PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 84 (2003) 543–553

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Transcriptional inhibition of the human insulin receptor gene by aldosterone

Consuelo Calle [a,*], Javier Campión [a], Moisés García-Arencibia [a], Begoña Maestro [a], Norma Dávila [b]

[a] *Department of Biochemistry and Molecular Biology, School of Medicine, Complutense University, Madrid 28040, Spain*
[b] *Biochemistry Unit, Puerta de Hierro Hospital, Madrid 28040, Spain*

Received 20 May 2002; accepted 17 January 2003

Abstract

In earlier studies, we reported reduced human insulin receptor (hIR) mRNA levels, insulin binding and insulin responsiveness in U-937 human promonocytic cells treated with aldosterone. The mechanism for this inhibition could be diminished IR gene transcription, since aldosterone did not affect hIR mRNA stability. All the effects were mediated by a downregulation of the mineralocorticoid receptor (MR, NR3C2) expressed at both the RNA and protein levels, suggesting that MR could act as a transcription factor that binds to hormone response elements in the hIR gene promoter. Indeed, MR has been shown to bind glucocorticoid response elements (GREs) in target genes. Given that five GREs have been characterized in the hIR promoter, we decided to test whether these elements could mediate the aldosterone-elicited inhibition of hIR expression detected by us in U-937 cells. In the present report, we demonstrate that aldosterone inhibits the activity of the hIR wild-type promoter by 23%, and causes 23 and 31% reductions in the activity of progressive deletions of this promoter comprised of fragments up to −1473 and −876 bp, respectively. This indicates that the −876 to −271 bp region of the hIR promoter may be sufficient for this transcriptional inhibition by aldosterone. We also provide evidence for direct MR interaction with some of the GREs of this promoter region, specifically with the cGRE1 and cGRE3, presumably as MR–MR homodimers, and with pGRE as a MR–GR heterodimer. This heterodimer may play the most relevant role and participate in the cross-talk between mineralocorticoids, glucocorticoids and insulin signalling in U-937 cells.

Keywords: Aldosterone; Mineralocorticoid receptor; Dexamethasone; Glucocorticoid receptor; Glucocorticoid response element; Heterodimerization; Human insulin receptor gene promoter; Transcriptional regulation; U-937 human promonocytic cells

1. Introduction

It is know that the genomic effects of mineralocorticoids and glucocorticoids are mediated by interaction with their receptors [1,2]. Both types of hormone passively diffuse through the cell membrane and bind the mineralocorticoid receptor (MR, NR3C2) and the glucocorticoid receptor (GR, NR3C1) in the cytoplasm/nucleus of the cell. The affinity of MR for glucocorticoids is almost identical to that for mineralocorticoids [3]. After binding, the activated receptors act as transcription factors, which bind to hormone response elements in the promoter region of responsive genes [4]. Since no selective mineralocorticoid response elements have been identified, MR and GR appear to promiscuously bind and transactivate glucocorticoid response elements (GREs) in the promoters of target genes [4,5]. Moreover, MR and GR seem to act as dimers, either homodimers or heterodimers [6–9], enhancing [7] or lowering [8] transcriptional activity.

Results from our laboratory have previously indicated in vivo and in vitro modulation of insulin receptor (IR) gene expression by glucocorticoids. Thus, we were able to demonstrate that IR mRNA levels were modulated in a tissue-specific manner in patients with Cushing's syndrome [10]. We also observed tissue-specific changes in IR mRNA concentrations in dexamethasone-treated rats [11]. In addition, we reported dose- and time-dependent dexamethasone stimulation of the two major species of the human insulin receptor (hIR) mRNA (11 and 8.5 kb in size) occurring in U-937 human promonocytic cells [12,13]. In these cells, dexamethasone failed to affect both hIR mRNA half-life and protein turnover, suggesting an effect on transcription [13].

We also reported the in vivo and in vitro modulation of IR gene and protein expression by mineralocorticoids. We

* Corresponding author. Tel.: +34-913941451; fax: +34-913941691.
E-mail address: consuelo@med.ucm.es (C. Calle).

0960-0760/03/$ – see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00072-4

noted a reduction in the number and affinity of hIRs in adipocytes isolated from the subcutaneous adipose tissue of a patient with primary hyperaldosteronism [14]. We also observed tissue-specific modulation of IR gene expression in a model of mineralocorticoid excess in the rat [15]. Further, we recently demonstrated dose- and time-dependent aldosterone inhibition of the two major species of hIR mRNA in U-937 cells [16]. RNA stability and protein turnover were both unaltered, suggesting an effect on transcription. Aldosterone also decreased the hIR number but not the affinity of this receptor, suggesting possible failure in the insulin responsiveness of the cells. When this possibility was explored, the maximal cellular response to insulin, both in terms of glucose transport and DNA synthesis, was decreased after aldosterone treatment [17]. The presence of MR in U-937 cells was also observed [16,17], and blockade of this receptor by the mineralocorticoid antagonist spironolactone indicated the involvement of this MR in the aldosterone-elicited inhibition of hIR mRNA levels [16].

The promoter of the hIR gene has been the subject of intense research. This promoter contains binding sites for many transcription factors including five GREs. These GREs were well characterized by Lee and co-workers [18,19] through transfection experiments and footprinting assays. Although these GREs did not match the canonical GRE sequence, they were able to confer a capacity for glucocorticoid-dependent transcriptional induction upon the hIR gene. Given the intimate relationship between MR and GR, these GREs could mediate the aldosterone-elicited inhibition of hIR gene expression and insulin action detected by us in U-937 cells.

On this setting, the present study was designed to extend our previous studies and investigate the possibility of a direct transcriptional effect of aldosterone on the promoter activity of the hIR gene in U-937 cells. In addition, we explored the interaction of the activated MR with each of the functional GREs previously described by Lee and co-workers [18,19] in the hIR promoter. In parallel, we examine the transcriptional effect of dexamethasone and binding of the activated GR with each of the GREs of this promoter.

2. Materials and methods

2.1. Cell culture and treatments

U-937 human promonocytic cells (mycoplasma-free) were grown in RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated foetal calf serum and antibiotics at 37 °C in a humidified 5% CO_2 atmosphere as described previously [12,20]. Besides the MR [16,17], these cells are naturally endowed with other receptors, including IR [12,21], and GR [22].

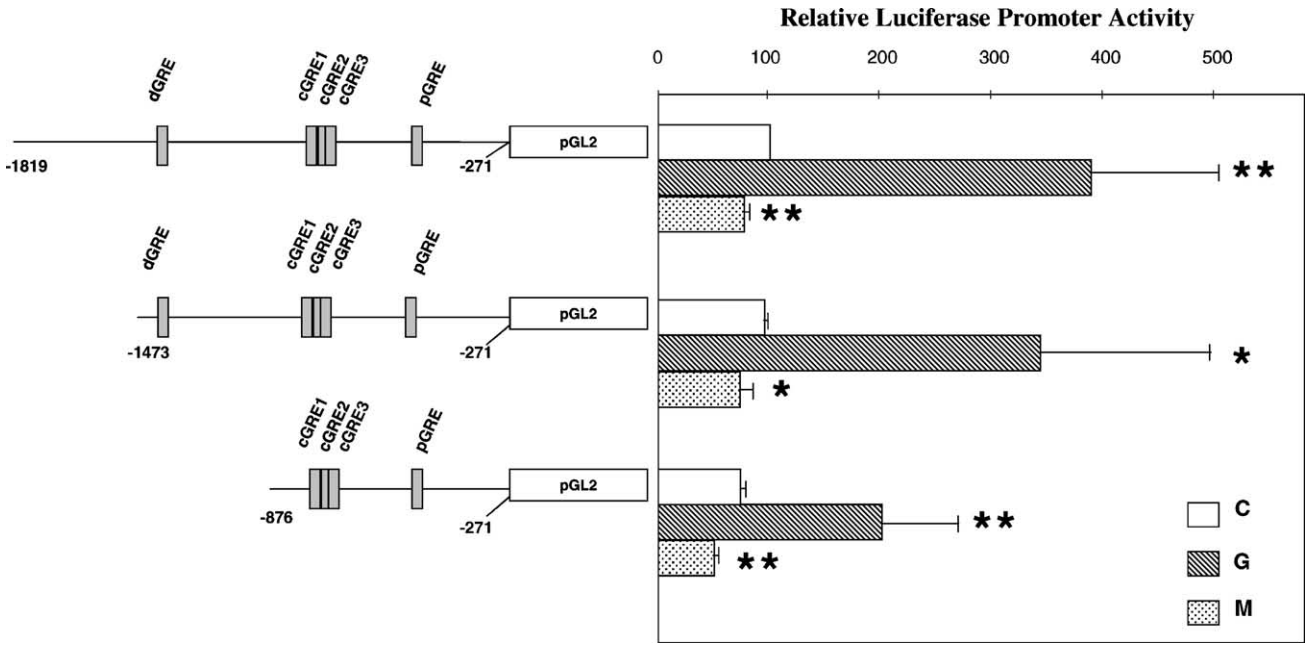

Fig. 1. Transcriptional effect of aldosterone and dexamethasone on the promoter activity of the human insulin receptor (hIR) gene in U-937 cells. The cells were transiently transfected with the phIR(−1819)-GL2 plasmid, which contains the −1819 to −271 promoter fragment of the hIR gene, considered as the wild-type promoter, and with phIR(−1473)-GL2 or phIR(−876)-GL2 plasmids, containing progressive 5′ deletions (up to −1473 and −876 bp, respectively) of the wild-type promoter. The transfected cells were left untreated (C), or were treated either with 5×10^{-6} M dexamethasone for 15 h (G) or with 10^{-9} M aldosterone for 24 h (M). Luciferase activity is given in arbitrary units relative to the value of 100 assigned to that of the wild promoter in untreated cells after correcting for transfection efficiency. Values are means ± S.E.M. of at least five experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. respective control.

2.2. Plasmids, transfections and analysis of luciferase activity

The −1819 to −271 promoter fragment of the hIR gene cloned in the BglII site of the pCAT3M vector was kindly provided by Drs. S.Y. Tsai (Department of Cell Biology, University of California) and G. Elberg (Baylor College of Medicine, Texas Medical Center). This promoter fragment, considered by these authors as the wild-type promoter, was subcloned into the BglII site of the pGL2-basic vector (Promega) to create the reporter plasmid phIR(−1819)-GL2 [23]. The orientation and integrity of the insert was confirmed by restriction analysis. Digestion of phIR(−1819)-GL2 plasmid with KpnI or XhoI generated the plasmids phIR(−1473)-GL2 and phIR(−876)-GL2, respectively.

Transient transfections were carried out by electroporation of 20×10^6 cells in RPMI 1640 medium with the Bio-Rad gene-pulser II, essentially as described previously [23]. The cells were electroporated at 250 V, 960 μF, in a volume of approximately 300 μl of RPMI containing 50 μg of each of the reporter plasmids described earlier, or 50 μg of the promoterless pGL2-basic vector, together with 50 μg of the pBluescript II KS(+/−) as a carrier. As a positive control, we used 25 μg of the pGL3-control vector (Promega), which includes SV40 promoter and enhancer sequences. All transfections were performed in the absence of MR and GR expression vectors due to the endogenous activity of both receptors in U-937 cells [16,17,22]. Transfection efficiency was determined using 12 μg of pCMV-βgal (Clontech), and by assessing β-galactosidase activity in the extracts [24]. After resting for 24 h, the transfected cells were left untreated,

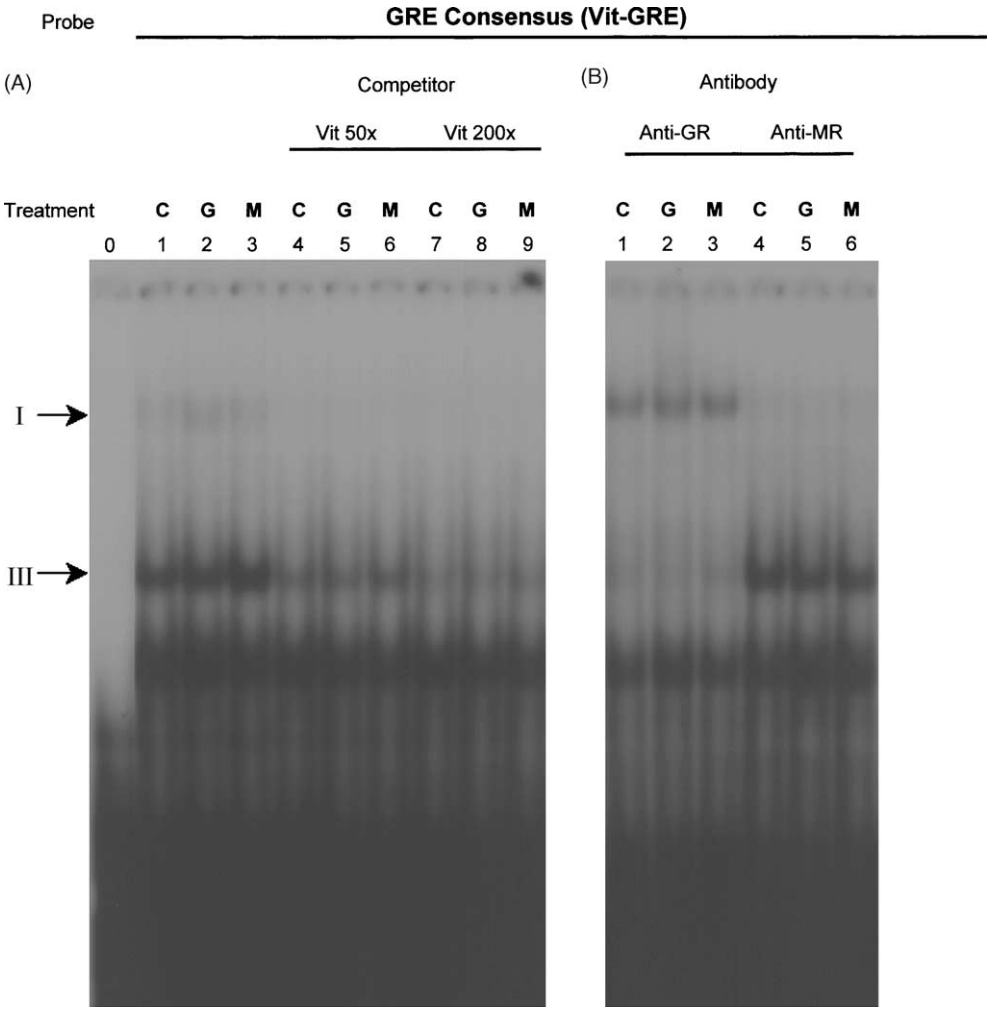

Fig. 2. Binding of the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) to Vit-GRE. EMSA was performed as described in Section 2. In brief, nuclear extracts of untreated U-937 cells (C), cells treated with 5×10^{-6} M dexamethasone for 15 h (G), and cells treated with 10^{-9} M aldosterone for 24 h (M), were incubated with the ^{32}P-labelled double stranded synthetic oligonucleotide Vit-GRE. (A) Lane 0, no nuclear extracts; lanes 1–3, nuclear extracts under the three treatment conditions; lanes 4–6 with 50× and lanes 7–9 with 200× excess of the unlabelled Vit-GRE oligonucleotide, respectively. (B) Lanes 1–3 with the specific anti-GR antibody (PA1-510A), and lanes 4–6 with the specific anti-MR antibody (MCR N-17), respectively. Arrows indicate the position of the complexes (I and III).

or treated with either 10^{-9} M aldosterone (Sigma) for 24 h, or 5×10^{-6} M dexamethasone (Sigma) for 15 h. These conditions of dose and time were selected on the basis of previously observed maximal effects in both aldosterone-elicited inhibition and dexamethasone-induced stimulation of hIR mRNA levels in U-937 cells [13,16]. These treatments were non-selective for MR or GR activation, given that the corticoids were supplied in the absence of antagonists to cells with endogenous MR and GR that could be activated by either hormone. Nevertheless, our purpose was to induce optimal transcriptional effects in each treatment by activation of both receptors.

The cells were then collected by centrifugation, and luciferase activity quantified following the instructions provided in the assay kit (Promega). Previous luciferase determinations revealed that the activity of the pGL3-control vector was about 100 times above the basal levels shown by the promoterless pGL2-basic vector, after correcting for transfection efficiency [23].

2.3. Electrophoretic mobility shift assays (EMSA)

Six oligonucleotides commercially synthesized by Cruachem: Vit-GRE, dGRE, cGRE1, cGRE2, cGRE3, and pGRE were used in these assays. Vit-GRE (5′GATCCAAAG TCAGAACACAGTGTTCTGATC3′) comprised the natural sequence of the oestrogen response element in the *Xenopus vitellogenin* A_2 gene mutated to obtain a perfect 3′ half-site GRE motif [25]. The rest of oligonucleotides comprised the following five GREs sequences of the hIR promoter, well characterised by Lee and co-workers [18,19] through transfection experiments and footprinting assays dGRE: from −1363 to −1335 bp (5′CCCTCCTCCCA TTGAGTTCTGGCTTTCCT3′); cGRE1: from −754 to

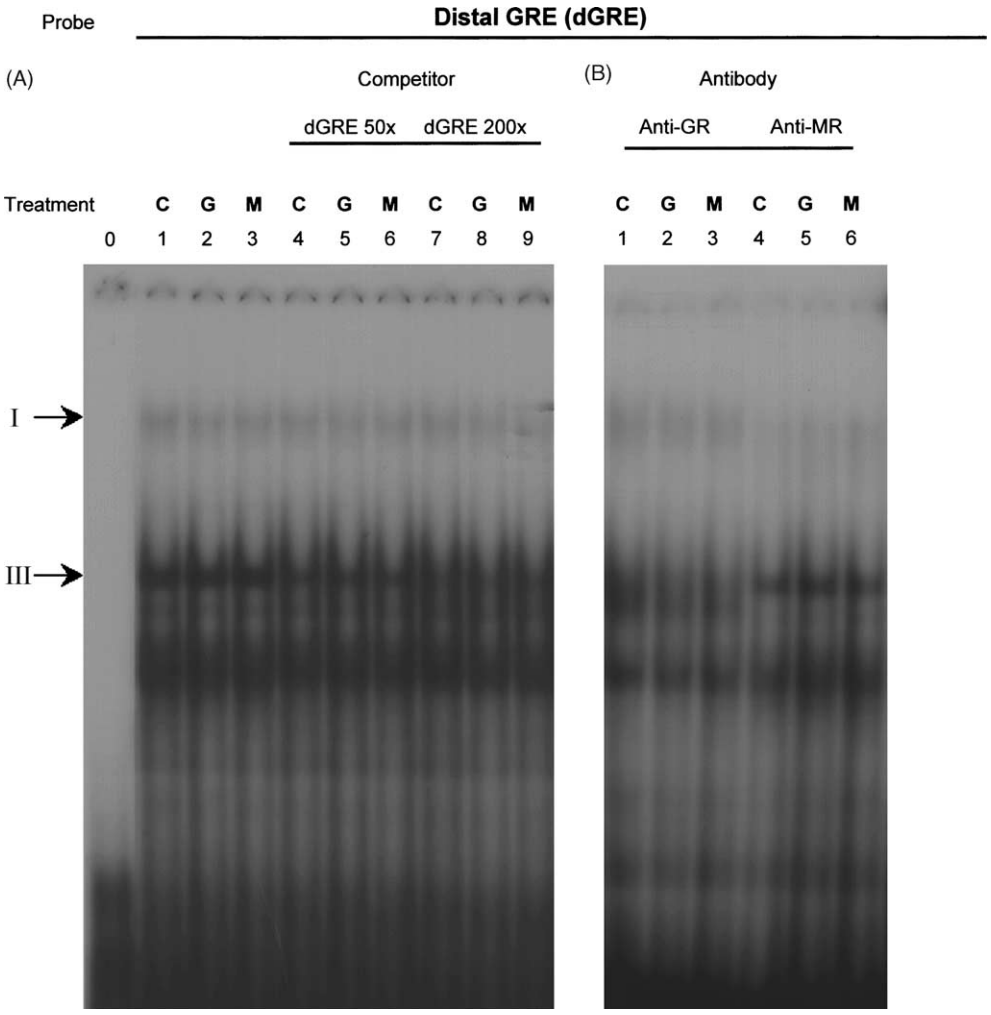

Fig. 3. Binding of the MR and GR to dGRE. EMSA was performed as described in Section 2. In brief, nuclear extracts of untreated U-937 cells (C), cells treated with 5×10^{-6} M dexamethasone for 15 h (G), and cells treated with 10^{-9} M aldosterone for 24 h (M), were incubated with the ^{32}P-labelled double stranded synthetic oligonucleotide dGRE. (A) Lane 0, no nuclear extracts; lanes 1–3, nuclear extracts under the three treatment conditions; lanes 4–6 with 50× and lanes 7–9 with 200× excess of unlabelled dGRE oligonucleotide, respectively. (B) Lanes 1–3 with the specific anti-GR antibody (PA1-510A), and lanes 4–6 with the specific anti-MR antibody (MCR N-17), respectively. Arrows indicate the position of the complexes (I and III).

−725 bp (5′CCTGTGGGGCGCCTCCGGGGGTCTGAAACT3′); cGRE2: from −707 to −689 bp (5′GTAGGGCGCGCGGATCTGG3′); cGRE3: from −680 to −656 bp (5′CTCGGTCCC**GGCGCG**CCC**AGGGCC**T3′); pGRE: from −363 to −341 bp (5′TCCCGGAGCCCGCAGATCGCGAC3′).

The annealed oligonucleotides (3.5×10^{-12} M) were 5′end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol) (NEN) with T4 polynucleotide kinase (Promega). Nuclear extracts were obtained as described by Schreibert et al. [26] from cells untreated or treated with either 10^{-9} M aldosterone for 24 h, or 5×10^{-6} M dexamethasone for 15 h. These extracts (10 μg) were incubated for 10 min on ice in a binding buffer containing 1×10^{-2} M Tris, pH 7.5, 8×10^{-2} M KCl, 10% glycerol, 1×10^{-3} M dithiothreitol, and 2 μg of poly(dI-dC) [27], in a total volume of 20 μl. Next, 0.1–0.5 ng (100,000 cpm) of each labelled oligonucleotide was added to the reaction mixture and incubation was continued for 20 min at room temperature. An excess of the corresponding unlabelled oligonucleotide (50×, or 200×) was added as a specific competitor in each EMSA. For a stricter test of the binding specificity of the protein–DNA complexes, we incubated the nuclear extracts with an anti-GR polyclonal antibody (PA1-510A) (Affinity Bioreagents) that recognises the N-terminal region of the human GR, or an anti-MR polyclonal antibody (MCR N-17) (Santa Cruz Biotechnology) that binds to the N-terminal region of the human MR. Incubation of the nuclear extracts with a non-immune serum had no effect. The protein–DNA complexes were resolved on 4% nondenaturating polyacrylamide gels at 4 °C in 0.25× TBE. The gels were then dried and examined by autoradiography.

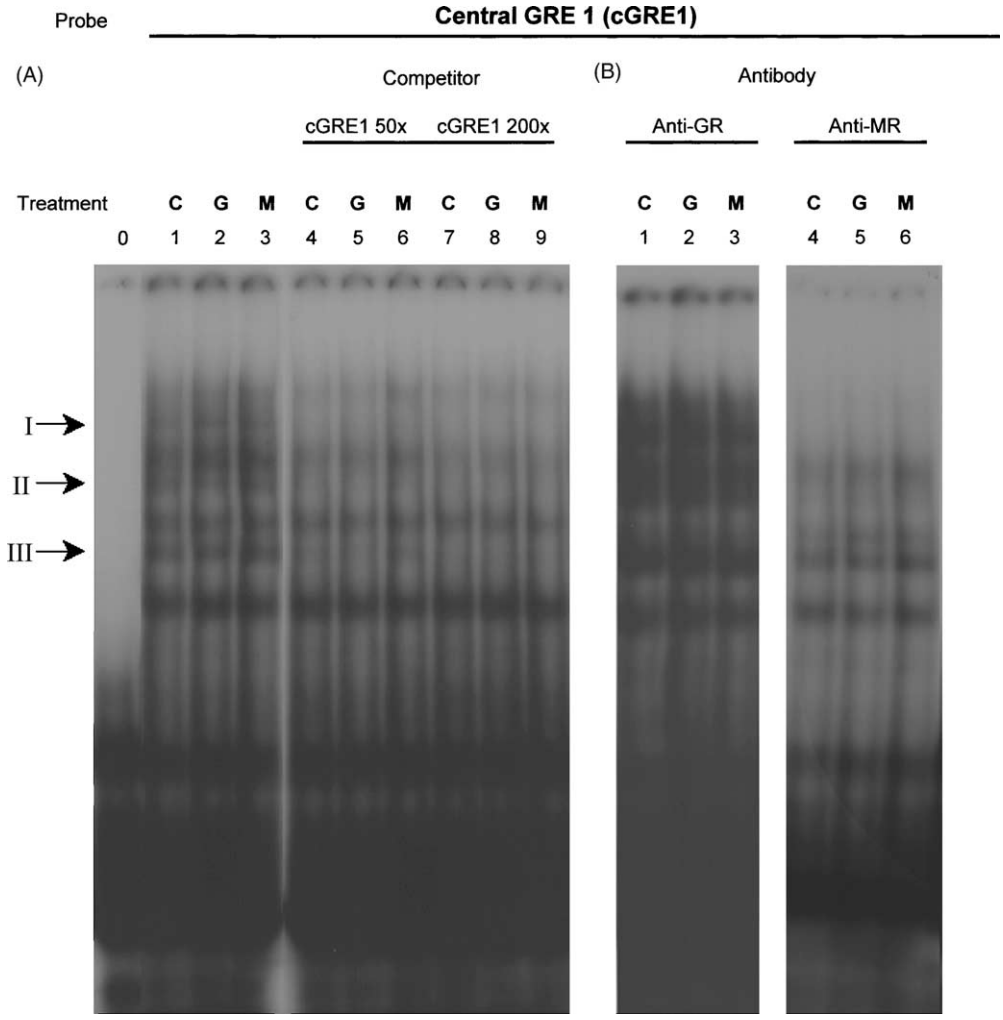

Fig. 4. Binding of the MR and GR to cGRE1. EMSA was performed as described in Section 2. In brief, nuclear extracts of untreated U-937 cells (C), cells treated with 5×10^{-6} M dexamethasone for 15 h (G), and cells treated with 10^{-9} M aldosterone for 24 h (M), were incubated with the ^{32}P-labelled double stranded synthetic oligonucleotide cGRE1. (A) Lane 0, no nuclear extracts; lanes 1–3, nuclear extracts under the three treatment conditions; lanes 4–6 with 50× and lanes 7–9 with 200× excess of unlabelled cGRE1 oligonucleotide, respectively. (B) Lanes 1–3, with the specific anti-GR antibody (PA1-510A). (C) Lanes 4–6, with the specific anti-MR antibody (MCR N-17). Arrows indicate the position of the complexes (I–III).

2.4. Statistical analysis

Unless otherwise stated, data are expressed as the mean ± S.E.M. The Student's *t*-test was used for the statistical comparisons. The threshold for significance was set at $P < 0.05$.

3. Results

To investigate the possibility of a direct transcriptional effect of aldosterone on the promoter activity of the hIR gene, U-937 cells were transiently transfected with a reporter plasmid encoding the hIR promoter spanning nucleotides −1819 to −271 (wild-type promoter) linked to the luciferase gene, phIR(−1819)-GL2. Treatment of the transfected cells with 10^{-9} M aldosterone for 24 h resulted in a 23% inhibition of the promoter activity of this wild-type promoter (Fig. 1). Treatment of the transfected cells with 5×10^{-6} M dexamethasone for 15 h induced a 288% increase in the luciferase activity of this promoter (Fig. 1).

To identify the hIR promoter fragment sufficient for this inhibition by aldosterone, we used, phIR(−1473)-GL2 and phIR(−876)-GL2, two plasmids with progressive deletions of the wild-type promoter, respectively showing 94 and 67% decreases in promoter activity (Fig. 1). These plasmids contained five and four of the GREs of the hIR promoter, respectively (Fig. 1). According with our previous results [28], we did not used the phIR(−577)-GL2 plasmid. This plasmid, with only the dGRE, shows low promoter activity (17%) coincident with that of the promoterless pGL2-basic, due to the removal of a particular region of four GC boxes located between −618 and −593 bp of the hIR promoter. This region has been shown to be essential for the promoter activity of this gene and thus must be conserved [29,30].

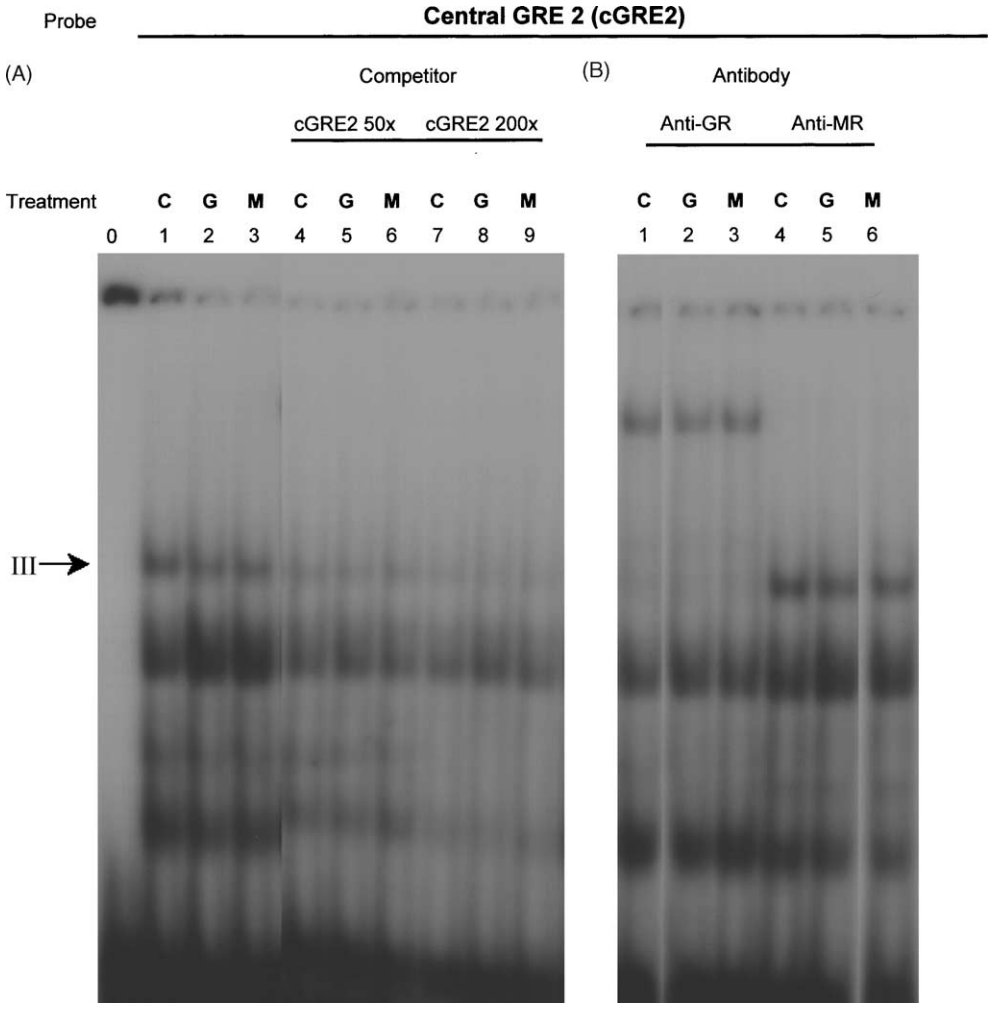

Fig. 5. Binding of the MR and GR to cGRE2. EMSA was performed as described in Section 2. In brief, nuclear extracts of untreated U-937 cells (C), cells treated with 5×10^{-6} M dexamethasone for 15 h (G), and cells treated with 10^{-9} M aldosterone for 24 h (M), were incubated with the ^{32}P-labelled double stranded synthetic oligonucleotide cGRE2. (A) Lane 0, no nuclear extracts; lanes 1–3, nuclear extracts under the three treatment conditions; lanes 4–6 with 50× and lanes 7–9 with 200× excess of unlabelled cGRE2 oligonucleotide, respectively. (B) Lanes 1–3 with the specific anti-GR antibody (PA1-510A), and lanes 4–6 with the specific anti-MR antibody (MCR N-17), respectively. Arrows indicate the position of complex III.

Once the cells were transfected with these plasmids and exposed to the treatments, luciferase determinations revealed that the promoter activity of the fragments spanning up to −1473 and −876 bp, were inhibited 23, and 31% respectively, by aldosterone (Fig. 1). In addition, the activities of the fragments spanning up to −1473 and −876 bp, were induced 260 and 174%, respectively, by dexamethasone (Fig. 1). These data together with those obtained with the wild-type promoter suggest that while dexamethasone needs at least the fragment spanning up −1473 bp for full transcriptional stimulation of the hIR gene, only the portion spanning up to −876 bp is needed for complete transcriptional inhibition by aldosterone.

We next explore the interaction of MR and GR with each of the functional GREs of the hIR gene promoter [18,19] by EMSA. As a positive control of MR binding, first we used the Vit-GRE as oligoprobe on nuclear extracts of untreated, aldosterone-treated or dexamethasone-treated cells. Vit-GRE has been previously demonstrated to specifically bind MR in this assay [31]. As shown in Fig. 2A, two major protein–DNA complexes (I and III) were formed under these three experimental conditions (lanes 1–3). Complex I was of less intensity than complex III, possibly reflecting differences in the expression level of MR and GR in U-937 cells [16,17,22]. The fact that the intensity of both complexes was apparently unmodified by the treatments may be explained by the background of both receptors in these cells. The addition of excess (50× and 200×) unlabelled oligonucleotide led to competition for both complexes (lanes 4–6, 7–9, respectively). Fig. 2B shows that the use of a specific antibody against MR blocked the formation of the protein–DNA complex I, indicating the presence of MR in this complex (lanes 4–6), while the addition of a specific antibody against GR caused supershifting of the protein–DNA complex III,

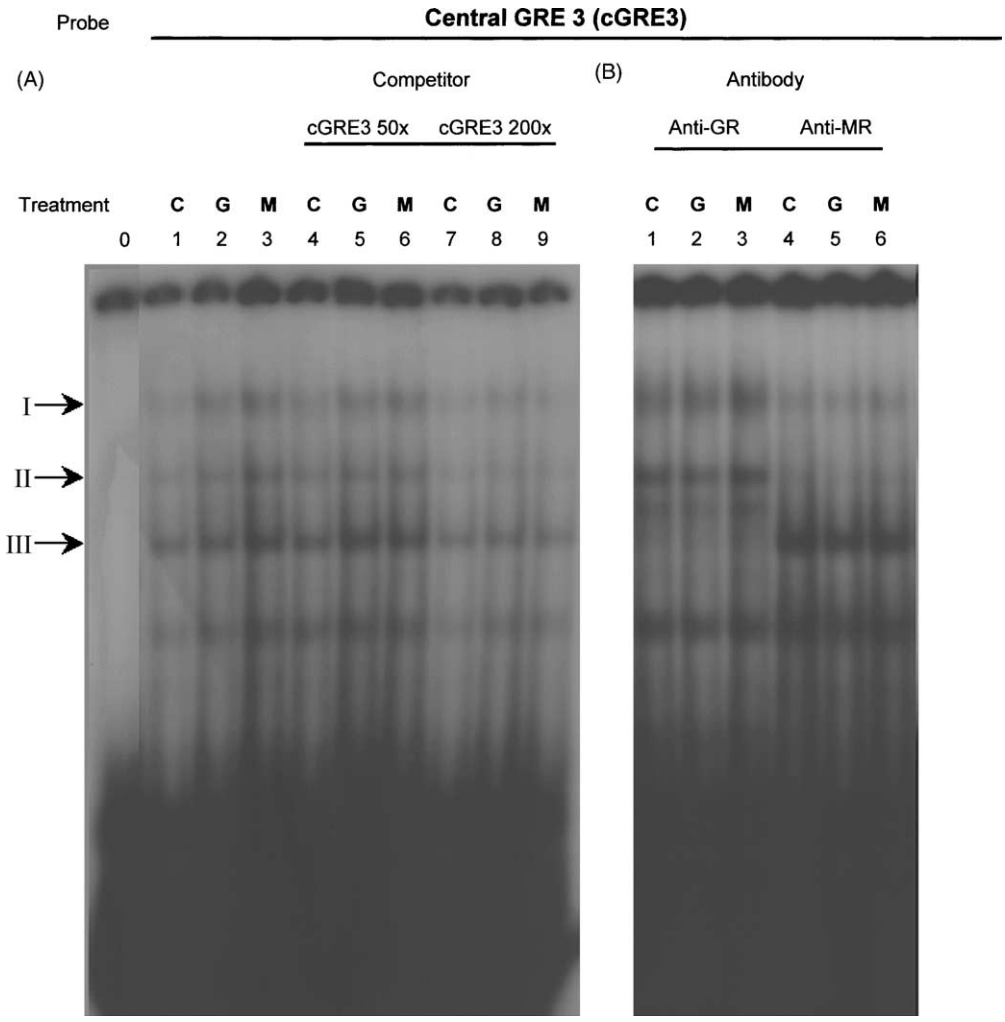

Fig. 6. Binding of the MR and GR to cGRE3. EMSA was performed as described in Section 2. In brief, nuclear extracts of untreated U-937 cells (C), cells treated with 5×10^{-6} M dexamethasone for 15 h (G), and cells treated with 10^{-9} M aldosterone for 24 h (M), were incubated with the ^{32}P-labelled double stranded synthetic oligonucleotide cGRE3. (A) Lane 0, no nuclear extracts; lanes 1–3, nuclear extracts under the three treatment conditions; lanes 4–6 with 50× and lanes 7–9 with 200× excess of unlabelled cGRE3 oligonucleotide, respectively. (B) Lanes 1–3 with the specific anti-GR antibody (PA1-510A), and lanes 4–6 with the specific anti-MR antibody (MCR N-17), respectively. Arrows indicate the position of the complexes (I–III).

indicating the presence of GR in this complex (lanes 1–3). Thus, Vit-GRE was able to bind two complexes with easily distinguishable electrophoretic mobilities due to the different molecular mass of MR and GR [1]. These complexes presumably corresponded to MR–MR and GR–GR homodimers [6–9]. This binding pattern was used as a control in subsequent experiments.

Using dGRE as the next oligoprobe, two main delayed complexes (I and III) were detected under the three experimental conditions (Fig. 3A, lanes 1–3). The intensity of both complexes was unmodified by the treatments. The addition of 50× and 200× excess unlabelled oligonucleotide gave rise to partial competition for the formation of complex III but not complex I (lanes 4–6, 7–9, respectively). Further, using specific antibodies (Fig. 3B), complex III was partially supershifted by the anti-GR antibody (lanes 1–3), while complex I was unaltered by the anti-MR antibody (lanes 4–6). It therefore appears that dGRE only partially binds GR, presumably as a GR–GR homodimer.

We then went on to test cGRE1, cGRE2 and cGRE3 as oligoprobes. The oligonucleotide cGRE1 gave rise to three main complexes (I–III) (Fig. 4A). The intensity of these complexes was unmodified by the treatments. The addition of excess (50× and 200×) unlabelled nucleotide (lanes 4–6, 7–9, respectively) resulted in competition for these complexes. Complex I appeared to be a MR–DNA complex (Fig. 4B), since it was blocked by the specific anti-MR antibody (lanes 4–6). However, neither complex II nor III were affected by either antibody (Fig. 4B) (lanes 1–3, 4–6, respectively). Thus, cGRE1 was only able to bind MR, presumably as a MR–MR homodimer.

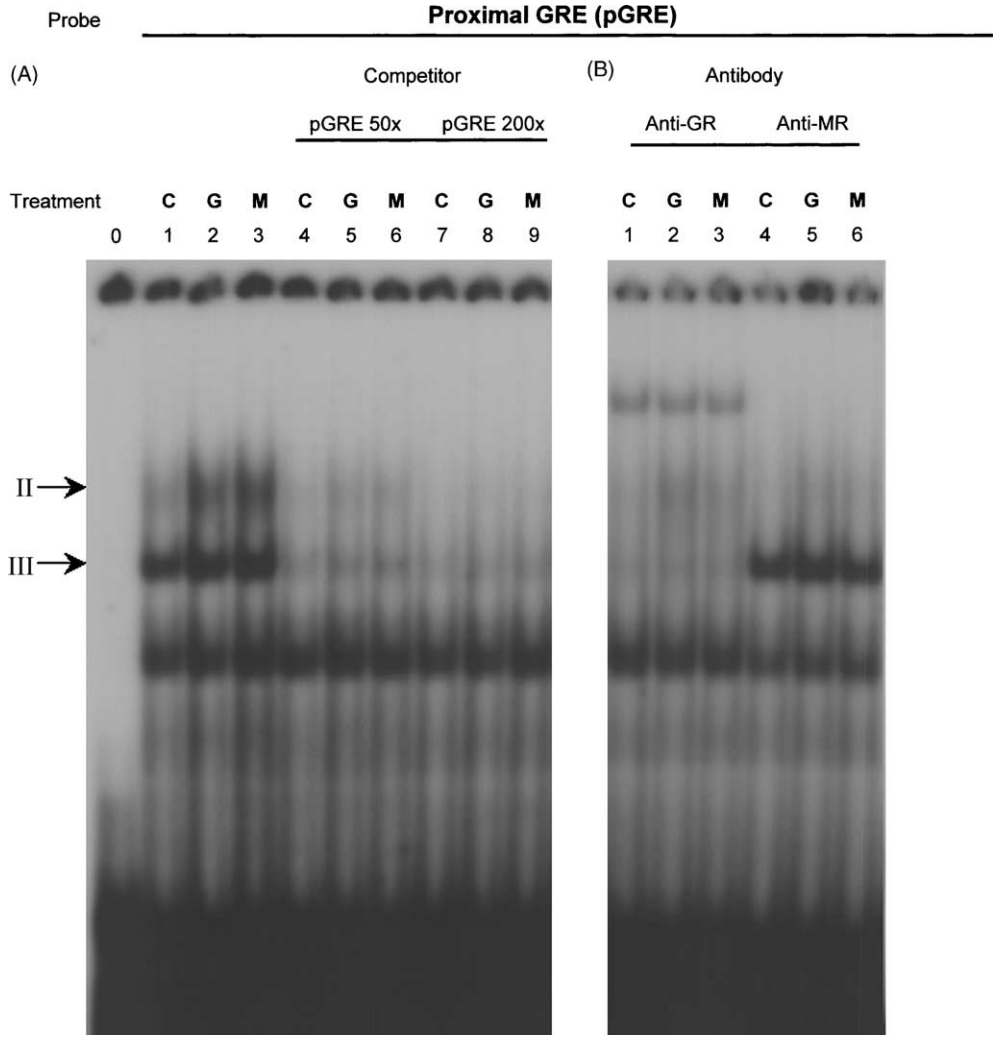

Fig. 7. Binding of the MR and GR to pGRE. EMSA was performed as described in Section 2. In brief, nuclear extracts of untreated U-937 cells (C), cells treated with 5×10^{-6} M dexamethasone for 15 h (G), and cells treated with 10^{-9} M aldosterone for 24 h (M), were incubated with the ^{32}P-labelled double stranded synthetic oligonucleotide pGRE. (A) Lane 0, no nuclear extracts; lanes 1–3, nuclear extracts under the three treatment conditions; lanes 4–6 with 50× and lanes 7–9 with 200× excess of unlabelled pGRE oligonucleotide, respectively. (B) Lanes 1–3, with the specific anti-GR antibody (PA1-510A), and lanes 4–6 with the specific anti-MR antibody (MCR N-17), respectively. Arrows indicate the position of the complexes (II and III).

When the binding pattern of the cGRE2 was analysed, only one GR–DNA complex (III) was observed (Fig. 5A). This complex was efficiently competed with the addition of unlabelled oligonucleotide (lanes 4–6, 7–9, respectively) and supershifted by the specific anti GR-antibody (Fig. 5B) (lanes 1–3), while addition of the anti-MR antibody had no effect (Fig. 5B) (lanes 4–6). Hence, cGRE2 was only able to bind GR, presumably as a GR–GR homodimer.

The cGRE3 binding pattern indicated three major complexes (I–III)(Fig. 6A), which showed enhanced intensity after treatment with either hormone. The formation of these complexes was competed with the addition of 200×, but not 50×, excess unlabelled oligonucleotide (lanes 4–6, 7–9, respectively). The specific anti-GR antibody caused a supershift of the protein–DNA complex III (Fig. 6B) (lanes 1–3), indicating the presence of GR in this complex. The use of the specific anti-MR antibody blocked the protein–DNA complex II but no complex I (Fig. 6B), possibly since cGRE3 includes two overlapping GRE elements, as will be indicated later. Therefore, cGRE3 appears to bind MR and GR, presumably as MR–MR and GR–GR homodimers.

The pGRE binding pattern included two major complexes (II and III) that were competed with the addition of the unlabelled oligonucleotide (Fig. 7A) (lanes 4–6, 7–9, respectively). Both hormone treatments enhanced the intensity of complex II. Addition of the anti-GR antibody supershifted the protein–DNA complexes II and III (Fig. 7B) (lanes 1–3), indicating the presence of GR in these two complexes. Moreover, the use of the anti-MR antibody blocked the formation of protein–DNA complex II, also indicating the presence of MR in this complex (Fig. 7B) (lanes 4–6). This complex II of intermediate electrophoretic mobility containing both MR and GR, appears to be a heterodimer [8,9]. Thus, pGRE was able to bind a MR–GR heterodimer and a GR–GR homodimer.

4. Discussion

In earlier studies [16,17], we observed that aldosterone reduced hIR gene expression by 30%, decreased the hIR number by 34% and was able to reduce several effects of insulin by 21–31% in U-937 cells. It would therefore appear that this aldosterone-elicited decrease in hIR expression and insulin action is the direct result of transcriptional inhibition of the hIR gene. Herein, we addressed this question and demonstrated that aldosterone inhibited the activity of the hIR wild-type promoter by 23%, and caused 23 and 31% reductions in the activity of progressive deletions of this promoter, consisting of fragments spanning up to −1473 bp and up to −876 bp, respectively (Fig. 1). This indicates that the −876 to −271 bp region of the hIR promoter may be sufficient for this transcriptional inhibition by aldosterone.

In this sense, we were able to observe that MR specifically recognized three of the four GREs of this region: cGRE1, cGRE3 and pGRE. In the case of cGRE1 and cGRE3, protein–DNA complexes I and II, respectively, probably representing MR–MR homodimers, were efficiently competed against by the addition of the corresponding unlabelled probe and blocked by the specific anti-MR antibody (Figs. 4 and 6). In the case of pGRE, we observed a protein–DNA complex II, probably representing a MR–GR heterodimer that was efficiently competed against by the addition of the unlabelled probe, blocked by the anti-MR antibody and supershifted by the anti-GR antibody. As is indicated in Table 1, cGRE1 has 5 conserved nucleotides of the 12 nucleotides of a consensus GRE element [32,33], cGRE3 has 4 and 5 in each of the two overlapping elements, while pGRE has 6. Thus, there has to be a minimal number of conserved nucleotides in the response element to achieve MR recognition. These nucleotides appear to be the two G, positions +2 and −5, and the C, position +5

Table 1
Comparison of the 3′ half-element and 5′ half-element of each of the five GREs of the human insulin receptor gene promoter (18, 19) with those of a consensus GRE element (32, 33)

-6 -5 -4 -3 -2 -1 0 0 0 +1 +2 +3 +4 +5 +6 5' G G T A C A n n n T G T T C T 3'	*consensus* GRE
5' C C C T C C T C C C A T T G A G T T C T G G C T T T C C T 3	dGRE3′ (-1363 / -1335 bp)
5' C C T G T G G G G C G C C T C C G G G G G T C T G A A A C T 3'	cGRE1 (-754 / -725 bp)
5' G T A G G G C G C G C G A T C T G G 3'	cGRE2 (-707 / -689 bp)
5' C T C G G T C C C G G C G C G C C C A G G G C C T 3'	cGRE3 (-680 / -656 bp)
5' T C C C G G A G C C C G C A G A T C G C G A C 3'	pGRE (-363 / -341 bp)

Base positions in the consensus GRE are indicated by numbers. Nucleotides identical to the consensus GRE are labelled by filled circles (●) or asterisks (★).

(Table 1) [33,34]. In addition, the relatively well conserved sequence of the pGRE, also includes the G, position −6, whose essential role in dimeric binding in GREs has been demonstrated [35].

Conversely, MR was not recognized by dGRE (Fig. 3). This dGRE has a sequence with 7 conserved nucleotides not including the G, position −5, that we have postulated as essential for MR recognition. Further, neither was MR recognized by cGRE2 (Fig. 5), perhaps due to the presence of three consecutive G, positions −3, −4, and −5, of the 5′ half-site (Table 1). This is consistent with mutational data reported by other authors which indicate that substitution of the T, position −4, for G in this position leads to a 50% decrease in receptor recognition [33,35].

The inducibility of the hIR promoter by dexamethasone was also tested. Dexamethasone induced a 288% increase in the activity of the wild-type promoter (Fig. 1), which is in agreement with the level of induction reported by Lee and co-workers [18,19] in rat 208 F cells. Moreover, this increase is in line with our previous observations of dexamethasone stimulation of hIR mRNA levels (230%), and high affinity IRs (250%) in U-937 cells [12,13]. In addition, the activities of the 5′deletions of this promoter (up to −1473 bp and up to −876 bp) were increased by 260 and 174%, respectively (Fig. 1). This indicates that the −1473 to −271 bp region of the hIR promoter that contains the five GREs may be sufficient for transcriptional induction by dexamethasone.

In this sense, we observed that GR specifically recognized dGRE, cGRE2, cGRE3 and pGRE (Figs. 3 and 5–7). In each case, a protein–DNA complex (III), probably representing a GR–GR homodimer was efficiently competed against by the addition of the corresponding unlabelled probe, and supershifted by the specific anti-GR antibody (Figs. 3 and 5–7). Moreover, in the case of pGRE, GR also recognised this element as a protein–DNA complex (II), probably representing a MR–GR heterodimer. The fact that GR was not recognized by cGRE1 may be attributable to a cell-specific difference and/or to the presence of three consecutive G, positions +1, +2, and +3, of the 3′ half-site (Table 1). A comparison of the consensus GRE element in Table 1 with the GREs of the hIR promoter, suggests that the minimal number of conserved nucleotides essential for GR recognition may be the G, position +2, and the C, position +5 [33–35].

Returning to the biological importance of the MR–GR heterodimer of the pGRE, in cells expressing both receptors, such as the cells used here, heterodimerization is favoured [6,9,27,36]. Thus, this heterodimer would play the most important role in the transcriptional modulation of the hIR gene by aldosterone and dexamethasone. Indeed, a MR–GR heterodimer mediates the transcriptional regulation by mineralocorticoids and glucocorticoids of the human Na/K-ATPase β1 gene promoter [27,37], the human Na/K-ATPase α1 gene promoter [38,39] and the rat 5-HT1A receptor gene promoter [36]. Moreover, the possibility that other factors could assist the formation of this heterodimer cannot be ruled out. Thus, upstream from the pGRE region (from −363 to −341 bp) there is a cluster of three GC boxes which coincide with two AP2-like sites (from −444 to −433 bp) and (from −436 to −425 bp) [40]. Given the previous description of cooperation of GR with AP-2 in the activation of other promoters [41–43], it is possible that MR and AP-2 also act together, though this remains to be demonstrated.

In conclusion, to our knowledge ours is the first demonstration of aldosterone causing the transcriptional inhibition of the hIR gene. This inhibition is consistent with our earlier observations that aldosterone led to reduced hIR mRNA levels, hIR number, and insulin-mediated effects in U-937 cells. It was also shown that this transcriptional inhibition of the hIR gene by aldosterone occurs by the direct interaction of MR with some of the functional GREs elements located in the hIR promoter, specifically with the cGRE1 and cGRE3 as MR–MR homodimers, and with pGRE as a MR–GR heterodimer. This heterodimer could be the most relevant and participate in the cross-talk between mineralocorticoids, glucocorticoids and insulin signalling in U-937 cells.

Acknowledgements

The authors are indebted to Drs. S.Y. Tsai and G. Elberg for their generous gift of the hIR promoter. J. Campión and B.Maestro were holders of fellowships from Gobierno de Navarra and Complutense University of Madrid, respectively. This work was supported by research funds from the DGES grants (PB 96-0573; BMC2000/0765) and the CAM, grant (08.6/0010/98).

References

[1] J.W. Funder, Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance, Annu. Rev. Med. 48 (1997) 231–240.
[2] A. Aranda, A. Pascúal, Nuclear hormone receptors and gene expression, Physiol. Rev. 81 (2001) 1269–1304.
[3] J.W. Funder, Aldosterone action: fact, failure and the future, Clin. Exp. Pharmacol. Physiol. 25 (1998) 547–550.
[4] R.E. Booth, J.P. Johnson, J.D. Stockand, Aldosterone, Adv. Physiol. Educ. 26 (2002) 8–20.
[5] F.M. Rogerson, P.J. Fuller, Mineralocorticoid action, Steroids 65 (2000) 61–73.
[6] D. Pearce, A mechanistic basis for distinct mineralocorticoid and glucocorticoid receptor transcriptional specificities, Steroids 59 (1994) 153–159.
[7] T. Trapp, R. Rupprecht, M. Castrén, J.M.H.M. Reul, F. Holsboer, Heterodimerization between mineralocorticoid and glucocorticoid receptor: a new principle of glucocorticoid action in the CNS, Neuron 13 (1994) 1457–1462.
[8] W. Liu, J. Wang, N.K. Sauter, D. Pearce, Steroid receptor heterodimerization demonstrated in vitro and in vivo, Proc. Natl. Acad. Sci. 92 (1995) 12480–12484.
[9] T. Trapp, F. Holsboer, Heterodimerization between mineralocorticoid and glucocorticoid receptors increases the functional diversity of corticosteroid action, Trends Pharmacol. Sci. 17 (1996) 145–149.

[10] M.A. Leal, P. Aller, A. Torres, A. Picardo, N. Dávila, C. Calle, Insulin receptor mRNA levels are modulated in a tissue-specific manner in Cushing's syndrome patients, Horm. Metab. Res. 26 (1994) 349–350.
[11] M.A. Leal, P. Aller, A. Más, M.C. Carranza, C. Calle, Tissue-specific changes in insulin receptor mRNA concentrations in dexamethasone-treated and adrenalectomized rats, Endocrinol. J. 41 (1994) 737–741.
[12] M.A. Leal, C. Cabañas, C. Rius, P. Aller, C. Calle, Modulation by dexamethasone of insulin binding and insulin receptor mRNA levels in U-937 human promonocytic cells, Biochemie 74 (1992) 545–549.
[13] M.A. Leal, P. Aller, C. Calle, Effect of dexamethasone on insulin receptor mRNA levels, RNA stability and isotype RNA pattern in U-937 human promonocytic cells, J. Endocrinol. Invest. 19 (1996) 530–534.
[14] M.C. Carranza, A. Torres, C. Calle, Disminución en el numero y en la afinidad de los receptores de insulina en el tejido adiposo subcutáneo de un paciente con hyperaldosteronismo primario, Rev. Clin. Esp. 188 (1991) 414–417.
[15] J. Campión, V. Lahera, V. Cachofeiro, B. Maestro, N. Dávila, M.C. Carranza, C. Calle, In vivo tissue-specific modulation of rat insulin receptor gene expression in an experimental model of mineralocorticoid excess, Mol. Cell Biochem. 185 (1998) 177–182.
[16] J. Campión, B. Maestro, F. Mata, N. Dávila, M.C. Carranza, C. Calle, Inhibition by aldosterone of insulin receptor mRNA levels and insulin binding in U-937 human promonocytic cells, J. Steroid Biochem. Mol. Biol. 70 (1999) 211–218.
[17] J. Campión, B. Maestro, S. Molero, N. Dávila, M.C. Carranza, C. Calle, Aldosterone impairs insulin responsiveness in U-937 human promonocytic cells via the downregulation of its own receptor, Cell Biochem. Funct. 20 (2002) 237–245.
[18] J.K. Lee, J.W.O. Tam, M.J. Tsai, S.Y. Tsai, Identification of *cis*- and *trans*-acting factors regulating the expression of the human insulin receptor gene, J. Biol. Chem. 267 (1992) 4638–4645.
[19] J.K. Lee, S.Y. Tsai, Multiple hormone response elements can confer glucocorticoid regulation on the human insulin receptor gene, Mol. Endocrinol. 8 (1994) 625–634.
[20] C. Sundstrom, K. Nilsson, Establishment and characterization of a human histiocytic lymphoma cell line (U-937), Int. J. Cancer 17 (1976) 565–570.
[21] A. Robert, G. Grunberger, J.L. Carpentier, J.M. Dayer, J.M. Orci, P. Gorden, The insulin receptor of a human monocyte-like cell line: characterization and function, Endocrinology 114 (1984) 247–250.
[22] D. Duval, P. Lynde, A. Hatzfeld, J. Hatzfeld, Dexamethasone-induced stimulation of arachidonic acid release by U-937 cells grown in defined medium, Biochim. Biophys. Acta 887 (1986) 204–213.
[23] B. Maestro, S. Molero, S. Bajo, N. Dávila, C. Calle, Transcriptional activation of the human insulin receptor gene by 1,25-dihydroxyvitamin D_3, Cell Biochem. Funct. 20 (2002) 227–232.
[24] C. Bonnerot, D. Rocancourt, P. Briand, G. Grimber, J.F. Nicolas, A β-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies, Proc. Natl. Acad. Sci. 84 (1987) 6795–6799.
[25] V. Kumar, P.C. Chambon, The estrogen receptor binds thightly to its responsive element as a ligand-induced homodimer, Cell 55 (1988) 145–156.
[26] E. Schreibert, P. Matthias, M.M. Müller, W. Schaffner, Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping badshift assay (PCBA), EMBO J. 7 (1988) 4221–4229.
[27] A. Derfoul, N.M. Robertson, J.B. Lingrel, D.J. Hall, G. Litwack, Regulation of the human Na/K-ATPase β1 gene promoter by mineralocorticoid and glucocorticoid receptors, J. Biol. Chem. 273 (1998) 20702–20711.
[28] B. Maestro, N. Dávila, M.C. Carranza, C. Calle, Identification of a vitamin D response element in the human insulin receptor gene promoter, J. Steroid Biochem. Mol. Biol. 84 (2003) 223–230.
[29] E. Araki, T. Murakami, T. Shirotani, F. Kanai, Y. Shinohara, F. Shimada, M. Mori, M. Shichiri, Y. Ebina, A cluster of four Sp1 binding sites required for efficient expression of the human insulin receptor gene, J. Biol. Chem. 266 (1991) 3944–3948.
[30] K. Yoshizato, T. Shirotani, N. Furukawa, T. Taguchi, H. Motoshima, T. Toyonaga, Y. Hirashima, J. Kawashima, Y. Ebina, M. Shichiri, E. Araki, Identification of a cis-acting element and a novel transacting factor of the human insulin receptor gene in HepG2 and rat liver cells, Biochem. Bioph. Res. Co. 280 (2001) 428–434.
[31] M. Lombès, N. Binart, M.E. Oblin, V. Joulin, E.E. Baulieu, Characterization of the interaction of the human mineralocorticoid receptor with hormone response elements, Biochem. J. 292 (1993) 577–583.
[32] M. Beato, G. Chalepakis, M. Schauer, E.P. Slater, DNA regulatory elements for steroid hormones, J. Steroid Biochem. 32 (1989) 737–747.
[33] C.C. Nelson, S.C. Hendy, R.J. Shukin, H. Cheng, N. Bruchousky, B.F. Koop, P.S. Rennie, Determinants of DNA sequence specificity of the androgen, progesterone and glucocorticoid receptors: evidence for differential steroid receptor response elements, Mol. Endocrinol. 13 (1999) 2090–2107.
[34] C. Scheidereit, H.M. Westphal, C. Carlson, H. Bosshard, M. Beato, Molecular model of the interaction between the glucocorticoid receptor and the regulatory elements of inducible genes, DNA 5 (1986) 383–391.
[35] S.K. Nordeen, B.J. Suh, B. Kuhnel, C.D. Hutchison, Structural determinats of a glucocorticoid receptor recognition element, Mol. Endocrinol. 4 (1990) 1866–1873.
[36] X.M. Ou, J.M. Storring, N. Kushwaha, P.R. Alberti, Heterodimerization of mineralocorticoid and glucocorticoid receptors at a novel negative response element of the 5-HT1A receptor gene, J. Biol. Chem. 276 (2001) 14299–14307.
[37] A. Derfoul, N.M. Robertson, D.J. Hall, G. Litwack, 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, Endocrine 13 (3) (2000) 287–295.
[38] V. Kolla, N.M. Robertson, G. Litwack, Identification of a mineralocorticoid/glucocorticoid response element in the human Na/K ATPase α1 gene promoter, Biochem. Bioph. Res. Co. 266 (1999) 5–14.
[39] V. Kolla, G. Litwack, Transcriptional regulation of the human Na/K ATPase via the human mineralocorticoid receptor, Mol. Cell Biochem. 204 (2000) 35–40.
[40] E. Wingender, X. Chen, E. Fricke, R. Geffers, R. Hehl, I. Liebich, M. Krull, V. Matys, H. Michael, R. Ohnhäuser, M. Prüβ, F. Schacherer, S. Thiele, S. Urbach, The TRANSFAC system on gene expression regulation, Nucleic Acids 29 (2001) 281–283.
[41] D.L. Wong, B.J. Siddall, S.N. Ebert, R.A. Bell, S. Her, Phenylethanolamine *N*-methyltransferase gene expression: synergistic activation by Egr-1, AP-2 and the glucocorticoid receptor, Brain Res. Mol. Brain Res. 61 (1–2) (1998) 154–161.
[42] P.A. Shaw, O. Chaparro, The 5′-flanking sequence and regulatory elements of the cystatin S gene, Biochem. Bioph. Res. Co. 261 (1999) 705–711.
[43] K. Hilger-Eversheim, M. Moser, H. Schorle, R. Buettner, Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control, Gene 260 (2000) 1–12.