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Estrogen receptor regulates MyoD gene expression by preventing AP-1-mediated repression

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ABSTRACT

Cell growth and differentiation are opposite events in the myogenic lineage. Growth factors block the muscle differentiation program by inducing the expression of transcription factors that negatively regulate the expression of muscle regulatory genes like MyoD. In contrast, extracellular clues that induce cell cycle arrest promote MyoD expression and muscle differentiation. Thus, the regulation of MyoD expression is critical for muscle differentiation. Here we show that estrogen induces MyoD expression in mouse skeletal muscle *in vivo* and in dividing myoblasts *in vitro* by relieving the MyoD promoter from AP-1 negative regulation through a mechanism involving estrogen receptor/AP-1 protein–protein interactions but independent of the estrogen receptor DNA binding activity.

Introduction

Cell growth and differentiation are mutually exclusive events in the myogenic lineage. Growth factors suppress muscle differentiation by inhibiting the expression of key myogenic factors (e.g., MyoD and Myogenin) [1]. MyoD gene repression involves the early genes *c-fos*, *c-jun*, and *c-myc* [2,3]. Accordingly, a negative cis-acting element with sequence homology to the cAMP responsive element (CRE) or to the phorbol ester (TPA)-responsive element (TRE) inhibits MyoD promoter activity through AP-1 binding in dividing myoblasts [4]. However, during differentiation AP-1 binding to this site is down-regulated allowing MyoD expression [4].

Members of the nuclear receptors superfamily regulate gene expression during cell differentiation in response to steroid hormones [1,5–7]. Although no hormone response elements have been identified on the MyoD promoter; it is known that testosterone, dexamethasone, and estrogens positively regulate MyoD expression in skeletal muscle [8–12]. However, the molecular mechanism leading to MyoD expression in response to steroid hormones is unknown. Since the MyoD CRE-like element binds AP-1 and this element shows sequence homology to DNA elements regulated by steroid hormones receptors, we determined the role of the estrogens on MyoD gene expression. Here, we show that estrogen positive effect on MyoD expression is mediated through a mechanism involving the interaction of the estrogen receptor (ER) with Fos–Jun heterodimers. ER prevents AP-1 binding to the CRE-like element of the MyoD promoter thus abrogating the AP-1-mediated negative effect on MyoD gene expression. The effect of the ER on the AP-1-mediated repression of the MyoD promoter is a clear example of the antagonistic effect between differentiation-inducing hormones and proliferation-inducing oncogenes.

Materials and methods

Animals. Procedures that involved mice were approved by institutional guidelines for animal care. Two weeks old Balb/c males were castrated and one month later injected every other day with 17β-estradiol (E_2) (100 μg) or testosterone (100 μg) during two weeks. Animals were sacrificed and RNA was prepared from skeletal muscle and heart.

Cell culture and DNA transfection. Mouse myoblast cells (G8) were grown on collagen-coated plates in DMEM supplemented with 10% heat inactivated fetal calf serum and 10% horse serum. Cells were transfected with 5 μg of the indicated reporter plasmid, 3 μg of internal control (RSV-β-GAL), and 1 μg of the indicated expression vector. CAT and β-galactosidase assays were performed as described [13,14]. Data were represented as the ratio of CAT activity/β-galactosidase activity.

Abbreviations: E2, 17β-estradiol; ER, estrogen receptor; TPA, 13-myristate-12-acetate; CRE, cAMP-response element; IgG, immunoglobulin G; CAT, chloramphenicol acetyl transferase; β-GAL, β-galactosidase

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Cell extracts. Nuclear extracts from mouse skeletal muscle (hind limbs) and G8 cells were prepared as reported [15,16]. Total extracts from G8 cells overexpressing the ER and/or c-Fos and c-Jun were prepared as described [17]. Protein determination was performed using Bio-Rad protein assay according to the manufacturer's indications. Extracts were kept at −70 °C until use.

Yeast whole cell extract. The yeast strain TGY14.1 transformed with the human ER expressing vector pYHEGO [18] was a generous gift from P. Chambon and D. Metzger. Yeast cells were grown as described [17], disrupted with a French-Press in a buffer containing 25 mM HEPES, pH 7.9, 0.4 M KCl, 20% glycerol, 0.5 mM DTT, 0.1 mM PMSF, leupeptine, aprotinin, and pepstatin (all at 0.5 μg/ml).

Electrophoretic gel mobility shift assay (EMSA). Labeled oligonucleotides (10^8 cpm/μg) were incubated with 5 μg of nuclear extract for 20 min at 4 °C in the presence of 1 μg of non-specific DNA competitor and with or without 20 M excess of unlabeled specific competitor oligonucleotide. For super-shift assays the nuclear extracts were incubated with the antibody for 1 h at 4 °C before the addition of the labeled probe, then the reaction was carried out as described [4].

Northern blotting. Total RNA, was separated by electrophoresis, blotted onto membranes, and crosslinked by UV light irradiation. After pre-hybridization, membranes were hybridized with 1–2 × 10^6 cpm/ml of the probe in 1% SDS, 1 M NaCl, and 10% dextran sulfate. After washing, membranes were exposed for autoradiography at −80 °C. For MyoD mRNA detection an oligonucleotide containing the sequence between 148 and 218 bp of the MyoD gene, was used.

Immunoprecipitation. Immunoprecipitation was carried out as described [19] using 10 μg of nuclear extracts and anti-ER (a kind gift of D. Metzger, INSERM, Strasburg) for 1 h on ice; anti-c-Fos antibody (Santa Cruz) was used for Western blot analysis.

Results and discussion

Estrogen induces MyoD expression in castrated mice and dividing myoblasts

Testosterone, dexamethasone, and estrogens have been shown to regulate MyoD expression [8–12]; however, the mechanism involved is unknown. Since, the MyoD promoter contains several half-palindrome sequences (GGTCA) that could mediate ER-induced responses, we determined the effect of E_2 on MyoD mRNA levels *in vivo*. Pre-pubertal male mice were castrated and one month later injected with E_2 or testosterone. MyoD mRNA in the hind limb muscle was detected in both adult males and females although lower levels were found in females (Fig. 1A). Castration resulted in a sharp decrease in the MyoD mRNA levels (Fig. 1A). Testosterone did not change MyoD mRNA levels; in contrast, E_2 induced MyoD mRNA to the levels found in females (Fig. 1A). No signal was detected in heart or liver, showing the tissue specificity of the MyoD transcripts.

In addition to the positive effect reported [12,11,20], our results suggest that the E2 mediated MyoD expression in males may be regulated by a cooperative effect between estrogen and testosterone signaling pathways since castration reduces levels of both estrogen and testosterone [21], and testosterone increased MyoD mRNA levels in myoblasts cultured in complete medium (Fig. 1B). However, the molecular mechanism by which testosterone upregulates MyoD expression remains to be elucidated.

Among the half-palindrome sequences for the ER (GGTCA) present on the MyoD promoter, the CRE-like element shows homology with sequences involved in mutual regulation by steroid hormone receptors and the oncogenes c-Fos and c-Jun. To determine whether estrogen regulates MyoD promoter activity by inducing ER binding to the CRE-like element, we competed the DNA–protein complex formed between the CRE-like element and muscle nuclear extracts using a series of wild-type or mutated oligonucleotides (Table 1) by EMSA. DNA–protein complex was competed by oligonucleotides containing an intact ERE half palindrome; CRE, CREΔG; oligonucleotides F, F1, and F3 from the chicken vitellogenin gene [22] and the wild-type ERE from the *Xenopus* vitellogenin gene or a mutant that destroys only one half of the palindrome (Fig. 2A). In contrast, disruption of the ERE half palindrome did not compete DNA binding (Fig. 2A, F2, F4, and F5). Oligonucleotides with unrelated binding sites (YY-1 and SP-1) did not compete for binding activity to the CRE-like element (Fig. 2A). These results suggest that a half palindrome of the ERE is required for DNA binding. In agreement with published data showing that ER DNA binding activity requires a full palindrome [17,23], neither the MyoD CRE-like element nor the mutated ERE element competed binding (Fig. 2B) while wild-type ERE did compete (Fig. 2B). Altogether, these

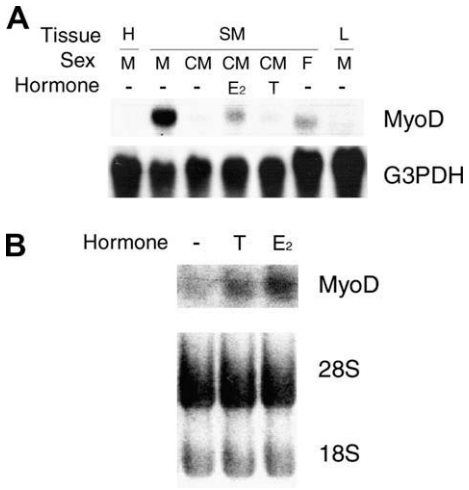

Fig. 1. Steroid hormones regulate MyoD expression. (A) Total RNA was prepared from heart (H), skeletal muscle (SM) and liver (L) of normal male (M), female (F), or castrated (CM) mice not treated (−) or treated with 17β-estradiol (E_2) or testosterone (T) and analyzed by Northern blot (10 μg) using specific probes for MyoD and G3PDH. (B) G8 myoblasts were untreated (−) or treated with 10^{-7} M testosterone (T) or 10^{-8} M E_2 (E_2). After 24 h cells were harvested and total RNA analyzed by Northern blot.

Table 1
Oligonucleotides used as probes or DNA-binding competitors.

Potential regulatory elements	Position[a]	Sequence[b]
CRE	−342 to −322	5′-GAGCACTGAGTCAGTACAG-3′
CREΔG	−342 to −322	5′-GAGCACTGAGGTCAGTACAG 3′
Oligo F	−213 to −182	5′-TACATTGAAACTCTGGTCAATCAGAAAAGG-3′
F1	−213 to −182	5′-TACATTGAAACTCTGG**AC**AATCAGAAAAGG-3′
F2	−213 to −182	5′-TACATTGAAACTCT**CTTCT**ATCAGAAAAGG-3′
F3	−213 to −191	5′-TACATTGAAACTCTGGTCAAT-3′
F4	−213 to −195	5′-TACATTGAAACTCTGGT-3′
F5	−191 to −182	5′-CAATCAGAAAAGG-3′
ERE	−310 to −314	5′-CAAGTCAGGTCACAGTGACCTGATCAAAGA-3′
ERE mut	−310 to −314	5′-CAAGTCAGG**T**CACAGTGA**C**CTGATCAAAGA-3′

The DNA binding elements are underlined.
The base changes used to create the mutated oligonucleotides are given in bold.
[a] Position is relative to the transcription start site.
[b] Only the upper strand is shown.