Differential Hormone-Dependent Transcriptional Activation and -Repression by Naturally Occurring Human Glucocorticoid Receptor Variants

Pieter de Lange, Jan W. Koper, Nannette A. T. M. Huizenga, Albert O. Brinkmann, Frank H. de Jong, Michael Karl, George P. Chrousos, and Steven W. J. Lamberts

Department of Internal Medicine III (P.d.L., J.W.K., N.A.T.M.H., F.H.d.J., S.W.J.L.)
Department of Endocrinology and Reproduction (A.O.B.)
Erasmus University
3015 GD Rotterdam, The Netherlands

Developmental Endocrinology Branch (M.K., G.P.C.)
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20892

The molecular mechanisms underlying primary glucocorticoid resistance or hypersensitivity are not well understood. Using transfected COS-1 cells as a model system, we studied gene regulation by naturally occurring mutants of the glucocorticoid receptor (GR) with single-point mutations in the regions encoding the ligand-binding domain or the N-terminal domain reflecting different phenotypic expression. We analyzed the capacity of these GR variants to regulate transcription from different promoters, either by binding directly to positive or negative glucocorticoid-response elements on the DNA or by interfering with protein-protein interactions. Decreased dexamethasone (DEX) binding to GR variants carrying mutations in the ligand-binding domain correlated well with decreased capacity to activate transcription from the mouse mammary tumor virus (MMTV) promoter. One variant, D641V, which suboptimally activated MMTV promoter-mediated transcription, repressed a PRL promoter element containing a negative glucocorticoid-response element with wild type activity. DEX-induced repression of transcription from elements of the intercellular adhesion molecule-1 promoter via nuclear factor-κB by the D641V variant was even more efficient compared with the wild type GR. We observed a general DEX-responsive AP-1-mediated transcriptional repression of the collagenase-1 promoter, even when receptor variants did not activate transcription from the MMTV promoter. Our findings indicate that different point mutations in the GR can affect separate pathways of gene regulation in a differential fashion, which can explain the various phenotypes observed. (Molecular Endocrinology 11: 1156–1164, 1997)

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INTRODUCTION

In man, response to glucocorticoids may vary considerably. Some individuals are quite sensitive to these steroid hormones, whereas others are relatively resistant. Hypersensitivity to glucocorticoids may be manifested by the development of cushingoid features after low-dose treatment (1). In primary glucocorticoid resistance, negative feedback of cortisol on the hypothalamic-pituitary-adrenal axis is decreased. The set point of this axis is set at a higher level with higher plasma concentrations of ACTH and cortisol (2–5). The diurnal rhythm of cortisol secretion remains intact, and the system also remains sensitive to external stressors such as acute hypoglycemia (2, 6). The elevated cortisol levels do not cause signs or symptoms of Cushing's syndrome, due to reduced response to cortisol in all target tissues.

In a number of reports, glucocorticoid resistance in humans has been correlated with mutations in the gene encoding the glucocorticoid receptor (GR) (7–10). The receptor is expressed throughout the body and plays a key role in both positive and negative regulation of gene expression (11–14). Because glucocorticoids play an important role in normal develop-

ment and in maintenance of basal and stress-related homeostasis, including regulation of various metabolic processes, central nervous system functions, and restraint of the inflammatory/immune reaction, altered GR function may have widespread consequences (2, 15).

Analysis of a number of healthy volunteers indicated the existence of two N-terminal GR receptor variants with a normal dissociation constant (16), carrying either an arginine to lysine change at position 23 of the protein (R23K) or an asparagine to serine change at codon 363 (N363S). In three individuals the R23K variant was accompanied by signs and symptoms of glucocorticoid resistance, whereas four other individuals with the same mutation were asymptomatic. The N363S variant was present in several members of a family with glucocorticoid resistance. The glucocorticoid-resistant members also had a GR gene microdeletion resulting in functional knock-out of the allele not containing the mutation. This variant had a normal capacity to activate transcription in a transfection assay (9). It was later reported to be present in two glucocorticoid-resistant small cell lung tumor cell lines (17, 18). Our recent studies suggest that the N363S variant is present in about 6% of the normal Dutch population and might be accompanied by an increased sensitivity to glucocorticoids (1). Furthermore, three different variant human GR forms with altered amino acids in their C termini have been reported to date: 1) a heterozygous isoleucine to asparagine change at codon 559 (I559N), which abolished detectable ligand binding and was found in a patient who presented with hypertension and oligospermia (10); 2) a homozygous aspartic acid to valine change at codon 641 (D641V), the dissociation constant of which was 3 times higher relative to the wild type receptor, occurring in a patient with severe hypertension and hypokalemia (7); and 3) a homozygous valine to isoleucine change at codon 729 (V729I), with a 2-fold higher dissociation constant relative to the wild type receptor, which was found in a young boy with isosexual precocity as a result of increased levels of adrenal androgens (8). These mutations are indicated in Fig. 1A. No mutations in the region involved in DNA binding have yet been reported in humans. The natural, non-ligand-binding β-isoform of the GR (human GRβ), the presence of which has not yet been related to disease, does not activate transcription in transfection assays (9). To obtain more insight into its mode of action, this isoform has been included in this investigation.

GR acts through several distinct mechanisms to activate or repress transcription. It can either bind directly to a specific DNA sequence, termed a positive or a negative (n) glucocorticoid response element (GRE) (19, 20), or it can interact with other transcription factors such as AP-1 (21, 22) and nuclear factor (NF)-κB (23–25) without itself being bound to DNA. GR variants may achieve a differential interaction with the transcription machinery and any of these factors. Furthermore, the abundance and activity of components of the transcription machinery and the interacting factors may differ among individuals. As a first step to-

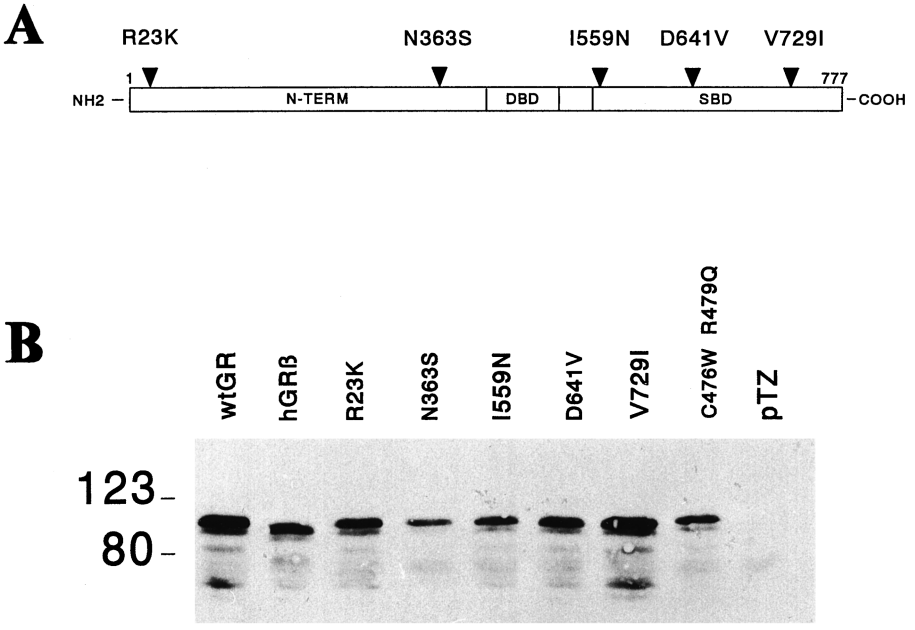

Fig. 1. Human GR Variants and Their Expression

A, Amino acid alterations reported in the hGR associated with glucocorticoid resistance (7, 8, 10, 16). B, Western Immunoblot analysis of the GR receptor variants. The expression of the indicated receptor variants was measured by transfecting COS-1 cells. hGRβ is a naturally occurring splice variant of the wild type GR (see Refs. 9 and 47). Size markers are β-galactosidase (123 kDa) and BSA (80 kDa).

ward explaining more precisely the varying phenotypic expression of the GR variants in primary glucocorticoid resistance, we examined their capacity to activate or repress transcription from several different promoters in COS-1 cells.

RESULTS

Expression of the Human (h) GR Mutant Constructs

First, we investigated whether transfection of expression plasmids containing the different GR variants as well as the naturally occurring splice variant hGRβ resulted in comparable intracellular levels of receptor protein. COS-1 cells were used in this and subsequent experiments because these cells contain little, if any, endogenous GR protein (Fig. 1B, pTZ control lane). Immunoblot analysis of lysates from COS-1 cells transfected with the GR variants showed that all GR constructs were properly expressed (Fig. 1B).

Activation of Transcription by hGR Variants

The R23K and N363S variants had a capacity to activate mouse mammary tumor virus (MMTV)-driven transcription similar to that of the wild type GR (see Table 1, column MMTV. The potency of the wild type GR to regulate transcription from the various promoters is set as 1). I559N did not activate transcription (Ref. 10 and Table 1), whereas the concentration of dexamethasone (DEX) required for half-maximal stimulation of LUC activity by the V729I variant was 12-fold higher (Fig. 2), similar to what has been published previously (8). The overall relative potency of the V729I variant to activate transcription from the MMTV promoter compared with the wild type GR at the suboptimal ligand concentrations was 0.08 (Table 1). The D641V variant was even less potent than the V729I variant (Fig. 2), with an overall relative potency of 0.04 (Table 1). The hGRβ-form, which is known not to activate transcription, was used as a negative control (Fig. 2).

Negative GRE-Mediated Repression of Transcription by the hGR Variants

An expression construct containing a region of the PRL promoter designated PRL3 (-247 to -214), comprising an nGRE fused upstream of a thymidine kinase (tk) promoter-LUC fusion gene (PRL3-tk-LUC), was cotransfected with the variant GR plasmids. At different ligand concentrations, repression of LUC transcription by the receptor variants was determined. In the presence of 10^{-7} M DEX, the wild type GR repressed transcription by 55% (Fig. 3). The capacity of the N-terminal variants (R23K and N363S) to repress transcription was comparable to the wild type GR (Table 1). The I559N and hGRβ variants did not repress transcription (Table 1). Surprisingly, the D641V variant

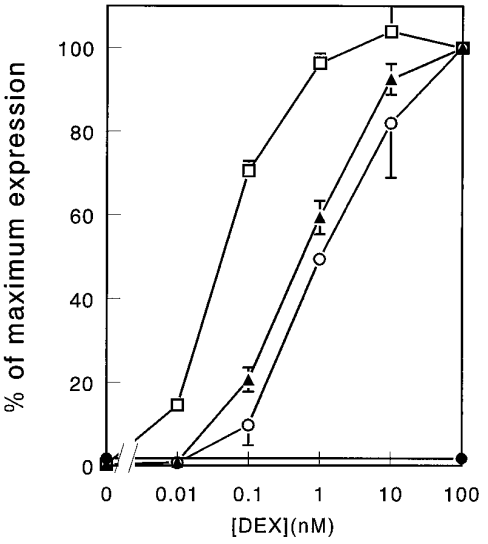

Fig. 2. Transcriptional Activation of the MMTV Promoter by Wild Type GR (□), D641V (○), V729I (▲), hGRβ (●)

Data are the means of two transfections carried out in triplicate; error bars represent standard deviations of the mean. Cells were cotransfected each time with the MMTV-LUC reporter construct and one of the GR expression vectors encoding the above mentioned variants and were subsequently treated with DEX at the indicated concentrations.

Table 1. Relative Potencies of the Various GR Mutants in Regulation of Transcription

	Promoter			
	MMTV	PRL-3	ICAM-1	COLL
wtGR	1	1	1	1
R23K	1	1	1	1
N363S	1	1	1	1
I559N	Inactive	Inactive	Inactive	0.002 (3.10^{-5}–0.03)[a]
D641V	0.04 (0.03–0.07)[a]	0.52 (0.07–2.8)[a]	4.6 (2.87–7.48)[a]	0.16 (0.10–0.26)[a]
V729I	0.08 (0.05–0.13)[a]	0.95 (0.15–6.6)[a]	0.3 (0.18–0.48)[a]	0.12 (0.07–0.19)[a]
hGRβ	Inactive	Inactive	Inactive	0.001 (3.10^{-5}–0.01)[a]

Regulation of transcription by the GR variants at different ligand concentrations as compared to the wild type GR was determined using parallel line statistics (45).
[a] 95% confidence limits.

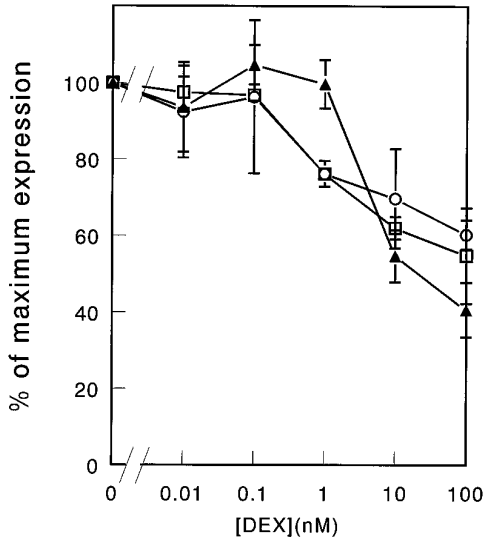

Fig. 3. Transcriptional Repression of PRL3-tk-LUC by the Wild Type GR (□), D641V (○), and V729I (▲)

Data are the means of three transfections carried out in triplicate; error bars represent standard deviations of the mean. Cells were cotransfected each time with the PRL3-tk-LUC reporter construct and one of the GR expression vectors encoding the above mentioned variants and were treated with the DEX concentrations indicated. The R23K and N363S variants repressed at wild type levels; the I559N and hGRβ variants did not repress (not shown).

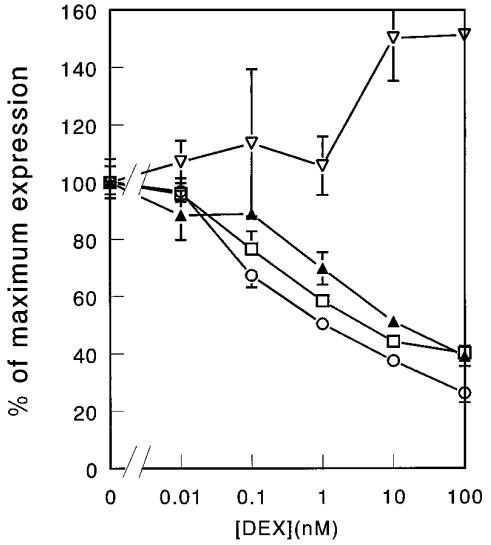

Fig. 4. Transcriptional Repression of p65-Activated ICAM-1-LUC by the Wild Type GR (□), D641V (○), V729I (▲) and C476W/R479Q (▽)

Data are the means of one representative transfection assay carried out in triplicate; error bars represent standard deviations of the mean. Cells were cotransfected with the ICAM-1-LUC reporter construct and the GR expression vectors encoding the variants mentioned above and were treated with DEX at the concentrations indicated.

repressed nearly as efficiently as did the wild type GR (Fig. 3). The overall relative potency of repression by D641V was 0.52 (Table 1). V729I, however, did not repress at 10^{-9} M DEX, whereas the wild type GR and D641V significantly repressed PRL3-tk-LUC transcription to 76% at this concentration (Fig. 3). At higher ligand concentrations, V729I repressed at wild type levels with an overall relative potency of 0.95 (Table 1).

Nuclear Factor (NF)-κB Transcriptional Repression by the hGR Variants

Without added p65 plasmid, the intercellular adhesion molecule (ICAM)-1 promoter had a basal activity that could not be repressed by active GR (data not shown), due to the virtual absence of endogenous NF-κB in COS-1 cells. A 6-fold induction of ICAM-1-LUC expression was observed when a p65 expression plasmid was cotransfected (data not shown). In the presence of 10^{-7} M DEX, the wild type GR repressed p65-induced expression by 57% (Fig. 4). The R23K and N363S variants repressed to a similar extent as did the wild type GR, but the I559N variant did not repress p65-induced expression (Table 1). To study subtle differences between the capacities of the variants to inactivate NF-κB, repression of the ICAM 1 promoter was also studied at lower ligand concentrations. The D641V variant repressed better than did the wild type GR (Fig. 4); the overall relative potency to repress the ICAM-1 promoter compared with the wild type GR at the measured ligand concentrations was 4.6 (Table 1). The V729I variant was slightly less effective compared with the wild type GR (Fig. 4), with a relative potency value of 0.3 (Table 1). Human GRβ did not repress at all ligand concentrations (Table 1).

Because the D641V variant repressed transcription from the ICAM-1 promoter more efficiently compared with the wild type GR, it might interact more efficiently with the p65 protein to prevent the interaction of the p65 protein with the ICAM-1 promoter. We studied repression by lower concentrations of the variants at 1.0 nM DEX, as this ligand concentration was most discriminative (Fig. 4). At receptor concentrations that were up to 5 times lower, we observed the same relative levels of repression of the ICAM-1 promoter (not shown). C476W/R479Q, an artificial GR variant that retained its full capacity to bind hormone (21), did not repress NF-κB activity at all; it even seemed to increase the activation of the ICAM-1 promoter at 10^{-8} M and 10^{-7} M DEX (Fig. 4).

Repression of AP-1 by the GR Variants

Repression of AP-1-mediated transcription from the collagenase $-517/+63$ promoter by the GR variants in the presence of increasing amounts of DEX was measured. In the presence of 10^{-7} M DEX, the wild type GR inactivated AP-1 by 84% (Fig. 5). The R23K and N363S variants repressed similarly as the wild type GR (Table 1). The I559N and hGRβ variants displayed a weak, hormone-dependent repression. The I559N

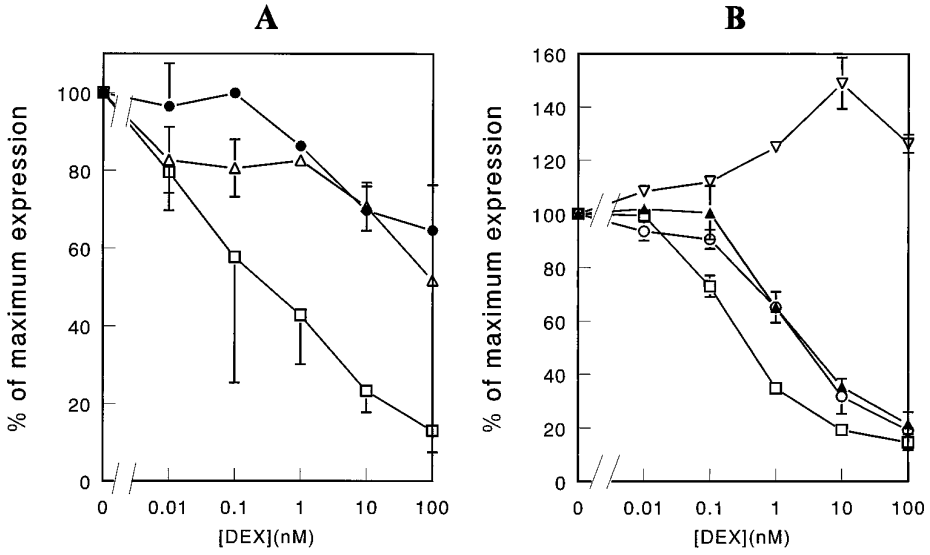

Fig. 5. Transcriptional Repression of COLL-LUC

A, Wild type GR (□, hGRβ (●), I559N (△). B, Wild type GR (□), D641V (○), V729I (▲), C476W/R479Q (▽). Data are the means of two transfections carried out in duplicate; error bars represent standard deviations of the mean. Cells were cotransfected with the COLL-LUC reporter construct and the GR expression vectors and were treated with DEX at the concentrations indicated. Simultaneously, cells were treated with 10^{-4} M TPA to activate endogenous AP-1.

variant repressed by 50% at 10^{-7} M DEX (Fig. 5A) with an overall relative potency compared with the wild type GR of 0.002 (Table 1). Human GRβ repressed by 40% at 10^{-7} M DEX with an overall relative potency compared with the wild type GR of 0.001 (Table 1).

The C476W/R479Q variant did not repress, as has been shown before (21). At 1, 10, and 100 nM DEX, this variant even induced expression from the collagenase promoter (Fig. 5B). At 10^{-7} M DEX, the D641V and V729I variants repressed as efficiently as did the wild type GR. However, these variants showed a shift in their dose-response curves (Fig. 5B), with overall relative potencies of 0.16 and 0.12, respectively (Table 1).

DISCUSSION

To obtain insight in what might be the functional significance of naturally occurring point mutations of the human GR gene, we set up transfection assays using different reporter constructs, the promoters of which were either positively or negatively regulated by GR.

There were no indications for reduced function of the R23K variant receptor because it activated MMTV transcription equally well as the wild type GR. The R23K amino acid substitution is well outside the τ_1-region of the transcriptional activation domain, which ranges from position 77 to 262 of the GR protein as determined in an *in vitro* system similar to the one used here (26); this explains the unaltered *in vitro* capacity of this variant to activate transcription. Carriers of the R23K variant receptor showed a variety of phenotypes, ranging from asymptomatic to severely glucocorticoid resistant. Thus, the presence of the mutation *per se* could not be correlated directly with glucocorticoid resistance (16). If it is a functional mutation, this would imply that at least one additional factor is involved in the resulting phenotype. Carriers of the N363S variant receptor showed a significantly higher increase of peripheral insulin levels in response to DEX than controls, suggesting increased sensitivity (1). At present it is not known which molecular mechanism underlies this observation.

Different modes of GR-mediated transcriptional repression have been reported (19–25), one of which requires binding of the receptor to an nGRE (19, 20). Different nGREs lack extensive homologies, but each nGRE is related to the GRE consensus element implicated in receptor binding and enhancer activity at positive GREs (20). The nGRE-bound GR does not activate, but represses, transcription of the downstream gene, as is shown to be the case for the POMC promoter (19) and the PRL promoter (20). The fact that the N-terminal amino acid alterations are positioned outside the τ_1-region does not necessarily imply that the capacity of the N-terminal GR variants to repress transcription by binding to an nGRE is unaltered. It is well known that the GR DNA-binding domain is involved in nGRE binding (20). The variants tested here have amino acid alterations residing well outside the DNA-binding domain. Since the effects of the N-terminal variants on PRL3tkLUC expression and MMTV-directed transcription did not differ from those of the wild type GR, there are no indications for disruption of conformation, or diminished binding to DNA due to the

altered amino acids. Results of this study suggest that repression by binding to the PRL nGRE requires hormone binding because the I559N variant, which has a very low, if any, ligand affinity, completely failed to repress transcription from the thymidine kinase promoter, most likely as a result of failure of this variant to translocate into the nucleus. The V729I variant repressed the PRL promoter relatively less efficiently at low ligand concentrations (Fig. 3), which could be anticipated from the similarly less efficient transcriptional activation of the MMTV promoter by this variant (Fig. 2), as both types of gene regulation are mediated by direct binding of the GR to response elements on the DNA. The D641V variant (7) showed a strongly reduced potency to activate MMTV-driven transcription compared with wild type GR (Fig. 2). These data are in line with the clinical observations concerning the propositus, who had a 7-fold elevation of free serum cortisol levels (7). However, this variant repressed transcription from the PRL promoter nearly at wild type levels (Fig. 3). This suggests that a different conformation of the GR is achieved when it is bound to an nGRE or that a different conformation is necessary for repression. The point mutation has an influence on the effective conformation for transcriptional activation, but not for this type of transcriptional repression.

GR has also been reported by several groups to play a role in repressing transcription regulated by NF-κB (23–25). NF-κB-responsive elements are required for the function of many cytokine promoters as well as other genes, including ICAM-1 (27, 28). A major form of NF-κB is composed of a dimer of p50 and p65 (RelA) subunits, and this complex is retained in the cytoplasm by repressor molecules that contain ankyrin repeat motifs (27–29). Recently it was shown that a large fraction of the NF-κB protein can be kept from entering the nucleus by interacting with IκBα, the transcription of which is stimulated by active GR (24, 25). COS-1 cells lack endogenous IκBα activity (23), indicating that in this system NF-κB is inactivated by a direct interaction with GR, a mechanism that has been postulated previously (23). The I559N and hGRβ receptor variants did not inactivate NF-κB at all. The capacity of the D641V variant to repress transcription was increased, whereas the V729I variant repressed slightly less efficiently as compared with the wild type GR (Fig. 4). At lower receptor concentrations, this difference in repression was maintained. This indicates that the binding capacities of the point mutants to p65 have not altered, but the conformation of the p65-GR complex differs depending on the GR variant.

The transcription factor AP-1, consisting of heterodimers of the various members of the Fos and Jun protein families, may play an essential role in converting extracellular signals into changes of the expression of specific genes involved in inflammation as well as cell growth and differentiation (for review see Refs. 30 and 31). AP-1 proteins share the bZIP-motif that allows the formation of homodimeric complexes with DNA. This motif is part of the target required for repression (32). Both the Fos and Jun proteins are at the receiving end of signal transduction pathways from the cell membrane to the nucleus. GR synergizes with Jun homodimers to activate AP-1 regulated promoters, whereas it represses transcription induced by Fos-Jun heterodimers without abolishing their binding to DNA (33, 34). Unliganded GR is associated with heat shock protein (hsp) 90 in the form of a heterohexamer containing the receptor, two molecules of hsp 90, and one molecule each of hsp 70, hsp 56, and hsp 26 (35–38). The receptor is thus kept in a ligand-friendly conformation. Ligand binding stimulates receptor activation, dissociation from hsp 90 (39, 40), and nuclear translocation, prerequisites for both activation and repression of transcription. The general inactivation of AP-1 we observed suggests that weak association of the ligand with the receptor is sufficient for dissociation of the heat shock proteins and subsequent binding of the receptor to AP-1. It has been shown that transcriptional repression of the collagenase promoter also occurs upon heat shock-induced nuclear import of GR in transfected CV-1 cells, even without addition of hormone (40). A receptor variant isolated from the human leukemic cell line ICR27TK.3 (41), carrying a leucine to phenylalanine change at position 753, which showed 14% hormone binding relative to the wild type GR, was 100-fold less active in transcriptional activation and did not reach wild type levels at 10^{-7} M DEX. However, it had full DEX-responsive AP-1-repressing activity. In addition, heat shock treatment of cells transfected with this variant resulted in full repression in the absence of ligand (22). This suggests that, in contrast to transcriptional activation, stably bound ligand is not necessary for transcriptional repression once the receptor is in the nucleus. The I559N and the hGRβ variants have an intact DNA-binding domain, which has been shown to be essential for inactivation of AP-1, more so than is the hormone binding domain (21). Interaction of GR with AP-1 in the cytoplasm would explain why certain receptor variants such as I559N and hGRβ may repress the collagenase promoter without stable ligand binding. It has been shown that the *in vivo* AP-1 footprint does not disappear in the presence of glucocorticoids (33), which would imply that the putative cytoplasmic GR/AP-1 complex does migrate into the nucleus and binds to AP-1 response elements but has lost its capacity to activate transcription. Because we have no indication for a more efficient repression of AP-1 by any of the GR variants we tested compared with the wild type GR, it is conceivable that the complex resides on the target promoter after inactivation by these variants. As is the case for MMTV transcriptional activation, the reduced transcriptional repression by the variants can directly be associated with their reduced ligand binding capacity.

The observed hormone-dependent induction of activity of the ICAM-1 promoter as well as the collagenase promoter by the C467W/R479Q variant is puzzling. Because this induction was not seen in the

absence of receptor (data not shown), the activation is somehow brought about directly by this receptor variant. It could be that this variant in its active state forms a complex with factors such as p65 or AP-1, thereby allowing a better interaction with the regulatory elements on the DNA rather than preventing it.

From the differential response of NF-κB and AP-1 to the variant receptors, one can conclude that different GR mutations may cause strong phenotypic differences due to the differential association of the GR mutants with several factors, which can either act as coactivators (42) or corepressors (43, 44). Recently, a protein representing the human homolog of the yeast E2 ubiquitin-conjugating enzyme, Ubc9, has been reported to interact with the wild type GR but not with the inactive artificial GR mutant C476W/R479Q (44). Furthermore, differences in phenotype between persons carrying the same receptor variant may be due to individual differences in the abundance and activity of the GR-associating factors.

Taken together, our data point toward an explanation as to why certain point mutations that reduce or even impair the transcriptional activation capacity of GR are not lethal to the subjects. Future research will focus on factors associating with the receptor (*e.g.* coactivators, corepressors as well as the hGRβ isoform), determining the fine-tuning of gene regulation that results in the observed variety of phenotypes.

MATERIALS AND METHODS

Materials

Dexamethasone was purchased from Pharmacin (Zwijndrecht, The Netherlands). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and D-luciferin were purchased from Sigma (St. Louis, MO). The Renaissance chemiluminescence Western blotting kit was obtained from Dupont NEN (Boston, MA).

Reporter Genes and Expression Vectors

Constructions of the majority of GR expression plasmids used in this study were previously described: pRShGRα and pRShGRβ (26), which were kindly provided by Dr. Ronald Evans (The Salk Institute, La Jolla, CA), pRShGRSer363 (9), pRShGRAsn559 (10), and pRShGRVal641 (7). The pRShGRIle729 expression plasmid (8) was a kind gift from Dr. Carl Malchoff (Farmington, CT). pRShGRTrp476Gln479, containing a GR variant generated by *Taq* polymerase errors during site-directed mutagenesis, which neither activates nor represses transcription (21), was used as a control. In this receptor variant, one of the coordinating cysteine residues in the second Zn-cluster of the DNA binding domain at position 476 was converted into a tryptophan; furthermore, it contains an arginine to glutamine change at position 479. This plasmid was a kind gift from Dr. Andrew Cato (Forschungszentrum, Karlsruhe, Germany). Construction of the plasmids pRShGRLys23 and pRShGRVal641-II is described in the following section.

The mouse mammary tumor virus-luciferase (MMTV-LUC) reporter plasmid was kindly provided by Organon (Oss, The Netherlands). The human collagenase 1-luciferase reporter plasmid (COLL-LUC) was a kind gift from Dr. Andrew Cato. The p65 expression plasmid was a kind gift from Prof. Carl Scheidereit (Max-Delbrück Center for Molecular Medicine, Berlin, Germany). The human intercellular adhesion molecule 1-luciferase (ICAM-1-LUC) reporter plasmid pHBLUC1.3 was kindly provided by Dr. Christian Stratowa (Bender & Co, GmbH, Vienna, Austria), and the bovine PRL-luciferase reporter plasmid (PRL3-tk-LUC) was a kind gift from Dr. Sam Okret (Huddinge Hospital, Huddinge, Sweden).

Construction of GR Plasmids

pRShGRLys23 was generated by overlap extension PCR (46), to replace the guanosine (G) residues at cDNA positions 198 and 200 for adenosine (A) residues using the primers: 5′-CCATTCACCACATTGGTGTG-3′ (outer forward primer, positioned 28 nucleotides (nt) 5′ of the unique *Kpn*I site), 5′-TTGCCTGACAGTAAACTGTG-3′ [outer reverse primer, cDNA nt position 1025–1045, numbering according to Hollenberg *et al.* (47)], 5′-CACATCTCCCTTTTCCTGCG-3′ (overlapping reverse primer, cDNA nt position 186–206), 5′-TTGCCTGACAGTAAACTGTG-3′ (overlapping forward primer, cDNA nt position 191–211), and the pRShGRα expression vector as a template. The G to A change at position 198 does not give rise to an amino acid change, and the G to A change at position 200 changes the arginine residue at codon 23 to a lysine residue. The thymine (T) residue at position 192 was replaced by a G residue to generate a *Cfo*I restriction site, without altering the encoded amino acid residue. The resulting fragment was digested with *Kpn*I and *Sal*I and was inserted into Bluescript plasmid (Stratagene, La Jolla, CA) digested with the same enzymes. The fragment was fully sequenced to confirm the presence of the desired point mutations and to exclude additional point mutations. After digestion of the recombinant plasmid with *Kpn*I and *Sal*I, the recombinant fragment was ligated back into pRShGRα. The expression vector obtained was confirmed by sequencing and was designated pRShGRLys23.

The pRShGRVal641-II plasmid was constructed as follows: The *Cla*I/*Xho*I fragment from the original pRShGRVal641 plasmid was inserted into Bluescript plasmid digested with the same enzymes. Similarly, the *Cla*I/*Xho*I fragment from pRShGRα was inserted into Bluescript plasmid. This latter plasmid was digested with *Cla*I/*Sau*I and replaced with the *Cla*I/*Sau*I recombinant fragment containing the desired A to T change at cDNA position 2054 without any further alterations. The *Cla*I/*Xho*I GR fragment containing the single-point mutation was finally inserted to replace the wild type fragment of pRShGRα. The resulting plasmid was designated pRShGRVal641-II.

In the previous sections, GR variants encoded by these plasmids have been referred to by their amino acid alterations, indicated by single letter code.

Cell Culture and Transfections

Monkey kidney (COS-1) cells were maintained in DMEM-Ham's F-12 tissue culture medium (Life Technologies, Gaithersburg, MD) supplemented with 5% charcoal dextran-treated FCS (Life Technologies). For transcription regulation studies, cells were plated at 1.0×10^5 cells per well (10 cm^2), grown for 24 h, and transfected overnight by calcium phosphate precipitation, as described previously (48). For MMTV-LUC and COLL-LUC measurements, cells were transfected with 250 ng GR expression plasmid and 250 ng reporter plasmid per well. For PRL3-tk-LUC and ICAM 1-LUC measurements, 500 ng GR expression plasmid and 1250 ng reporter plasmid were added; in the ICAM-1 studies 75 ng p65 expression plasmid were also added. pTZ carrier DNA was added to a total amount of 5 μg DNA/well. After transfection, experimental media were added. After an incubation period of 24 h, cells were harvested for the LUC assay, as described previously (49).

Western Immunoblot Analysis

Whole-cell lysate was prepared by resuspending the cell pellet from a well (10 cm^2) in 200 μl 40 mM Tris-HCl, pH 7.4 (Boehringer, Mannheim, Germany), 1 mM EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.08% (wt/vol) SDS (all from Merck, Amsterdam, The Netherlands), 0.6 mM phenylmethylsulfonylfluoride (Sigma), and 0.5 mM bacitracin (Aldrich, Axel, The Netherlands) at 4 C. The lysate was centrifuged (10 min, 1700 $\times$ *g*), and GR protein was immunoprecipitated from the supernatant using monoclonal antibody F52 (50) coupled to goat anti-mouse agarose beads. Immunoprecipitated GR protein was used for Western immunoblot analysis, essentially as described previously (48). The polyclonal rabbit antiserum 57 (Affinity Bioreagents, Golden, CO) was used as the primary antibody to identify the GR in a chemiluminescence protein detection method, performed as described by the manufacturer (Dupont NEN).

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Address requests for reprints to: P. de Lange, Ph.D., Department of Internal Medicine III, Room Bd277, Erasmus University, Dijkzigt University Hospital, 40 Dr. Molewaterplein, 3015 GD Rotterdam, The Netherlands.

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