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Identification of functional glucocorticoid response elements in the mouse FoxO1 promoter ☆

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ABSTRACT

Glucocorticoids stimulate muscle atrophy through a cascade of signals that includes activation of FoxO transcription factors which then upregulate multiple genes to promote degradation of myofibrillar and other muscle proteins and inhibit protein synthesis. Our previous finding that glucocorticoids upregulate mRNA levels for FoxO1 in skeletal muscle led us to hypothesize that the FoxO1 gene contains one or more glucocorticoid response elements (GREs). Here we show that upregulation of FoxO1 expression by glucocorticoids requires the glucocorticoid receptor (GR) and binding of hormones to it. In cultured C2C12 myoblasts dexamethasone did not alter FoxO1 mRNA stability. Computational analysis predicted that the proximal promoter of the FoxO1 gene contained a cluster of eight GRE half sites and one highly conserved near-consensus SRE; the cluster is found between −800 and −2000 bp upstream of the first codon of the FoxO1 gene. A reporter gene constructed using the first 2 kb of the FoxO1 promoter was stimulated by dexamethasone. Removal of a 5′ domain containing half of the GREs reduced reporter gene activity and removal of all GREs in this region ablated activation by dexamethasone. Restriction fragments of the cluster of 8 upstream GREs bound recombinant GR in gel shift assays. Collectively, the data demonstrate that the proximal promoter of the FoxO1 gene contains multiple functional GREs, indicating that upregulation of FoxO1 expression by glucocorticoids through GREs represents an additional mechanism by which the GR drives glucocorticoid-mediated muscle atrophy. These findings are also relevant to other physiological roles of FoxO1 such as regulation of hepatic metabolism.

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1. Introduction

FoxO1 is one of a family of four forkhead O transcription factors that is known to be involved in diverse cellular processes that include proliferation, differentiation, regulation of metabolism and control of bone formation [1–3]. An important determinant of transcriptional activity of FoxO1 is inhibitory phosphorylation by Akt in response to IGF-1 or other growth factors [4], because such phosphorylation excludes FoxOs from the nucleus. In skeletal muscle, expression levels of FoxO1 relate inversely to muscle size [5], and FoxO1 and FoxO3A proteins have been found to determine the expression levels of several negative regulators of muscle mass [6–11]. Upregulation of FoxO1 expression is a conserved feature of diverse conditions that result in muscle atrophy including paralysis, glucocorticoid administration, and immobilization [12–15]. In parallel, during muscle atrophy, FoxOs are activated through mechanisms that include reduced Akt activity, thereby driving increased expression of FoxO-regulated genes for muscle atrophy (atrogenes).

Elevated levels of glucocorticoids are one well recognized cause of muscle atrophy. Increases in glucocorticoid levels occur in states of physiologic stress such as starvation, confinement, stroke, or diabetes [16], or through administration of glucocorticoid medications [17]. We have previously reported that FoxO1 expression is upregulated by glucocorticoids in rat skeletal muscle [15]. The glucocorticoid receptor (GR) is a member of the steroid hormone receptor family of nuclear receptors [18]. The classical mechanism of signaling through the GR involves its binding to glucocorticoid response elements in promoters and enhancers of target genes

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resulting in either repression or upregulation of transcription [18]. We hypothesized that the FoxO1 gene contained one or more GREs through which glucocorticoids determined FoxO1 transcription. The goal of the present study was to test this possibility in cultured myoblasts reporter genes and gel shift assays.

2. Methods

2.1. Cell culture

Mouse C2C12 cells (ATCC, Rockland, MD) were maintained in DMEM containing 10% fetal bovine serum (FBS) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (growth media) at 37 °C in a humidified atmosphere containing 5% carbon dioxide. Differentiation was initiated by replacing growth media with media containing 2% horse serum. Primary cultures of human skeletal muscle myoblasts (Lonza, Walkersville, MD) were maintained following the manufacturer's recommended procedures.

2.1.1. Incubation of cells and extraction of RNA

Cells were seeded into wells of 6-well plates at 3×10^6 per well in DMEM supplemented with 10% FBS and incubated overnight in this medium. Cells were then incubated for 48 h in DMEM containing 2% horse serum to induce differentiation. To test effects of dexamethasone on mRNA levels, dexamethasone, dexamethasone alone, or together with the GR antagonist RU486 or vehicle (ethanol) was added and cells were incubated for 48 h. For determination of mRNA half-life, medium was supplemented with actinomycin (5 μg/ml) at various times before processing for isolation of total RNA. For RNA isolation, cells were disrupted with Qiashredder columns (QIAGEN, Valencia, CA). Total RNA was extracted from cell lysates using RNAeasy columns (QIAGEN) after digestion on the column of residual genomic DNA with ribonuclease-free deoxyribonuclease (DNase; QIAGEN).

2.1.2. Small interfering RNA (siRNA) transfection

siRNA against-GR and non silencing random siRNA (negative control) were purchased from Ambion (Invitrogen, Carlsbad, CA). Cells were cultured in 6-well plates and transfected with either 15 nM non silencing random siRNA or 15 nM GR-siRNA using PepMute Plus siRNA transfection reagent (SignaGen Laboratories, Ijamsville, MD), following the manufacturer's recommended procedures. Cells were then treated with either vehicle or dexamethasone under differentiating conditions.

2.1.3. Real-time PCR

Real time PCR was performed as described previously [19,20]. Briefly, total RNA was used to prepare cDNA libraries with the high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Libraries were diluted 25-fold with water. Real-time PCR was performed using Taqman 2× PCR buffer (Applied Biosystems) and an Applied Biosystems 7500 thermocycler using Taqman Assay on Demand probes (Applied Biosystems). Changes in expression were calculated using the $2^{-\Delta\Delta Ct}$ method (33) using 18S RNA for a loading control. For experiments testing effects of dexamethasone on FOXO1 mRNA levels the control group used for these calculations was the vehicle-treated group; for mRNA half-life studies, of the control group was the mRNA level at time zero.

2.2. Western blotting

C2C12 cells or human skeletal muscle myoblasts were seeded in 100-mm tissue culture plates. The following day cells were treated with dexamethasone for 48 h. Media was removed and cells were covered with 500 μl of cell lysis buffer (Cell Signaling, Danvers, MA) containing 5 μl/ml of protease inhibitor cocktail (Sigma–Aldrich). Lysates were cleared by centrifugation, and protein concentrations of the supernatant fractions were determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins (40 μg/lane) were resolved by SDS–PAGE on 8% gels and then transferred onto polyvinyl difluoride membranes. Membranes were blocked with 5% milk. Primary antibodies were used were: FoxO1 (1:1000; Cell Signaling, Boston, MA) and β-tubulin (1:2000; Abcam, Cam Cambridge, MA). The secondary antibody was a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (MP Biomedical, Solon, OH). Immunostaining was visualized by enhanced chemiluminescence and captured on photographic film. The densities of protein bands were measured by using an AlphaImager 2200 Gel Doc system (Alpha Innotech, San Leandro, CA).

2.3. Cloning of the FoxO1 promoter and construction of reporter genes

A 2119 bp fragment of the FoxO1 promoter (−1984 to +135) was amplified from mouse skeletal muscle genomic DNA by PCR using a Platinum pfx DNA polymerase (Invitrogen) with the following PCR conditions: initial denaturation at 95 °C for 4 min; amplification using cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 2.5 min. The final extension was at 72 °C for 10 min. The primers were: forward, 5′ TTA CAA AGG AGC ACC AGA GG-3′; reverse, 5′-TGG TCG AGT TGG ACT GGT TA-3′. PCR products (FoxO1-P1) were cloned into pCR2.1-TOPO TA (Invitrogen), and the cDNA inserts were verified by DNA sequencing.

Reporter genes expressing firefly luciferase under the control of upstream regions of the FOXO1 promoter were constructed by restriction enzyme digestion of segments of interest (Fig. 3B) using pCR2.1-TOPO TA-FoxO1-P1 as a template. Inserts were excised with restriction enzymes and were subcloned into pGL2-Promoter (Promega) at these same sites. pGL2-Promoter expresses firefly luciferase under the control of a minimal promoter.

2.4. Reporter gene assays

C2C12 cells plated in 96-well plates and grown to 80–90% confluence were transfected as indicated in the figure legends with reporter genes and, in some cultures, expression vectors for wild type GR (PS6R GR WT) or mutant GR (PS6R GR C460A) [21]. Transfection was performed using Lipofectamine 2000 according to the manufacturers recommended procedures. After approximately 5–6 h, the transfection mixture was removed and replaced with differentiation media; 48 h later, media was supplemented with dexamethasone or vehicle (ethanol) as indicated. After overnight incubation, a Dual Luciferase Assay (Promega) was performed following the manufacturer's recommended procedures and measured by a Berthold 96-well plate luminometer. Firefly luciferase activities were normalized relative to renilla luciferase. Luciferase activities were then normalized relative to mean values for vehicle-treated cells.

2.5. Gel shift assays

cDNA fragments generated by restriction enzyme digestion of pGL2-basic-FoxO1-P1 were resolved by electrophoresis on agarose gels and isolated by excision of bands of the correct size and purification using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). Gel shift assays were conducted using the LightShift Chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL) according the manufacturer's recommended procedures. Recombinant GR protein was purchased from Merck (Merck KGaA, Darmstadt, Germany).

2.6. Statistics

Data are expressed as mean values ± SEM. Statistical analysis was performed using Prism 4.0c software (GraphPad Software, San Diego, CA). Comparisons between two means were performed using a Student's *t* test. Comparisons among multiple means were performed using one-way ANOVA with a Newman–Keuls test *post hoc* to determine the significance of differences between specific pairs of means. A $p < 0.05$ was considered significant.

3. Results

We began by testing whether dexamethasone upregulated FoxO1 expression in differentiating C2C12 myotubes. Incubation of these cells with dexamethasone increased FoxO1 mRNA by approximately 3-fold and FoxO1 protein by more than 2-fold (Fig. 1A and B). In parallel experiments, primary cultures of human myoblasts were subjected to differentiating conditions and the effects of dexamethasone were examined. FoxO1 protein levels were significantly increased in primary myoblast cultures by nearly 2-fold (Fig. 1C).

The role of the GR in upregulation of FoxO1 by dexamethasone was examined in additional experiments. In gene knockdown studies, siRNA against the GR completely blocked upregulation of FoxO1 mRNA levels by dexamethasone in differentiating C2C12 cells (Fig. 2A). Similarly, the GR antagonist RU486 completely prevented upregulation of FoxO1 mRNA levels by dexamethasone (Fig. 2B). To test the possibility that changes in mRNA levels might be due, in part, to altered mRNA stability rather than changes in the rate of transcription, changes in FoxO1 mRNA levels over time were monitored after inhibiting transcription with actinomycin. Incubation of differentiating C2C12 cells with dexamethasone did not alter the rate of decay of FoxO1 mRNA levels in these cells (Fig. 2C). Thus, upregulation of FoxO1 mRNA levels by glucocorticoids involves primarily increased transcription.

These findings suggested the presence of one or more functional GREs in the FoxO1 gene. To examine the possibility that the proximal promoter of the FoxO1 gene contained GREs, the sequence of the first 2.5 kb upstream of the first codon of the mouse FoxO1 gene was subjected to computational analysis using TESS. This analysis identified 8 potential GRE half sites, and two possible GREs each of which contained two half-sites. One potential GRE (shown at ~−800 in Supplemental Fig. 1B, and highlighted with a bold underline in Supplemental Fig. 1A) consisted of two GR binding sites separated by a 3 bp spacer; this GRE was conserved between mouse and human FoxO1 promoters, and was similar to a consensus SRE (steroid response element). A second highly conserved GRE half site was also observed further upstream (Supplemental Fig. 1A and B). Nine of the 10 GREs were clustered between −800 and −2200 bp upstream of the first codon of the FoxO1 gene. To construct a luciferase reporter gene with which to examine the function of these GREs, the first 2 kb of the FoxO1 promoter, containing 8 of the putative GREs, was cloned into pGL2-Basic-Luc

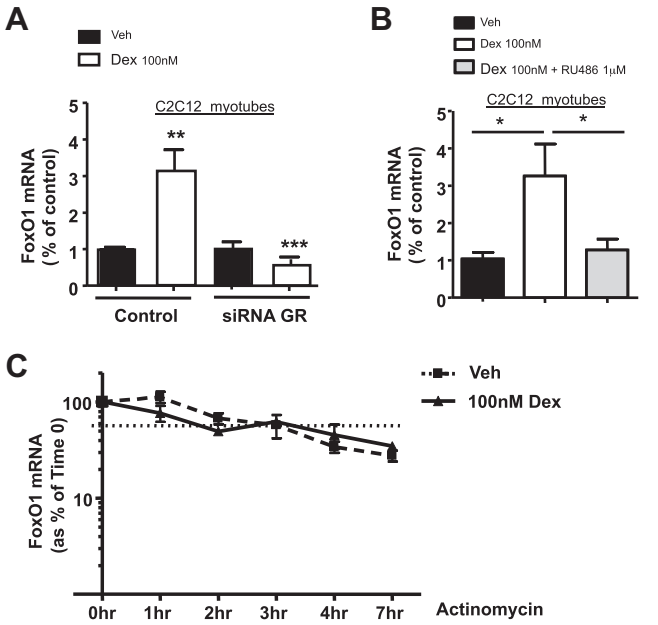

Fig. 2. Dexamethasone increases FoxO1 mRNA transcription through binding of dexamethasone to the glucocorticoid receptor. A. C2C12 myotubes were transfected with either a scrambled siRNA (control) or one targeting mouse glucocorticoid receptor (GR), differentiated for 48 h, then incubated for 48 h with dexamethasone (Dex) or vehicle (Veh) after which mRNA levels for FoxO1 were determined by real time PCR. B. C2C12 cells differentiated as in A were incubated for 48 h with Dex, Dex plus RU486, or vehicle as indicated; FoxO1 mRNA levels were then determined by real time PCR. C. C2C12 myotubes differentiated as in A were incubated overnight with Dex or vehicle. The following day, actinomycin (5 μg/ml) was added and mRNA levels of FoxO1 were determined at several time points thereafter, as indicated in the figure. Data are mean ± SEM for at least two separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $N = 3$.

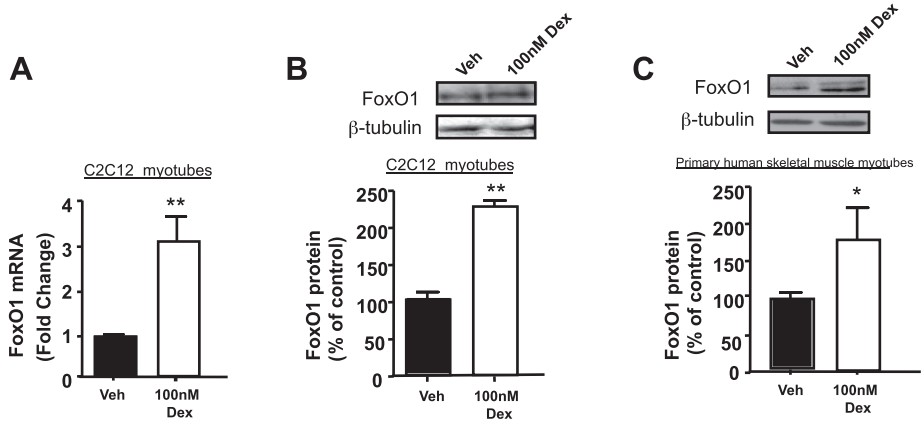

Fig. 1. Dexamethasone increases FoxO1 mRNA and protein expression in cultured myoblasts. (A) C2C12 myoblasts were differentiated for 48 h then incubated with dexamethasone (Dex) or vehicle (Veh, ethanol) for 48 h after which FoxO1 mRNA levels were determined by real time PCR. (B) C2C12 cells were incubated as in panel A after which FoxO1 protein levels were determined by Western blotting. The inset shows a representative Western blot while the graph shows results of scanning densitometry analysis of FoxO1 band intensity on Western blots using β-tubulin for normalization of band intensity. (C) Primary cultures of human myoblasts were differentiated for 48 h then incubated for 48 h with dexamethasone or vehicle after which FoxO1 protein levels were determined by Western blotting. Figures show mean values ± SEM for at least two separate experiments; $N = 3$. *, $p < 0.05$; **, $p < 0.01$.

(Supplemental Fig. 1B). Restriction digests of this clone were used to generate additional reporter genes to test the function of GREs in subsegments of the full length clone. A map of the restriction sites employed and truncation mutants generated is shown in Supplemental Fig. 1B.

The effect of dexamethasone on luciferase expression for the full length clone of the FoxO1 promoter (pGL2-Promoter-FoxO1-P1) was examined after transfection of this reporter into C2C12 cells. A dose-dependent increase in reporter gene activity was observed as dexamethasone concentration increased from 1 to 100 nM (Fig. 3A). Co-transfection of C2C12 cells with this reporter gene together with a mutant GR lacking the ability to bind DNA (GR C460A) revealed that dexamethasone did not significantly increase reporter gene activity; in cells transfected with the reporter gene and the same expression vector encoding wild-type GR, a significant increase in reporter gene activity was observed when cells were incubated with dexamethasone (Fig. 3B). Thus, the first 2 kb of the mouse FoxO1 promoter confers glucocorticoid responsiveness, and binding of the GR to one or more DNA sequences in this region of the FoxO1 promoter is necessary for glucocorticoid effects on transcription.

To localize regions of the FoxO1 promoter containing putative GREs, additional experiments were conducted evaluating the effects of dexamethasone on activity of truncation mutants of the full length reporter gene. Deletion of the cluster of GREs between −800 and −2000 bp in part (pGL2-basic-FoxO1-P6) diminished glucocorticoid responsiveness while removal of all GREs (pGL2-basic FoxO1-P4) eliminated dexamethasone responsiveness. Unexpectedly, subcloning this region into luciferase reporter genes with an SV40 minimal promoter in its entirety, or as either of two fragments, did not confer dexamethasone responsiveness to reporter genes (reporters pGL2-SV40-FoxO1-P3, P5, P7, Supplemental Fig. 1B and Fig. 3C).

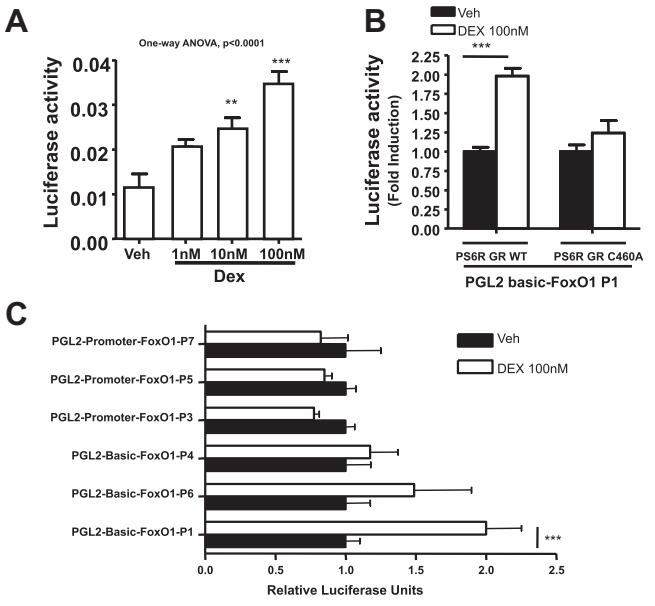

Fig. 3. The FoxO1 promoter contains a cluster of functional GREs. (A) C2C12 cells were transfected with pGL2-FoxO1-P1 and TK-Renilla then incubated for 48 h with vehicle or the indicated concentration of dexamethasone (Dex) after which luciferase activity was determined. (B) C2C12 cells were transfected with pGL2-FoxO1-P1 and TK-Renilla, and with plasmids expressing wild type GR or GR-C460A, cultured under differentiating conditions for 48 h, then incubated overnight with vehicle or dexamethasone after which luciferase activity was determined. (C) C2C12 cells were transfected with the indicated reporter gene and TK-Renilla, cultured for 48 h under differentiating conditions, then incubated overnight with vehicle or dexamethasone after which luciferase activity was determined. Data are mean ± SEM for at least two separate experiments. * $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $N = 6$.

To determine whether the GR bound to DNA containing the putative GREs, gel shift assays were conducted using recombinant GR and restriction fragments of the full length FoxO1 promoter clone. Significant binding was observed for each of the 2 fragments containing a portion of the putative GRE cluster between −800 and −1926 bp (EP2 and EP3, Fig. 4A and B), supporting the existence of one or more GR binding sites within these sequences. Analysis of the fragment containing a single GRE just upstream of the mouse transcriptional start site did not show a clear shifted GR-DNA complex (EP4, Fig. 4A and B). No binding was seen for a fragment lacking any putative GREs located between bases −786 and −428 (EP4, Fig. 4A and B).

4. Discussion

The above data support the conclusions that FoxO1 is a glucocorticoid-responsive gene whose expression is regulated by binding of the GR to GREs within the proximal promoter. These GREs are clustered between −800 and approximately −2000 bp upstream of the first codon of the FoxO1 gene and include two GREs that are highly conserved between mice and humans. These conclusions are supported by findings that dexamethasone increased mRNA levels for FoxO1 without altering FoxO1 mRNA degradation, that the GR was necessary for upregulation, and that upregulation was prevented by RU486, which is a GR antagonist. Further support is provided by findings that the first 2 kb of the mouse FoxO1 promoter confer glucocorticoid responsiveness to reporter genes, and that deletion of the sequences containing the cluster of putative GREs abrogates glucocorticoid responsiveness. Deletion of a portion of the cluster of putative GREs reduced, but did not completely remove, glucocorticoid responsiveness suggesting that there are at least two GREs within the region spanning from −800 to −2000 kb upstream of the first codon of the FoxO1 gene. While reporter genes containing part or all of the cluster of GREs without flanking downstream sequences did not confer glucocorticoid responsiveness to reporter genes, these sequences bound recombinant GR in gel shift assays. The reasons for these discrepant findings are not clear but possible explanations include the presence of inhibitory sequences within the cluster of GREs, or synergistic interactions between the upstream cluster of GREs and the more downstream putative single GRE located near the transcriptional start site. Taken together, the weight of the evidence indicates that the proximal FoxO1 promoter contains functional GREs through which glucocorticoids upregulate FoxO1 transcription.

Transcriptional control of the expression of FoxO1 by the GR adds an additional layer to the multiple mechanisms by which glucocorticoids regulate transcriptional programs in skeletal muscle resulting in diminished skeletal muscle mass, reduced muscle protein synthesis and catabolism of muscle proteins. Glucocorticoids reduce phosphorylation of FoxO1 by Akt [4] resulting in translocation of FoxO1 to the nucleus and regulation of multiple genes encoding proteins linked to muscle atrophy including MAFbx [7], MuRF1 [8], REDD1 [9], and 4EBP1 [11]. Transactivating GREs have been found in the promoters for MuRF1 [8] and REDD1 [22], and FoxO1 and GR exert synergistic effects on transcription of MuRF1 [8] and, most likely, REDD1 [22]. The discovery that KLF15 mRNA expression is upregulated through GREs in its promoter and that KLF15 increases transcription of MAFbx, MuRF1, and REDD1, as well as that of FoxO1 and FoxO3A [22], supports the existence of a third transcriptional pathway by which glucocorticoids upregulate expression of atrogenes through KLF15. One might expect synergy between KLF15 and GR in upregulating FoxO1 transcription, although this possibility has not been tested.

Collectively, these findings indicate an intricate transcriptional network through which initial effects of glucocorticoids on

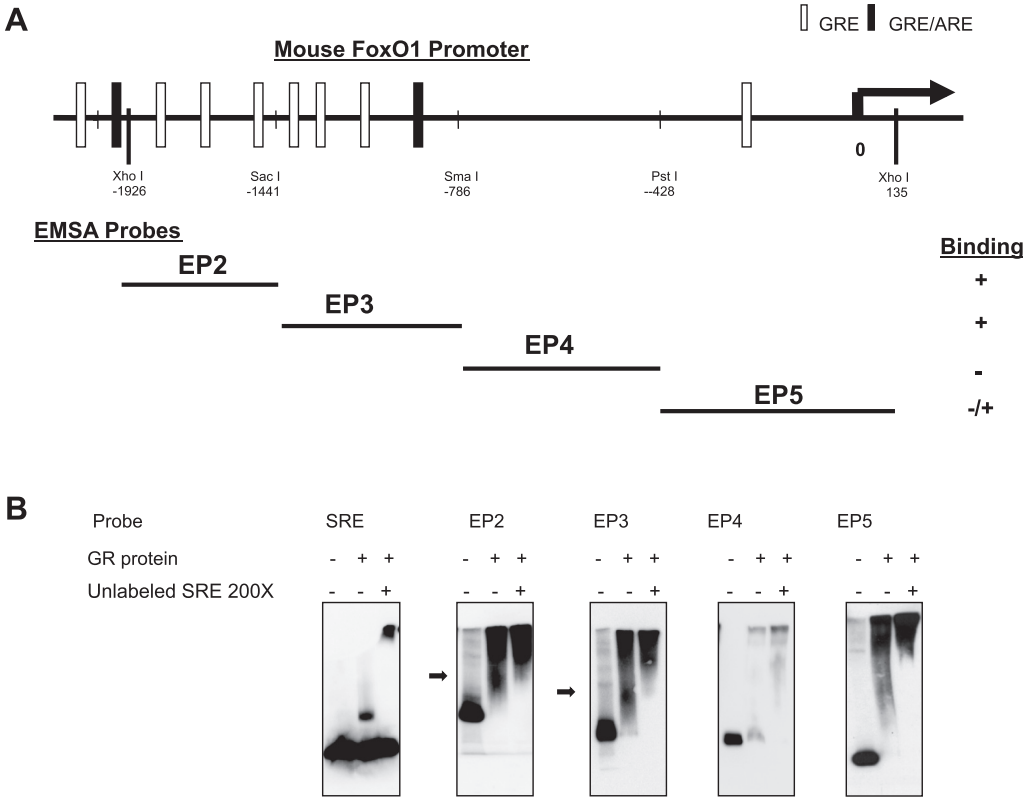

Fig. 4. A region of the FoxO1 promoter rich in GREs binds to the GR. (A) The map depicts the location of restriction fragments of the mouse FoxO1 promoter and their position relative to the cluster of GRE within this promoter sequence. The dark bars show the location of highly conserved GREs while light bands show positions of less well conserved GREs. (B) Gel shift assays evaluating binding of recombinant GR protein (20 ng) to each of the restriction fragments (1 pmol) shown in A is shown. Images are representative gel shift assays for each of at least two separate experiments. Binding of a consensus SRE (steroid response element) to GR served as a positive control.

transcriptional activity of FoxOs at promoter regions of genes linked to skeletal muscle atrophy is amplified through glucocorticoid-mediated upregulation of FoxO1 and KLF15 and subsequent synergistic effects of GR, FoxOs and KLF15 on such promoters. Promoters for MuRF1 and REDD1 contain binding sites for all three of these transcriptional regulators [9,11,22], but it should be noted that while the promoter for MAFbx contains FoxO [7] and KLF15 [22] binding sites it has to date not been found to have functional GREs. Absence of GREs from certain promoters controlling genes may fine tune gene expression changes in response to glucocorticoids to precise and specific adjustments of the catabolic response of skeletal muscle to conditions that induce atrophy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.080.

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