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The N-Terminal Domain of the Mineralocorticoid Receptor Modulates both Mineralocorticoid Receptor– and Glucocorticoid Receptor–Mediated Transactivation from Na/K ATPase β1 Target Gene Promoter

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Mineralocorticoid and glucocorticoid hormones activate the expression of the Na/K ATPase β1 through direct binding of the mineralocorticoid receptor (MR) and glucocorticoid receptors (GR) to a mineralocorticoid- and glucocorticoid-responsive element in the β1 promoter region, but activation of the β1 promoter is inhibited by coexpression of both receptors. Here, using a series of mutated and chimeric receptors, we show that the N-terminal region of MR mediates an inhibitory effect on MR and GR activation from the β1 promoter, in CV-1 cells. Deletion of the N-terminal region of MR (1–603) enhanced MR activation fourfold. Activation by chimeric MR, in which the N-terminus of GR replaces the N-terminal region of MR, was threefold that of wild-type MR. In addition, whereas coexpression of wild-type MR and GR was inhibitory, coexpression of chimeric MR and wild-type GR was nearly equal to that of MR. By contrast, mutated GR lacking its N-terminal region (1–420) was less efficient than the wild type in activating this promoter. These results demonstrate that the N-terminal domains of MR and GR have opposite transactivation properties and that MR region 1–603 is indeed inhibitory for both MR- and GR-mediated regulation of the Na/K ATPase β1 gene promoter.

Key Words: Mineralocorticoid receptor; glucocorticoid receptor; Na/K ATPase.

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Introduction

The mineralocorticoid hormone aldosterone plays a central role in the regulation of Na homeostasis, extracellular fluid volume, and blood pressure. In target epithelia, Na reabsorption is mediated by the epithelial Na channel, which controls the entrance of Na at the apical cell membrane and its extrusion toward the extracellular space by the basolateral Na/K ATPase pump. Na/K ATPase is an ubiquitous membrane oligomeric protein responsible for the transport of sodium and potassium ions across the plasma membrane in an adenosine triphosphate-dependent manner (for review see ref. *1*). It is composed of two subunits, the α-subunit (113 kDa), which mediates the catalytic activity, and the smaller glycosylated β-subunit (35 kDa), which is involved in the localization of the enzyme to the plasma membrane, protein folding, and stabilization of the K-bound form of the enzyme *(2)*. Na/K ATPase is encoded by a multigene family and isoforms have been described for both α- and β-subunits *(3,4)*. Whereas the α1 and β1 isoforms are expressed in most tissues, α2, α3, α4, β2, and β3 expression occur in a tissue-specific manner *(4–6)*.

In amphibians, aldosterone has been shown to increase rapidly the activity of the Na/K ATPase α1 and β1 at the transcriptional level *(7)*. Following a lag period of 20–60 min, aldosterone stimulates Na reabsorption by activating preexisting channels and pumps during an early phase, which reaches a maximum 2–4 h after the addition of hormone. A late phase, starting 3–6 h posthormone induction, is characterized by an increase in the number of channels and pumps *(8)*. In mammalian cells, conflicting data exist regarding an early transcriptional effect of aldosterone in vivo. However, in the late phase, regulation of the activity of Na/K ATPase α1 and β1 at the transcriptional level has been described following hormone induction in mineralocorticoid target cells, such as cardiomyocyte and the kidney cortical collecting duct isolated from intact rats. In

addition, regulation of the expression of Na/K ATPase by glucocorticoids has been reported in the colon *(9–11)*.

Aldosterone and glucocorticoids bind to the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), which are both members of the nuclear receptor superfamily *(12)*. MR and GR are ligand-activated transcription factors that bind specific hormone response elements in the regulatory regions of target genes, leading to the expression of specific hormone effects in a target cell *(13)*. MR is highly homologous to GR and binds aldosterone and cortisol with similar affinity in vitro *(14)*. In addition, MR has been shown to regulate gene expression by binding to glucocorticoid response elements (GREs) of various artificial promoters in vitro *(15–17)*. However, MR regulation of natural target gene promoters is not well studied. Although the effect of aldosterone on the Na/K ATPase mRNA synthesis is mediated entirely by MR in the rat kidney cells NRK-52-E *(10)*, Na transport is optimal in amphibian and certain mammalian epithelia at doses that occupy both MR and GR *(18)*. Previously, we identified a functional mineralocorticoid response element (MRE) GRE capable of activating transcription from the Na/K ATPase β1 gene promoter in response to both aldosterone and glucocorticoids. We have shown that both MR and GR regulate the activity of the Na/K ATPase β1 gene promoter; however, coexpression of MR and GR inhibited activation of transcription from this promoter *(19)*.

To gain insight into the mechanism underlying the inhibition of regulation of the Na/K ATPase β1 gene promoter by MR and GR coexpression, in this study, we examined the contribution of various regions of the MR and GR to this regulation. Using deletion constructs, we show that both the ligand-binding domain (LBD) and the N-terminal domain of GR positively modulate GR-induced regulation of the Na/K ATPase β1 gene promoter. By contrast, when the N-terminal region of MR was deleted, activation of the β1 gene promoter was more than threefold higher than that of the wild-type MR and was similar to that induced by GR. In addition, chimeric receptor analyses demonstrate that the MR N-terminal domain is responsible for inhibition of transcription from the Na/K ATPase β1 gene promoter when both MR and GR are coexpressed. Together, our data indicate that the N-terminal domain of MR modulates an inhibitory effect on MR- and GR-mediated regulation of the Na/K ATPase β1 gene promoter.

Results

Expression of Epitope-Tagged Wild-Type and Mutated Receptors

To begin studies of MR-mediated activation of gene expression, MR was epitope tagged (Flag) at the very amino terminus (Fig. 1A). Full-length MR or MR with an N-terminal deletion was cloned by polymerase chain reaction (PCR) techniques in frame with a Flag epitope tag in the modified Flag-pcDNA3 expression vector to produce Flag-MR and Flag-carboxy-terminal-MR (Ct-MR). Similarly, full-length GR and GR with an N-terminal deletion (Ct-GR) were generated. In addition, using a domain swap strategy, we generated chimeric receptors in which the N-terminal domains of MR and GR are exchanged to produce chimeric MR and chimeric GR (Fig. 1A).

Twenty micrograms of these Flag constructs or the Flag-pcDNA3 empty vector, used as a control, was transiently expressed in CV-1 cells. Following 1 h of hormone treatment, cells were harvested at 48 h posttransfection and processed for immunofluorescence using the M2 monoclonal antiflag antibody. Representative fields illustrated in Fig. 1B show that recombinant Flag-receptors are expressed to a similar level in CV-1 cells, but immunofluorescence is not detected in cells transfected with the empty vector Flag-pcDNA3. In the absence of hormone, Flag-receptors are cytoplasmic and nuclear, whereas fluorescence is detected predominantly in the nucleus for both MR and GR constructs, following treatment of cells with 10 n*M* aldosterone or 10 n*M* triamcinolone acetonide (TA), respectively (Fig. 1B, C). These results indicate that wild-type and mutated MR and GR are expressed in CV-1 cells and undergo nuclear translocation in the presence of hormones, as expected.

Effect of N-Terminal Domain on MR-Induced Activation

We have shown previously that both MR and GR regulate the activity of the human Na/K ATPase β1 promoter in vitro, in a hormone-dependent manner *(19)*. This regulation involves a 21-bp sequence at position –650 to –630 within the human Na/K ATPase β1 promoter that represents a true MRE/GRE, which is required for both MR and GR activation *(19)*. To examine the role played by various domains of the MR in this regulation, 0.3 μg of human full-length MR or 0.3 μg of MR with an N-terminal domain deletion (Flag-MR, Flag-CtMR) was transiently transfected in CV-1 cells. These cells are known to express very little, if any, endogenous MR and GR *(14,20)*. To measure receptor activity, 3 μg of a reporter plasmid encoding the human β1 gene promoter, spanning nucleotides –1141 to +490, linked to the luciferase gene (pHβ1-1141LUC) was cotransfected.

Following treatment with 10 n*M* aldosterone for 48 h, expression of the wild-type human MR resulted in a significant increase in the activation of the luciferase gene. Interestingly, when the N-terminal region was deleted, Ct-MR activation of the β1 gene promoter was 390 ± 5.5% that of the wild-type set arbitrarily to 100% ($n = 3$, $p < 0.01$) (Fig. 2A). This difference represents more than a threefold increase, suggesting an inhibitory role for the N-terminal region of the human MR. To demonstrate further the inhibitory function of the N-terminal domain of MR, we examined the effect mediated by coexpression of the full-length MR along with the carboxy-terminal MR, Ct-MR. CV-1

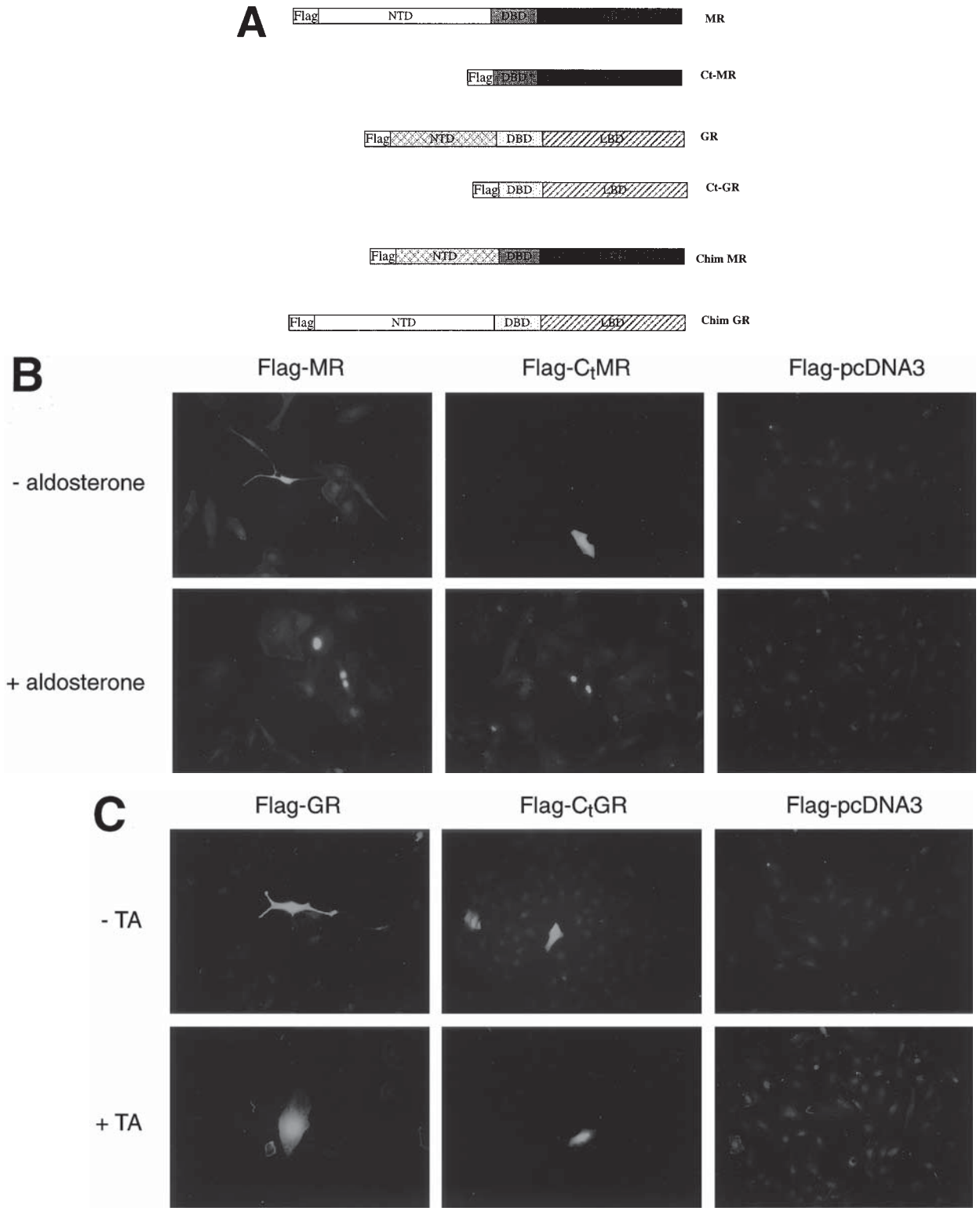

Fig. 1. Expression analysis of Flag-tagged wild-type and mutated MR and GR. (**A**) Schematic representation of the Flag-tagged human wild-type MR and GR, the N-terminal deletion constructs, and the chimeric receptors generated in Flag-pcDNA3. (**B**) Twenty micrograms of Flag-tagged wild-type MR, mutated Ct-MR, or the empty vector Flag-pcDNA3 used as a control were transiently transfected in CV-1 cells for 48 h. Cells were treated with either vehicle or 10 n*M* aldosterone for 1 hour, and processed for immunofluorescence using the mouse monoclonal anti-Flag M2 antibody. (**C**) Flag-tagged wild-type GR, mutated Ct-GR, or the empty vector Flag-pcDNA3 were transfected in CV-1 cells, treated with either vehicle or 10 n*M* TA for 1 h and then processed for immunofluorescence as described for (**B**) [N-terminal domain (NTD), DNA binding domain (DBD), and ligand-binding domain (LBD)].

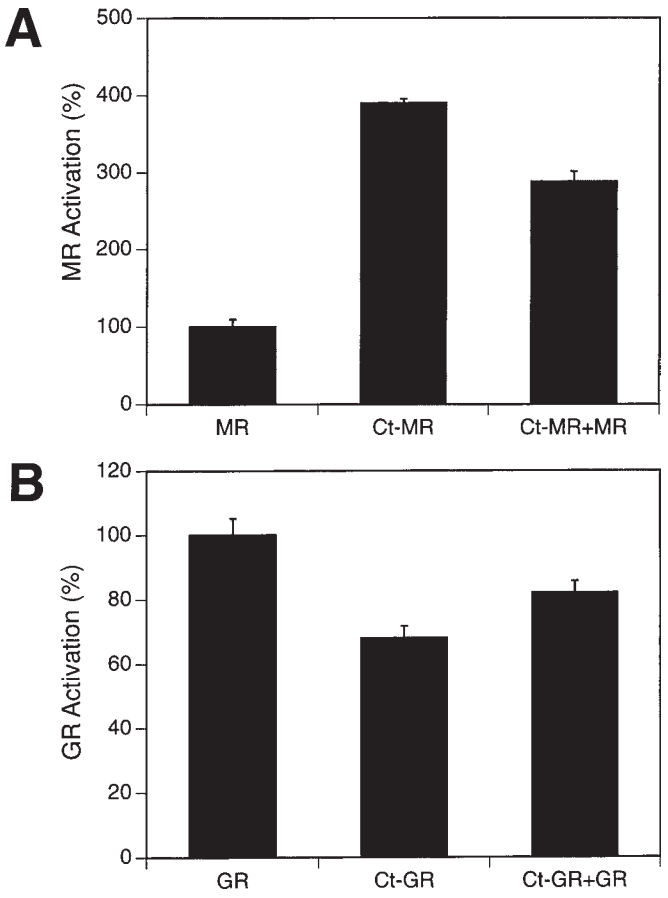

Fig. 2. Activation of the Na/K ATPase β1 promoter by Flag-tagged wild-type and mutated MR and GR in CV-1 cells. (**A**) CV-1 cells were transfected with 0.3 mg of each indicated receptor in the presence of 3 μg of Hβ1-LUC containing the human Na/K ATPase β1 gene promoter and treated with 10 n*M* aldosterone for 48 h. Activation levels obtained in the absence of receptor used as a control experiment (Flag-pcDNA3 empty vector) were subtracted from values obtained in the presence of receptor, then expressed as percentage of the MR activation set arbitrarily at 100%. The results are represented as an average of three experiments ±SD (p <0.01). (**B**) CV-1 cells were transfected as in (A) with 0.3 μg of either the indicated Flag-GR constructs or the empty vector Flag-pcDNA3, and cells were treated with 10 n*M* TA. Activation levels obtained in the absence of receptor (Flag-pcDNA3 empty vector) used as a control experiment were subtracted from values obtained in the presence of receptor, then expressed as the percentage of the GR activation set arbitrarily at 100%. The results are represented as an average of two experiments ±SD (p <0.03).

cells were transfected as already discussed with 0.3 μg of Flag-MR and 0.3 μg of Flag Ct-MR along with the reporter plasmid β1-1141LUC encoding human Na/K ATPase β1 gene promoter. As shown in Fig. 2A, activation of transcription by coexpression of MR and Ct-MR was 288 ± 13.4% vs 100% for MR alone ($n = 3, p < 0.01$). This difference does not reflect variations in receptor levels because receptor content was corrected when a single receptor was expressed and total DNA was kept constant at 6 μg. The result indicates that Ct-MR is more transcriptionally active than the wild-type MR in regulating the Na/K ATPase β1 promoter and demonstrates the inhibitory effect mediated by the N-terminal domain of MR. By contrast, in GR experiments conducted as already described, activation by the mutated receptor Ct-GR was only 68 ± 3.7% that of the wild-type GR set arbitrarily to 100% ($n = 2, p < 0.03$) (Fig. 2B). Coexpression of wild-type GR and Ct-GR resulted in an activation representing 82 ± 3.5% of that of the wild-type GR ($n = 2, p < 0.03$) (Fig. 2B). These results suggest that Ct-GR is less active than wild-type GR and that the N-terminal domain of GR contributes positively to the activation of the β1 promoter. Together, these data indicate that the GR and MR N-terminal domains have opposite effects and suggest that the N-terminal region of MR mediates a suppressor effect on the activation of Na/K ATPase β1 gene expression in CV-1 cells.

Analysis of Chimeric Receptor Homodimers

Because of the high homology between MR and GR in their DNA-binding domain (DBD) and ligand binding domain (LBD) we hypothesized that if the N-terminus of GR replaced the N-terminus of MR, activation of β1 promoter by the resulting chimeric MR would be higher than that of the wild-type MR. Using a domain swap strategy, we generated chimeric receptors, in which the N-terminal domains of the MR and GR are exchanged to produce chimeric MR and chimeric GR (Fig. 1A). We examined the expression of chimeric receptors in CV-1 cells by immunofluorescence. Representative fields illustrated in Fig. 3 show that chimeric MR is expressed in CV-1 cells as detected by the antiflag antibody, but no immunofluorescence is detected in cells transfected with the empty vector Flag-pcDNA3. Chimeric MR is cytoplasmic and nuclear in the absence of hormone. Following treatment of cells with 10 n*M* aldosterone, fluorescence is detected predominantly in the nucleus. Similar results are shown for chimeric GR following treatment with 10 n*M* TA (Fig. 3). Western blot analysis showed that chimeric MR and chimeric GR are expressed as proteins with a molecular weight of 97 and 110 kDa, respectively, as detected by the anti-Flag M2 monoclonal antibody. Specificity of the MR and chimeric GR proteins was confirmed by using the polyclonal anti-MR (αMR), recognizing the N-terminal region of MR. Specificity of GR and chimeric MR was demonstrated by using an anti-GR (αGR) antibody, recognizing the N-terminal and the of GR (data not shown).

To examine transactivation properties of these receptors, 0.3 μg of chimeric MR or chimeric GR was transiently expressed in CV-1 cells in the presence of 3 μg of the luciferase reporter plasmid driven by the Na/K ATPase β1 gene promoter. Similarly, wild-type MR or GR was expressed in CV-1 cells for comparison. Cells were treated for 48 h with either 10 n*M* aldosterone or 10 n*M* TA according to the type of LBD present within the chimeric receptor. As shown in Fig. 4, activation of gene expression by the

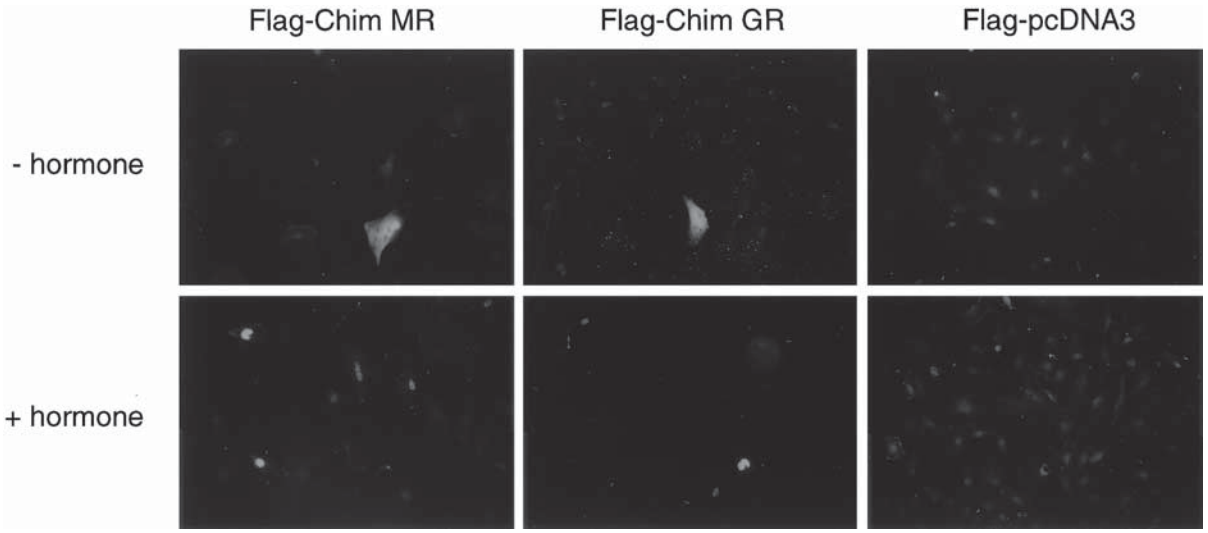

Fig. 3. Sub-cellular localization of chimeric MR and chimeric GR. CV-1 cells plated on glass cover slips were transfected for 48 h with 20 μg of Flag-tagged chimeric MR, chimeric GR, or the empty vector Flag-pcDNA3. At 47 h posttransfection, cells were treated with vehicle, 10 n*M* aldosterone, or 10 n*M* TA for 1 h, and processed for immunofluorescence using the mouse monoclonal anti-Flag antibody, M2.

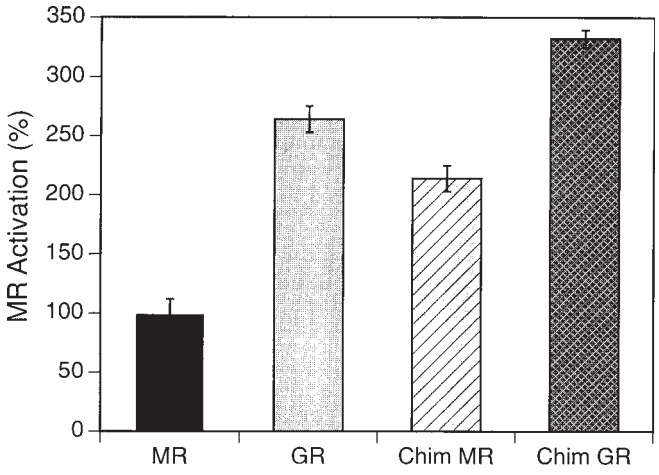

Fig. 4. Activation of β1 promoter by chimeric MR and GR. CV-1 cells were transfected with 0.3 μg of either of the indicated Flag-constructs in the presence of 3 μg of Hβ1-LUC. Cells transfected with 0.3 μg of Flag-pcDNA3 empty vector were used as a control. Cells were treated for 48 h with either 10 n*M* aldosterone in MR and chimeric MR transfections or 10 n*M* TA in GR and chimeric GR transfections. Activation levels obtained in the absence of receptor in control experiments (Flag-pcDNA3 empty vector) were subtracted from values obtained in the presence of Flag-receptor, then expressed as a percentage of the MR activation set arbitrarily at 100%. The results are represented as an average of three experiments ± SD (p <0.04).

chimeric GR containing the MR N-terminal region was 332 ± 7.3% that of MR set at 100% and nearly equivalent to the activation mediated by wild-type GR estimated at 264 ± 11% ($n = 3, p < 0.04$). These results indicate that the MR N-terminal region is not repressive in the context of chimeric GR and suggest that the LBD of GR is able to antagonize the MR N-terminal inhibitory activity. However, transactivation by the chimeric MR was 214 ±11% that of the wild-type MR ($n = 3, p < 0.04$) and similar to that induced by the wild-type GR (214 ±11% and 264 ±11%; $n = 3, p <$ 0.04). These data indicate that the N-terminal region of GR retains its positive modulatory effect within the chimeric MR and increases activation of the Na/K ATPase β1 gene promoter by twofold when compared to wild-type MR.

Role of MR N-Terminus in Context of Receptor Heterodimers

Previously, we have shown that inhibition of the β1 promoter activity observed when MR and GR are coexpressed in CV-1 cells correlates with the formation of MR/GR heterodimers as detected by supershift experiments (*19*). To examine whether the N-terminal region of MR within the MR/GR heterodimer mediates this inhibition, we conducted cotransfection experiments of wild-type and chimeric receptors along with the Na/K ATPase β1 gene promoter, coupled to luciferase. CV-1 cells were transfected with either 0.3 μg of wild-type GR plus 0.3 μg of chimeric MR or 0.3 μg wild-type MR in the presence of the 0.3 μg chimeric GR. Control experiments were carried out with 0.3 μg of wild-type MR plus 0.3 μg of wild-type GR as well as individual MR and GR expression. When a single receptor was expressed, the amount of receptor was corrected and total DNA was kept constant. Cells were treated for 48 h with 10 n*M* aldosterone, 10 n*M* TA, or both, and luciferase expression from the reporter plasmid was measured. As shown in Fig. 5, activation by coexpressed wild-type MR and wild-type GR (MR/GR) was only 49 ± 2% that of MR ($n = 3, p < 0.01$). Coexpression of the wild-type GR with a chimeric MR containing the N-terminal domain of GR induced an activation that was 161 ± 14% that of the

MR expressed alone ($n = 3$, $P < 0.01$). In addition, coexpression of MR with chimeric GR, which contains the N-terminal domain of MR, activated transcription to a level of only 80 ± 5% of that of MR (100%; $n = 3$, $P < 0.01$). These data suggest that the N-terminal region of MR mediates MR inhibition of GR-induced regulation from the Na/K ATPase β1 gene promoter when both receptors are expressed.

Discussion

Aldosterone regulation of sodium reabsorption is mediated, in part, through the transmembrane Na/K ATPase *(1,21,22)*. Early aldosterone effects on Na transport are thought to be mediated by regulating preexisting pumps followed by upregulation of the expression of the Na/K ATPase α- and β-subunits at the transcriptional level. In vitro, both mineralocorticoid and glucocorticoids enhance the expression of the α1 and β1 genes at the mRNA level *(18)*. We have shown previously that MR and GR upregulate the activity of the Na/K ATPase β1 gene promoter through direct binding to an MRE/GRE. However, gene expression was inhibited by the MR/GR heterodimers formed when both receptors were coexpressed *(19)*. In the present study, using mutated and chimeric receptors, we show that the N-terminal region of MR is inhibitory for both MR homodimers and MR/GR heterodimers.

Because deletion of the activation function-2 (AF-2) located in the LBD at the C-terminus of steroid receptors has been shown to result in a constitutively active receptor, independently from hormone activation *(24,25)*, we analyzed only N-terminal AF-1 mutated receptors. This approach revealed that the mutated receptor Ct-MR, which spans amino acids 603–984, was four times more potent than the wild-type MR. This result indicates that the Ct-MR homodimers are transcriptionally more active than those of the wild-type MR and suggests an inhibitory role for the N-terminal region of MR corresponding to amino acid sequence 1–602. Similar data have been reported for mutated rat MR lacking the N-terminal region *(17)*, indicating that the inhibitory activity modulated by the MR N-terminal region is conserved between rat and human MR. Our data are supported by a recent study by Govindan and Warriar *(26)* that identified in proximity to the AF-1 site located between position 328 and 382 a suppressive domain extending from residues 59–247 of human MR. Deletion of this domain enhanced activation of transcription from the mammary tumor virus promoter in HelA cells *(26)*.

Whereas transcriptional activity of the mutant receptor Ct-MR was fourfold higher in comparison with that of wild-type MR, only a threefold difference in the activation of gene expression was obtained when mutant Ct-MR and wild-type MR were coexpressed. This further demonstrates that Ct-MR is more transcriptionally active than wild-type MR and that the latter is not capable of effectively competing for activation of the β1 gene promoter when coexpressed with Ct-MR. However, the intermediate level of activation may reflect either formation of two classes of homodimers competing for GRE-binding sites in the promoter region or the formation of MR/Ct-MR heterodimers inducing an intermediate transcriptional activation from the Na/K ATPase β1 gene.

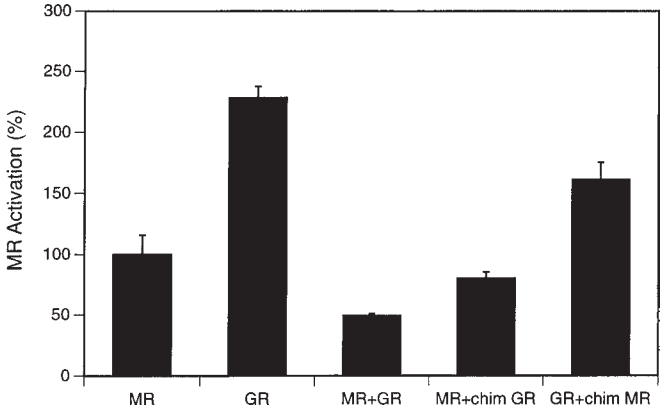

Fig. 5. Inhibition of the β1 promoter activity by wild-type and chimeric receptor heterodimers. CV-1 cells were transfected with 0.3 mg of the wild-type MR plus 0.3 mg of GR, 0.3 mg of wild-type MR plus 0.3 mg of chimeric GR, or 0.3 mg wild-type GR plus 0.3mg chimeric MR, in the presence of Hβ1-LUC. Cells were treated for 48 h with 10 n*M* aldosterone plus 10 n*M* TA. Activation levels obtained in the absence of receptor in control experiments (Flag-pcDNA3 empty vector) were subtracted from values obtained in the presence of receptor, then expressed as a percentage of the MR activation set arbitrarily at 100%. The results are represented as an average of three experiments ±SD ($p < 0.01$).

By contrast, activation of Na/K ATPase β1 gene promoter by the mutated receptor Ct-GR containing amino acids 421–777 was 25% lower than that of the full-length GR, and activation levels were reduced by only 15% when Ct-GR and wild-type GR were coexpressed. This result indicates that Ct-GR dimers are less active than wild-type GR homodimers and suggests that the N-terminal region of GR has a positive effect on transactivation of Na/K ATPase β1 gene promoter. Earlier studies have implicated the N-terminal GR domain in GR self-synergy *(27–30)* whereas the MR N-terminal region prevents MR from self-synergizing at multiple GREs *(29,31)*, resulting in lower levels of MR transactivation from various promoters. In our studies, human GR activation of transcription from the β1 promoter was two- to threefold higher than that induced by MR, similar to other reports *(16,17,29)*. Thus, the inhibitory activity of the N-terminal region of MR may result from disruption of synergy from the Na/K ATPase β1 gene promoter, which contains three MRE/GRE sequences (at positions –1048, –650, and –276).

Opposite effects of the N-terminal region of MR and GR on the activation of the β1 gene promoter activity were further confirmed by the chimeric receptor analyses. Chimeric MR containing the N-terminal domain of GR induced

more than a twofold increase in the activation of the β1 gene promoter when compared to the wild-type MR. In addition, activation by chimeric MR was nearly equivalent to that of wild-type GR, further demonstrating that the MR N-terminal region is indeed inhibitory. An increase in the MR activation by similar chimeric MR constructs has been described by others in the neuronal SK-N-MC and RN33B cells as well as the epithelial and nonepithelial kidney cells, LLC-PK1 and CV-1 *(29,32)*. The consistency of this result for chimeric MR within cells originating from different tissues indicates that the inhibitory activity of the N-terminal region of the MR is inherent to the receptor itself. To explore the role of the N-terminal region of MR in inhibition of the β1 gene promoter activity by MR/GR heterodimers, we coexpressed wild-type and chimeric receptors. While activation of the β1 gene promoter by wild-type MR and GR heterodimers was reduced by 50% relative to wild-type MR alone, we found that coexpression of the chimeric MR and wild-type GR not only reversed inhibition but increased activation threefold.

By contrast, chimeric GR containing the N-terminal region of MR was unable to reduce activation of the β1 gene promoter when compared to wild-type GR. Although a reduction in activation of gene expression by similar chimeric GR constructs has been reported *(29,32)*, these differences may be owing to the use of artificial promoters vs natural promoter containing other *cis*-acting elements that may interfere with chimeric GR activity. In addition, our data suggest the possible implication of other MR regions in the modulation of the inhibitory activity of the MR N-terminal domain in the regulation of the Na/K ATPase β1 gene promoter. This hypothesis is supported by our observation that only 80% of MR activation was induced when chimeric GR and wild-type MR were coexpressed, whereas chimeric GR activation, when expressed alone, was threefold that of MR.

Two studies by others have shown that MR and GR can form heterodimers *(16,17)*. Consistent with our results, an inhibitory activity was mediated by MR/GR heterodimers in CV-1 cells, whereas a synergistic effect on transcription was obtained in the neuroblastoma SK-N-MC cells. The opposite transactivation properties of MR/GR heterodimers in the kidney and the brain cells may result from differential interactions with a tissue-specific factor. It is conceivable that the synergistic activity of MR/GR heterodimers may reflect a coordinate action of GR and MR in the brain to accommodate a range of ligand concentrations, providing additional flexibility and complexity to the biological responses. By contrast, the inhibitory activity of MR/GR heterodimers in the kidney may constitute a mechanism for the exclusion of GR from MR target gene network.

In conclusion, we have shown that the inhibition of activation of the natural Na/K ATPase β1 promoter by MR/GR heterodimer, formed when MR and GR are coexpressed, is modulated by the N-terminal domain of MR in vitro in CV-1 cells. The significance of the regulation of the Na/K ATPase by MR/GR heterodimers in vivo remains to be evaluated. In vivo, MR and GR are coexpressed in several tissues, such as the brain, heart, vascular smooth muscle, and leukocytes *(33,34)*, for which both genomic and nongenomic aldosterone effects on sodium transport have been described *(35,36)*. The importance of tissue-specific factors in the regulation by MR is illustrated by the differential control of the expression of the Na/K ATPase by steroids in vivo *(9,11,37)*. Although expression of α1- and β1-subunits is regulated by aldosterone in the rat kidney and the heart *(9,37)*, glucocorticoids were shown to control their expression in the rat colon, a classic mineralocorticoid target tissue. This suggests that in classic mineralocorticoid target tissues, such as the kidney, in addition to the inhibition of the access of the glucocorticoid cortisol to MR by 11β-HSD2 *(22)*, other mechanisms may be implicated in determining receptor specificity. Heterodimerization of MR with other nuclear receptors and interaction of specific cellular factors with MR N-terminal and LBDs may constitute important factors in the modulation of MR regulation of Na/K ATPase β1 gene expression.

Materials and Methods

Plasmids

Expression plasmids for the human MR (RshMR) and the human GR (RshGR) were a generous gift from Dr. R. M. Evans. Construction of the luciferase plasmids containing the human Na/K ATPase β1 gene promoter, pHβ1-1141LUC, was previously described *(38)*. β-galactosidase expression plasmid (Rsv-β-gal) and 7ZF$^+$ were purchased from (Promega Madison, WI).

Generation of Epitope-Tagged Wild Type, Mutated, and Chimeric MR and GR

Full-length human MR or GR was subcloned by PCR in the *Kpn*I/*Xho*I restriction sites of the pcDNA3 (Invitrogen, CA), in frame with an upstream Flag epitope, to generate Flag-MR and Flag-GR. Modification or the pcDNA3 to Flag-pcDNA3 has been described previously *(39,40)*. Similarly, regions corresponding to the N-terminal domains A/B of MR and GR (amino acids 1–603 and 1–420, respectively) or the C-terminal C/D-E/F domains (amino acids 604–984 and 421–778, respectively) were cloned in the *Kpn*I/*Eco*RV or the *Eco*RV/*Xho*I restriction sites of Flag-pcDNA3, respectively. In addition, a domain swap strategy, in which the N-terminal domains of the receptors are exchanged, was used to produce the chimeric receptors (Chim MR, Chim GR). The structure of these plasmids was confirmed for all constructs by sequencing.

Cell Culture and Transfections

CV-1 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 100 U/mL of

penicillin, and 100 μg/mL of streptomycin. Cells were washed three times with phosphate-buffered saline (PBS) and plated overnight on 6-well plates in DMEM supplemented with dextran/charcoal-stripped, steroid-free FBS (Hyclone, UT). Cells were transfected by the calcium phosphate method with 3 μg of Hβ1-LUC containing the human Na/K ATPase β1 gene promoter and either 0.3 μg of Flag-MR or 0.3 μg of Flag-GR constructs, or 0.3 μg of each receptor expression plasmid. In addition, 1.5 μg of β-galactosidase expression plasmid (Rsv-β-gal) was included and the 7Zf+ plasmid filler DNA up to a total of 6 μg. In transfections containing a single receptor, 0.3 μg of Flag-pcDNA3 empty vector was used to correct for receptor DNA content, and total DNA was maintained at 6 μg.

Cells were incubated with DNA precipitates for 18 h, then washed and treated for 48 h with 10 n*M* aldosterone in MR transfections, 10 n*M* TA in GR transfections, or 10 n*M* aldosterone plus 10 n*M* TA when both MR and GR were cotransfected. Sixty-six to 72 h posttransfection, cells were harvested and cell extracts were assayed for luciferase activity.

For immunofluorescence, CV-1 cells were plated on glass cover slips in 100-mm plates. Cells were transfected by the calcium-phospate method for 48 h with 20 μg of each Flag construct or the empty Flag-pcDNA vector. Cells were treated with either vehicle or the indicated hormone for 1 h, and cells were washed with PBS and then fixed in a 4% paraformaldehyde solution for 20 min at room temperature. Cells were washed again and stored in PBS at 4°C for immunofluorescence staining.

Luciferase Assays and Statistics

Whole-cell extracts were prepared from transfected CV-1 cells in reporter lysis buffer and 20-μL extracts were assayed for luciferase activity, as recommended by the manufacturer (Promega). Luciferase values were all normalized to β-galactosidase activity measured on 20-μL extracts, as recommended by the manufacturer (Promega). For each experiment, cells were cotransfected with an equal amount (1.5 μg) of Rsv-β-galactosidase reporter plasmid (pRsv-β-gal), and each transfection was carried out in triplicate. β-galactosidase activity was evaluated by measuring the absorbance of *o*-nitrophenol at 420 nm produced by transformation of the substrate *o*-nitrophenyl-β-D-galactopyranoside by the β-galactosidase enzyme contained in cell extracts. Differences in the β-galactosidase activity were expressed as a fraction (highest activity divided by the other values). Luciferase values were then normalized to β-galactosidase activity by multiplying the luciferase value by the corresponding β-galactosidase ratio for the same transfection. Luciferase activation obtained in the absence of receptor (Flag-pcDNA3) was used as a control and subtracted from values obtained in the presence of Flag-receptor construct, and then expressed as a percentage of the MR activation set arbitrarily at 100%. The results are presented as an average of two to three independent transfection experiments ± 50 (S.D). Statistical significance by one-way analysis of variance was calculated using Excel 98 statistical analysis ($P < 0.05$).

Immunofluorescence

CV-1 cells plated out on glass cover slips were processed for immunofluorescence using the mouse monoclonal anti-Flag antibody M2 as a primary antibody. Cells were permeabilized with PBS-0.2% Triton X-100, washed with PBS-0.2% gelatin, and then incubated with the mouse monoclonal anti-Flag antibody (M2, 1:200) (VWR Scientific, Bridgeport, NJ) for 1–1.5 h at 37°C. Cells were washed and incubated with antimouse fluorescein IgG (Vector, Burlingame, CA) for 30 min at 37°C. Following several washes, cells were counterstained with DAPI (1:2000) for 10 min at room temperature. Cover slips were washed in water prior to air-drying and mounting of slides. Receptors were visualized by indirect fluorescence microscopy using appropriate filters and a Sarastro 2000 confocal laser-scanning microscope (Molecular Dynamics, Sunnyvale, CA).

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