A New Function for the C-terminal Zinc Finger of the Glucocorticoid Receptor

REPRESSION OF RelA TRANSACTIVATION*

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Glucocorticoids inhibit NF-κB signaling by interfering with the NF-κB transcription factor RelA. Previous studies have identified the DNA-binding domain (DBD) in the glucocorticoid receptor (GR) as the major region responsible for this repressive activity. Using GR mutants with chimeric DBDs the repressive function was found to be located in the C-terminal zinc finger. As predicted from these results the mineralocorticoid receptor that contains a C-terminal zinc finger identical to that of the GR was also able to repress RelA-dependent transcription. Mutation of a conserved arginine or a lysine in the second zinc finger of the GR DBD (Arg-488 or Lys-490 in the rat GR) abolished the ability of GR to inhibit RelA activity. In contrast, C-terminal zinc finger GR mutants with mutations in the dimerization box or mutations necessary for full transcriptional GR activity were still able to repress RelA-dependent transcription. In addition, we found that the steroid analog ZK98299 known to induce GR transrepression of AP-1 had no inhibitory effect on RelA activity. In summary, these results demonstrate that the inhibition of NF-κB by glucocorticoids involves two critical amino acids in the C-terminal zinc finger of the GR. Furthermore, the results from the use of mineralocorticoid receptor and anti-glucocorticoids suggest that the mechanisms for GR-mediated repression of NF-κB and AP-1 are different.

Glucocorticoid hormones regulate many different biological processes via a specific intracellular receptor, the glucocorticoid receptor (GR), which is present in most cell types. The GR is a member of the superfamily of nuclear receptors, which all contain three main functional domains (1, 2). After binding of hormone to the C-terminal ligand binding domain (LBD) and dissociation of heat shock proteins, the GR homodimerizes and interacts with specific DNA sequences termed glucocorticoid response elements (GREs) through its central DNA binding domain (DBD). The transcriptional activity of GR is mainly dependent on the r1 domain localized in the N-terminal part of the protein (3, 4). The highly conserved DBD contains two zinc fingers and in each of them a zinc ion is tetrahedrally coordinating four cysteine residues. One function of the DBD is to discriminate between different response elements, thus determining target genes to be activated (5, 6). This function is achieved by a few amino acids localized in the C-terminal part of the N-terminal zinc finger, the so-called the P box. A second subdomain termed the D box in the N-terminal knuckle of the C-terminal zinc finger has been shown to harbor amino acid residues important for homodimerization (7). DNA binding of the ligand-activated GR results in an increased rate of formation of transcriptionally competent pre-initiation complexes. This is thought to be achieved by protein-protein interactions between the receptors and different components of the transcriptional machinery (8, 9). Besides the more well studied transcriptional activation process, the GR can repress transcription via different mechanisms (10). The GR has been shown to bind to overlapping DNA response elements for other transcription factors leading to repression (11–13). These GR binding elements have been termed negative GREs. Inhibition of gene expression by glucocorticoids can also occur in the absence of GR DNA binding. The most well studied system for this is the repression by the GR of genes activated by the AP-1 transcription factor complex. In this case there is evidence for a direct physical association between the proteins present in AP-1 and the GR (14–16). Although direct binding of the GR to DNA is not necessary, the DBD has in some cases been shown to be essential for this interaction (15). Furthermore, the composition of the AP-1 complex determines whether the GR will cause a positive or a negative effect on AP-1 controlled transcription. In addition to the ability of GR to interfere with AP-1 controlled transcription, we and other (17–20) have shown that the GR also can repress NF-κB signaling.

NF-κB is an inducible transcription factor complex that plays an essential role in the inflammatory and immune responses (21). It is activated by a diverse range of signals including the pro-inflammatory cytokines tumor necrosis factor-α and interleukin-1 as well as phorbol esters, physical or oxidative stress, and bacterial and viral proteins. In most cells the NF-κB is composed of a heterodimer between RelA (p65) and NFκB1 (p50), where the RelA protein is responsible for the transactivation potential. In the non-activated state the NF-κB is sequestered in the cytoplasm through the interaction with the inhibitory protein IkB. During activation, the IkB protein becomes phosphorylated and degrades allowing NF-κB to translocate to the nucleus where it binds to specific DNA elements and subsequently regulates genes involved in inflammatory and immune responses (22–24).

Glucocorticoids have potent immunosuppressive effects and are commonly used in the clinic to suppress different immunological and inflammatory responses. Different molecular mech-

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The abbreviations used are: GR, glucocorticoid receptor; GRE, glucocorticoid response element; AR, androgen receptor; DBD, DNA binding domain; LUC, luciferase; MR, mineralocorticoid receptor; PR, progesterin receptor; RARs, retinoic acid receptor α; TRβ, thyroid receptor β; PCR, polymerase chain reaction.
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Anisomes have been suggested to be involved in this process including inhibition of AP-1 and NF-κB (25). In an earlier report (19) using transient transfections of GR and NF-κB responsive reporter genes, we have shown a mutual transcriptional inactivation between the GR and the NF-κB protein RelA. Direct DNA binding of GR is not required for the NF-κB repression to occur, since a GR, in which the P box had been mutated so that it no longer recognized a GRE, still was able to repress. Instead we showed that the GR and the RelA can directly or indirectly interact with each other in vitro and mutually interfere with transcriptional activity. Using deletion mutants and chimeric receptors, we demonstrated that the GR DBD is the major GR domain responsible for repression of RelA activity. In addition, an alternative mechanism for glucocorticoid repression of NF-κB activity has been suggested, which involves induction of IxBo by GR leading to the sequestering of NF-κB in the cytoplasm (26, 27).

In this report we investigated the subdomain and critical amino acids in the GR DBD involved in glucocorticoid repression of the NF-κB protein RelA. In addition, we investigated the role of GR dimerization and transactivation for the repression of RelA activity. Finally, we analyzed the ability of glucocorticoid antagonists to cause repression of NF-κB activity.

EXPERIMENTAL PROCEDURES

Reagents—Deep Vent® DNA polymerase and T4 DNA ligase were from New England Biolabs. Media, antibiotics, fetal bovine serum, and Lipofectin® were purchased from Life Technologies, Inc. Dexamethasone and aldosterone was obtained from Sigma. The monoclonal antibody number 7 (28) followed by secondary horseradish peroxidase-labeled anti-mouse antibody (Amersham Corp.) according to the suggested protocols.

RESULTS

The C-terminal Zinc Finger of the GR DBD Is Responsible for the Interference with RelA Transactivation—We and others (19, 20) have previously shown that the DBD of the GR is of crucial importance for the ability of GR to interfere with the NF-κB-mediated response, since deletion of the GR DBD or replacement of the GR DBD with the corresponding TRß DBD abolished repression. To further determine if a particular region in the GR DBD is responsible for the functional interference with NF-κB activity, GR mutants were created in which individual parts of the DBD were replaced by the corresponding regions of the GRnx expression plasmid. The C-terminal zinc finger alone (GggtG) or the linker region together with the C-terminal zinc finger and the linker region, and C-terminal zinc finger region of GR (g) or TRß (t), respectively. The expression plasmids GR(GggtG) (32) and GTG (33) in this report named GgggG and GtttG, respectively, were used for the amplification of the different DBD regions. The first PCR reaction contained 1 ng of plasmid DNA, 250 μM each dNTP, 1 μM primers, 1 × reaction buffer, and 1 unit of Deep Vent® DNA polymerase. The PCR program contained a 30-s denaturation step at 94 °C, a 30-s annealing time at 58 °C, and a 30-s extension at 72 °C, for 25 cycles. Plasmid DNA templates and a 30-s extension at 72 °C, for 25 cycles. Plasmid DNA templates and a 30-s extension at 72 °C, for 25 cycles. Plasmid DNA templates and a 30-s extension at 72 °C, for 25 cycles.
(36), this would suggest that the MR is able to repress RelA-mediated transactivation. Indeed, in transfection experiments performed as above, the MR activated by 10 nM aldosterone repressed RelA-mediated transactivation as efficiently as the GR (data not shown). This also shows that the four amino acids outside the second zinc finger that differ between the MR and GR DBDs are not critical for the repressive capacity.

An Intact GR Dimerization Box Is Not Required for Repression of RelA-mediated Transactivation—A major function for the C-terminal zinc finger in the GR DBD is to contribute to receptor homodimerization, a prerequisite for the receptor to bind DNA and transactivate efficiently (7, 37). This function is achieved by the D box region which is localized in the N-terminal knuckle of the C-terminal zinc finger. Since dimerization and NF-κB repression functions are localized in the same zinc finger of the GR, we tested if GR dimerization is a prerequisite for repression of RelA transactivation. For this purpose we used a GR mutant (D4X) in which three amino acids out of five in the D box have been mutated (Fig. 2A) (38). Transfection experiments with the D4X mutant demonstrated that mutations in the D box did not impair the ability of GR to repress RelA transcriptional activity (Fig. 2B). This shows that receptor dimerization is not a prerequisite for GR-mediated repression of RelA. As previously shown, the D4X mutant harbored no significant transcriptional activity (Fig. 2C).

Identification of GR Amino Acids Involved in Repression of RelA-mediated Transactivation—Previous studies have shown that in addition to GR, the estrogen receptor, progestin receptor (PR), and the androgen receptor (AR) also repress NF-κB activity (39–41). In contrast, the TRβ, the retinoic acid receptor α isoform (RARα), and the ecdysone receptor are un-

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**Fig. 1. Importance of the GR DBD C-terminal zinc finger for repression of RelA-mediated transactivation.** A, schematic representation of the DNA binding domains of the different GR mutants with chimeric GR/TRβ DBDs used for identifying regions in the GR DBD important for repression of RelA activity. B, COS-1 cells were transiently transfected with the 3xNF-κB(IC)tk-LUC reporter plasmid together with expression plasmids coding for RelA and the various GR/TRβ DBD mutants. After transfection, cells were treated with 1 μM dexamethasone for 24 h, and luciferase activity was determined. The wild type GR (GgggG) repressed RelA activity by 50–60% in the presence of hormone as compared with the luciferase activity in the absence of hormonal treatment. This level of repression was given the nominal value of 100, and repression by the GR mutants was expressed relative to this nominal value. Values are mean ± S.D. from three independent experiments.

**Fig. 2. GR amino acids involved in repression of RelA-mediated transactivation.** A, schematic representation of the tip of the C-terminal zinc fingers from the different GR mutants used showing the amino acids that were mutated to identify the critical residues for RelA repression. B, COS-1 cells were transiently transfected with the 3xNF-κB(IC)tk-LUC reporter plasmid together with expression plasmids coding for RelA and the various GRs with mutations in the C-terminal zinc finger. After transfection, cells were treated with 1 μM dexamethasone for 24 h, and luciferase activity was determined. The wild type GR (GgggG) repressed RelA activity by 50–60% in the presence of hormone as compared with the luciferase activity in the absence of hormonal treatment. This level of repression was given the nominal value of 100, and repression by the GR mutants was expressed relative to this nominal value. Values are mean ± S.D. from four independent experiments. C, COS-1 cells were transiently transfected with the (GRE)2tk-LUC reporter plasmid together with expression plasmids coding for the various GR mutants. After transfection, cells were treated with 1 μM dexamethasone for 24 h, and luciferase activity was determined. The induction factor for the wild type GR (GgggG) was given the nominal value of 100, and the induction levels of the GR mutants were expressed relative to this nominal value. Values are mean ± S.D. from four independent experiments. D, expression levels for the wild type and mutant GRs were determined by Western blotting after transfection into COS-1 cells.

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able to repress RelA-mediated transactivation (19). An amino acid sequence comparison of the C-terminal zinc fingers of repressive and non-repressive receptors revealed that the arginine and the lysine in position 488 and 490 in the rat GR are conserved only in the repressing receptors, suggesting an important role for these amino acids in the repressive activity (Table I). To test the importance of these amino acids for GR-mediated repression of RelA activity, we exchanged these amino acids in the rat GR to a glutamine and a glutamic acid, respectively (R488Q and K490E, respectively), and tested the ability of the mutated GRs for their ability to repress RelA transactivational activity in COS-1 cells. Transfection experiments showed that both GR mutants, R488Q and K490E, had lost almost all their repressive activity as compared with the wild type GR (Fig. 2B). In contrast, the GR mutants N491A and LS7 with substitutions of amino acids that are not conserved among the repressive receptors (Fig. 2A, Table I) had retained their ability to repress RelA activity. None of these C-terminal zinc finger GR mutants except the N491A mutant could transactivate the GRE tk-LUC reporter gene. The N491A mutant retained approximately 50% transactivational activity as compared with the wild type GR (Fig. 2C). The inability of the R488Q and K490E mutant to repress the RelA activity was not due to poor expression of the receptor proteins, since Western blot analysis of the transfected cells showed that the expression levels of the mutated receptors were the same as for the wild type GR (Fig. 2D). These results demonstrate that the arginine and the lysine residues in positions 488 and 490 in the rat GR (corresponding to positions 469 and 471 in the human GR) are critical for GR-mediated inhibition of RelA-dependent transactivation.

**Differen** Ability of Glucocorticoid Antagonists to Promote GR-dependent Repression of RelA-mediated Transactivation—The steroid analogs RU486 and ZK98299 are antagonists of GR transactivation but are able to stimulate GR transrepression of AP-1 activity (38). Furthermore, we and others (19, 20, 40) have previously shown that RU486 can also act as a partial agonist for GR- and PR-mediated repression of NF-κB activity. We tested if the steroid analog ZK98299 could inhibit NF-κB activity in analogy to RU486. As shown in Fig. 3A, 10 nM RU486 repressed RelA activity to a level that was approximately 40% the repression obtained with 10 nM dexamethasone in contrast, no repression was observed with 10 or 100 nM ZK98299. This was not due to the lack of biological activity of ZK98299, since it could inhibit GR transactivation as efficiently as RU486 (Fig. 3B). These results suggest that RU486 and ZK98299 induce GR to states with different competence to repress RelA activity.

**DISCUSSION**

The recent discovery of an inhibitory cross-talk between the NF-κB and GR signaling pathways has provided one molecular mechanism by which glucocorticoids exert their potent anti-inflammatory effects (17, 19, 20). The inhibition of NF-κB activity by estrogen receptor, PR, and AR has also been reported (39–42). Here we show that the MR also has the ability to repress RelA activity in a co-transfection assay. Thus, all steroid receptors are able to repress NF-κB in contrast to nuclear receptors from the RAR/TR subfamily, suggesting that a distinct structural determinant within the steroid receptor subfamily is responsible for the repression of NF-κB. The importance of the DBD in these receptors for the repressive activity has been demonstrated in several previous studies, where it was shown that deletion or replacement of the whole DBD resulted in the loss of the repressive activity (19, 20, 39–41). To identify which subdomain in the GR DBD is responsible for the repression of RelA activity, we have used GR mutants in which various parts of the GR DBD have been replaced with the corresponding regions of the non-repressive TRβ DBD. Our results demonstrate that most of the repression of NF-κB activity could be attributed to the C-terminal zinc finger of the GR DBD. This localizes a new function to this finger, which previously has been known mainly to harbor functions important for dimerization and transactivation (7, 37). Analysis of two C-terminal zinc finger GR mutants with substitutions of the arginine (amino acids 488 in the rat GR) and lysine (amino acids 490 in the rat GR) to a glutamine and a glutamic acid, respectively, confirmed the importance of this finger and identified two critical basic residues for repression of NF-κB activi-

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**TABLE I**

Comparison of the amino acid sequences in the tips of the second zinc finger of RelA repressive and some non-RelA repressive nuclear receptors

| Receptors | C-terminal zinc finger tip | RelA repression |
|-----------|---------------------------|----------------|
| GR        | CIIKIRKNC                 | Yes            |
| MR        | V                         | Yes            |
| PR        | T F                       | Yes            |
| ER        | N S                       | Yes            |
| RARα      | N VT NR                   | No             |
| TRβ       | V VT NQ                   | No             |
| EcdR      | EMMYMY RM                 | No             |

2 J. Liden, unpublished observations.
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ity. No particular function has previously been attributed to the arginine residue 488 (corresponding to amino acid 469 in the human GR) with regard to dimerization or interaction with DNA, since substitution of this residue to a glutamine did not impair DNA binding (34). The lysine residue 490 (corresponding to amino acid 471 in the human GR), on the other hand, is involved in making contact with the phosphate backbone (43). Interestingly, these two residues are conserved only in the C-terminal zinc finger of steroid receptors, consistent with the observation that only members of this subfamily of nuclear receptors seem to be able to repress NF-κB. Mutation of either of these two amino acids resulted in a significantly decreased transcriptional activity of GR (this study and Ref. 34). However, no correlation between transcriptional activation by GR and transrepression of RelA activity exists, since the two GR mutants D4X and LS7, which lack most of their transcriptional activity, were fully active with regard to repression of RelA activity. This is also in line with previous data showing that GR mutants with a deletion of the major transactivation domain r1 or a substitution of the P box by that of the TRβ were still repressive (19, 20). Thus, glucocorticoid induction of the NF-κB inhibitor IκBα which previously has been suggested as a mechanism controlling NF-κB inhibition by glucocorticoids cannot explain why transcriptionally deficient GR mutants can still repress NF-κB (26, 27). In addition, it has been shown in monocyte U937 cells that inhibition of ICAM-1 gene expression by glucocorticoids occurs in the absence of protein synthesis (44). More recently, several studies also reported that NF-κB could be inhibited in osteoblast U2-OS cells (39), alveolar epithelium-like A549/S cells (45), kidney epithelial NRK-52E cells (46), and aortic endothelial BAEC cells (47) in the absence of IκBα induction. Collectively, these data argue for a general mechanism for glucocorticoid inhibition of NF-κB which involves GR transrepression via protein–protein interaction between non-DNA binding GR and NF-κB transactivation factors as initially suggested (17, 19, 20). However, induction of IκBα may play a more significant role in some specific cell types such as lymphocytes (47).

Several results reported here together with previous observations indicate that the mechanisms by which GR inhibits NF-κB and AP-1 signaling pathways are different. Most notable is that MR, as reported in this study, is an efficient inhibitor of NF-κB activity. This finding identifies a new function for the GR C-terminal zinc finger. Furthermore, using C-terminal zinc finger GR mutants and antagonists, we further characterized the mechanism by which GR represses NF-κB, and we obtained evidence that the mechanisms by which GR inhibits NF-κB and AP-1 have different features. This knowledge could be very useful in the search for new GR ligands with selective activity for GR transrepression of different signaling pathways. Such ligands could be a useful tool for basic research regarding mechanisms of glucocorticoid action and possibly in clinical use with less side effects.
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