Glucocorticoid-induced tethered transrepression requires SUMOylation of GR and formation of a SUMO-SMRT/NCoR1-HDAC3 repressing complex

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Upon binding of a glucocorticoid (GC), the GC receptor (GR) can exert one of three transcriptional regulatory functions. We recently reported that SUMOylation of the GR at position K293 in humans (K310 in mice) within the N-terminal domain is indispensable for GC-induced evolutionary conserved inverted repeated negative GC response element (IR nGRE)-mediated direct transrepression. We now demonstrate that the integrity of this GR SUMOylation site is mandatory for the formation of a GR-small ubiquitin-related modifiers (SUMOs)-SMRT/NCoR1-HDAC3 repressing complex, which is indispensable for NF- κ B/AP1-mediated GC-induced tethered indirect transrepression in vitro. Using GR K310R mutant mice or mice containing the N-terminal truncated GR isoform GR α -D3 lacking the K310 SUMOylation site, revealed a more severe skin inflammation than in WT mice. Importantly, cotreatment with dexamethasone (Dex) could not efficiently suppress a 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin inflammation in these mutant mice, whereas it was clearly decreased in WT mice. In addition, in mice selectively ablated in skin keratinocytes for either nuclear receptor corepressor 1 (NCoR1)/silencing mediator for retinoid or thyroid-hormone receptors (SMRT) corepressors or histone deacetylase 3 (HDAC3), Dex-induced tethered transrepression and the formation of a repressing complex on DNA-bound NF- κ B/AP1 were impaired. We previously suggested that GR ligands that would lack both (+)GRE-mediated transactivation and IR nGRE-mediated direct transrepression activities of GCs may preferentially exert the therapeutically beneficial GC anti-inflammatory properties. Interestingly, we now identified a non-steroidal antiinflammatory selective GR agonist (SEGRA) that selectively lacks both Dex-induced (+)GRE-mediated transactivation and IR nGRE-mediated direct transrepression functions, while still exerting a tethered indirect transrepression activity and could therefore be clinically lesser debilitating on long-term GC therapy.

glucocorticoid receptor | SUMOylation |
 NF- κ B/AP1-mediated GC-induced tethered repression

Glucocorticoids (GC) are widely used in clinical treatments to suppress inflammatory and allergic disorders. However, various associated undesirable side effects limit their therapeutic usefulness (1). Upon GC binding, the GC receptor (GR) regulates the expression of target genes either by transcriptional activation through direct binding to (+)GRE DNA binding sites (DBS) (2), direct transrepression through binding to evolutionary conserved inverted repeated negative response element (IR nGRE DBSs) (3), or tethered indirect transrepression mediated through interaction with transactivators such as NF- κ B/activator protein 1 (AP1)/STAT3 bound to their cognate DBSs (4, 5). The beneficial antiinflammatory effects are generally ascribed to tethered transrepression, whereas many of the undesirable side effects appear to be related to transactivation (1) and direct transrepression (3). Tethered transrepression of NF- κ B and AP1 by the GR has been proposed to result from alteration in the assembly of coactivator (6–8) or from interference with serine-2 phosphorylation at the C-terminal domain of RNA polymerase II (9, 10). We recently

dissected the molecular mechanisms involved in GR-mediated GC-induced IR nGRE-mediated direct transrepression, which revealed that GR SUMOylation in its N-terminal domain (NTD) at K293 (K310 in mice), as well as the subsequent formation of a small ubiquitin-related modifier (SUMO)-associated SMRT/NCoR1-HDAC3 repressing complex, are instrumental in direct transrepression (11). This led us to investigate whether the same GR SUMOylation could also be implicated in GC-induced tethered transrepression. We report here the molecular mechanism underlying GC-induced tethered indirect transrepression and demonstrate the important role played by SUMOylation of the GR in this transrepression. We also report preliminary data illustrating how the elucidation of the molecular mechanisms underlying the three main functions of the GR could be used in searches for selective GR agonists (SEGRAs), which would selectively exert the beneficial antiinflammatory activities of glucocorticoids while being devoid of their (+)GRE and IR nGRE-mediated debilitating activities.

Results

SUMOylation of the GR Is Not Required for Binding to DNA-Bound NF- κ B, AP1, and STAT3, but Is Mandatory for Tethered Transrepression and Is Involved in GC Antiinflammatory Effects. We previously demonstrated that the SUMOylation site located at K293 in the GR NTD is mandatory for dexamethasone (Dex)-induced IR nGRE-mediated direct transrepression (11). The report of Gross et al. (12), showing that the GR α -D3 isoform (Fig. 1A), which

Significance

The antiinflammatory property of natural glucocorticoids (GCs) was demonstrated more than 60 years ago. Since then, synthetic GCs have been widely used to combat inflammatory and allergic disorders. However, multiple severe undesirable side effects associated with long-term GC treatments, as well as induction of glucocorticoid resistance associated with such treatments, limit their therapeutic usefulness. In the present study, we unveiled the molecular mechanism underlying the GC-induced GC receptor (GR)-mediated tethered indirect transrepression. This knowledge paves the way to the future educated design and screening of drugs, collectively named selective GR agonists, which would exhibit the major therapeutically beneficial properties of GCs, but would be devoid of undesirable debilitating effects upon prolonged GC therapy.

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See Commentary on page 1115.

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Fig. 1. SUMOylation of the GR is mandatory for tethered transrepression and involved in GC antiinflammatory effects. (A) Schematic presentation for GR FL, GR K293R, GR 336, and GR ABCD. (B) Quantitative qRT-PCR for transcripts of endogenous NF-κB/AP1 DBS-containing genes in GRwt and GRα-D3 MEFs, treated with vehicle, IL1β (5 ng/mL), and Dex (0.5 μM) for 6 h. (C) ChIP assays performed with GRwt and GRα-D3 MEFs treated with vehicle, IL1β (5 ng/mL), and Dex (0.5 μM) for 1 h. qPCR analyses were performed on NF-κB/AP1 DBS regions, as indicated. (D) As in B, but using ear extracts from GRwt or GRα-D3 mice topically treated with vehicle, TPA (1 nmol/cm²), and Dex (6 nmol/cm²) for 3 d. (E) As in D, but for GRwt or GR K310R mice topically treated with TPA and Dex for 5 d. (F) As in C, but using GRwt and GR K310R mouse dorsal epidermis treated as in D for 6 h. Values are mean ± SEM. *P < 0.05.

does not contain this NTD SUMOylation site, could not inhibit the activity of an NF-κB-responsive reporter gene, prompted us to investigate whether the K293 GR SUMOylation site could also be involved in GR tethered binding and transrepression. Upon transfection of Cos-1 cells with GR 336 (GRα-D3) or GR K293R mutants, there was no or little Dex-induced tethered repression of Cos-1 cell endogenous genes *TNFα* (NF-κB/AP1 DBS), matrix metalloproteinase 13 (*MMP13*) (AP1 DBS), and suppressor of cytokine signaling 3 (*Socs3*) [STAT3-binding sites (SBEs)] (Fig. S1 A and B; note that the IR nGRE present in *Socs3* is not functional in Cos-1 cells; see Fig. S1 I and J). Unexpectedly, ChIP assays following the expression of GR, GR K293R, and GR 336 (GRα-D3) into Cos-1 cells revealed that all three were associated with p65 (NF-κB) and c-jun (AP1) bound to cognate NF-κB and AP1 DBSs present in *TNFα*, *MMP13*, and thymic stromal lymphopoietin (*TSLP*) genes (Fig. S1C). However,

an association of silencing mediator for retinoid or thyroid-hormone receptors (SMRT) and/or nuclear receptor corepressor 1 (NCoR1) within repressing complexes could be readily detected with GR but not with GR K293R and GR 336 (Fig. S1C), and a concomitant association of SUMO1, SUMO2/3, histone deacetylase 3 (HDAC3), and HDAC2 with these complexes was also observed with GR but not with GR 336 or GR K293R (Fig. S1 C and D). Most notably, a competition between SUMOylated GR (associated with NCoR1, SMRT, and SUMOs) and the non-SUMOylated GR mutant K293R showed that the affinity of SUMOylated GR for NF-κB (p65) and AP1 (c-jun) was higher than that of the non-SUMOylated GR K293R, as a 10 times excess of non-SUMOylated GR K293R was required to fully prevent p65 and c-jun binding to SUMOylated GR WT associated with corepressors and SUMOs (Fig. S1G). Similarly, SUMOylation of GR was not required for tethered binding to

STAT3 bound to the SBE of the endogenous Cos-1 cell *SOCS3* gene (Fig. S1F), but was mandatory for its tethered repression (Fig. S1B). In contrast, as expected, the direct binding of GR K293R or GR 336 to the *TSLP* IR1 nGRE was severely decreased, while SMRT and/or NCoR1 and SUMO1 were associated with GR but not with GR K293R or GR 336 (Fig. S1E). Interestingly, phosphorylation of the GR NTD may stimulate, under limiting Dex concentration (10 nM) (11), the SUMOylation of NF- κ B-bound GR, and the binding of SMRT and NCoR1 corepressors, as suggested by their decrease relative to GR upon mutation of NTD phosphorylation sites (GR 55A; Fig. S1H).

To validate the above data in vivo, studies were performed with *GR α -D3* and *GR K310R* SUMOylation mutant mice (11). In *GR α -D3* mouse embryonic fibroblasts (MEFs), there was no or little Dex-induced repression of IL1 β -induced NF- κ B- and/or AP1-mediated expression of proinflammatory genes (Fig. 1B). Moreover, ChIP assays performed on *TSLP* NF- κ B, *IL6* NF- κ B, and *MMP13* AP1 elements revealed that on IL1 β + Dex treatment of *GR α -D3* mutant MEFs, the *GR α -D3* isoform, but not SUMOs and corepressors, was associated to p65 or c-jun bound to their cognate DBS, whereas SUMOs/corepressors association did occur in WT MEFs (Fig. 1C). Most notably, a 3-d 12-*O*-tetradecanoylphorbol-13-acetate (TPA) topical application on ears revealed a more severe skin inflammation in *GR α -D3* than in WT mice in which it was strongly decreased on topical Dex cotreatment, whereas in marked contrast, this treatment was inefficient in *GR α -D3* mice (Fig. S2A, Left, and B, Upper). Ac-

cordingly, using extracts from these mouse ears, analyses of proinflammatory genes revealed that, upon topical Dex treatment, TPA-induced activation of transcription by NF- κ B and/or AP1 was repressed in WT but not in *GR α -D3* mice (Fig. 1D). Interestingly, after a 5-d TPA topical treatment, a more severe ear inflammation was also observed in *GR K310R* mutants than in WT mice, and this inflammation, which was clearly suppressed upon Dex cotreatment in WT mice, was decreased in *GR K310R* mice, albeit to a lesser extent (Fig. S2A, Right, and B, Lower). Transcript analyses using ear extracts from these mice also indicated a decrease in Dex-induced repression of TPA-induced genes in *GR K310R* mice compared with WT mice (Fig. 1E). Importantly, ChIP assays carried out on dorsal skin of *GR K310R* mutant mice treated with TPA and Dex for 6 h revealed a strong, but not full, decrease in the SUMOs and SMRT/NCoR1 association with GR bound to NF- κ B (p65)/AP1 (c-jun) DBSs of several genes, to which the binding of GR on its own was unaffected (Fig. 1F). This partial decrease most likely reflects the formation of a repressing complex on the weak SUMOylation site located at GR K294 (K277 in human) (11) as ChIP assays (Fig. 1F) revealed (i) that all components of the repressing complex (GR, SMRT, NCoR1, SUMO1, SUMO2/3) were associated on NF- κ B/AP1 sites of *MMP13*, *COX2*, and *TNF α* genes in mouse epidermis extracts from *GR K310R* mice, (ii) that such an association did not exist in *GR α -D3* MEFs (Fig. 1C), and (iii) that the NCoR1/SMRT ratio is similar in repressing complexes found in WT and *K310R* mice. We therefore conclude that the

Fig. 2. NCoR1 and SMRT corepressors as well as HDAC3 are required for GC-induced GR-mediated tethered transrepression in vivo. (A) qRT-PCR for transcripts of proinflammatory genes in ear epidermis of WT and [SMRT/NCoR1]^{ep-/-} mice, treated with vehicle, TPA (1 nmol/cm²), and Dex (6 nmol/cm²) as indicated for 18 h. (B) qPCR analyses of ChIP assays performed with dorsal epidermis of WT and [SMRT/NCoR1]^{ep-/-} mice treated as in A but for 6 h, showing the association of indicated proteins to NF- κ B or AP1 DBS in promoter regions of genes as indicated. (C) As in A, but using HDAC3^{ep-/-} mice. (D) As in B, but using HDAC3^{ep-/-} mice. Values are mean $\pm$ SEM. **P* < 0.05.

SUMOylation at K310 (and to a much lesser extent at K294) within the mouse GR (K293 and K277 in human) is required for GC-induced tethered transrepression in vitro and in vivo but not for binding of the GR to DNA-bound NF- κ B or AP1. Of note, the above observations also conclusively demonstrated that the SUMOylation site located at GR position K703 cannot assemble a repressing complex.

Interestingly, in contrast to its IR nGRE-mediated repressive effect (11), and in agreement with Nissen and Yamamoto (10), the ligand binding domain (LBD)-deleted GR (GR ABCD) could not repress the IL1 β -induced NF- κ B/AP1-mediated transactivation of *TNF α* and *MMP13* genes (Fig. S14). Of note, ChIP assays showed that GR ABCD could not bind to DNA-bound p65 or c-jun (Fig. S1K), indicating that the GR LBD is required for tethered interaction with NF- κ B/AP1.

NCoR1 and SMRT Corepressors and HDAC3 Are Required for GC-Induced GR-Mediated Tethered Transrepression in Vivo. Using mice selectively mutated for *SMRT* and/or *NCoR1*, we previously reported that the corepressors SMRT and NCoR1 are required for GC-induced IR nGRE-mediated direct transrepression (11). We use these mutant mice to determine whether these corepressors are also required for GC-induced tethered transrepression. Upon topical Dex treatment of [*SMRT/NCoR1*]^{ep/-} mouse ears, TPA-induced NF- κ B- and AP1-mediated activation of transcription of *TNF α* , *COX2*, *MMP13*, and *IL6* genes was not significantly repressed (Fig. 2A). ChIP assays using dorsal epidermis of the same mice showed that tethered binding of GR to either NF- κ B or AP1 DBS was not affected by the lack of NCoR1/SMRT (Fig. 2B). Transcript analyses of genes containing NF- κ B and/or AP1 binding sites were also performed using MEFs from *NCoR1*^{-/-} or *SMRT*^{-/-} mice. In all cases, the presence of one of the two corepressors was sufficient to ensure Dex-induced tethered repression (Fig. S34). Note that a case of corepressor substitution (11) was observed on the AP1 site located in the *MMP13* promoter (Fig. S3B). Thus, NCoR1 and/or SMRT corepressors are required for GC-induced tethered indirect transrepression, but not for tethered association of the GR to NF- κ B or AP1 bound to their cognate DBSs.

We have shown that HDAC3 is indispensable for GC-induced IR nGRE-mediated direct repression in vivo, the binding of which to IR nGRE is mediated by the corepressors NCoR1/SMRT (11). Using mutant mice in which *HDAC3* was selectively ablated in epidermal keratinocytes, we found that the expression of proinflammatory genes was strongly increased upon TPA topical treatment compared with their expression in WT mice (Fig. 2C). In addition, Dex did not repress TPA-induced expression of these proinflammatory genes in *HDAC3*^{ep/-} mice, whereas these genes were readily repressed in WT mice (Fig. 2C). Importantly, using mouse dorsal epidermis, ChIP assays showed that on TPA + Dex treatment, the ablation of HDAC3 did not affect the association of NCoR1 and SMRT to GR bound to NF- κ B/AP1 (Fig. 2D), whereas the mutation of NCoR1 and SMRT did prevent the binding of HDAC3 to GR (Fig. 2B). Altogether these results demonstrated that HDAC3 plays an important role in GC-induced tethered transrepression in vivo.

TIF2 (GRIP1/SRC2) Coactivator Is Not Indispensable for GC-Induced GR-Mediated Tethered Transrepression in Vivo. It has been reported (13) that the transcriptional coactivator steroid receptor coactivator 2 (*SRC2*) [initially known as transcriptional mediator/intermediary factor 2 (*TIF2*) and glucocorticoid receptor-interacting protein 1 (*GRIP1*); hereafter called *TIF2*] is instrumental in GC-induced tethered transrepression in bone marrow-derived macrophages. To further investigate the function of *TIF2* in this transrepression, we prepared according to Reilly et al. (14) bone marrow-derived and peritoneal macrophages from WT and *TIF2*^{-/-}-null mice (15). Surprisingly, upon lipopolysaccharides

(LPS) and Dex cotreatment, all tested LPS-induced proinflammatory genes were similarly repressed in WT and *TIF2*^{-/-} bone marrow-derived and peritoneal macrophages (Fig. 3A and B). However, ChIP assays revealed that upon LPS + Dex treatment, TIF2 was associated with the repressing complexes containing GR, SMRT, and/or NCoR1 present on the *TNF α* NF- κ B site and the *MMP13* AP1 site, but not on the *IL6* NF- κ B site (Fig. 3C). As expected, in bone marrow-derived macrophages from *TIF2*^{-/-}-null mice, no TIF2 was associated with these repressing complexes, whereas both GR and SMRT/NCoR1 were present in all three cases (Fig. 3C).

To further study in vivo the possible antiinflammatory role of *TIF2* in TPA-induced skin inflammation, we used epidermis from *TIF2*^{ep/-} mice in which *TIF2* was ablated in keratinocytes. Upon topical treatment of mouse ears, the same Dex-induced repression of TPA-induced gene expression was observed in epidermis from WT and *TIF2*^{ep/-} mice (Fig. 3D). Furthermore, when ears of WT and *TIF2*^{-/-} mice were topically treated with TPA alone or TPA + Dex for 10 d, the TPA-induced skin inflammation was efficiently and similarly reduced by Dex topical treatment in both WT and *TIF2*^{-/-} mice (Fig. S4). Of note, transcript analyses of TPA-induced proinflammatory genes in ear extracts revealed similar repression by Dex in WT and *TIF2*^{-/-} mice (Fig. 3E). From these in vitro and in vivo data, we conclude that, even though ChIP assays indicated that TIF2 could be, in some instances, associated with the repressing complex that is instrumental in GC-induced tethered transrepression of inflammatory genes, it is unlikely that *TIF2* is actually involved in tethered transrepression, the magnitude of which is not affected in *TIF2*^{-/-}-null and *TIF2*^{ep/-} mice.

GR-Mediated Transactivation, Direct Transrepression, and Tethered Transrepression Are Similarly Activated by GC and Inhibited by RU486, but Differentially Respond to Dissociated GR Ligands (SEGRAs). That upon binding of the glucocorticoid Dex, the GR could exert three functions, raises the question as to whether Dex is similarly efficient at inducing the activity of the GR, irrespective of its functions. A549 cells, as well as WT mouse-derived MEFs, were treated with increasing Dex concentrations without or with IL1 β (to activate NF- κ B/AP1) (Fig. 4A). Dex could efficiently and similarly (i) activate transcription of the (+)GRE serum and glucocorticoid-regulated kinase 1 (*SGK1*) gene, (ii) directly repress transcription of the IR1 nGRE *TSLP* gene, and (iii) indirectly repress transcription of the NF- κ B/AP1 *MMP13* and *TNF α* genes. Similar experiments were performed with WT mice through i.p. injection of increasing doses of Dex with or without LPS. In mouse liver, transactivation of *REDD1* [a (+)GRE gene], direct repression of *TNFRSF19* (an IR nGRE gene), and tethered indirect repression of *MMP13* and *TNF α* genes exhibited similar dose-response curves to reach a maximum at ~1 mg Dex/kg (Fig. 4B). Thus, the capability of Dex to potentiate the GR appears to be the same for triggering either one of its three activities. ChIP assays further showed that, for a given Dex concentration, the same relative amount of GR was bound to a (+)GRE, to an IR nGRE, and to NF- κ B/AP1 bound to cognate DBSs (Fig. 4C). The same amount of SUMO1 relative to that of GR was also bound in the case of direct and indirect tethered repression (Fig. 4C).

Interestingly, all three Dex-induced GR functions were inhibited upon ear topical treatment with RU486 (RU) (Fig. 4D and E), but ChIP assays revealed that different mechanisms were at work as (i) for transactivation, RU prevented the binding of GR to (+)GRE DBSs, as reported (16) (Fig. 4F, a), (ii) for direct transrepression, RU prevented the GR NTD SUMOylation required for SUMO/corepressors-mediated binding of GR to IR nGREs (17) (Fig. 4F, b), and (iii) for tethered transrepression, RU also prevented the GR NTD SUMOylation required for SUMO/corepressor-mediated

Fig. 3. The TIF2 (GRIP1/SRC2) coactivator is not indispensable for GC-induced GR-mediated tethered transrepression in vivo. (A) qRT-PCR for transcripts of proinflammatory genes in bone marrow-derived macrophages of WT and *TIF2* $^{-/-}$ mice. Cells were treated with vehicle, LPS (10 ng/mL), and Dex (0.5 μ M) for 1 h. (B) As in A, but using peritoneal macrophages. (C) qPCR analyses of ChIP assays performed with bone marrow-derived macrophages of WT and *TIF2* $^{-/-}$ mice, treated with vehicle, LPS (10 ng/mL), and Dex (1 μ M) for 1 h. (D) As in A, but using ear epidermis of WT and *TIF2* $^{ep-/-}$ mice, treated with vehicle, TPA (1 nmol/cm 2), and Dex (6 nmol/cm 2) for 18 h. (E) As in D, but with ear extracts of WT and *TIF2* $^{-/-}$ mice treated as indicated. Values are mean $\pm$ SEM.

repression of NF- κ B/AP1 activities, but did allow tethered binding of GR to NF- κ B/AP1 (Fig. 4G and Fig. S5 A, a and b).

The beneficial antiinflammatory effects of GC therapy have been classically ascribed to tethered transrepression, whereas a number of its debilitating (undesirable) side effects appear to be related to both (+)GRE transactivation and IR nGRE direct transrepression (2, 5). This led to the quest for dissociated GR ligands (also known as SEGRA or SGRM for selective GR agonist or selective GR modulator, respectively), which would preferentially induce tethered transrepression. Such ligands have not yet been found, even though a partially dissociated steroidal GR ligand (RU24858), selectively lacking the (+)GRE-mediated transactivation activity, was characterized (Fig. 4 F, a and Fig. S5 A, c) (1, 3). However, in addition to tethered transrepression, RU24858 still induces IR nGRE-mediated direct transrepression (3), in keeping with our present data showing that it does not inhibit GR SUMOylation (Fig. 4 F, b and G and Fig. S5 A, c).

Interestingly, potent antiinflammatory nonsteroidal compounds (NSCs) that may exhibit reduced side effects have been more recently identified (18, 19). We therefore investigated whether a related NSC GR ligand (designated hereafter as CpdX) that efficiently promotes GR nuclear translocation (Fig. 5A) could be a truly dissociated GR ligand. A topical treatment of mouse ears showed that CpdX could induce tethered NF- κ B/AP1-mediated transrepression (Fig. 4E) and also alleviate a topically TPA-

induced ear inflammation (Fig. 5B), but in both cases less efficiently than Dex. In contrast, it did not trigger (+)GRE-mediated transactivation nor IR nGRE-mediated transrepression, including that mediated by the IR1 nGRE located in exon 6 of the *GR* gene (Fig. 4D) (17). Furthermore, ChIP assays using GR-transfected Cos-1 cells revealed that, on CpdX treatment, GR did bind (albeit less efficiently than on Dex treatment) together with SUMO1, SMRT, and NCoR1 corepressors to NF- κ B and AP1 bound to their DBSs (*TSLP* and *MMP13* genes; Fig. 4G), but not to the (+)GRE of the *SGK1* gene (Fig. 4 F, a) and the IR1 nGRE of the *GR* gene (Fig. 4 F, b and Fig. S5 A, d). Moreover, treatment of A549 cells with CpdX did not induce (+)GRE-mediated transactivation (*SGK1*, *SPDR*, *SLC19A2*, and *FKBP5* genes; Fig. S5 B, a and d) or IR nGRE-mediated direct transrepression (*GEM* and *GR* genes; Fig. S5 B, b and f), whereas cotreatment with Dex and an excess of CpdX somewhat decreased (+)GRE-mediated transactivation (Fig. S5 B, a and d) and IR nGRE-mediated direct transrepression (Fig. S5 B, b and f), thus suggesting that CpdX could bind to the GR but is inefficient at inducing the GR transconformations required for exerting (+)GRE-mediated and IR nGRE-mediated functions. Note that under identical conditions, CpdX coadministration did not affect the Dex-induced expression of the (+)GRE genes dual specificity protein phosphatase 1 (*DUSP1*) and glucocorticoid-induced leucine zipper (*GILZ*) (Fig. S5 B, e). On the other hand, CpdX appears to be about 50–60% as

Fig. 4. GR-mediated transactivation, direct transrepression, and tethered transrepression are similarly activated by Dex and inhibited by RU486, but differentially respond to dissociated GR ligands. (A) qRT-PCR for *SGK1*, *TSLP*, *TNF α* , and *MMP13* transcripts in A549 cells or MEFs, treated for 6 h as indicated. The values correspond to Dex-induced activation or repression. (B) As in A, but for *Redd1*, *TNFRSF19*, *TNF α* , and *MMP13* genes expressed in liver of WT mice i.p. injected with LPS (10 μ g) and Dex for 4 h. (C) qPCR analyses of ChIP assays performed with A549 cells treated for 1 h, as indicated, showing the binding of indicated proteins to (+)GRE, IR1 nGRE, and AP1/NF κ B DBSs. (D) As in A, but for (+)GRE and IR nGRE-containing genes using ear extracts from WT mice topically treated for 18 h with Dex, RU486, and CpdX (6 nmol/cm²) as indicated; 36 nmol/cm² RU486 was used for Dex+RU486. (E) As in D, but for proinflammatory genes upon TPA (1 nmol/cm²) topical treatment with or without Dex, RU486, and CpdX, as indicated. (F) As in C, but performed with Cos-1 cells transfected with GR and treated with Dex, RU486, RU24858, and CpdX (1 μ M) as indicated for 1 h, showing the association of indicated proteins on the (+)GRE of the *SGK1* gene or the IR1 nGRE of the *GR* gene, as indicated; 6 μ M RU486 was used for Dex+RU486. (G) As in F, but on the NF- κ B DBS of the *TSLP* gene and the AP1 DBS of the *MMP13* gene, upon IL1 β cotreatment (5 ng/mL). Values are mean $\pm$ SEM.

Fig. 5. CpdX could relieve TPA-induced skin inflammation. (A) Immunofluorescence analyses of Cos-1 cells transfected with GR and treated with vehicle, Dex, or CpdX (0.5 μM) for 1 h. (B) Appearance of BALB/cByJ mouse ears before and after 5-d treatment, as indicated (Left); H&E-stained ear sections (Right). (Scale bar, 50 μm.)

efficient as Dex for GR-mediated tethered transrepression of *IL1β* (Fig. S5 B, c), which is likely to reflect both a lower efficiency at promoting the binding of GR to NF-κB/AP1 and at inducing the assembly of a tethered repressing complex (Fig. 4G).

Discussion

SUMOylation of the Full-Length GR Generates Repressing Complexes That Are Instrumental in Tethered Transrepression and GC-Induced Antiinflammatory Processes. The discovery that GC-induced IR nGRE-mediated direct repression by the GR involves the formation of a SMRT/NCoR1 repressing complex initiated by SUMOylation of the GR NTD (11) prompted us to investigate whether a similar mechanism could be instrumental in tethered indirect repression known to involve an interaction between the GR and p65(NFκB)/c-jun(AP1)/STAT3 activators (4). Interest-

ingly, upon transfection in cells activated by IL1β (NF-κB/AP1) or IL6 (STAT3), SUMOylation was not required for tethered binding of GR to p65 (NF-κB), c-jun (AP1), and STAT3 (Fig. S1 C–F). On the other hand, GR ABCD could not bind on its own (Fig. S1 A and K), thus showing that the LBD is necessary for tethered binding, whereas it is dispensable for binding to IR nGREs (11). Notably, this tethered binding does not require the formation of a repressing complex in vivo, as it occurs readily in MEFs expressing the *GRα-D3* isoform or in epidermal keratinocytes of the mouse mutant *GR K310R* (Fig. 1 B and E). Importantly, competition experiments (Fig. S1G) revealed that, upon SUMOylation, the interaction of GR with NF-κB/AP1 (and possibly with other factors involved in tethered repression) is strengthened, thus indicating that SUMOylation, which leads to the formation of an active repressing complex, could be

preceded by tethered binding of non-SUMOylated GR to NF- κ B/AP1/STAT3.

In vivo experiments with mice and MEFs expressing only the *GR α -D3* isoform, and with mice bearing the GR SUMOylation K310R mutation confirmed, at the transcript level, the requirement of GR SUMOylation for tethered repression (Fig. 1 *A*, *C*, and *D*). In addition, using mice in which both *SMRT* and *NCoR1*, or *HDAC3*, were selectively ablated in epidermal keratinocytes, we demonstrated that both *SMRT/NCoR1* and *HDAC3* are indispensable in GC-induced tethered transrepression in vivo (Fig. 2). Moreover, experiments carried out with mice topically treated with TPA showed that TPA-induced skin inflammation was drastically reduced by administration of Dex to WT, but not to *GR α -D3* mice, whereas it was reduced to a lesser extent in *K310R* mutants (Fig. S2), most likely because a repressing complex was assembled on the accessory SUMOylation site located at K294 (K277 in human), therefore leading to the conclusion that GR SUMOylation is instrumental in GC-induced repressing mechanisms involved in tethered repression. Note, however, that it is not excluded that additional factors could be marginally involved in these repressing mechanisms (13, 20). Further experiments, performed with *GR K310R* and *GR K310R/K294R* double mutant mice to study the effect of GC on a variety of actual inflammatory models, will establish the importance of GR SUMOylation in tethered repression and more generally in GC-induced anti-inflammatory processes.

Which Mechanisms Allow a Single GR to Exert Three Main Functions Under the Control of a Single GC Ligand. We have shown that in cells in culture (Fig. 4*A*) and in mouse liver (Fig. 4*B*), Dex could efficiently induce (*i*) the transactivation of (+)GRE genes, (*ii*) the direct repression of IR nGRE genes, and (*iii*) the tethered indirect transrepression of NF- κ B/AP1/STAT3-containing genes. The efficiency of Dex at inducing the GR activity appears to be similar for triggering either of its three functions and to promote the binding of GR to a (+)GRE, to an IR nGRE, or to transactivators (e.g., NF- κ B/AP1/STAT3) bound to their cognate DBSs (Fig. 4*C*). Thus, additional mechanisms must operate to enable the GR to selectively recognize a (+)GRE DBS, an IR nGRE DBS, or p65 (NF- κ B)/c-jun (AP1) proteins. As the GR ABCD is a constitutive activator of transcription (11, 21), GR binding to a (+)GRE only requires a GC-dependent conformational modification of the LBD, which unmasks the DNA binding domain (DBD), whereas the binding of GR to an IR nGRE requires a GC ligand, which by binding to the LBD, allows the unmasking of both the DBD and the NTD SUMOylation site, therefore allowing the formation of a SMRT/NCoR1 repressing complex on the IR nGRE (11). In this respect, note that GR ABCD, but not its SUMOylation mutant GR ABCD K293R, is readily SUMOylated to assemble a repressing complex on an IR nGRE (Fig. S5 *A*, *e*), whereas the (+)GRE-mediated activation of transcription by GR ABCD is unaffected by the K293R mutation (11). In the case of (+)GREs, this unmasking allows on its own an efficient binding of the GR DBD to a (+)GRE, which appears to be incompatible with SUMOylation of the NTD, whereas the much weaker binding of the GR DBD to an IR nGRE (22) requires the subsequent SUMOylation of the NTD, as well as the formation of the repressing SMRT/NCoR1 complex, to stably anchor the GR on the IR nGRE.

On the other hand, for tethered repression, the presence of the LBD plays an essential role, as it is required first for the binding of GR to p65/c-jun/STAT3 bound to their DBSs (Fig. S1*K*) and subsequently for its GC-dependent SUMOylation, which is mandatory for assembling the repressing complex involved in tethered repression of NF- κ B/AP1/STAT3 target genes (Fig. 4*G*). Of note, the tethered binding of the liganded GR with p65 (NF- κ B)/c-jun (AP1)/STAT3 requires both the integrity of the GR DBD (10, 23) and LBD domains (Fig. S1*K*), and is not

prevented by the GC antagonist RU486 (Fig. 4*G* and Fig. S5 *A*, *b*), nor by a mutation of the SUMOylation site (Fig. S1 *C–F*). However, the subsequent NTD SUMOylation and the formation of a repressing complex that results in a tighter binding to p65/c-jun are GC dependent (Fig. S1*G*), therefore suggesting that the NTD SUMOylation site is in some way masked by the LBD, as it is the case for IR nGRE-mediated transrepression (11).

Interestingly, such unmaskings of the NTD SUMOylation site may involve not only a ligand-dependent conformational modification of the LBD, but also the phosphorylation of sites located in the NTD. Indeed, upon mutation of five such phosphorylation sites, as well as upon addition of selective inhibitors of two cognate kinases (JNK and GSK-3 β), ChIP assays revealed that under limiting Dex concentration (10 nM), there was a decrease in the binding of SUMOylated GR on an IR nGRE (11), as well as on p65/NF- κ B bound to its cognate DBS (Fig. S1*H*). Of note, the loss of NTD phosphorylation did not affect the binding of SUMOylated GR ABCD on the IR nGRE (11). It appears therefore that SUMOylation of the GR NTD could be controlled, not only by GC, but also through protein kinases that have been implicated in inflammation and homeostasis (24, 25).

Thus, for all three GR main functions, it appears that the initial step is common and consists in the binding of the GC ligand, which in all three cases, due to a GC-induced conformational modification of the LBD, may result in the unmasking of the NTD/DBD (ABCD) domains. Subsequently, the unveiled GR may bind efficiently to a (+)GRE (26), loosely to an IR nGRE (22) or through tethering to either p65, c-jun, or STAT3. It is only then that, in the two latter cases, SUMOylation and assembly of a repressing complex may occur, thereby strengthening the binding of the GR to an IR nGRE DBS, or through tethering to either p65, c-jun, or STAT3. Most interestingly, with such operational mechanisms, there might be no need for predestined nuclear pools of SUMOylated GR, as SUMOylation may occur only upon binding of the GR to either IR nGREs or p65 (NF- κ B)/c-jun (AP1)/STAT3 accessible targets.

To conclude, the multiple GC-dependent functions of the GR appear to be accounted for both by the versatility of its DNA binding domain, which can selectively recognize widely different regulatory targets [(+)GRE, IR nGRE, NF- κ B/AP1/STAT3], and by the SUMOylation of its NTD, which considerably increases its potential to negatively control gene expression.

Are Truly Antiinflammatory SEGAs in Sight? It is now well accepted that tethered indirect transrepression accounts for many of the beneficial antiinflammatory effects of glucocorticoids, whereas (+)GRE-mediated transactivation and IR nGRE-mediated direct repression are responsible for most of the clinically debilitating effects (1, 3). We report here that a compound (CpdX), which is related to a novel nonsteroidal antiinflammatory SEGAs named ZK245186 (19) or Mapracorat (18), cannot induce the transactivation and direct transrepression functions of the GR while still inducing its tethered indirect transrepression activity and antiinflammatory properties in vivo (Figs. 4 *D* and *E* and 5*B*). Together with the ZK245186/Mapracorat compound, CpdX may therefore be a truly antiinflammatory SEGAs, which might be devoid of most of the debilitating effects of present day natural and synthetic glucocorticoids. Note, however, that even though a treatment with CpdX on its own does not induce (+)GRE-mediated transactivation nor IR nGRE-mediated direct transrepression in A549 cells, a coadministration of CpdX can inhibit, albeit not efficiently, the Dex-induced transactivation of (+)GRE genes (Fig. S5 *B*, *a* and *d*), as well as the direct transrepression of IR nGRE genes (Fig. S5 *B*, *b* and *f*). Thus, the administration of CpdX in vivo could possibly inhibit the induction of the (+)GRE antiinflammatory genes *DUSP1* and *GILZ* by physiological GCs (1, 27). This possibility is, however, unlikely as Dex-induced expression of *DUSP1* and *GILZ* is not affected by CpdX addition to A549 cells

(Fig. S5 B, e). On the other hand, our present finding that CpdX inhibits the IR1 nGRE-mediated Dex-induced repression of *GR* expression suggests that CpdX administration could be therapeutically beneficial, as it may increase the level of *GR* in inflammatory cells (Fig. S5 B, f). Future studies *in vivo* are required to determine to which extent the administration of CpdX may affect the expression of (+)GRE and IR nGRE genes by interfering with endogenous GCs, and also to investigate whether administering CpdX when endogenous GCs are at their lowest diurnal levels (i.e., during the rest phase of the circadian cycle) could be beneficial (28, 29).

Materials and Methods

Mice. *GR α -D3*, *GR K310R*, *NCoR1/SMRT*, and *HDAC3* mutant mice are described in ref. 11. The breeding, maintenance, and experimental manipulation of mice were approved by the animal care and use committee of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC)/Institut Clinique de la Souris (ICS).

The ChIP assay, RNA isolation, and qPCR analyses were performed as in ref. 3. Primers are available on request.

Chemical Compound. The chemical compound of CpdX is (*R*)-5-(4-(5-fluoro-2-methoxyphenyl)-2-hydroxy-4-methyl-2-(trifluoromethyl)pentylamino)isobenzofuran-1(3H)-one.

Statistics. Data are represented as mean $\pm$ SEM of at least three independent experiments and were analyzed by Microsoft Excel statistics software using the Student *t* test. *P* < 0.05 was considered significant.

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- Clark AR, Belvisi MG (2012) Maps and legends: The quest for dissociated ligands of the glucocorticoid receptor. *Pharmacol Ther* 134(1):54–67.
- Meijising SH, et al. (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. *Science* 324(5925):407–410.
- Surjit M, et al. (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* 145(2):224–241.
- Ratman D, et al. (2013) How glucocorticoid receptors modulate the activity of other transcription factors: A scope beyond tethering. *Mol Cell Endocrinol* 380(1–2):41–54.
- Langlais D, Couture C, Balsalobre A, Drouin J (2012) The Stat3/GR interaction code: Predictive value of direct/indirect DNA recruitment for transcription outcome. *Mol Cell* 47(1):38–49.
- Rogatsky I, Luecke HF, Leitman DC, Yamamoto KR (2002) Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts. *Proc Natl Acad Sci USA* 99(26):16701–16706.
- Cruz-Topete D, Cidlowski JA (2015) One hormone, two actions: Anti- and pro-inflammatory effects of glucocorticoids. *Neuroimmunomodulation* 22(1–2):20–32.
- Glass CK, Saijo K (2010) Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nat Rev Immunol* 10(5):365–376.
- Luecke HF, Yamamoto KR (2005) The glucocorticoid receptor blocks P-TEFb recruitment by NFkappaB to effect promoter-specific transcriptional repression. *Genes Dev* 19(9):1116–1127.
- Nissen RM, Yamamoto KR (2000) The glucocorticoid receptor inhibits NFkappaB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. *Genes Dev* 14(18):2314–2329.
- Hua G, Paulen L, Chambon P (2016) GR SUMOylation and formation of an SUMO-SMRT/NCoR1-HDAC3 repressing complex is mandatory for GC-induced IR nGRE-mediated transrepression. *Proc Natl Acad Sci USA* 113:E626–E634.
- Gross KL, Oakley RH, Scoltock AB, Jewell CM, Cidlowski JA (2011) Glucocorticoid receptor alpha isoform-selective regulation of antiapoptotic genes in osteosarcoma cells: A new mechanism for glucocorticoid resistance. *Mol Endocrinol* 25(7):1087–1099.
- Chinenov Y, et al. (2012) Role of transcriptional coregulator GRIP1 in the anti-inflammatory actions of glucocorticoids. *Proc Natl Acad Sci USA* 109(29):11776–11781.
- Reilly MM, Pantoja C, Hu X, Chinenov Y, Rogatsky I (2006) The GRIP1:IRF3 interaction as a target for glucocorticoid receptor-mediated immunosuppression. *EMBO J* 25(1):108–117.
- Gehin M, et al. (2002) The function of TIF2/GRIP1 in mouse reproduction is distinct from those of SRC-1 and p/CIP. *Mol Cell Biol* 22(16):5923–5937.
- Heck S, et al. (1994) A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J* 13(17):4087–4095.
- Ramamoorthy S, Cidlowski JA (2013) Ligand-induced repression of the glucocorticoid receptor gene is mediated by an NCoR1 repression complex formed by long-range chromatin interactions with intragenic glucocorticoid response elements. *Mol Cell Biol* 33(9):1711–1722.
- Baiula M, et al. (2014) Mapracorat, a selective glucocorticoid receptor agonist, causes apoptosis of eosinophils infiltrating the conjunctiva in late-phase experimental ocular allergy. *Drug Des Devel Ther* 8:745–757.
- Schäcke H, et al. (2009) Characterization of ZK 245186, a novel, selective glucocorticoid receptor agonist for the topical treatment of inflammatory skin diseases. *Br J Pharmacol* 158(4):1088–1103.
- Uhlenhaut NH, et al. (2013) Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. *Mol Cell* 49(1):158–171.
- Godowski PJ, Rusconi S, Miesfeld R, Yamamoto KR (1987) Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* 325(6102):365–368.
- Hudson WH, Youn C, Ortlund EA (2013) The structural basis of direct glucocorticoid-mediated transrepression. *Nat Struct Mol Biol* 20(1):53–58.
- Liden J, Delaunay F, Rafter I, Gustafsson J, Okret S (1997) A new function for the C-terminal zinc finger of the glucocorticoid receptor. Repression of RelA transactivation. *J Biol Chem* 272(34):21467–21472.
- Beck IM, et al. (2009) Crosstalk in inflammation: The interplay of glucocorticoid receptor-based mechanisms and kinases and phosphatases. *Endocr Rev* 30(7):830–882.
- Beurel E, Grieco SF, Jope RS (2015) Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. *Pharmacol Ther* 148:114–131.
- Luisi BF, et al. (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 352(6335):497–505.
- Hübner S, Dejager L, Libert C, Tuckermann JP (2015) The glucocorticoid receptor in inflammatory processes: Transrepression is not enough. *Biol Chem* 396(11):1223–1231.
- Biddie SC, Conway-Campbell BL, Lightman SL (2012) Dynamic regulation of glucocorticoid signalling in health and disease. *Rheumatology (Oxford)* 51(3):403–412.
- Buttgereit F, Smolen JS, Coogan AN, Cajoche C (2015) Clocking in: chronobiology in rheumatoid arthritis. *Nat Rev Rheumatol* 11(6):349–356.