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Mutagenesis of the glucocorticoid receptor in mice ☆

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Abstract

The glucocorticoid receptor is an ubiquitously expressed transcription factor involved in the regulation of many different physiological processes. Activated by glucocorticoids the receptor regulates transcription positively or negatively either by direct binding to DNA or by protein–protein interactions. In order to define the role of the receptor during development and in physiology several mutations have been generated in the mouse. Mice with a disrupted glucocorticoid receptor gene die shortly after birth due to respiratory failure indicating an important role of the receptor in lung function. Transcription of genes encoding gluconeogenic enzymes in the liver is decreased, proliferation of erythroid progenitors is impaired and the HPA axis is strongly upregulated. To analyze molecular mechansims of glucocorticoid receptor action in vivo a point mutation has been introduced into the mouse genome which allows to separate DNA-binding-dependent from DNA-binding-independent actions of the receptor. Mice homozygous for the point mutation survive indicating that DNA-binding of the receptor is not required for survival. Induction of glucoconegenic enzymes and proliferation of erythroid progenitors however is impaired. Interestingly, repression of corticotropin releasing factor (CRF) synthesis is maintained, whereas proopiomelanocortin (POMC) expression is upregulated. Since mice with a disrupted glucocorticoid receptor gene die shortly after birth attempts using the Cre/loxP-recombination system are made to bypass early lethality and to study the function of the receptor in defined cell types of adult animals.

1. Introduction

Already more than half a century has passed since the discovery of glucocorticoids (GC) and their role in the regulation of glucose metabolism [1]. Meanwhile it is known that these steroid hormones are involved in the regulation of a wide range of physiological processes, mainly in the maintenance of homeostasis under basal and stressful conditions. The whole range of actions is mediated through binding to two distinct intracellular transcription factors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) [2,3]. GCs have higher affinity to MR than to GR, however specificity of receptor action is achieved by their different expression pattern. Whereas GR is ubiquitously expressed, MR is mainly found in epithelial cells of the kidney, the large intestine and in neurons of the limbic system. In cells, expressing both receptors low concentration of GCs will predominantly activate the MR whereas for GR occupation higher levels are required [4]. In aldosterone responsive cells of the kidney and colon activation of the receptors by GCs is prevented by enzymatic inactivation [5]. In order to determine the importance of GR for development and physiology various mutations of the receptor have been generated in mice. This review will summarize the results obtained by analyzing these mutant mice.

2. Transcriptional control by the glucocorticoid receptor

The glucocorticoid receptor is a ligand-regulated transcription factor which is a member of the nuclear hormone receptor superfamily [3,6]. Nuclear receptors are characterized by a modular structure including a

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DNA binding domain (DBD), a ligand binding domain (LBD) and two transactivation motifs (AF1 and AF2). In the absence of ligand the LBD of steroid receptors associate with heat shock proteins keeping the receptor in an inactive form. Ligand binding releases the heat shock complex and activates the receptor. In the classical model GR subsequently transactivates transcription by binding with its DBD to conserved palindromic recognition sequences, termed glucocorticoid responsive elements (GREs, Fig.1, [7]). Transactivation is probably mediated through interaction of DNA-bound GR-homodimers with the basic transcriptional machinery, coactivators and other transcription factors [6]. However, the idea that GR exclusively induces gene expression had to be modified, when genes were identified that are negatively regulated by glucocorticoids. Transcriptional repression can be mediated by GR binding to negative response elements (nGRE) as in the POMC promoter (Fig.1, [8]) or by competitive binding with other transcription factors to composite elements like in the proliferin gene [9].

Unexpectedly, in 1990 several groups found that GR also regulates transcription by protein–protein interaction without binding directly to DNA [10–12]. This has been well demonstrated in the case of AP-1 regulated genes, like the collagenase-3 gene, whose transcriptional induction by proinflammatory cytokines can be repressed by GR (Fig.1). Whether the interaction between GR and AP-1 is direct or needs an intermediary factor is controversial and might vary from gene to gene [13]. The activated receptor also interferes with functions of other transcription factors like NF-κB, CREB and GATA-1 [14–17]. GR functions not always as a negative regulator, but can also synergize with other factors in transcriptional activation as in the case of Jun homodimers or Stat-5 (Fig.1) [17,18].

Recently, an interference of GR signalling with the Jun amino-terminal kinase (JNK) signal transduction pathway has been shown [19]. GR blocks JNK mediated phosphorylation of c-Jun at Ser-63/73, thereby preventing the activation of AP-1 by JNK. Reciprocally, JNK can inhibit GR action by phosphorylating the receptor at Ser-246 [20].

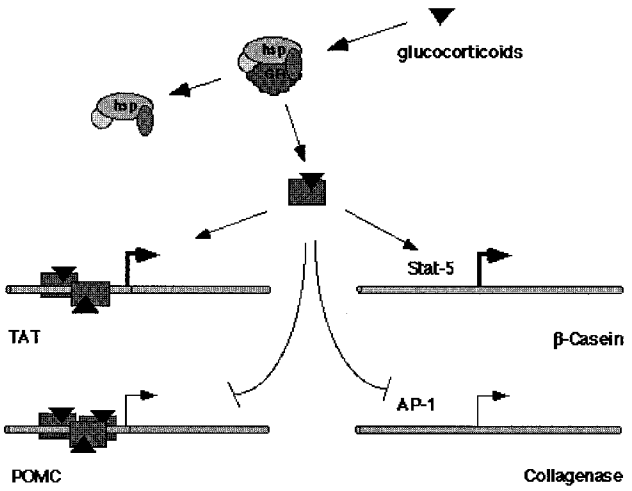

Fig. 1. The glucocorticoid receptor regulates transcription using different modes of action. In the absence of ligand GR is associated with several heat shock proteins in an inactive complex. After glucocorticoid binding activated receptor regulates transcription by DNA binding-dependent or -independent mechanisms. The tyrosine aminotransferase (TAT) gene expression is positively regulated by specific binding of GR to GREs, transcription of the proopiomelanocortin (POMC) gene is repressed by binding of the receptor to a negative GRE. Regulation of the β-casein and collagenase genes involves protein–protein interaction between GR and Stat-5 or AP-1, respectively. Whereas in the case of the β-casein gene the actions of GR and Stat-5 are synergistic, GR is repressing the AP-1-induced expression of the collagenase gene.

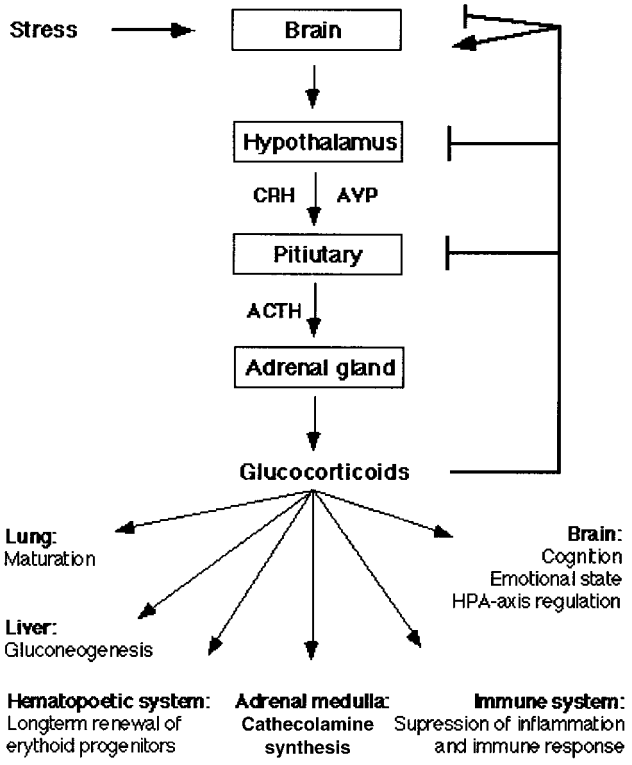

Fig. 2. Glucocorticoids regulate gene expression in many different cell types. Synthesis and secretion of GCs are tightly regulated by the hypothalamus–pitiutary–adrenal (HPA) axis. Once released from the adrenal cortex GCs regulate gene expression of specific target genes in many different cell types. A few relevant examples of physiological processes which are influenced by GCs and described in the text are given. Via a negative feedback mechanism at the level of the brain and the pituitary GCs control their own synthesis. CRF: corticotropin releasing factor, AVP: arginine vasopressin, ACTH: adrenocorticotropin.

3. Glucocorticoids and physiology

GCs are synthesized and released into the circulation by the adrenal gland [1], (Fig. 2). Upon binding to GR they act on a variety of different cell types and regulate transcription of specific target genes. Prolonged changes in GC concentration have severe pathological consequences, such as in patients with Cushing's syndrome, a human condition characterized by chronically elevated GC levels. Therefore, GC levels are tightly controlled by an endocrine cascade, the hypothalamus–pituitary–adrenal axis (HPA, [21]). Two neuropeptides, corticotropin releasing factor (CRF) and vasopressin (AVP) are secreted from the hypothalamus, which stimulate synthesis and release of adrenocorticotropin (ACTH) from anterior-pituitary cells into the circulation. ACTH then stimulates adrenocortical GC production and secretion. Finally, GCs control their own production by inhibiting ACTH and CRF release via negative feedback exerted at the levels of the brain and pituitary.

GCs are indispensable for the maintenance of homeostasis and their coordinate actions allow the body to respond to internal and environmental changes. Emotional or physical stress can stimulate GC secretion by activating the HPA axis. For example interleukins produced in response to infection or injury will induce the release of CRF from the hypothalamus and so enhance adrenal GC secretion [22].

GCs induce the mobilisation of energy resources by acting on many different cell types. In hepatocytes, for example, they stimulate transcription of gluconeogenic enzymes like phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase), serine dehydrogenase (SDH) or tyrosine aminotransferase (TAT). In adipocytes they increase lipolysis and in peripheral tissues they inhibit glucose uptake.

GCs also protect the body from an excessive response to stressful events. Through interaction with AP-1 and NF-κB GR can repress the expression of pro-inflammatory cytokines, thus suppressing inflammation and immune response [23], a property widely used in medical therapy.

In the brain GCs have been suggested to influence emotions and cognitive processes like learning and memory [24–26]. Although the effects of physiological GC concentrations on the brain are complex and not yet well understood, chronically elevated GC levels appear to impair brain function. This is supported by existing correlations between elevated GC levels and the occurrence of pathological disorders like depressive illness or by the observation that memory deficits correlate with elevated GC levels in elderly healthy humans [27].

GCs are not only involved in adult physiology but also in developmental processes. Important roles are suggested, e.g. for final lung maturation, chromaffin cell differentiation and erythroblast proliferation [28–30].

4. Glucocorticoid receptor function is essential for survival

One strategy to learn more about GC actions is to

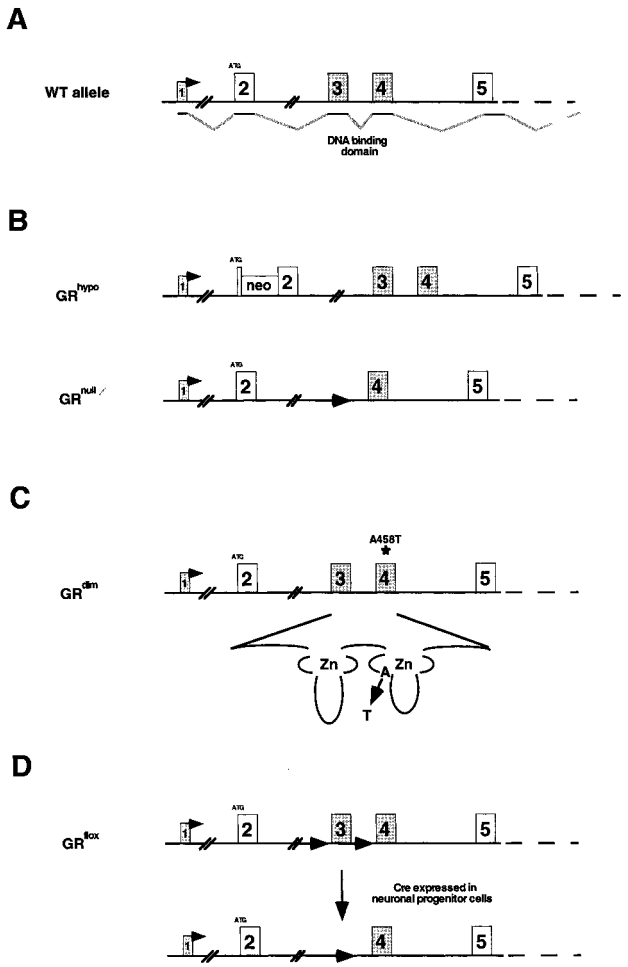

Fig. 3. Glucocorticoid receptor mutations in the mouse Several mutation have been generated in mice to analyse GR function in the living mammalian organism. (A) Schematic representation of GR gene structure. Exon III (3) and IV (4) encode the DNA binding domain of the receptor. (B) Top panel: scheme of a hypomorphic GR allele (GR^{hypo}) which has been generated by inserting a neomycin resistance cassette into exon II immediately after the initiation start codon (ATG). Bottom panel: inactivation of the GR gene by deleting exon III (Gr^{null}). The arrow shows a loxP recognition sequence. (C) Substitution of alanine 458 to threonine in the D-loop of the second zinc finger results in a dimerisation-defective receptor, which lost its ability for cooperative DNA-binding (GR^{dim}). (D) Somatic mutagenesis of the GR using the Cre/loxP recombination system. The GR gene has been modified with two Cre recombinase recognition sites (arrows), which are flanking the third exon (GR^{flox}). Cre-mediated recombination between the loxP sites excises exon III and thereby inactivates the gene. By expressing the Cre recombinase in neuronal precursor cells deletion of exon III could be restricted to the nervous system.

address the in vivo role of their receptor. In humans several GR mutations have been described, which lead to congenital GC resistance [31]. However, a loss-of function of the gene has so far not been observed suggesting that GR may be indispensible for life. In order to inactivate GR function in mice the GR gene has been mutated via homologous recombination in ES-cells. Two independent mutant mouse lines were generated, one carrying an insertion in the GR gene, the other one a deletion (see Ref. [32] and unpublished data). The insertion was obtained by introducing a neomycin resistance cassette into exon II, the deletion by removing exon III. Since mice homozygous for the insertion mutation still express two unusual GR transcripts, the mutated allele has been named GR^{hypo} (for hypomorphic, Fig.3(B)). Deletion of exon III, which encodes the first zinc finger of the DNA binding domain leads to a complete inactivation of the receptor (GR^{null} allele, Fig.3(B)). All the phenotypes observed in GR^{hypo} mice are also present in GR^{null} mice, however with higher penetrance and intensity in the GR^{null} mice.

Mutant mice die in the first minutes after birth, due to atelactasis of the lungs (Ref. [32] and unpublished data). This indicates that GR function is indeed required for survival although the molecular mechanism for the development of the atelactasis still has to be elucidated. Analysis of lung surfactant proteins SF-A, SF-B and SF-C, which are activated prior to birth by a number of factors including GCs [28,33] did not show major differences in mRNA expression levels between wild type and $GR^{hypo/hypo}$ mutants [32]. One explanation could be that reduction of the amiloride-sensitive $Na+$ channel (ENaC) activity may result in an incomplete removal of fluid from the lung around birth, thus explaining the atelactasis. However since mice with an inactivated ENaC α-subunit die later than GR mutants do, reduction of ENaC expression can not be the only explanation for the lung failure [34]. Interestingly, all $GR^{null/null}$ mice die immediately after birth, whereas 20% of $GR^{hypo/hypo}$ mice are surviving until adulthood, suggesting that the observed residual transcription of the hypomorphic allele is responsible for a limited GR activity, which is sufficient for survival of some $GR^{hypo/hypo}$ mice.

Maturation and proliferation defects could also be observed in other organs. $GR^{null/null}$ mice show reduced keratinisation of skin epidermis at birth (unpublished data). In the haematopoetic system the in vitro proliferation of erythroid progenitor cells is impaired in the mutants, which is most likely also manifested in vivo (unpublished data). The identification of genes, whose misregulation in mutant mice give rise to the observed defects will enhance the understanding of the role of GR in proliferation and differentiation processes.

Surprisingly, GR does not seem to be required for the development of adrenal medulla chromaffin cells. Chromaffin cells producing the catecholamines adrenalin and noradrenalin are derived from neural crest cells, which also have the potential to develop into sympathetic neurons. In vitro cell culture experiments have shown that sympathoadrenal progenitors derived from the adrenals differentiate into sympathetic neurons in the presence of nerve growth factor and into chromaffin-like cells in the presence of GCs [29]. A two step model has been postulated, in which an early GC signal initiates chromaffin cell differentiation and a subsequent signal the activation of chromaffin cell specific gene expression [35]. The presence of differentiated chromaffin cells in both mutants argues against an essential role of GC signalling in chromaffin cell development [36]. Although the cells are scattered in the gland, their number is not reduced in mutant newborns. However GC is necessary for the expression of chromaffin cell specific genes, since PNMT expression is abolished in the mutant animals.

Since $GR^{null/null}$ and most $GR^{hypo/hypo}$ mice die shortly after birth the role of GR in physiology could be addressed only in embryos and newborns. At birth, neonates have to pass a short period of starvation and hypogylcemia. To maintain glucose homeostasis gluconeogenesis is activated by different signals including GCs and glucagon [37]. $GR^{hypo/hypo}$ mice express strongly reduced mRNA levels of the gluconeogenic enzymes serine dehydrogenase (SDH) and tyrosine aminotransferase (TAT) at birth as well as weaker levels of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate kinase (PEPCK, [32]). The remaining transcription of these is probably under the control of other transcription factors such as CREB, which responds to glucagon-increased cAMP levels. A recent analysis of different GRE-reporters in transgenic mice even argues against an essential role of GCs in the neonatal gluconeogenic gene induction [38].

The absence of GR in mutant animals should theoretically give rise to an impaired GR mediated feedback regulation at the PVN and the anterior pituitary level resulting in an activated HPA-axis is expected. Indeed mutant animals show elevated GC plasma concentrations, probably due to a marked increase in ACTH synthesis and secretion by the pituitary [32]. CRF peptide expression is elevated in the PVN, but not the expression of arginine vasopressin (AVP), suggesting that CRF and not AVP is the main mediator of GR controlled feedback regulation. Interestingly, the feedback regulation is established during fetal development, since the expression of proopiomelanocortin (POMC, encodes ACTH) mRNA in the pituitary and the expression of CRF mRNA in the PVN are already strongly upregulated at E16,5 [39]. Increased ACTH levels in surviving GR^{hypo} mutants associate with adre-

Table 1
Phenotypic consequences of different glucocorticoid receptor mutations. n.d.: not determined

	GR^{null}-GR^{hypo}	GR^{dim}	
		dimerisation dependent	dimerisation independent
Lung	respiratory failure		no respiratory failure
Skin	impaired maturation		
Liver	reduced gluconeogenic enzyme expression	lost inducibility of gluconeogenic enzyme expression	
Thymus	no in vitro T-cell apoptosis	no in vitro T-cell apoptosis	
Erythroid progenitors	impaired proliferation	impaired proliferation	
HPA axis	up regulation of POMC and CRF expression	up regulation of POMC	no changes in CRF expression
Adrenal cortex	hypertrophy		no hypertrophy
Adrenal medulla	no PNMT expression		normal PNMT expression
Collagenase-3	n.d.		normal repression
GRE activity in transfections	n.d.	no activity	

nal hypertrophy, hyperplasia and an overproduction of steroidogenic enzymes, but no obvious pathological consequences of elevated GC levels like growth retardation, muscle atrophy or altered fat disposition have been observed. These symptoms can be observed in human patients with chronically elevated GC levels. The discrepancy, however, is not surprising since mutant animals contain strongly reduced GR levels.

5. DNA-binding of the glucocorticoid receptor is not essential for survival

GR regulates transcription through DNA binding-dependent and -independent mechanisms. To study the in vivo relevance of DNA binding a point mutation was introduced into the mouse GR gene (GR^{dim}, Fig. 3(C)), which allows to distinguish between these two modes of action [40]. The mutation was obtained by an amino acid substitution in the D loop (A458T) of the second zinc finger. Previous experiments in cell-culture had shown that the mutation prevents GR dimerisation and hence abolishes cooperative binding to GREs, whereas it only slightly affects transrepression of AP-1 induced gene expression [41]. Surprisingly, mice homozygous for the GR^{dim} allele survive showing normal differentiation of skin and lung [40]. This indicates that DNA binding of GR is not essential for survival.

To demonstrate the absence of DNA-binding dependent transcriptional regulation several experiments were performed with $GR^{dim/dim}$ mice. Transient transfection of GRE-dependent reporters into embryonic fibroblast showed a strongly decreased GRE activity of the reporters in mutant cells compared to wildtype cells. Band-shift experiments with liver extracts from mutant animals showed hardly any binding of the receptor to classical GREs. Both observations were confirmed by the failure to induce tyrosine aminotransferase (TAT) mRNA expression in the liver with dexamethasone. As predicted from cell culture experiments transrepression of AP-1 via protein–protein interactions is still intact in $GR^{dim/dim}$ mice. TPA-induced expression of collagenase-3 and gelatinase B was repressed by dexamethasone in mutant and in wildtype cells with equal efficiency. It is likely, that the mutated GR can also interact with other transcription factors like NFκB, but this still requires experimental demonstration. In conclusion, $GR^{dim/dim}$ mice are extremely useful for distinguishing GR-mediated actions, which require DNA binding of the receptor from actions, which do not require DNA binding. The analysis of the HPA axis in $GR^{dim/dim}$ mice demonstrates that complex regulatory systems can make use of both mechanisms. Whereas CRF expression in the PVN is not altered in mutant mice, the expression of POMC and prolactin mRNA in the pituitary is strongly upregulated. In agreement with this finding nGREs have only been detected in the promoter regions of the POMC and prolactin genes, but not in the CRF gene [8,42]. For GC mediated repression of CRF production other mechanisms like interaction of GR with Nur77 or CREB may therefore be responsible [43].

The anti-inflammatory and immunosuppressive effects of glucocorticoids which are suggested to be mainly mediated by interactions between GR and AP-1 or NFκB are of particular medical interest. A careful analysis of $GR^{dim/dim}$ mice will determine which genes encoding cytokines or other proinflammatory mediators are indeed regulated by these interactions in vivo. This will be important information for the development of new anti-inflammatory drugs with the goal to minimizing side-effects of the currently used glucocorticoid derivates.

Table 1 summarizes the phenotypical consequences of different GR mutations in mice. Physiological pro-

cesses which need dimerisation of the receptor are distinguished from processes in which the dimerisation function is not required.

6. Future perspectives

The inactivation of GR in the mouse demonstrated an important role of the receptor for proliferation and differentiation processes during mouse development as well as for HPA axis regulation. Mutant mice die shortly after birth preventing the analysis of adult animals. With the help of $GR^{dim/dim}$ mice many but not all of the physiological functions of GR can be studied in adult mice. For this reason tissue-specific somatic mutations of the receptor are highly desirable. These not only allow circumvention of early lethality, but also study of the GR function in one tissue without having indirect effects, caused by its inactivation in another tissue. To address GR function in the nervous system mutagenesis of the GR gene was restricted to neuronal progenitor cells using the Cre/loxP-recombination system ([44,45], $GR^{flox/flox}$; Cre, Fig.3(D)). The tissue-specific mutants survive and initial results indicate an impaired HPA axis regulation leading to increased corticosterone levels. In contrast to the already described mutants, GR signalling is intact outside the nervous system. Due to elevated glucocorticoid levels animals show retarded growth, altered fat distribution and osteoporosis, symptoms which can be observed in patients with Cushing syndrome. By analysing the $GR^{flox/flox}$; Cre mice the role of GR signalling in the brain can be addressed thereby increasing our knowledge of GC function in cognition and emotion.

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