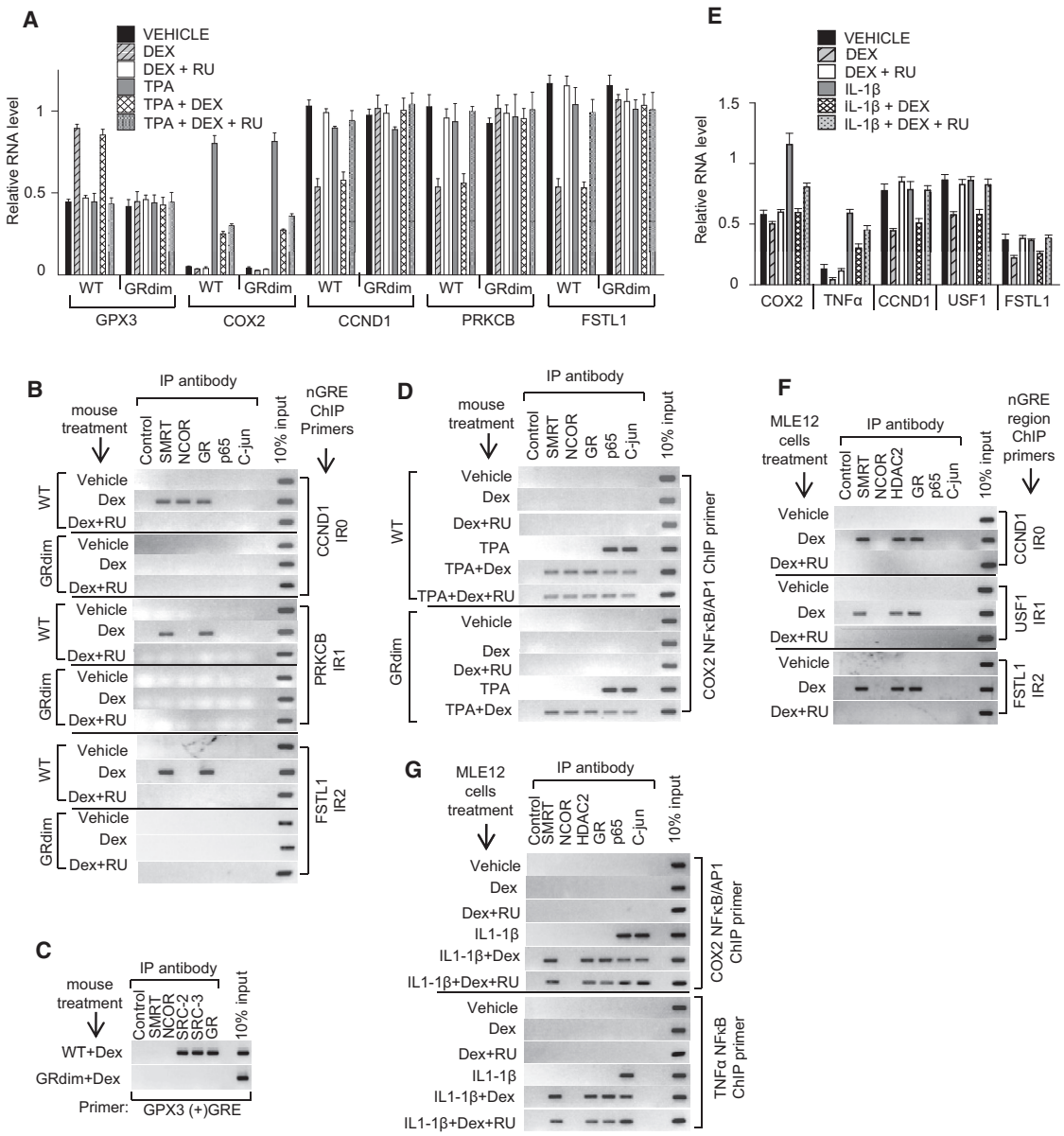

Figure 4. Differential Effects of GRdim Mutation and RU486 Treatment on Tethered and IR nGRE-Mediated Transrepression

(A) Q-RT-PCR of gene transcripts in WT and GRdim mutant mice treated as indicated for 6 hr.

(B) ChIP analysis of epidermis from WT and GRdim mice, treated for 6 hr as indicated, showing GR and corepressor recruitment to IR0, IR1, and IR2 nGREs of CCND1, PRKCB and FSTL1 genes.

(C) ChIP analysis of GPX3 (+)GRE in epidermis from WT and GRdim mouse, treated for 6 hr as indicated.

(D) ChIP analysis of epidermis from WT and GRdim mouse, treated for 6 hr as indicated, showing binding to the COX2 NFκB and AP1 regions.

(E) Q-RT-PCR of gene transcripts in MLE12 cells treated for 6 hr as indicated.

(F) ChIP analysis of MLE12 cells, treated as indicated for 2 hr, showing binding to the IR nGRE regions of CCND1, USF1 and FSTL1 genes.

(G) ChIP analysis of MLE12 cells, treated as indicated for 2 hr, showing binding to COX2 NFκB and AP1 regions, and TNFα NFκB region.

Values are mean ± SEM.

See also Table S5.

in Table S3). Note, however, that many of the NFκB and AP1 binding site-containing genes that encode regulatory components (e.g., cytokines) of the immune system, also contain IR nGREs (Table 2B and Table S3), while genes encoding anti-apoptotic proteins (Bcl2 and Bcl-XL), as well as mitogenic proteins involved in cell cycle progression at the G1/S phase (Cyclin D1 and CDK4) can also be GC-transrepressed via IR nGREs (Table 2 and Table S3).

DISCUSSION

GC-Induced Direct Transrepression Is Mediated by a Family of IR nGREs Present in Numerous Genes

We have discovered a widespread conserved family of "negative" palindromic GC-response elements (IR nGREs) that mediate transrepression by direct binding of GC-agonist-liganded GR which assembles a repressing complex through association of SMRT/NCoR corepressors and HDACs. GC-induced IR nGRE-mediated direct transrepression is distinct from GC-induced tethered indirect transrepression, as: (1) tethering GREs do not contain DNA binding sites for GR per se, but instead binding sites for other DNA-bound transregulators (e.g., NFκB and AP1) that recruit GR, (2) the GRdim mutation that does not affect tethered transrepression, abolishes IRnGRE-mediated direct transrepression, (3) cotreatment with RU486 relieves GC-induced IRnGRE-mediated direct transrepression, whereas tethered transrepression is not or only slightly affected, (4) IR nGREs appear to act as silencer elements, on which the GC-induced assembly of a repressing complex precludes the interaction between an enhancer and the proximal promoter region.

Investigating in vitro whether the integrity of the TSLP nGRE in which a 1 bp spacer separates the Inverted Repeated motifs (IR1 nGRE) is essential for its function, has shown that no (IR0 nGRE) or a 2 bp (IR2 nGRE) spacer is "tolerable." Moreover, one base changes at any position of the "canonical" repeated motifs of the TSLP IR1 nGRE and of its IR2 nGRE derivative is, with one exception, functionally tolerable in vitro. Mouse and human genome−wide analyses revealed the presence of hundreds of mouse and human ortholog genes containing conserved canonical IR0, IR1 and IR2 nGREs (Tables 1A and 1B). In no case are these nGREs located in the near vicinity (<100 bp) of binding sites for regulatory factors (i.e., there is no evidence that IR nGREs are composite sites), and most of them are conserved throughout vertebrates (mammals, chicken and zebra fish, our unpublished data). A number of "IR0," "IR1," and "IR2" nGRE-containing genes expressed in mouse epidermis, intestinal epithelial cells or liver, were analyzed for (1) repression by Dex-treatment, (2) prevention of this repression by RU486 cotreatment, (3) association of their IR nGREs with liganded-GR and corepressors, and (4) repressing activity of their nGREs in vitro, which taken all together represent the signature of IR nGRE-mediated transrepression. This analysis demonstrates that IR0, IR1, and IR2 nGRE-containing genes can be efficiently transrepressed by agonist-liganded GRs bound to their nGREs together with corepressors. Moreover, not only the tissue-specific expression of these genes is epigenetically controlled but, when expressed, their GC-induced transrepression is also epigenetically controlled (Table 1B and Figure 3). Assuming that the mouse/human genes that we have randomly selected are representative, our data (Tables 1A and 1B) indicate that the expression of approximately 600 mouse/human ortholog genes could be negatively controlled through nGRE-mediated transrepression in epidermis, intestinal epithelium and liver. Therefore, provided the remaining 400 mouse/human ortholog genes that contain canonical IR nGREs are expressed in other tissues, it is likely that the expression of all (∼1000) of the ortholog genes listed in Table S1 are negatively controlled in the mouse by GC-induced IR nGRE-mediated transrepression. Note, in this respect, that most of the IR nGRE-containing genes present among the GC downregulated genes characterized in the Reddy et al. (2009) RNA seq study of human A549 cells treated with Dex (Table S2), are different from those identified by us in epidermis, intestinal epithelial cells and liver (Table 1B). Similarly, only 7 out of the 313 human IR1 and IR2 nGRE-containing genes identified by ChIP-seq analysis of GR DBS in Dex-treated A549 cells (Reddy et al., 2009)(see Table S4) are present among the ∼1000 human/mouse ortholog genes listed in Table S1. In fact, the actual number of genes whose expression could be negatively controlled by GC-induced IR nGRE-mediated transrepression might be much higher, as the existence of functionally tolerable single base changes in IR nGREs of human and/or mouse ortholog genes could result in underestimating by several hundreds the actual number of genes containing functional IR nGREs. In this respect, we note that most of the IR nGREs present among the GC downregulated genes characterized by Reddy et al. (2009) exhibit canonical repeated motifs, whereas the nGREs of their mouse orthologs bear tolerable single base changes, and therefore are not present in Table S1 (data not shown).

Structural studies are obviously required to unveil the detailed mechanism that underlies GC agonist-induced IRnGRE-mediated direct transrepression. The role played by an agonistic GC in this transrepression cannot be simply to ensure the translocation of the GR into the nucleus, as the GC antagonist RU486, known to promote such a translocation, acts as an antagonist of IR nGRE-mediated transrepression. Moreover, ChIP assays in vivo (Figure 1 and Figure S1) and in vitro (Figure 2) show that binding of a GR corepressor complex to IR nGREs requires the presence of a glucocorticoid agonist which "normally" is known to induce a GR conformational change that allows the formation of a GR coactivator complex that binds to a (+)GRE (Figure S1F). Whether the IR nGRE DNA binding site may possibly act as a conformational "allosteric" effector of GR, enabling it to bind corepressors in the presence of a GC-agonist, and to which extent binding of the latters may strengthen the binding of the GR to IR nGREs (see Figure 1I and Figure S7B), remains to be seen.

Physiological and Pathophysiological Importance of GC-Inducible Direct Transrepression by IR nGRE-Containing Genes

GCs that act as end-effectors of the HPA (hypothalamus-pituitary-adrenal) axis, are secreted by adrenal glands in a circadian and stress-related manner. They influence the functions of virtually all organs and tissues throughout life span, and are essential for maintenance of their homeostasis and important biological activities, such as intermediary metabolism, immune and inflammatory reactions, as well as circadian clock and stress systems (Chrousos, 2009; Nader et al., 2010). Our ontology search (Table S3) has revealed that a number of IR nGRE-containing genes are involved in such functions. We focus here on a few examples illustrated in the present study.

Our data (Figures 3H and 3I, and Table 2A) unequivocally demonstrate that GC-induced IR nGRE-mediated repression of

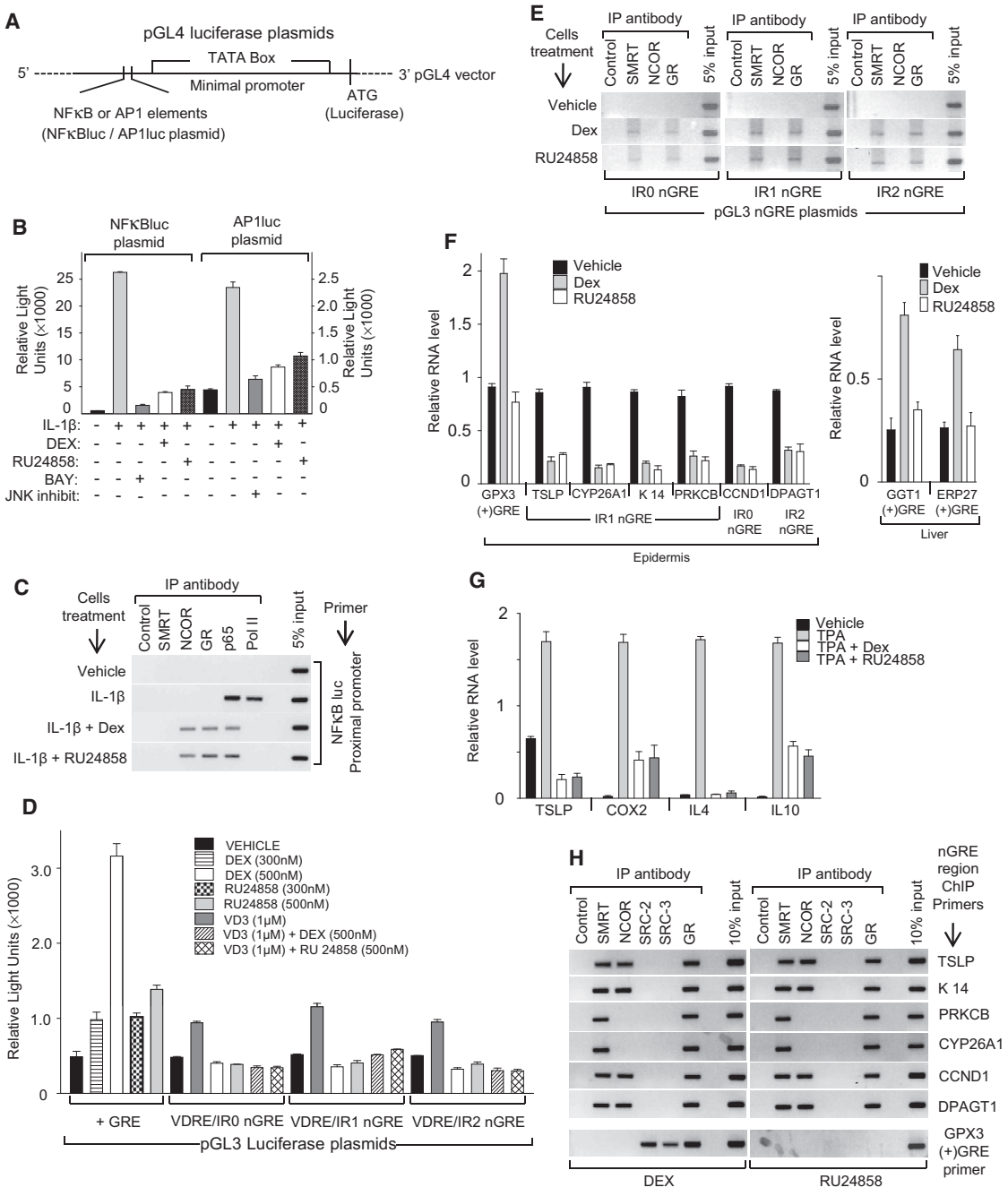

Figure 5. The Dissociated Glucocorticoid RU24858 Induces Repression of IR nGRE-Containing Genes

(A) NFκBluc and AP1luc luciferase reporter plasmids.

(B) Dex and RU24858 similarly repress NFκB and AP1-driven transcription in vitro. NFκBluc and AP1luc transfected A549 cells were treated as indicated for 6 hr, followed by luciferase assay. BAY, NFκB-specific inhibitor BAY 11-7082; "JNK inhibit," JNK inhibitor II.

(C) ChIP analysis showing, upon IL-1β addition, binding of NFκB p65 and Pol II to the proximal promoter region of NFκBluc transfected in A549 cells. Addition of Dex or RU24858 resulted in similar GR tethered complexes, while Pol II was released.

(D) A549 cells transfected with various pGL3 vector-1 (Figure 2A)-based plasmids as indicated, were treated as indicated for 6 hr, followed by luciferase assay.

(E) ChIP analysis showing binding of SMRT and GR to IR nGREs of pGL3 VDRE/IR0/IR1/IR2 nGRE vector1 (see Figures 2A and 2E) transfected in A549 cells treated with Dex and RU24858 for 6 hr.

(F) Q-RT-PCR of gene transcripts from WT dorsal epidermis topically-treated with Dex or RU24858 for 18 hr (left panel) and from liver of WT mice IP-injected with Dex or RU24858 for 18 hr (right panel).

(G) Q-RT-PCR of gene transcripts showing that Dex and RU24858 similarly downregulate expression of cytokines in vivo, in dorsal skin of WT mice topically treated for 18 hr with TPA and either Dex or RU24858, as indicated.

both the insulin precursor gene (in pancreas β cells) and insulin receptor gene (in liver) are early stress-induced events. This ensures that, upon occurrence of a stress, an elevation of blood glucose level will rapidly follow the surge of GC secretion, thereby providing the increased nutrition of brain, heart and skeletal muscles, required for the central coordination of stress response (Chrousos, 2009). On the other hand, under conditions of chronic stress, GC-induced IR nGRE-mediated repression of the insulin receptor gene may cause insulin resistance and lead to diabetes. Our study also reveals the existence of functional IR1 nGREs in ACTH receptor (melacortin 2 receptor, MC2R) gene, and ACTH receptor accessory protein (MRAP) gene (Figure 3H, Table 2A, and Table S3), thereby adding, at the adrenal level, another step to the closed negative feedback loop that resets the HPA axis by regulating the synthesis of secreted GCs through GR-mediated repression of CRH and POMC gene expression in hypothalamus and pituitary, respectively (Dostert and Heinzel, 2004). We also found GC-induced IR nGRE-mediated repression for the Reverbα gene (Figures 3H and 3I and Table 2A) and the RORα gene (Table 1B and Figure S4), which both intervene in the control of the circadian timing system, and are likely to play an important role in communications of the molecular Clock and stress systems with intermediary metabolism, which are fundamental for survival (Duez and Staels, 2008; Nader et al., 2010; and references therein).

Importantly, it is known from previous studies that the expression of a number of genes is decreased upon GC therapy (Table 2A and references therein), but the underlying pathophysiological molecular mechanisms were often unknown. Our data demonstrate that, upon GC therapy, decrease of keratins 5 and 14 in skin, as well as those in Cyclin D1 and CDK4, are due to GC-induced IR nGRE-mediated repression (Table 2A and Figure 3). Hsd11b2 is another important gene that is repressed by IR1 nGRE-mediated GC-treatment in both skin and colon tissues (Figure 3A, and data not shown). The 11β-HSD2 enzyme (encoded in Hsd11b2 gene) is responsible for inactivating glucocorticoids in mineralocorticoid receptor (MR) target tissues (Gross and Cidlowski, 2008). In the absence of this enzyme, GCs (corticosterone in mice) activate MR despite the absence of aldosterone, resulting in hypertension (Stewart et al., 1996). That Hsd11b2 is an IR1 nGRE-containing gene provides a possible molecular explanation for GC therapy-induced hypertension.

Toward Improved Anti-Inflammatory Dissociated Glucocorticoid Agonists

Our study indicates that previous attempts, aimed at identifying GC analogs exhibiting a dissociated profile likely failed because such GCs had kept their IR nGRE-mediated repression activity. Thus, improved screenings for anti-inflammatory dissociated GCs should look for compounds that would repress gene expression through tethered transrepression, while lacking IR nGRE-mediated transrepression and (+)GRE-mediated transactivation activities. Our luciferase reporter plasmids could be useful for such screenings, as well as for characterizing the mode of action of some non-GC-derived compounds that may exhibit some of the beneficial therapeutic activities of dissociated GCs, but be devoid of their detrimental effects (De Bosscher and Haegeman, 2009).

Conclusion

That a single hormone (cortisol in human and corticosterone in rodents) bound to a single nuclear receptor, the glucocorticoid receptor, can finely and coordinately tune transcription of thousands of genes involved in vital functions, in essentially all cells throughout the life span of vertebrates, remains an amazing conundrum to be solved at the molecular level. We demonstrate here the existence of a novel mechanism of control of gene expression by GCs, namely GC-induced transrepression through direct binding of agonist-liganded GR associated with SMRT/NCoR corepressors to an evolutionary conserved family of "simple" negative DNA binding sites (IR nGREs), unrelated to the "simple" (+)GRE binding site family. Such a mechanism which, to our best knowledge, has no precedent in the nuclear receptor field, introduces a new paradigm for GR action through which variations in levels of a single ligand can concomitantly differentially turn on or off two sets of genes widely differing in their response element DNA sequences [(+)GRE and IR nGRE, respectively]. This possibility remarkably suits GR signaling, as GC adrenal secretion varies in a circadian and stress-related fashion. Indeed, increases in GC levels will concomitantly turn on the expression of (+)GRE-containing genes, and turn off the expression of IR nGRE-containing genes, whereas decreases in GC levels will have the opposite effects on the two sets of genes, thus enabling them to synergistically contribute to the control of given physiological events (e.g., stress-induced hyperglycemia). Whether the different cell-specific GR isoforms (Gross and Cidlowski, 2008) generate additional specificity in these controls remains to be seen, as well as the possible existence of similar mechanisms of control of gene expression by other members of the NR superfamily.

EXPERIMENTAL PROCEDURES

Additional details on methods are available online in Extended Experimental Procedures.

Mice

For topical treatment, 1 nmole (nm)/cm^2 MC903, at-RA or TPA; 6 nm/cm^2 FA, Dex or RU24858; and 90 nm/cm^2 RU486 were used. For systemic use, 100 ng/kg body weight active Vit D3, 8 mg/kg Dex and 64 mg/kg RU486 was intraperitoneally injected. GRdim mice were from the European Mouse Mutant Archives (EM:02123). Breeding, maintenance and experimental manipulation of mice were approved by the Animal Care and Use Committee of the IGBMC.

ChIP Assay

Isolated epidermis and intestinal epithelial cells were crosslinked in 1% formaldehyde followed by ChIP assay, as reported (Vaisanen et al., 2005).

(H) ChIP analysis of epidermis from WT mice topically-treated with Dex and RU24858 for 18 hr, showing binding to IR nGRE or (+) GRE regions of genes as indicated.

Values are given as the mean ± SEM.

Table 2. Side Effects Generated by GC Therapy Are Related to Those Produced by GC-Induced Transrepression of IR nGRE-Containing Genes

(A) IR nGRE-Containing Genes Whose GC-Induced Transrepression Could Generate Side Effects Related to Those Produced by GC Therapy (see also Table S3)

Debilitating Side Effects upon GC Therapy	Gene Symbol	Gene Name	a	b	References
Skin atrophy, bruising, thinning, brittle skin, and disturbed wound healing (Schacke et al., 2002)	*Krt 14, Krt 5*	Keratin 14 (IR1), Keratin 5 (IR1)	+	–	Ramot et al., 2009, This study (see Figures 3A and 3H)
	TGFβ1	Transforming growth factor beta 1 precursor (IR1)	+	–	Frank et al., 1996
	Smad4	SMAD family member 4 (IR2)	–	+	Chen et al., 2000
	Tnc	Tenascin C (IR2)	+	–	Fassler et al., 1996
	Trpv3	Transient receptor potential cation channel subfamily V member 3 (IR2)	–	+	Cheng et al., 2010
	Ccnd1	Cyclin D1 (IR0)	+	+	This study (see Fig. S3A)
	Cdk4	Cyclin-dependent kinase 4 (IR2)	+	+	Rogatsky et al., 1999
Impaired skeletal growth and osteoporosis (Schacke et al., 2002, Kleiman and Tuckermann, 2007)	*Tnfrsf11b*	Osteoprotegerin (IR2)	+	+	Sasaki et al., 2001
	Bcl2	Bcl- 2 (IR1)	+	–	Mocetti et al., 2001
	Bcl2l1	Bcl- XL (IR1)	+	–	Lu et al., 2007
	TGFβ1	Transforming growth factor beta 1 precursor (IR1)	–	+	Geiser et al., 1998
	Smad 4	SMAD family member 4 (IR2)	–	+	Tan et al., 2007
	Ghr	Growth hormone receptor (IR1)	+	+	Gevers et al., 2002
	Gnas	Adenylate cyclase stimulating G-alpha protein (IR1)	–	+	Weinstein et al., 2004
	Wnt5a	Wingless-related MMTV integration site 5A (IR1)	–	+	Yang et al., 2003
	Ahsg	Alpha -2- HS- glycoprotein precursor (IR0)	–	+	Szweras et al., 2002
	Col11a2	Collagen, type XI, alpha 2 chain precursor (IR2)	–	+	Li et al., 2001
Hyperglycemia and diabetes (Schacke et al., 2002, Kleiman and Tuckermann, 2007)	*Ins*	Insulin precursor (IR1, IR2)	+	+	Delaunay et al., 1997, This study (see Figure 3H)
	Insr	Insulin receptor (IR1)	+	+	Caro and Amatruda., 1982, This study (see Fig. 3H)
Muscle atrophy/myopathy (Schakman et al., 2008a)	*ctnnb1*	Beta –catenin (IR1)	+	+	Schakman et al., 2008b
	Akt1	Protein kinase B (IR2)	–	+	Schakman et al., 2008a
	Tpm2	Tropomysin beta chain (IR1)	–	+	Ochala et al., 2007
Impaired HPA axis and adrenal insufficiency (Schacke et al., 2002)	*Mc2r*	ACTH receptor (IR1)	+	+	Chida et al., 2007, This study (see Figure 3H)
	Mrap	ACTH receptor accessory protein (IR1)	+	+	Metherell et al., 2005, This study (see Figure 3H)
Circadian rhythm disorder, metabolic syndrome, bipolar disorder, and mania (Bechtold et al., 2010, Duez and Staels, 2008)	*Clock*	Circadian locomoter output cycle kaput protein (IR1)	–	+	Roybal et al., 2007
	Nr1d1	Reverbα (IR1)	+	–	Torra et al., 2000; Preitner et al., 2002; This study (see Figure 3H)
	Rora	RORα (IR2)	+	–	This study (see Figures S4B and S4C)
Anxiety and depression (Schacke et al., 2002)	*Ucn2*	Urocortin 2 (IR1)	+	+	Chen et al., 2004, 2006; Neufeld-Cohen et al., 2010
	Crhr2	Corticotropin releasing hormone receptor 2 (IR1)	–	+	Bale et al., 2000
Hypertension (Schacke et al., 2002)	*Hsd11b2*	Corticosteroid 11-beta-dehydrogenase isozyme 2 (11β-HSD2) (IR1)	+	+	Stewart et al., 1996, This study (see Figure 3A)

Table 2. *Continued*

(B) IR nGRE- Containing Genes Involved in GC-Anti-Inflammatory Therapy (see also Table S3)

Gene Symbol	Gene Name*	Gene Symbol	Gene Name*
C1qb	Complement C1q subcomponent subunit B Precursor (IR2)	*Il16*	Interleukin-16 Precursor (IR1)
C1ql1	C1q-related factor Precursor (IR1)	*Il17rb*	Interleukin-17 receptor b (IR1)
C3	Complement C3 Precursor (IR1)	*Il11ra1*	Interleukin-11 receptor (IR1)
Cfd	Complement factor D Precursor (IR1)	*Il17b*	Interleukin-17b Precursor (IR1)
Il6	Interleukin-6 Precursor (IR1)	*Il17f*	Interleukin-17f Precursor (IR1)
Il20	Interleukin-20 Precursor (IR2)	*Il28a*	Interleukin-28a Precursor (IR1)
Ccr10	C-C chemokine receptor type 10 (IR2)	*Il28b*	Interleukin-28b Precursor (IR1)
Stat3	Signal transducer and activator of transcription 3 (IR2)	*Il24*	Interleukin-24 Precursor (IR1)
Nfatc1	Nuclear factor of activated T-cells, cytoplasmic 1 (IR1)	*Il34*	Interleukin-34 Precursor (IR1)
Il8ra	Interleukin-8 receptor a (IR1)	*Il1rn*	Interleukin-1 receptor antagonist protein Precursor (IR2)
Il12rb1	Interleukin-12 receptor b1 (IR1)	*Il22ra1*	Interleukin-22 receptor a1 (IR1)
Il17ra	Interleukin-17 receptor a (IR1)	*TSLP*	Thymic Stromal Lymphopoietin (IR1)

[a] Genes whose expression is known to be decreased upon GC treatment. [b]Genes, whose decreased expression is known to generate effects related to those produced by GC treatment. (IR0), (IR1), and (IR2) indicate the type of nGRE motif present in that gene. Asterisk denotes that all of these genes also contain NFκB and AP1 binding sites.

Nuclear Run-on, EMSA, 3C and Luciferase Assays

Nuclear Run-on and EMSA (Carey and Smale, 2001), 3C (Liu and Garrard, 2005) and Luciferase assays (Promega kit) were as described.

Real-Time PCR

Total RNA was reverse transcribed using hexamers, followed by Q-PCR, as reported (Li et al., 2005).

siRNA Treatment

ON-TARGETplus SMARTpool siRNAs against SMRT (L-020145-01-0050) and NCOR (L-003518-00-0050, Dharmacon) were transfected into A549 cells using Lipofectamine 2000 (Invitrogen), according to manufacturer's instruction.

Statistics

Data are represented as mean ± SEM of at least three independent experiments, and were analyzed using sigmastat (Systat Software) by the Student t test. $p < 0.05$ was considered significant.

Bioinformatics Analysis

hg19 (human) and mm9 (mouse) repeat masked genome assembly was used to identify genome wide distribution of IR nGRE motifs. Gene functional annotation was performed using DAVID program. Details in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and six tables and can be found with this article online at doi:10.1016/j.cell.2011.03.027.

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