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DNA Binding of the Glucocorticoid Receptor Is Not Essential for Survival

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Summary

Transcriptional regulation by the glucocorticoid receptor (GR) is essential for survival. Since the GR can influence transcription both through DNA-binding-dependent and -independent mechanisms, we attempted to assess their relative importance in vivo. In order to separate these modes of action, we introduced the point mutation A458T into the GR by gene targeting using the Cre/loxP system. This mutation impairs dimerization and therefore GRE-dependent transactivation while functions that require cross-talk with other transcription factors, such as transrepression of AP-1-driven genes, remain intact. In contrast to *GR*$^{-/-}$ mice, these mutants termed *GR*^{dim} are viable, revealing the in vivo relevance of DNA-binding-independent activities of the GR.

Introduction

Nuclear hormone receptors are known to modulate gene transcription, upon binding of their cognate ligand, by activation as well as by repression (Beato et al., 1995). One of the best-studied members of the nuclear hormone receptor superfamily is the glucocorticoid receptor (GR), which plays an important role in physiology and development. Transactivation by the GR requires binding of receptor dimers to specific palindromic sequences in the *cis*-regulatory region of target genes called glucocorticoid response elements (GREs). The ability to dimerize depends on the D loop, a stretch of five amino acids located in the DNA-binding domain (DBD). Several contacts made by D loop residues at the dimerization interface stabilize receptor dimers and thereby allow cooperative DNA binding (Dahlman-Wright et al., 1991; Luisi et al., 1991).

Whereas the mechanism of transactivation is well characterized, transrepression of target genes by steroid hormones is much less understood. Most genes that are negatively regulated by the GR do not contain a classical GRE, and therefore, distinct modes of action using different classes of response elements, namely negative, composite, and tethering GREs, have been proposed to account for transrepression (Diamond et al., 1990; Miner et al., 1991). Negative GREs require DNA binding of the GR as exemplified by the *POMC* gene (Drouin et al., 1993). At composite elements (e.g., in the proliferin gene), the GR has to contact DNA together with another transcription factor whereas at tethering elements repression is mediated by protein–protein interaction without direct DNA binding of the GR. Of particular relevance are tethering interactions for genes that are regulated by AP-1 and NF-κB. Interstitial collagenase (collagenase type I and collagenase-3), whose expression is modulated via AP-1 (Angel et al., 1987; Gack et al., 1994; Schreiber et al., 1995), was one of the first genes for which repression by interaction with another transcription factor at a tethering element was demonstrated (Jonat et al., 1990; Schüle et al., 1990; Yang Yen et al., 1990; König et al., 1992). The repressive effect is probably mediated by GR monomers, and mutational analysis showed that the ligand-binding domain (LBD) and the DBD of the GR participate in this mode of action. By introducing point mutations into the GR, it has been shown that transactivation can be dissociated from transrepression in cell culture (Heck et al., 1994). For example, a GR with an amino acid exchange located in the D loop (A458T) fails to bind DNA and cannot transactivate GRE-dependent promoters in cell transfection studies. However, repression of the AP-1-dependent collagenase promoter by the mutant GR is almost as effective as by the wild-type receptor (Heck et al., 1994). In addition to the cross-talk with AP-1, protein–protein interaction between GR and NF-κB was also demonstrated and proposed to account for many of the immunosuppressive effects of glucocorticoids (Ray and Prefontaine, 1994; Caldenhoven et al., 1995; Scheinman et al., 1995b; Heck et al., 1997). Since similar regions of the GR are involved, the mechanism of transrepression seems to be a common regulatory principle of transcriptional control. However, induction of IκB by glucocorticoids has been proposed as an alternative mechanism for repression of NF-κB by glucocorticoids (Auphan et al., 1995; Scheinman et al., 1995a).

The generation of GR-deficient mice demonstrated that functions of the GR are essential for survival (Cole et al., 1995; F. Tronche and C. Kellendonk, personal communication). *GR*$^{-/-}$ mice die shortly after birth and display a number of severe abnormalties, including atelectasis of the lungs, impaired expression of gluconeogenic enzymes in the liver, derepression of the hypothalamus–pituitary–adrenal (HPA) axis, hyperplasia of the adrenal cortex, loss of glucocorticoid-dependent thy-

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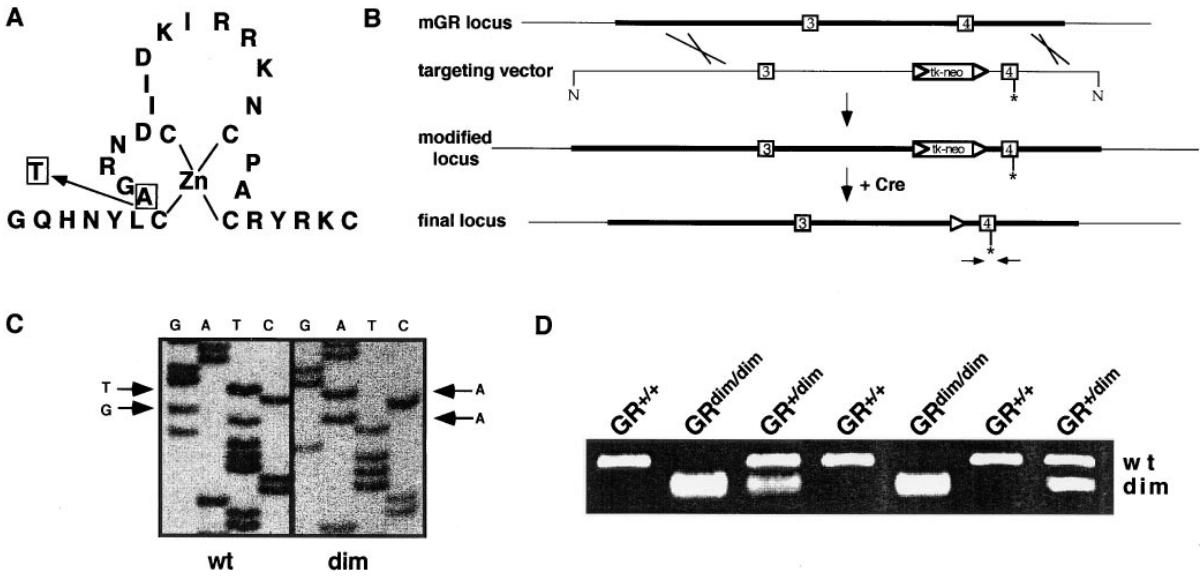

Figure 1. Generation of GR^{dim} Mice by Introduction of the Point Mutation A458T into the GR

(A) Amino acid sequence of the second zinc finger in the DNA-binding domain of the GR; the exchange of alanine 458 to threonine is indicated.

(B) Targeting strategy for homologous recombination. The asterisk marks the position of the point mutation in exon 4. The modified locus represents the structure of the *GR* locus after homologous recombination, and the final locus marks the structure after subsequent recombination of the two loxP sites (triangles) by Cre recombinase.

(C) Brain RNA was amplified by RT-PCR using primers located in sequences corresponding to exon 3 and 5; the resulting fragments from the wild-type allele (wt) and from the mutated allele (dim) were subcloned and sequenced. The two mutated bases are indicated by arrows.

(D) Genotyping of the offspring resulting from a heterozygous intercross by PCR. A 0.24 kb fragment was amplified using the primers indicated by arrows in (B) and digested with the restriction enzyme BsrGI for which a new recognition site had been introduced by the point mutation. The two 0.12 kb fragments generated from the GR^{dim} allele were separated from the uncut fragment by gel electrophoresis.

mocyte apoptosis, and impaired proliferation of erythroid progenitor cells. In order to dissect the importance of GR functions dependent on DNA binding and those mediated by protein–protein interaction, we have generated mice carrying a dimerization-defective GR. These mutants lack inducibility of GRE-dependent genes and show an impairment of several important physiological functions of the GR. Nevertheless, they are viable, indicating that activities of the GR dependent on DNA binding are not essential for survival.

Results

Generation and Analysis of GR^{dim} Mice

In cell transfection studies, the point mutation A458T was shown to abolish DNA-binding-dependent transactivation but not transrepression mediated by protein–protein interactions (Figure 1A) (Heck et al., 1994). To introduce this mutation into the GR via homologous recombination in ES cells, a Cre/lox-based targeting strategy (Gu et al., 1993) was employed to remove the selection cassette from the modified allele after homologous recombination and thus to guarantee faithful expression. A targeting vector containing 12.1 kb of homologous sequences from the *GR* gene, including the mutation in exon 4 and a loxP-tymidine kinase/neomycin phosphotransferase (*loxP-tk/neo*) selection cassette, was constructed and electroporated into ES cells. Homologously recombined clones were identified and transiently transfected with an expression plasmid for Cre recombinase to remove the *tk/neo* cassette (Figure 1B). The ES cell clones obtained are distinct from wild-type cells only by the point mutation and a stretch of approximately 50 bp around the remaining loxP site in intron 3. Two ES cell clones were used to generate chimeras, germline-transmitting mice were crossed with C57Bl/6 mice, and the resulting heterozygous mice were interbred. The presence of the point mutation was confirmed by RT-PCR with primers located in exons 3 and 5 and subsequent sequencing (Figure 1C). GR protein levels were found unchanged as demonstrated by Western blot analysis of brain and liver extracts as well as by immunohistochemistry of the hippocampus (data not shown). This indicates that the mutation had no effect on expression of the GR. Because the introduced mutation is in the dimerization domain, we named the mouse strain GR^{dim}.

Using a PCR-based strategy for genotyping (Figure 1D), we found an almost Mendelian ratio of the three genotypes with 21% homozygous mutants ($GR^{dim/dim}$) among the first 240 offspring. This was surprising since $GR^{-/-}$ mice are not viable and die shortly after birth due to respiratory failure (Cole et al., 1995). In contrast, $GR^{dim/dim}$ mice survive. They do not show any signs of impaired lung function such as atelectasis, and both sexes are fertile. From these findings, we conclude that the mutation does not affect viability. Taking into account that DNA-binding-dependent transactivation of target genes is widely considered to be the main mechanism of GR action, this result is highly interesting and raises the question of which GR properties are required for survival.

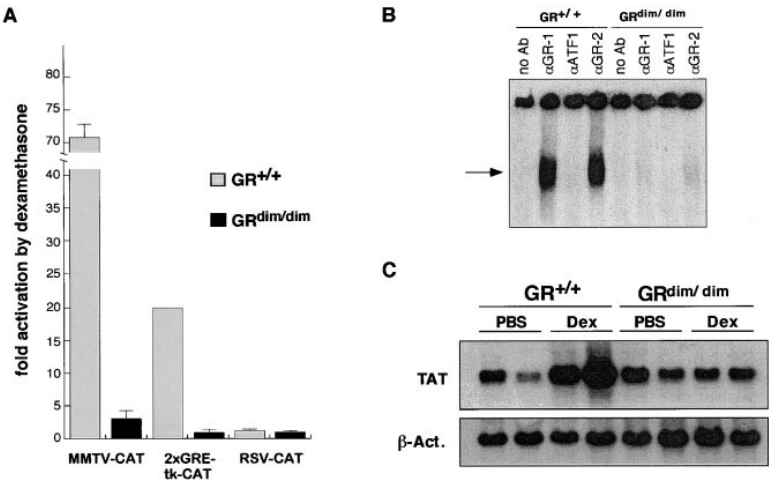

Figure 2. Analysis of DNA-Binding-Dependent Transactivation in $GR^{dim/dim}$ Mice

(A) Induction of GR-regulated promoters by dexamethasone in immortalized embryonic fibroblasts from $GR^{+/+}$ and $GR^{dim/dim}$ mice (3T3). Cells were transfected with 7.5 μg of *MMTV-CAT* or *2xGRE-tk-CAT* reporter plasmid or *RSV-CAT* plasmid as a control and treated with or without 10^{-7} M dexamethasone as described. *CAT* assays of cell extracts were performed and quantified using a phosphorimager. Each value of the diagram, showing fold activation of reporter gene expression in dexamethasone-treated versus untreated cells, represents the mean ± SD of at least two independent experiments performed with two $GR^{+/+}$ and two $GR^{dim/dim}$ cell lines.

(B) Analysis of GRE binding in $GR^{+/+}$ and $GR^{dim/dim}$ mice. Bandshift experiments with liver nuclear extracts from mice of both genotypes and a *2xGRE* were performed as described previously (Schmid et al., 1989). To obtain supershifts of the protein–DNA complexes (indicated by an arrow), antibodies against the GR-LBD (Cole et al., 1995) or ATF1 as a control were included in the assay.

(C) Induction of *TAT* mRNA by glucocorticoids in liver was analyzed. $GR^{+/+}$ and $GR^{dim/dim}$ mice were intraperitonally injected with 10 μg/100 g dexamethasone (Dex) or PBS and killed after 2 hr. Liver RNA was isolated, and expression of *TAT* and β-actin mRNAs as controls was analyzed by Northern blot.

$GR^{dim/dim}$ Mice Have Lost the Ability to Transactivate Gene Transcription by Cooperative DNA Binding

In order to confirm the absence of DNA-binding-dependent transcriptional activation in $GR^{dim/dim}$ mice, we analyzed induction of GRE-dependent transcription by dexamethasone in immortalized embryonic fibroblasts obtained from $GR^{+/+}$ and $GR^{dim/dim}$ mice. Cells were transiently transfected either with a *MMTV-CAT* or a *2xGRE-tk-CAT* reporter and cultured in the absence or presence of dexamethasone. In $GR^{+/+}$ cells, *MMTV-CAT* was activated 71-fold and *2xGRE-tk-CAT* 20-fold by dexamethasone. In contrast, two independent cell lines derived from $GR^{dim/dim}$ mice exhibited only minimal activation (Figure 2A). This demonstrates that DNA-binding-dependent transcriptional regulation of GRE-dependent genes is indeed abolished in $GR^{dim/dim}$ mice.

To show that the loss of transactivation is due to impaired DNA binding, we performed band shift experiments with liver nuclear extracts obtained from $GR^{+/+}$ and $GR^{dim/dim}$ mice. In extracts derived from $GR^{+/+}$ mice, we obtained supershifts with a high affinity *2xGRE* (Schmid et al., 1989) using two different antibodies directed against the LBD of the GR (Cole et al., 1995). However, binding in extracts from $GR^{dim/dim}$ mice was dramatically reduced (Figure 2B). No supershift was obtained without antibody or with an antibody directed against the transcription factor ATF1. It is noteworthy that the GR antibodies used in this study recognize the mutant GR protein on a Western blot of nuclear extracts with equal affinity. Thus, we conclude that DNA binding in $GR^{dim/dim}$ mice is strongly impaired.

A good paradigm to test transcriptional activation of endogenous genes by the GR is inducibility of gluconeogenic enzymes by glucocorticoids, a process known to be GRE-dependent (Nitsch et al., 1990). $GR^{+/+}$ and $GR^{dim/dim}$ mice were injected with dexamethasone and analyzed for mRNA expression of tyrosine aminotransferase (*TAT*) in liver (Figure 2C). As shown previously, *TAT* mRNA is induced by dexamethasone in wild-type mice (Ruppert et al., 1990). However, in $GR^{dim/dim}$ mice no induction was observed although basal *TAT* mRNA levels were unaltered. Similar results were obtained for other gluconeogenic enzymes, such as serine dehydrogenase and phosphoenolpyruvate carboxykinase (data not shown). Taken together, these findings are consistent with the transfection data obtained in fibroblasts and confirm that transcriptional control by the glucocorticoid receptor dependent on GRE binding is absent in $GR^{dim/dim}$ mice.

The Repressing Function of the Glucocorticoid Receptor Is Retained in $GR^{dim/dim}$ Mice

In addition to the absence of GRE binding activity, a main characteristic of the D loop mutation A458T in cultured cells was the ability of the mutant receptor to regulate transcription negatively by protein–protein interaction (Heck et al., 1994). To show that this property of the GR was indeed unaffected in $GR^{dim/dim}$ mice, we studied AP-1-mediated repression of the collagenase-3 gene by the GR (Jonat et al., 1990; König et al., 1992). Primary embryonic fibroblasts were isolated from $GR^{+/+}$ and $GR^{dim/dim}$ mice, treated with either dexamethasone, phorbol ester, or a combination of both and analyzed for collagenase-3 mRNA expression (Figure 3A). Only very little expression could be detected in untreated cells or after treatment with dexamethasone alone, whereas treatment with phorbol ester, which is known to induce transcription via AP-1 (Angel et al., 1987; Gack et al., 1994; Schreiber et al., 1995), led to a strong up-regulation. This induction could be significantly reduced by addition of dexamethasone in $GR^{+/+}$ and $GR^{dim/dim}$

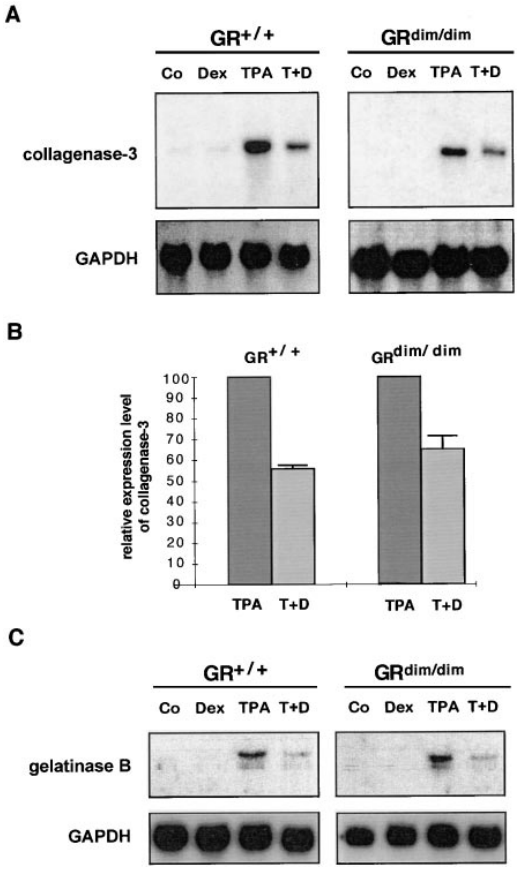

Figure 3. Analysis of DNA-Binding-Independent Transrepression in $GR^{dim/dim}$ Mice

(A) Induction of collagenase-3 expression by phorbol ester (TPA) and subsequent repression by dexamethasone was analyzed in primary embryonic fibroblasts. Fibroblasts were isolated from $GR^{+/+}$ and $GR^{dim/dim}$ embryos of the same litter at day E14.5 and treated with 10^{-6} M dexamethasone (Dex), 10^{-7} M phorbol ester (TPA), or both (T + D). RNA was isolated 6 hr after treatment, and levels of collagenase-3 and *GAPDH* mRNAs as controls were analyzed by Northern blot. The bands obtained were quantified using a phosphorimager system.

(B) The results of three independent experiments using independent isolates of $GR^{+/+}$ and $GR^{dim/dim}$ fibroblasts are summarized. The relative expression level of collagenase-3 after TPA as well as combined TPA/dexamethasone (T + D) treatment is depicted.

(C) Induction of gelatinase B expression by phorbol ester (TPA) and repression by dexamethasone were analyzed in primary embryonic fibroblasts as described above.

cells. Data obtained in three independent sets of experiments demonstrated that repression in the absence of DNA binding was nearly as efficient in $GR^{dim/dim}$ cells as in $GR^{+/+}$ cells (Figure 3B). In order to obtain additional evidence that the repressing function of the GR is retained in $GR^{dim/dim}$ mice, we analyzed in a similar way mRNA expression of gelatinase B (Gum et al., 1996), another gene that is inducible in an AP-1-dependent manner (Figure 3C). Using cells from two different $GR^{dim/dim}$ mice, we found that repression of gelatinase B mRNA expression by dexamethasone is also unimpaired in the mutants. Taken together, these results show that the repressing function of the GR dependent on cross-talk of the GR with transcription factors such as AP-1 is retained in $GR^{dim/dim}$ mice.

The Regulation of the HPA Axis Depends on Both DNA-Binding-Dependent and -Independent Functions of the GR

Since GRE-dependent gene activation is obviously not essential for viability, it was important to define which GR properties were retained in $GR^{dim/dim}$ mice. Therefore, we characterized several physiological functions involving regulation by the GR.

A physiological system to look for negative GR functions is the autoregulatory circuit of the HPA axis. This system is activated during stress and controls secretion of glucocorticoids by the adrenal gland. In a neuroendocrine cascade, the hypothalamic hormone corticotropin-releasing factor (CRF) stimulates synthesis and secretion of adrenocorticotrophe hormone (ACTH) from the anterior pituitary. Consequently, glucocorticoids are released and inhibit the system by negative feedback via the GR. In the hypothalamus and the anterior lobe of the pituitary, this feedback regulation is exerted both at the level of mRNA synthesis and hormone secretion (Birnberg et al., 1983). As distinct molecular mechanisms are thought to account for these different levels of control, we analyzed components of the HPA axis for changes in $GR^{dim/dim}$ mice.

To assess the mechanism of feedback regulation at the level of the hypothalamus, we measured CRF immunoreactivity in the median eminence, the site where the hormone is released into the portal blood system of the anterior pituitary. CRF, which is considered the main stimulus for ACTH secretion, is subject to repression by the GR, and consequently, expression is strongly increased in $GR^{-/-}$ mice (Reichardt and Schütz, 1996; unpublished data). In contrast, in $GR^{dim/dim}$ mice CRF expression was not found to be altered as determined by microdensitometry (Figures 4A and 4B). However, in the anterior lobe of the pituitary, we found a strong elevation in proopiomelanocortin (*POMC*) mRNA expression (Figure 4C and 4D) as well as a 2.2-fold increase of ACTH immunostaining (Figures 4E and 4F). This shows that in contrast to CRF expression in the hypothalamus, feedback inhibition of *POMC*/ACTH by the GR in the pituitary is abolished at the level of transcription. Taken together, these results demonstrate different requirements of DNA binding for transcriptional regulation by the GR.

In addition to *POMC* in the anterior lobe, mRNA expression of other hormones in the pituitary, such as prolactin and *POMC* in the neurointermediate lobe, is also regulated by the GR. Whereas prolactin is negatively regulated by the GR (Sakai et al., 1988), *POMC* in the neurointermediate lobe is under positive control of the GR most likely by an indirect mechanism (Reichardt and Schütz, 1996). In order to characterize the regulation of these two genes by the GR in more detail, we performed an RNase protection assay (Figure 4G). In contrast to $GR^{-/-}$ mice where a strong decrease in *POMC*

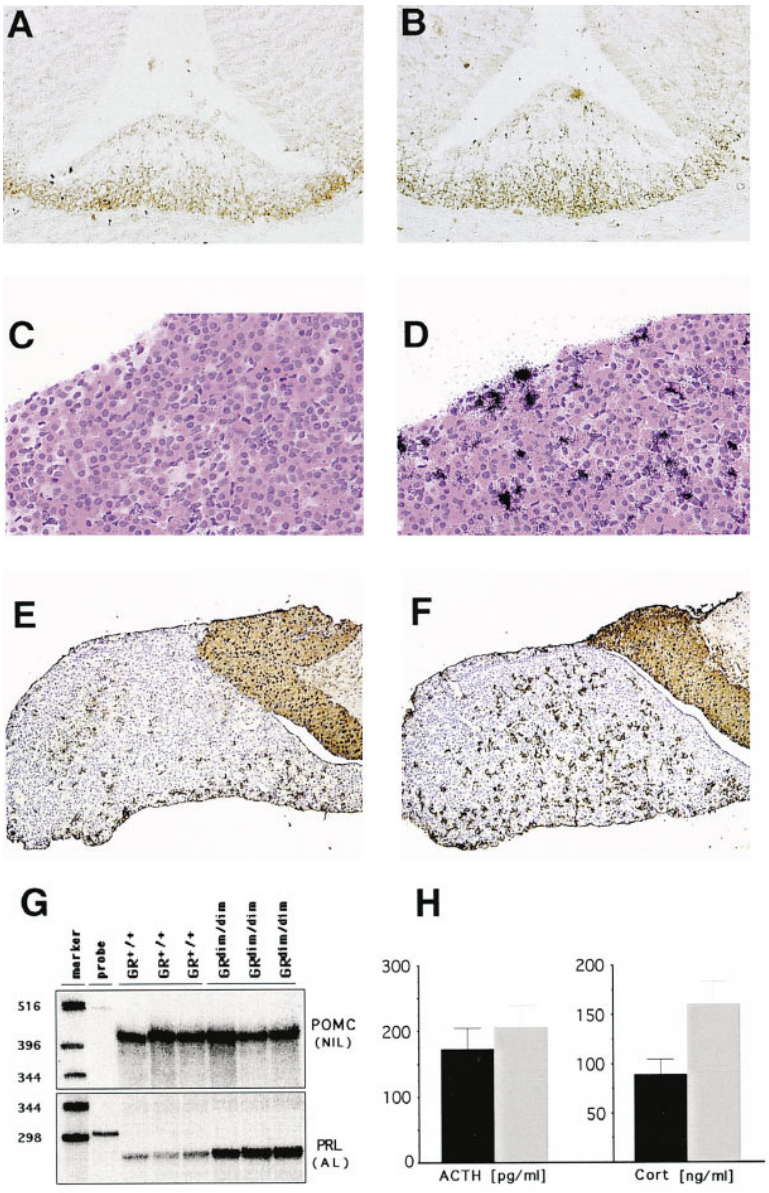

Figure 4. Analysis of Components of the HPA Axis in $GR^{+/+}$ and $GR^{dim/dim}$ Mice

(A and B) Immunostaining of the median eminence in newborn mice for CRF peptide expression. Optical densities of specifically stained structures were determined by microdensitometry (wt, 0.0313 ± 0.023; $GR^{dim/dim}$, 0.0245 ± 0.0116; n = 4).

(C and D) In situ hybridization of the anterior lobe of the pituitary for *POMC* mRNA expression.

(E and F) Immunostaining of the pituitary for ACTH peptide expression in the anterior lobe of the pituitary. Quantification of specific immunoreactivity (optical density × percentage of stained area) was obtained by microdensitometry (wt, 4.693 ± 1.746; $GR^{dim/dim}$, 10.416 ± 3.8624; n = 4)

(G) RNase protection analysis of pituitary mRNA expression for proopiomelanocortin (*POMC*) in the intermediate lobe (NIL) and prolactin (PRL) in the anterior lobe (AL). Total RNA (0.5 μg) was used in each lane. Molecular size markers and the position of protected fragments are indicated. The bands obtained were quantified using a phosphorimager system.

(H) Serum concentrations of ACTH and corticosterone (CORT) were determined by RIA. In the Student's t-test, the increase in the serum level of corticosterone was significant (p = 0.017; n = 12/8), but not in the case of ACTH (p = 0.483; n = 9/8).

mRNA expression had been observed in the neurointermediate lobe (Reichardt and Schütz, 1996), no alterations were found in $GR^{dim/dim}$ mice. However, prolactin mRNA expression was elevated 3.1-fold in $GR^{dim/dim}$ mice, which is comparable to the increase seen in $GR^{-/-}$ mice (unpublished data). Again these findings demonstrate that gene regulation by the GR is affected differently in $GR^{dim/dim}$ mice.

As $GR^{-/-}$ mice show a strong elevation of hormone levels in the serum and dramatic alterations in the adrenals (Cole et al., 1995), we also analyzed $GR^{dim/dim}$ mice for such alterations. Thereby we found the serum level of corticosterone (Figure 4H) and mRNA expression of side-chain cleavage enzyme (*SCC*, Figures 5C and 5D), one of the key steroidogenic enzymes in the adrenal cortex, up-regulated in $GR^{dim/dim}$ mice, whereas no change was found in the serum level of ACTH (Figure 4H) and adrenal morphology (Figures 5A and 5B).

Development and Function of the Adrenal Medulla Are Not Impaired in $GR^{dim/dim}$ Mice

In the adrenal medulla, the GR is involved, among others, in the control of catecholamine synthesis (Jiang et al., 1989). In $GR^{-/-}$ mice, the medulla is disorganized, and mRNA expression of phenylethanolamine-N-methyltransferase (*PNMT*), an enzyme involved in adrenalin synthesis, is impaired (Cole et al., 1995). In contrast, in $GR^{dim/dim}$ mice the development of the adrenal medulla is unaltered as deduced from the lack of histological abnormalties in adult mice (Figure 5A and 5B). To compare *PNMT* mRNA expression in the adrenals of $GR^{+/+}$, $GR^{-/-}$, and $GR^{dim/dim}$ mice, a RNase protection assay was

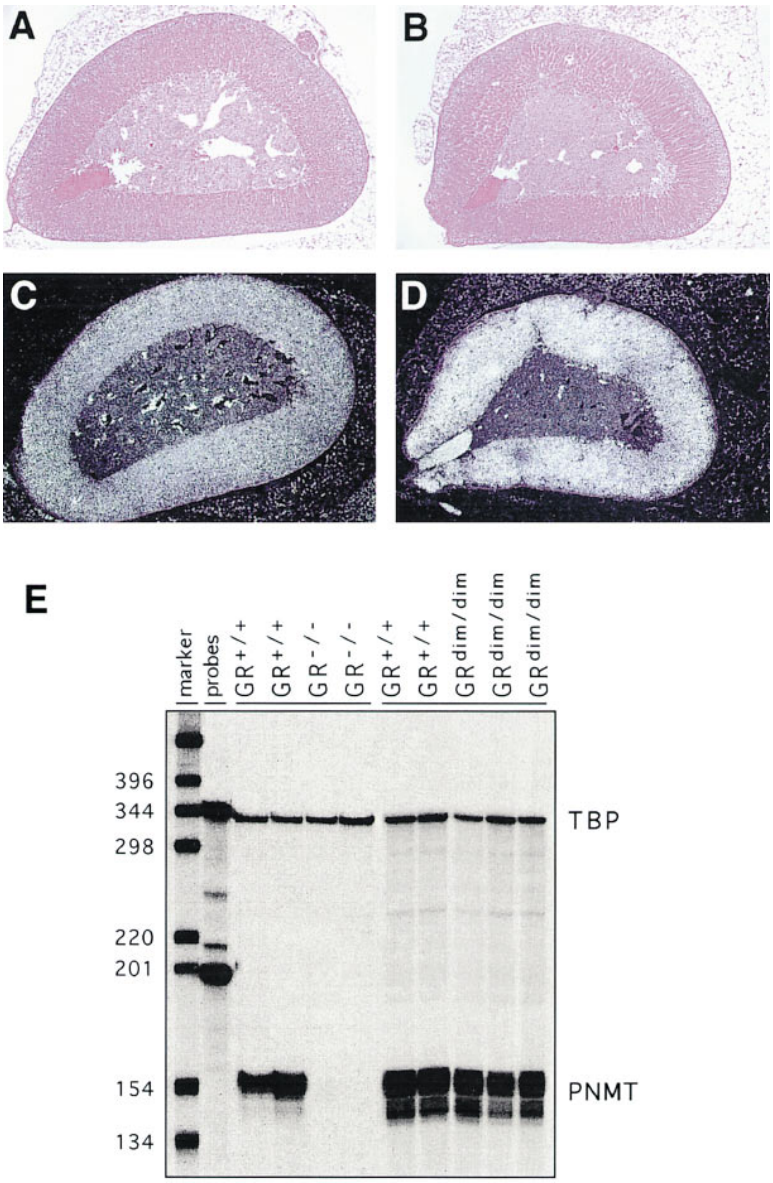

Figure 5. Analysis of Development and Function of the Adrenal Gland in $GR^{+/+}$ and $GR^{dim/dim}$ Mice

(A and B) Morphology of the adrenal gland (hematoxylin-eosin staining).

(C and D) In situ hybridization of the adrenal gland for *SCC* mRNA expression.

(E) RNase protection analysis of adrenal mRNA expression of *PNMT* and *TBP* as controls in adult mice. Total RNA (5 μg) was used in each lane. Molecular size markers and the positions of the protected fragments are indicated. For comparison, RNA from $GR^{-/-}$ mice was included in this experiment.

performed. Interestingly, whereas in the $GR^{-/-}$ mice no *PNMT* mRNA could be detected at all, expression in $GR^{dim/dim}$ mice was unaffected (Figure 5E).

DNA Binding of the GR Is a Prerequisite for Glucocorticoid-Mediated Thymocyte Apoptosis and Long-Term Proliferation of Erythroblasts

The GR is thought to control T-lymphocyte development and differentiation by glucocorticoid-induced apoptosis. Whether the molecular mechanism of apoptosis is a consequence of DNA-binding-dependent transcriptional activation or is due to repression of survival factors by the GR has been a controversial topic for years (Nazareth et al., 1991; Beato et al., 1995; Helmberg et al., 1995; Chapman et al., 1996). To address this question, we analyzed glucocorticoid-mediated apoptosis of T cells in GR^{dim} mice. Thymocytes were isolated and cultured in the presence or absence of dexamethasone, and after staining with propidium iodide, the DNA content as a measure of apoptosis was analyzed by FACS (Figures 6A and 6B). Whereas in $GR^{+/+}$ and $GR^{+/dim}$ mice apoptosis was strongly induced, indicated by massive cell death, T-lymphocytes from $GR^{dim/dim}$ mice were refractory to dexamethasone. To investigate the consequence of the loss of glucocorticoid-dependent apoptosis, we analyzed the CD4/CD8 thymocyte profile in $GR^{dim/dim}$ mice. Interestingly, in contrast to the clear phenotype observed for apoptosis, we could not detect any difference in the relative abundance of T-lymphocyte subtypes (data not shown).

An important physiological function of the GR in erythropoiesis is the induction of long-term proliferation of erythroid progenitor cells, and in vitro experiments had indicated that this most likely requires the DNA binding activity of the GR (Wessely et al., 1997). To analyze

GR$^{dim/dim}$ mice for this function, fetal livers from *GR*$^{+/+}$ and *GR*$^{dim/dim}$ mice were isolated and erythroid cells cultured in vitro. After 12 days, cells from *GR*$^{+/+}$ mice had rapidly proliferated while those prepared from *GR*$^{dim/dim}$ mice had not significantly increased in number (Figure 6C). Cytospins of the cultures from *GR*$^{+/+}$ mice contained mainly outgrowing erythroblasts whereas those from mutants were composed of mature erythrocytes and mast cells (Figure 6D). Thus, GRE-dependent activation of target genes is required for the proliferation of erythroid progenitor cells.

Discussion

DNA Binding of the GR Is Not Required for the Survival of *GR*$^{dim/dim}$ Mice

We have generated mice by gene targeting carrying a point mutation (A458T) of the GR known to impair dimerization and DNA binding. We were able to show that transcriptional regulation dependent on DNA binding is dispensable for survival of *GR*$^{dim/dim}$ mice. Despite the impairment of several important physiological functions of the GR, the mutation does not reduce viability. Interestingly, *GR*$^{dim/dim}$ mice do not show lung atelectasis, the cause of death in *GR*$^{-/-}$ mice. Since in these mutants functions dependent on GRE binding are no longer present, cross-talk of the GR with other transcription factors is most likely reponsible for survival of *GR*$^{dim/dim}$ mice.

A Mutation in the GR Allows Separation of DNA-Binding-Dependent and -Independent Functions

Using well-known paradigms for transactivation dependent on DNA binding of the GR, namely induction of GRE-containing reporters in embryonic fibroblasts and gluconeogenic enzymes in liver by dexamethasone, we could demonstrate loss of transcriptional activation by the GR. Additionally we were able to show that DNA binding of the mutant GR is dramatically impaired. In contrast, repression of other transcription factors, such as AP-1, by the GR, was shown to be intact in the case of phorbol ester–induced collagenase-3 and gelatinase B expression. These experiments thus indicate that *GR*$^{dim/dim}$ mice separate GRE-binding-dependent transactivation from transrepression via cross-talk of the GR with other transcription factors. The demonstration that CRF expression as well as inhibition of collagenase-3 and gelatinase B transcription by glucocorticoids were unaffected by the mutation presents in vivo evidence for DNA-binding-independent gene repression. In the future, it will be interesting to extend the analysis of negative regulation by the GR to other transcription factors, such as NFκB (Caldenhoven et al., 1995; Scheinman et al., 1995b; Heck et al., 1997), CREB (Imai et al., 1993), and GATA-1 (Chang et al., 1993), which are also targets of GR-mediated regulation.

The HPA Axis Represents a Complex Regulatory Network Involving Different Modes of Action of the GR

The analysis of the HPA axis revealed that the GR can operate via different mechanisms in the same physiological system. The level of CRF peptide in the median eminence was unaffected by the mutation; however, *POMC* mRNA expression was increased several-fold, demonstrating the complexity of the regulatory network of feedback inhibition. The fact that expression of CRF in *GR*$^{dim/dim}$ mice is not elevated suggests a mechanism of repression based on protein–protein interaction. Possible targets for such cross-talk are the transcription factors CREB and Nur77; for both, the *CRF* promoter contains response elements (Seasholtz et al., 1988; Murphy and Conneely, 1997). In the case of *POMC*, mRNA expression in *GR*$^{dim/dim}$ mice is strongly increased and thereby provides evidence that repression of *POMC* in vivo requires DNA binding activities of the GR. Obviously, binding of the GR to the negative GRE (nGRE) in the promoter (Drouin et al., 1993) is a prerequisite for repression of basal *POMC* gene transcription, although we cannot exclude that other mechanisms of the GR are operative in the repression of stimulated transciptional activity (Philips et al., 1997). Another pituitary gene for which an nGRE had been described is the prolactin gene (Sakai et al., 1988). We found prolactin mRNA expression to be increased more than 3-fold in *GR*$^{dim/dim}$ mice, providing an in vivo experiment that demonstrates DNA-binding-dependent repression of this gene. Together with the results obtained for the regulation of the *POMC* gene, these findings strengthen the concept of nGRE-dependent gene control as one posssible mechanism for GR-mediated transrepression.

As expected from the elevated *POMC* mRNA levels, ACTH in the anterior lobe of the pituitary was also elevated. Interestingly, despite the 2.2-fold increase in ACTH immunoreactivity in the anterior pituitary, we found no statistically significant change in the ACTH serum level, which is in sharp contrast to the more than 10-fold elevation of ACTH in *GR*$^{-/-}$ mice. This might be explained by a DNA-binding-independent mechanism involved in the regulation of ACTH secretion. The fact that derepression of *POMC* expression and ACTH release after adrenalectomy follow different time courses supports this interpretation (Birnberg et al., 1983). A summary of how the different activities of the GR contribute to the regulation of the HPA axis is given in Figure 7.

In contrast to *GR*$^{-/-}$ mice, the analysis of *GR*$^{dim/dim}$ mice revealed normal organization of the adrenal medulla as well as unaltered *PNMT* expression. Although *PNMT* is one of the classical examples for genes regulated by a GRE (Ross et al., 1990), we conclude that at least basal expression of *PNMT* is independent of the DNA-binding activity of the GR.

Glucocorticoid-Dependent Apoptosis in Thymocytes and Proliferation of Erythroblasts Is Mediated by the Transactivation Function of the GR

Glucocorticoids are potent inducers of thymocyte apoptosis. However, direct target genes are unknown, and the molecular mechanism by which the GR regulates apoptosis is still controversial (Nazareth et al., 1991; Helmberg et al., 1995; Chapman et al., 1996). By the generation of *GR*$^{dim/dim}$ mice, we have now provided compelling evidence that glucocorticoid-mediated apoptosis of thymocytes requires DNA binding of the GR. Interestingly, despite the pronounced effects of the mutation

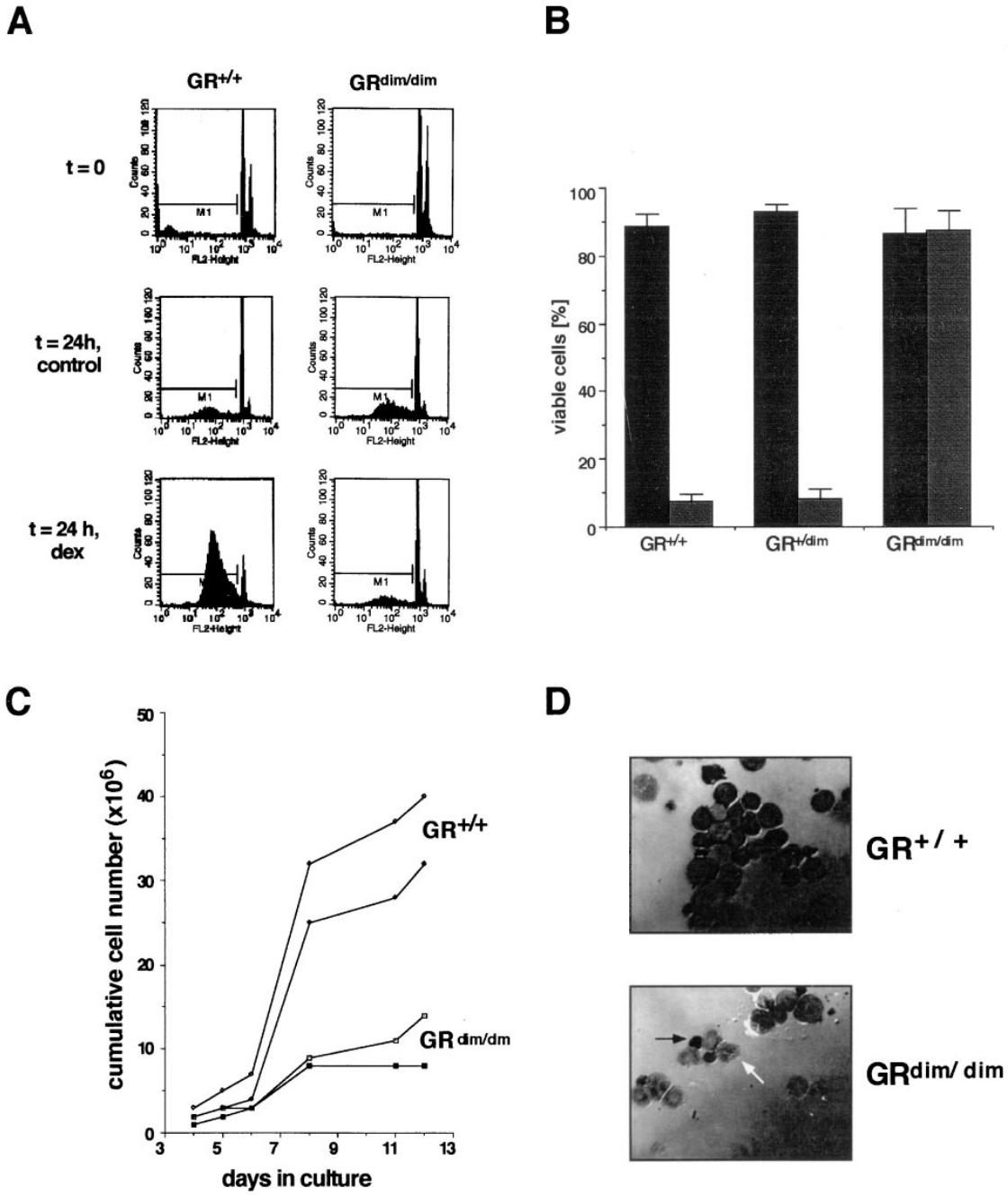

Figure 6. Analysis of Thymocyte Apoptosis and Proliferation of Erythroid Progenitors in $GR^{+/+}$ and $GR^{dim/dim}$ Mice

(A) Thymocytes were isolated and cultured in vitro for the indicated time in the presence or absence of 10^{-6}M dexamethasone (dex). The cells were stained with propidium iodide and analyzed by FACS. M1 indicates sub-*G1* DNA, which was considered as an indication of cell death. The results from one representative experiment are shown.

(B) Thymocytes with a DNA content above a threshold level were considered viable and counted. The percentage of viable cells after 24 hr treatment with dexamethasone (black bars) or in controls (gray bars) is shown. In this experiment, results obtained for $GR^{+/dim}$ mice were included.

(C) Erythroid progenitor cells were isolated at day E14.5 from livers of $GR^{+/+}$ and $GR^{dim/dim}$ embryos and cultured as described. Cumulative cell numbers were determined by cell counting and are individually depicted.

(D) Cytospins of cultures derived from $GR^{+/+}$ and $GR^{dim/dim}$ embryos. Mature erythrocytes (black arrow) and mast cells (white arrow) are indicated.

on T cell apoptosis, we were not able to detect differences in the CD4/CD8 thymocyte profile, which is in contrast to observations made in mice carrying a *GR* antisense transgene (King et al., 1995). To analyze regulation of the immune system and thymocyte functions further in $GR^{dim/dim}$ mice, in vivo studies are in progress.

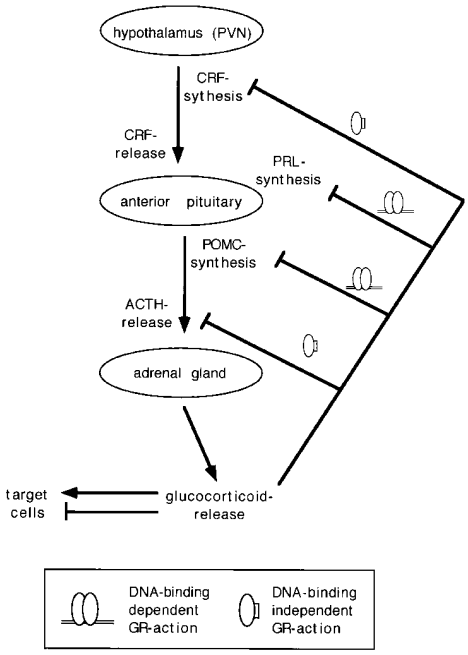

Figure 7. Proposed Model for the Feedback Regulation of the HPA Axis by the GR

CRF synthesis in the paraventricular nucleus (PVN) of the hypothalamus and ACTH secretion from the anterior pituitary are most probably mediated by a DNA-binding-independent mechanism. In contrast, repression of *POMC* synthesis and *PRL* synthesis in the anterior pituitary requires DNA binding by the GR.

The GR plays an important role in erythropoiesis, especially in the long-term proliferation of erythroblasts (Wessely et al., 1997). Using mutants with altered DNA binding specifity, it had been shown that, most likely, binding to a GRE was essential for this function. Here we provide additional evidence for this finding by the demonstration that long-term proliferation is abolished in erythroid progenitor cells derived from $GR^{dim/dim}$ mice that are devoid of GRE-binding activity.

$GR^{dim/dim}$ Mice Open up the Opportunity to Develop New Pharmacological Drugs with High Selectivity

Application of glucocorticoids in medical therapy is often hampered by unfavorable side effects of presently available drugs. In the search for GR ligands with only a limited range of physiological effects, $GR^{dim/dim}$ mice may now serve to develop new and better drugs that specifically interfere with GR functions present in these mice. First attempts to develop such GR ligands acting preferentially through the DNA-binding-independent repressing function of the GR have already been successfully made in a cell culture system (Vayssiére et al., 1997), and thus, approaches using $GR^{dim/dim}$ mice seem promising. We conclude that the generation of mice carrying a DNA-binding-deficient GR not only offers the possibility of addressing questions on the molecular mechanism and the physiological role of the GR, but may also contribute to the development of new approaches in the therapy of major diseases.

Experimental Procedures

Gene Targeting

GR^{dim} mice were generated by homologous recombination in the E14/1 ES cell line using the Cre/loxP system. λ phage clones containing exon 4 of the murine *GR* gene have been isolated from a mouse ES cell library (Kaestner et al., 1994). A targeting vector (pmGR2-tv) was contructed from 12.1 kb of homologous sequences, a *tk/neo* selection cassette flanked by two loxP sites and a genomic 0.9 kb HindIII fragment containing exon 4 in which the point mutation had been introduced by site-directed mutagenesis. Targeting vector (20 μg) was electroporated into 10^7 ES cells, and stably transfected clones were isolated after selection with 350 μg/ml G418. Homologously recombined clones identified by Southern blot were transiently transfected with 20 μg of a Cre expression plasmid, and after treatment with 1 μM gancyclovir, surviving clones were picked and analyzed for recombination of the two loxP sites. Positive mutant clones were used to produce chimeric animals by blastocyst injection, which were then mated to C57Bl/6 for germline transmission. Heterozygous mice ($GR^{+/dim}$) were intercrossed to generate $GR^{dim/dim}$ mice. Embryos and adult mice were genotyped by PCR using two primers: 5′-primer (GTGTCTTGATGATAGTCTGCTC) and 3′-primer (CCATTACCTTCCAGGTTCATTC). PCR reactions were carried out for 35 cycles (94°C, 1 min; 58°C, 1 min; 72°C, 1 min) in a buffer containing 2 mM $MgCl_2$. To distinguish between both alleles, an aliquot of the PCR reaction was digested with the enzyme BsrGI and analyzed on an agarose gel. The wild-type allele gave a 0.24 kb band whereas, in the case of the targeted allele, two smaller bands of approximately equal size were produced due to the new restriction site introduced by the point mutation.

RNA Analysis

Total RNA from cells and tissues was isolated after homogenization in guanidinium thiocyanate. For Northern analysis, the RNA was separated in formaldehyde-containing agarose gels and hybridized according to Ruppert et al., 1990. The cDNA probes for TAT, collagenase-3, gelatinase B, β-actin, and *GAPDH* were described previously (Ruppert et al., 1990; Gack et al., 1994; Schreiber et al., 1995).

RNAse protection analysis was performed as previously described (Kaestner et al., 1989) using [^{32}P]-αUTP-labeled antisense RNA probes. The probes were hybridized overnight against an appropriate amount of total RNA at 54°C in 80% formamide, and excess probe was removed by digestion with RNAses A and T1. The protected fragments were analyzed on a denaturing 6% polyacrylamide gel after exposure to an autoradiographic film. For the analysis of *PNMT* expression, a probe was synthesized from a plasmid containing a 152 bp ClaI/SmaI fragment from the third exon of the mouse gene. The probes for *POMC* and *PRL* represented cDNA fragments of the respective genes of 0.9 kb and 0.6 kb, respectively.

For RT-PCR analysis, first-strand cDNA synthesis was performed at 42°C for 50 min using 2 μg of total RNA as template and hexanucleotides as primers. To check for the presence of the mutation in *GR* mRNA from the targeted allele, a PCR reaction with 1/10 of the cDNA was carried out for 35 cycles (94°C, 1 min; 64°C, 1 min; 72°C, 1 min) in a buffer containing 1.5 mM $MgCl_2$ using two primers: 5′-primer (CTGGTGTGCTCCGATGAAGC) and 3′-primer (GAGACT CCTGCAGTGGCTTG).

Cell Culture

Primary mouse embryonic fibroblasts were isolated and immortalized as described (Todaro and Green, 1963; Brüsselbach et al., 1995). Each primary fibroblast culture was isolated from a single E14.5 mouse embryo, and each 3T3 fibroblast line was immortalized from an individual primary culture. Cells were cultured in DMEM containing 10% FCS. Transfection of immortalized fibroblasts using the calcium phosphate method, followed by chloramphenicol acetyltransferase (*CAT*) assays, was performed as described (Oehler and Angel, 1992). In brief, 5×10^5 cells were seeded, and 24 hr later, the DNA precipitate was added. After 12 hr, the culture medium was removed and cells were kept in fresh medium. Cells were mock treated or treated with dexamethasone (10^{-7} M) 12 hr later and

incubated for an additional 12 hr. The tranfected reporter gene constructs *MMTV-CAT*, *2xGRE-tk-CAT*, and *RSV-CAT* have been described previously (Cato et al., 1988; Strähle et al., 1988; Oehler and Angel, 1992).

In Situ Hybridization

Tissues were dissected quickly and fixed in 4% paraformaldehyde (pH 7.2) overnight, dehydrated through an ethanol series, cleared in toluene, and embedded in paraffin. Sections (5 μm) from each tissue were sealed on TESPA-treated slides. In situ prehybridizations and hybridizations were carried out as described in Wilkinson, 1992. Hybridizations were done overnight at 58°C in 50% formamide using [^{35}S]-αUTP-labeled antisense RNA probes at a concentration of 60 ng/ml. The first posthybridization wash was performed at 62°C and the second at 68°C, both in 50% formamide. The final wash in 0.1× SSC at 55°C was done before dehydration of the sections. Slides were then dipped in Kodak NTB2 emulsion diluted 1:1 with water, exposed at 4°C for 2–8 days, and developed using Kodak D19 developing solution and Kodafix at 15°C for 4 min. Sections were stained using eosin and hematoxylin and visualized using a Zeiss Axiophot microscope. The probes for *POMC* and *SCC* were described previously (Cole et al., 1995; Reichardt and Schütz, 1996).

Immunohistochemistry

Embryos and tissues were immerse-fixed in Bouin solution for 3 days at 4°C, dehydrated through an ascending alcohol series, and embedded in paraffin. Immunostaining was performed on serial sections of 7 μm using polyclonal antibodies against human CRF or ACTH as primary antibodies. Visualization of antigen–antibody complexes was achieved with goat anti-rabbit IgG conjugated with horseradish-peroxidase and 3,3′-diaminobenzidine as substrate. Optical density of specifically stained structures was measured with the IBAS image processing system (Kontron, Germany) according to the method described (Bock et al., 1991).

Hormone Measurements

Male mice at 3 months of age were sacrificed in the morning around 9:00 AM using CO_2, and the blood was collected from the inferior vein. Hormone concentrations in the serum were measured using commercially available RIA kits according to the suppliers' instructions (ICN Biomedicals, Meckenheim, Germany).

Analysis of Thymocyte Apoptosis

The thymus was isolated from 6–12 week old mice, teased in RPMI medium, and expelled trough a nylon mesh into a centrifugation tube. Thymocytes were centrifuged for 10 min at 800 rpm, washed twice, and the concentration adjusted to 3.0×10^6 cells/ml. Thymocytes were cultured in RPMI medium supplemented with 10% FCS, β-mercaptoethanol, glutamate, streptomycin/penicillin for 24 hr at 37°C in the presence or absence of 10^{-6}M dexamethasone. Subsequently, cells were stained with propidium iodide and analyzed by flow cytometry as described elsewhere (Nicoletti et al., 1991). The reduction in DNA content was taken as a measure for apoptosis.

Analysis of Erythroid Progenitor Outgrowth

Individual fetal livers were isolated at day E14.5, dispersed into single cell suspension, and expanded in modified CFU-E medium supplemented with SCF, hEpo, IGF-1, and dexamethasone. Outgrowth kinetics of erythroid progenitors were monitored by daily cell countings using an electronic cell counter (Schaerfe Systems, Germany) and depicted as cumulative cell numbers. For morphological analysis, cells were cytocentrifuged onto slides, stained with histological dyes and neutral benzidine, and photographed using a CCD camera and a blue filter so that mature erythrocytes appear darkly stained.

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