p300/cAMP-response-element-binding-protein ('CREB')-binding protein (CBP) modulates co-operation between myocyte enhancer factor 2A (MEF2A) and thyroid hormone receptor–retinoid X receptor

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Thyroid hormone receptors (TRs) and members of the myocyte enhancer factor 2 (MEF2) family are involved in the regulation of muscle-specific gene expression during myogenesis. Physical interaction between these two factors is required to synergistically activate gene transcription. p300/cAMP-response-element-binding-protein ('CREB')-binding protein (CBP) interacting with transcription factors is able to increase their activity on target gene promoters. We investigated the role of p300 in regulating the TR–MEF2A complex. To this end, we mapped the regions of these proteins involved in physical interactions and we evaluated the expression of a chloramphenicol acetyltransferase (CAT) reporter gene in U2OS cells under control of the α-myosin heavy chain promoter containing the thyroid hormone response element (TRE). Our results suggested a role of p300/CBP in mediating the transactivation effects of the TR–retenoid X receptor (RxR)–MEF2A complex. Our findings showed that the same C-terminal portion of p300 binds the N-terminal domains of both TR and MEF2A, and our *in vivo* studies demonstrated that TR, MEF2A and p300 form a ternary complex. Moreover, by the use of CAT assays, we demonstrated that adenovirus E1A inhibits activation of transcription by TR–RxR–MEF2A–p300 but not by TR–RxR–MEF2A. Our data suggested that p300 can bind and modulate the activity of TR–RxR–MEF2A at TRE. In addition, it is speculated that p300 might modulate the activity of the TR–RxR–MEF2A complex by recruiting a hypothetical endogenous inhibitor which may act like adenovirus E1A.

Key words: cell cycle, myogenesis, thyroid hormone receptor (TR).

INTRODUCTION

The interplay among different classes of transcription factors is critical for inducing an appropriate, tissue-specific transcriptional programme. Recently, the p300/cAMP-response-element-binding-protein ('CREB')-binding protein (CBP) transcriptional co-activators, molecules that interact with transcription factors but are not directly involved in DNA binding, were characterized [1]. p300 was shown to interact with many classes of transcription factor, dramatically increasing their activity on target gene promoters. p300 activity is blocked by the product of DNA tumour virus oncogene E1A, which interacts with and sequesters it [2]. Basic helix-loop-helix and myocyte enhancer factor 2 (MEF2) proteins are among the p300-interacting molecules. In particular, the myogenic determinant MyoD transcription factor of the basic helix-loop-helix class was shown to require p300 for the full activation of promoters of specific myogenic genes [3–5] or of the p21/cyclin-dependent kinase inhibitor protein (CIP) [6,7].

A further analysis of the MyoD–p300 interaction complex demonstrated the importance of the MEF2C transcription factor as well. At the nuclear level, MEF2 proteins are mediators of external stimuli. Whereas MEF2 factors are not able to activate the myogenic pathway by themselves, on the other hand they are able to improve the efficiency of myogenic conversion induced by MyoD. Moreover, the p38 members of the mitogen-activated protein kinase family are able to modulate the activity of MEF2 by phosphorylation [8,9]. Therefore, MEF2 and p300 are both co-activators of specific genetic programs mediated by external stimuli.

A further category of p300-interacting factors is that of nuclear hormone receptors. Ligand-bound hormone receptors, such as thyroid hormone receptor (TR) or retinoic acid receptor, in a heterodimerization state with the ubiquitous component retinoid X receptor (RxR), show high-affinity binding for p300, which in turn potently enhances their transcriptional activity on target promoters [10].

Recently, it was also suggested that MEF2 and TR functionally interact, binding to each other *in vivo* and *in vitro* [11]. This interaction activates transcription of the α-myosin heavy chain (α-myosin HC) promoter, a target gene that is regulated by the metabolic state of the organism [12]. In fact, cardiac myocytes express two forms of myosin HC, α and β. The α form is up-regulated by thyroid hormone (T3), while the β form is up-regulated during stress. It was shown that the relative expression of either of the two forms is controlled in a reciprocal manner, one being up-regulated while the other is down-regulated.

In this study, we sought to determine whether TR–RxR and MEF2A were modulated by p300, based on the results indicating that TR and MEF2A were separately activated by p300. We provided evidence that the interaction between TR and MEF2A is modulated by p300.

Abbreviations used: α-myosin HC, α-myosin heavy chain; CAT, chloramphenicol acetyltransferase; CBP, cAMP-response-element-binding-protein ('CREB')-binding protein; GST, glutathione S-transferase; MEF2, myocyte enhancer factor 2; RxR, retinoid X receptor; T3, thyroid hormone; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

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EXPERIMENTAL

Construct preparation

The thyroid hormone response element (TRE) plasmid was generated by inserting the TRE sequence of the α-myosin HC promoter into a chloramphenicol acetyltransferase (CAT) reporter plasmid (Promega). For mammalian expression vectors, prTR (rat TR) was obtained from Dr Seigo Izumo (Beth Israel Hospital, Harvard Medical School, Boston, MA, U.S.A.). RxR was obtained from Dr Ron Evans (The Salk Institute, La Jolla, CA, U.S.A.). pcDNA3 p300 was obtained from Dr David Livingston (Dana Farber Cancer Institute, Boston, MA, U.S.A.), while CBP was provided by Dr Ron Evans. pMEF2A and MEF2C were provided by Dr Ye-Thaa Yu and Dr John McDermott (University of Tennessee, Nashville, TN, U.S.A., and York University, Toronto, Ontario, Canada, respectively). The p300 mutants were kindly provided by various colleagues. The p300 mutants 1–670, 670–1194 and 1135–2414 were in pCI vectors and the mutants 1572–2414 and 1869–2414 were in pcDNA3 vectors.

For prokaryotic expression experiments the following plasmids were generated after PCR amplification: pGEX3X-MEF2A wild-type, pGEX3X-MEF2A 1–86, pGEX3X-MEF2A 1–103, pGEX3X-MEF2A 1–196, pGEX3X-MEF2A 127–507, pGEX3X-MEF2A 274–373 and pGEX3X-MEF2A 196–507. The TR gene was instead cloned in pGEX5X-3, generating the following plasmids: pGEX5X-3-TR wild-type, pGEX5X-3-TR 1–126, pGEX5X-3-TR 121–410 and pGEX5X-3-TR Δ 215–343. pCMV M2 FLAG-TR and pcDNA3T7TAG-MEF2A were also generated by PCR amplification for *in vivo* immunoprecipitation studies.

In vitro transcription/translation and protein–protein interaction assays

Supercoiled DNA plasmids (1 μg) were transcribed/translated *in vitro* in the presence of [^{35}S]methionine with the TnT coupled reticulocyte lysate system (Promega), according to the manufacturer's suggestions. Expression and purification of glutathione S-transferase (GST) fusion polypeptides were performed as described previously [13]. The GST fusion proteins were analysed by SDS/polyacrylamide gel to determine their amount and integrity.

For protein–protein interaction assay, glutathione beads (Sigma) coated with the GST fusion protein (4 μg) were reacted with 2 μl of [^{35}S]methionine-labelled translation product in 200 μl of binding buffer containing 20 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA and 0.5 % Nonidet P-40. The reaction was allowed to proceed for 1 h at 4 °C with gentle rocking, after which the glutathione beads were collected by brief centrifugation and subjected to three rounds of washing with 1 ml of binding buffer/wash. The beads were resuspended in 10 μl of Laemmli loading buffer and boiled for 5 min. Eluted proteins were resolved by SDS/PAGE; the gel was treated with 1 M sodium salicylate/12 % propan-2-ol, dried and exposed for autoradiography.

Electrophoretic mobility-shift assay

In vitro-translated wild-type p300, TR and MEF2A were incubated with ^{32}P-labelled TRE-DR4 (5′-AGCTTCAGGTCACA-GGAGGTCAGA-3′; 100000 c.p.m.) for 30 min at room temperature in a 25 μl reaction containing 100 mmol of KCl, 6 % glycerol, 10 mmol of Tris, pH 8.0, 0.05 % Nonidet P-40, 1 mmol of dithiothreitol and 20 μM T3 (Sigma).

The protein–DNA complexes were resolved by native PAGE (4.5 % gel) in 0.5 × Tris/borate/EDTA and visualized by autoradiography.

Cell transfection and co-immunoprecipitations

Transfections of COS-7 cells were performed following the calcium phosphate precipitation protocol [14]. Co-transfections were carried out with 5 μg of each DNA plasmid (pcDNA3T7TAG-MEF2A and pCI-p300; pCMV M2 FLAG-TR and pCI-p300; pcDNA3T7TAG-MEF2A and pCMV M2 FLAG-TR). COS-7 cells were centrifuged at 4 °C, and the pellet was lysed by incubation in 100 μl of lysis buffer (50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 % Triton X-100, 0.1 mM Na_3VO_4, 1 mM PMSF and 10 mg/ml leupeptin) for 30 min at 4 °C. The lysate was centrifuged and the supernatant was recovered.

Protein extracts were incubated in the presence of 1 μg of monoclonal anti-T7TAG (Novagen), 3 μg of monoclonal anti-M2 FLAG (Kodak) or 3 μg of rabbit polyclonal anti-p300 in lysis buffer for 2 h at 4 °C. A 50 % slurry of Protein G–agarose or Protein A–agarose (200 μl; Sigma) was added and incubated for 1 h at 4 °C. The agarose resins were collected by brief centrifugation and subjected to three rounds of washing with 1 ml of lysis buffer/wash. Proteins were resolved by SDS/PAGE (6 % gels) and transferred to PVDF membranes (Millipore) in Tris/glycine buffer (25 mM Tris, 192 mM glycine, 5 % methanol and 0.05 % SDS). The membranes were blocked with 5 % skimmed milk in TBS-T buffer (2 mM Tris, 13.7 mM NaCl and 0.1 % Tween-20, pH 7.6), incubated with polyclonal anti-p300 antibody, monoclonal anti-T7TAG or monoclonal anti-M2 FLAG, and then washed with TBS-T. The membranes were then incubated with anti-mouse or anti-rabbit Ig coupled with horseradish peroxidase (Amersham Biosciences) and washed with TBS-T. The presence of secondary antibody bound to the membrane was detected using the ECL system (Dupont NEN).

Chromatin immunoprecipitation

C2C12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20 % fetal bovine serum (growth medium). Cells (5×10^5) were plated in 10 mm plates 24 h before transfection and then transiently tranfected with pCMV M2 FLAG-TR, pcDNA3T7TAG-MEF2A and pCI-p300 (4 μg of each plasmid) by Fugene 6 (Roche), according to the manufacturer's protocol. After transfection (24 h), the cells were washed with PBS, and Dulbecco's modified Eagle's medium supplemented with 2 % fetal bovine serum (differentiation medium) was added. Then, 24 h later, transfected cells were fixed by adding formaldehyde directly to culture medium to a final concentration of 1 %, and incubated for 10 min at 37 °C. The fixed cells were scraped with ice-cold PBS containing protease inhibitors (1 mM PMSF and 1 μg/ml leupeptin), pelleted for 4 min at 700 ***g*** at 4 °C, and resuspended in 200 μl of SDS lysis buffer (1 % SDS, 10 mM EDTA and 50 mM Tris/HCl, pH 8.1) for 10 min on ice. The lysates were sonicated to reduce DNA length between 200 and 1000 bp. Debris was removed by centrifugation for 10 min at 9800 ***g*** at 4 °C, and the supernatant fraction diluted 10-fold in chromatin immunoprecipitation dilution buffer (0.01 % SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl, pH 8.1, 150 mM NaCl, 1 mM PMSF and 1 μg/ml leupeptin). A portion of this chromatin solution was kept as a template for PCR positive control. To reduce non-specific background, the chromatin solution was pre-cleared with 80 μl of salmon sperm DNA/Protein A–agarose slurry or salmon sperm DNA/Protein G–agarose slurry for 30 min at 4 °C with agitation. Beads were

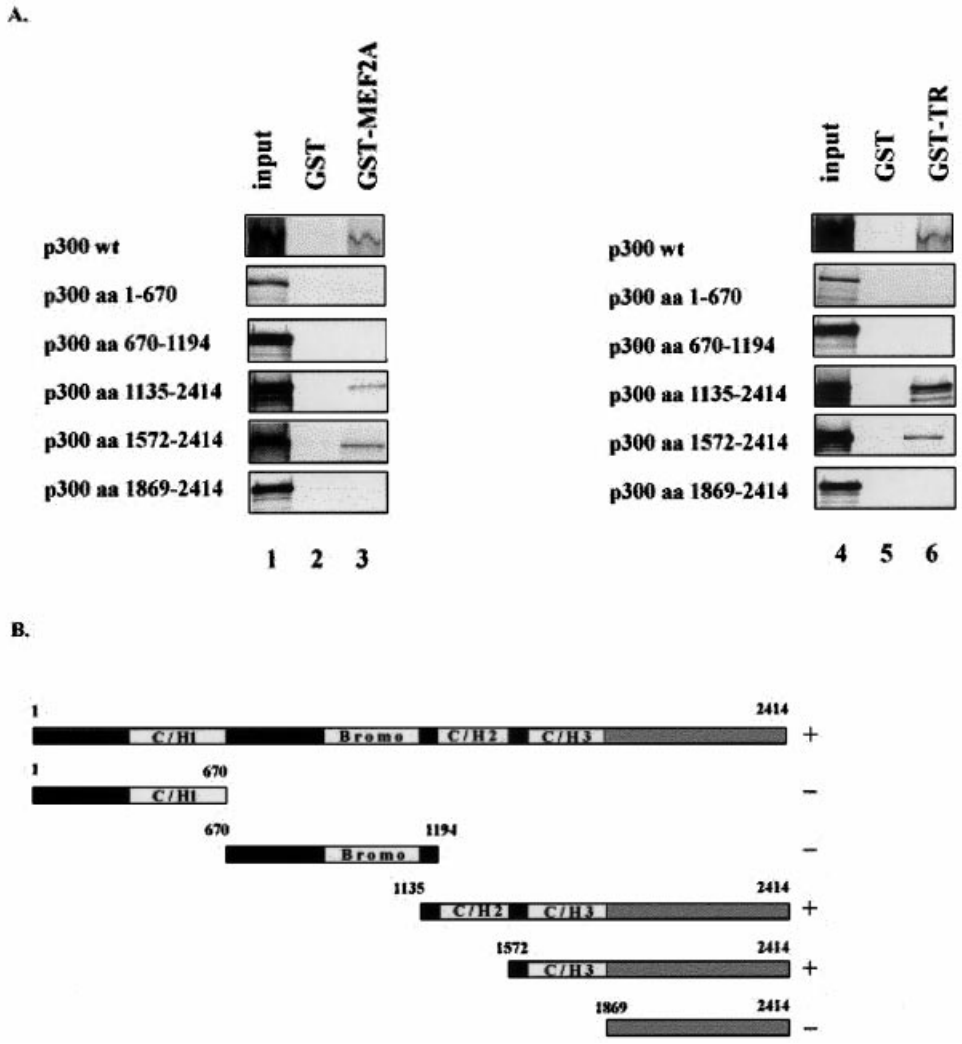

Figure 1 p300 deletion mutants: *in vitro* binding with TR and MEF2A

(**A**) Wild-type p300 (wt) and p300 mutants (as shown in **B**) were translated in rabbit reticulocyte lysates, labelled with [^{35}S]methionine (lanes 1 and 4) and incubated with 4 μg of purified GST-MEF2A wt (lane 3) and GST-TR wt (lane 6) or GST (lanes 2 and 5) for 1 h at 4 °C. Protein complexes were collected on glutathione–agarose beads, washed and boiled in SDS sample buffer. Proteins were resolved by SDS/PAGE. (**B**) Schematic representation of p300 deletion mutants.

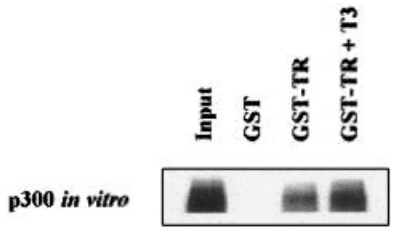

Figure 2 T3's effect on p300/TR *in vitro* binding

p300 wt was translated in rabbit reticulocyte lysates, labelled with [^{35}S]methionine (lane 1) and incubated with 4 μg of purified GST (lane 2) and wild-type GST-TR (lanes 3 and 4) for 1 h at 4 °C. Protein complexes were collected on glutathione–agarose beads, washed and boiled in SDS sample buffer. Proteins were resolved by SDS/PAGE. Lane 1 shows the input used in the experiment.

pelleted by brief centrifugation at 1800 ***g***, and the supernatant fraction was collected.

Immunoprecipitations were performed with 10 μg of anti-p300 polyclonal antibody, anti-T7TAG monoclonal antibody (Novagen) or anti-M2 FLAG monoclonal antibody (Kodak), incubated overnight at 4 °C with rotation. Equal volumes of the supernatant fractions were saved for a no-antibodies control, and incubated with 3 μl of normal rabbit or normal mouse serum. Immune complexes were collected with 60 μl of salmon sperm DNA/Protein A–agarose slurry or salmon sperm DNA/Protein G–agarose slurry, for 1 h at 4 °C with rotation. Beads were pelleted by centrifugation and washed for 3 min on a rotating platform with 1 ml of each of the listed buffers: low-salt immune complex wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.1, and 150 mM NaCl), high-salt immune complex wash buffer (as for the low-salt buffer

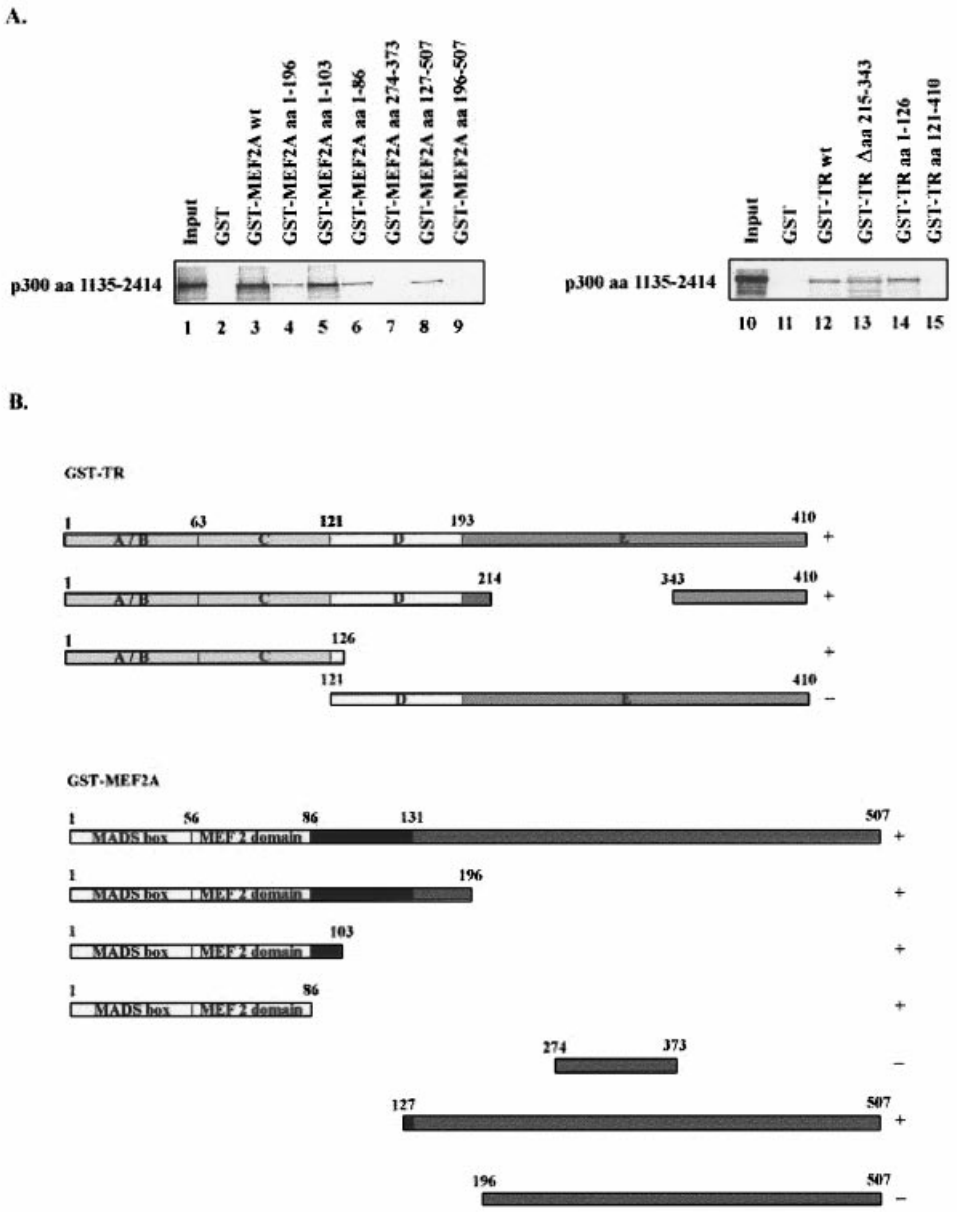

Figure 3 TR and MEF2A deletion mutants: *in vitro* binding with p300 amino acids 1135–2414

(**A**) p300 amino acids 1135–2414 (lanes 1 and 10) was translated in rabbit reticulocyte lysates, labelled with [^{35}S]methionine and incubated with 4 μg of purified to wild-type GST-MEF2A (wt) and GST-TR wt (lanes 3 and 12), and several deletion mutants of GST-MEF2A and GST-TR (lanes 4–9 and 13–15), shown in (**B**). GST fusion protein was used as a negative control (lanes 2 and 11). (**B**) Schematic representation of MEF2A and TR deletion mutants.

but with 500 mM NaCl), LiCl immune complex wash buffer (0.25 M LiCl, 1 % Nonidet P-40, 1 % deoxycholate, 1 mM EDTA and 10 mM Tris/HCl, pH 8.1) and 1 × Tris/EDTA (two washes). Immune complexes were eluted by adding 250 μl of elution buffer (1 % SDS and 0.1 M $NaHCO_3$) to pelleted beads, vortexing briefly to mix and incubating at room temperature for 15 min with rotation. Beads were pelleted, and the eluates carefully transferred to other tubes. This step was repeated a second time and eluates were combined. We added 20 μl of 5 M NaCl to the combined eluates, and cross-links were reversed by incubating at 65 °C for 4 h. After incubation, 10 μl of 0.5 M EDTA, 20 μl of 1 M Tris/HCl, pH 6.5, and 20 μg of proteinase K were added to the eluates, and incubated for 1 h at 45 °C. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Pellets were washed with 1 ml of 70 % ethanol, air dried and resuspended in 20 μl of sterile double-distilled water. We used 5 μl of the resuspended DNA as a template for the PCR using the following primers, corresponding to regions 3901–3925 and 4476–4500 of the *Mus musculus* α-myosin HC gene promoter region (NCBI GI no. 1621436), respectively: 5′-GCCTGAAGCTATGCAGATAGCCAGG-3′ and 5′-CCTGTCAGATCTCTGACCGGCCTGA-3′.

Cell culture and CAT assays

U2OS cells were plated on 60 mm tissue-culture plates and grown in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% fetal calf serum. The medium was changed 18 h after plating and the cells were kept for 3 h in medium containing 5% stripped fetal calf serum and then transfected by the calcium phosphate co-precipitation method. Stripped fetal calf serum was prepared as described by Samuels et al. [15]. After 5 h of incubation under stirring, the resin was removed by centrifugation at 1000 ***g*** (10 min). An additional quantity of fresh resin was added at a concentration of 50 mg/ml to the residual serum, and the serum was incubated for an additional 15–18 h. Finally, the resin was removed by centrifugation at 1000 ***g*** (10 min). This step was performed twice. U2OS cells were transfected by the calcium phosphate co-precipitation method. The precipitated DNA contained 1 μg of N1-CAT reporter, 1 μg of pSV2 b-gal (Promega) and 1.5 μg of each expression vector, and salmon sperm carrier DNA (Stratagene) was added to bring the total amount transfected to 25 μg. After 12–18 h of incubation in medium containing the precipitated DNA, the cells were washed and cultured in fresh medium containing 2% stripped horse serum with or without T3 (100 nM) for 48 h before harvesting. The quantities of cell extracts used for CAT assays were normalized to β-galactosidase activity. CAT and β-galactosidase assays were performed as described elsewhere [16]. The results were quantified using a Phosphorimager. The data are means $\pm$ S.E.M. from six experiments.

RESULTS

p300 physically associates *in vitro* with TR and MEF2A

Our study was focused on understanding whether TR and MEF2A were able to bind p300. To demonstrate the possible interaction between p300 and these two proteins, *in vitro*-translated wild-type p300 was incubated with GST-MEF2A, GST-TR and GST alone (as a negative control). *In vitro*-translated wild-type p300 was able to bind both proteins *in vitro* (Figure 1A). Moreover, when *in vitro*-translated wild-type p300 and TR were incubated with T3, the binding of TR was increased (Figure 2), probably due to the change in conformation of TR on binding T3.

To identify the region of p300 that bound to TR–MEF2A, we used a set of p300 deletion mutants translated *in vitro* (Figure 1B). When the *in vitro*-translated product of p300 wild-type and five deletion mutants were incubated with equal amounts of GST-MEF2A, GST-TR and GST alone (negative control), two fragments of p300 were able to bind TR and MEF2A, amino acids 1135–2414 and 1572–2414, whereas the 1869–2414 fragment did not bind. This showed that both TR and MEF2A proteins bound to a region of p300 between amino acids 1572 and 1868 (Figure 1).

We also identified the region of MEF2A and TR required to bind p300 by generating a panel of mutants, representing different regions of MEF2A and TR, expressed as GST fusion proteins (see Figure 3B). These were immobilized on glutathione–agarose beads and incubated with *in vitro*-translated p300, amino acids 1135–2414, as before. The results showed that p300 bound to the N-terminal 195-amino acid fragment of MEF2A (Figure 3A) and to the N-terminal 120 amino acids of TR (Figure 3A).

Interaction between p300, TR and MEF2A *in vivo*

To test whether p300, MEF2A and TR formed a ternary complex *in vivo*, COS-7 cells were transiently co-transfected with p300, MEF2A and TR. The transiently transfected cells were immunoprecipitated using antibodies against T7TAG-MEF2A, M2 FLAG-TR or p300, followed by Western-blot analysis with antibodies against the other two proteins. This showed that all three proteins could form a ternary complex in intact cells (Figure 4).

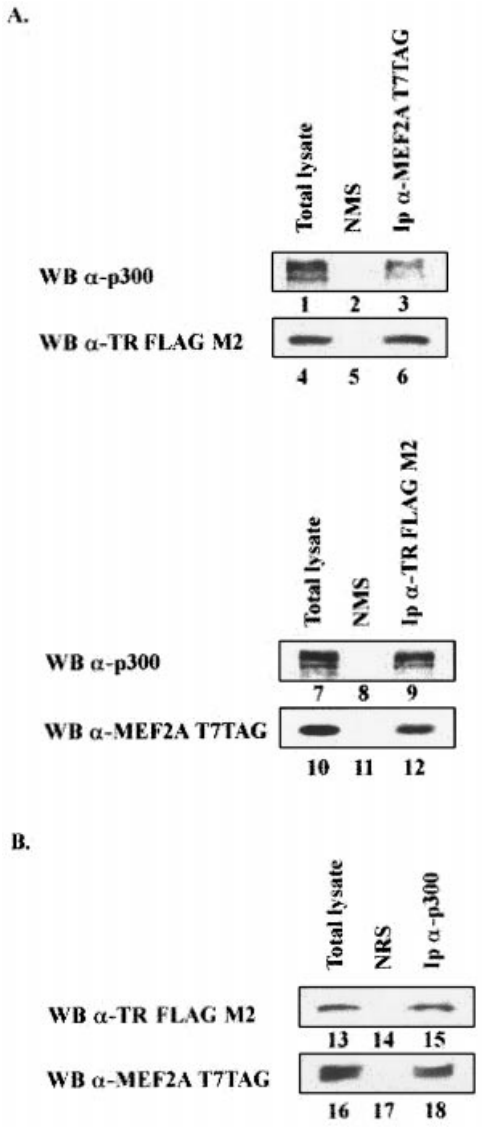

Figure 4 Association of MEF2A, TR and p300 *in vivo*

(**A**) Proteins were precipitated with normal mouse serum (NMS; lanes 2, 5, 8 and 11), anti-T7TAG (lanes 3 and 6) or anti-M2 FLAG (lanes 9 and 12) monoclonal antibodies by COS-7 lysate containing 800 μg of total cellular proteins, resolved by SDS/PAGE, transferred to PVDF membranes and immunoblotted with anti-p300 polyclonal antibody (lanes 1–3 and 7–9) or anti-M2 FLAG (lanes 4–6) or anti-T7TAG (lanes 10–12) monoclonal antibodies. (**B**) Proteins were precipitated with normal rabbit serum (NRS; lanes 14 and 17) or p300 polyclonal antibody (lanes 15 and 18) by COS-7 lysate containing 800 μg of total cellular proteins, resolved by SDS/PAGE, transferred to PVDF membranes and immunoblotted with anti-M2 FLAG (lanes 13–15) or anti-T7TAG (lanes 16–18) monoclonal antibodies. WB, Western blot.

Electrophoretic mobility-shift assay

To further investigate the ternary complex between p300, MEF2A and TR, we performed electrophoretic mobility-shift

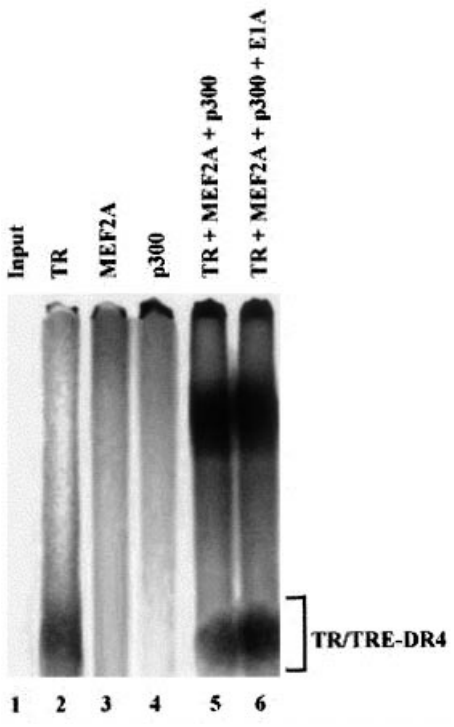

Figure 5 Association of MEF2A, TR and p300

Equal count of *in vitro*-translated wild-type TR, MEF2A or p300 were incubated with ^{32}P-labelled TRE-DR4 in a binding buffer containing 20 μM T3 (lanes 2, 3 and 4, respectively): as expected, only TR could bind to TRE-DR4. Moreover, a supershift of the labelled probe was observed in the presence of all these factors (lane 5) and the addition of E1A did not affect this slow-moving band (lane 6).

assays, using ^{32}P-labelled TRE-DR4 double-stranded DNA as a probe. As shown in Figure 5, labelled TRE-DR4 was shifted in the presence of TR and super-shifted in the presence of TR, MEF2A and p300. The addition of E1A did not affect this slow-moving band (Figure 5, lane 6).

Chromatin immunoprecipitation

Following chromatin immunoprecipitation on lysates of differentiating C_2C_{12} cells co-transfected with pCMV M2 FLAG-TR, pcDNA3T7TAG-MEF2A and pCI-p300, using antibodies against M2 FLAG-TR, T7TAG-MEF2A and p300, respectively, the DNA recovered from the immune complexes was shown by PCR to contain the α-myosin HC promoter region containing the TRE-DR4 sequence, known to bind TR (Figure 6). These data strengthen the hypothesis of the interaction between p300, MEF2A and TR in a ternary complex at the level of the α-myosin HC promoter.

CAT assay

The possible role of these transcription factors and cofactors in the regulation of transcription at a promoter containing TR-recognition sites upstream of CAT was investigated by co-transfection into U2OS cells (Figure 7). Overexpression of TR caused a CAT activity that was approx. 3-fold higher than the basal level, whereas MEF2A caused a stimulation of approx. 2-fold. Overexpression of TR plus RxR caused a stimulation of CAT activity of 7-fold, whereas addition of MEF2A to the TR–RxR complex caused a 17-fold stimulation over basal CAT level. Adding p300 did not further increase CAT activity. However, co-transfection of p300 plus adenovirus E1A with TR–RxR–MEF2A decreased activity, whereas E1A alone had no effect (Figure 7).

These data show that there are at least two possible complexes: one formed in the absence of p300 which does transactivate but which is E1A-insensitive, and another formed in the presence of p300 that is E1A-sensitive.

DISCUSSION

Recent studies have already shown that TR and MEF2A interact with each other physically [11]. More precisely, the DNA-binding

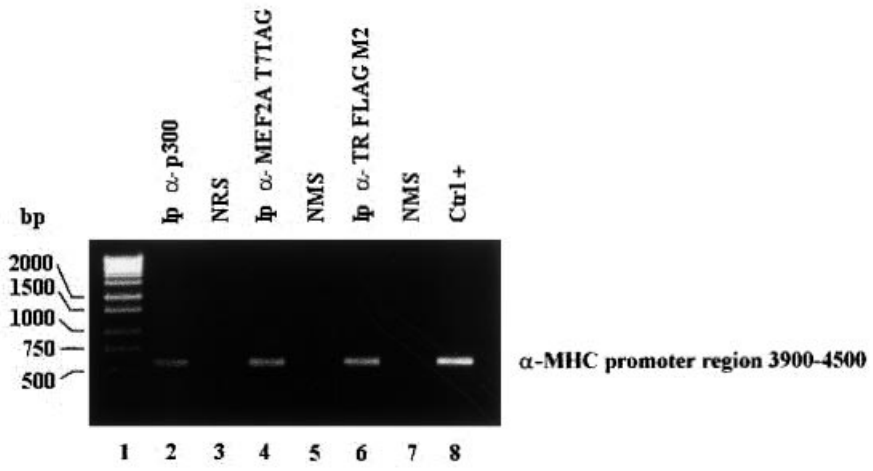

Figure 6 Chromatin immunoprecipitation followed by PCR

Lysates of differentiating C2C12 cells co-transfected with pCMV M2 FLAG-TR, pcDNA3T7TAG-MEF2A and pCI-p300, were immunoprecipited using antibodies against p300 (lane 2), T7TAG-MEF2A (lane 4) and M2 FLAG-TR (lane 6) by the chromatin immunoprecipitation method, and the DNA recovered used as a template for PCR, to amplify a 600 bp region of the α-myosin HC promoter region containing the TRE-DR4 sequence. No-antibody controls were performed using normal rabbit serum (NRS; lane 3) or normal mouse serum (NMS; lanes 5 and 7), and a fraction of the total chromatin solution was used as a positive control (lane 8). Lane 1, molecular mass markers.

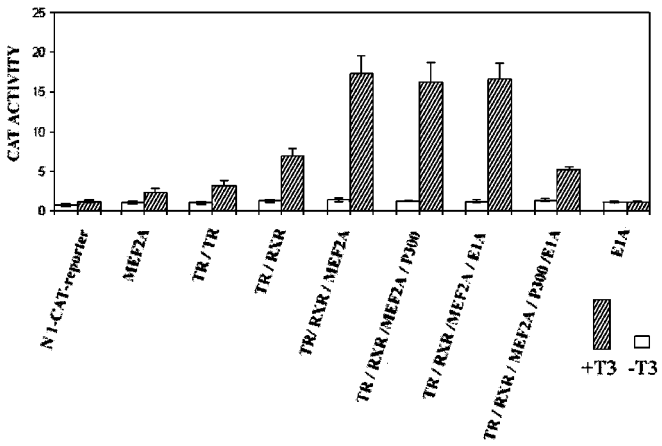

Figure 7 p300 can recruit transcription factors that negatively control the expression of muscle-specific genes

U2OS cells were transiently co-transfected with α-myosin HC-CAT reporter construct and expression vectors for MEF2A, TR, p300 and E1A in the absence or presence of T3.

domain of TR, called the C domain, interacts with MEF2A through the MADS [MCM1, AGAMOUS, DEFICIENS and SRF (serum response factor)] domain [11]. This interaction, described *in vitro*, has been confirmed *in vivo* when these two proteins are overexpressed. We analysed whether p300 plays any role in the interaction between TR–RxR and MEF2A. Previous reports established interactions between TR–RxR and MEF2A [11], MEF2C and p300 [17], and p300 and TR–RxR [18]. Our findings indicate that the N-terminal A/B and C domains of TR (first 195 amino acids) and the MADS and MEF2 domains of MEF2A (first 120 amino acids) are involved in the interaction with the same C-terminal portion of p300, between amino acids 1572 and 1868, which contain a cysteine/histidine-rich domain [5]. Interestingly, this region is known to be involved also in MyoD interaction, another myogenic factor [3]. No other region of p300 was able to bind MEF2A and TR.

Our *in vivo* studies indicated the possibility of a ternary complex involving the three proteins, but were not able to define the stoichiometry of the complexes. The bandshift assay and chromatin immunoprecipitations provided further support for the formation of complexes between TR, MEF2A and p300 on the TRE-DR4 element. Moreover, the bandshift assay also showed that the addition of E1A did not affect the labelled TRE-DR4 slow-moving band. Thus either E1A binding does not dissociate p300 from this complex, or p300 is not part of the complex under these conditions. For instance, it could acetylate TR and/or MEF2A, stabilizing the complex.

p300 is a large protein, and its ability to interact simultaneously with a number of factors has led to its description as a transcriptional adapter protein [1,19]. It has been demonstrated that p300 has a role as co-activator for the transcriptional activation mediated by the heterodimer of T3 receptor and 9-*cis*-retinoic acid receptor (TR–RxR) in the context of chromatin [18]. Our results suggest a role for p300/CBP as a modulator of transcription activation by the TR–RxR–MEF2A complex on the TR element found in the α-myosin HC gene. Interestingly, in presence of p300, activity of the TR–RxR–MEF2A complex was almost completely abolished by E1A. However, E1A had no effect on the complex if p300 was not present. This may mean that the role of p300 is to recruit additional components to the complex, but these components are not required for basal activity in a reporter system. Thus there are at least two possible complexes: one formed in the absence of p300 which does transactivate but is E1A-insensitive, and another formed in the presence of p300 that is E1A-sensitive. A possible role for p300 could be in targeting a hypothetical regulator of transcription on the α-myosin HC promoter that negatively controls gene expression. As such, E1A could mimic the action of the hypothetical cellular regulator by interacting with p300. The evidence that E1A alone is not able to inhibit drastically the transcriptional

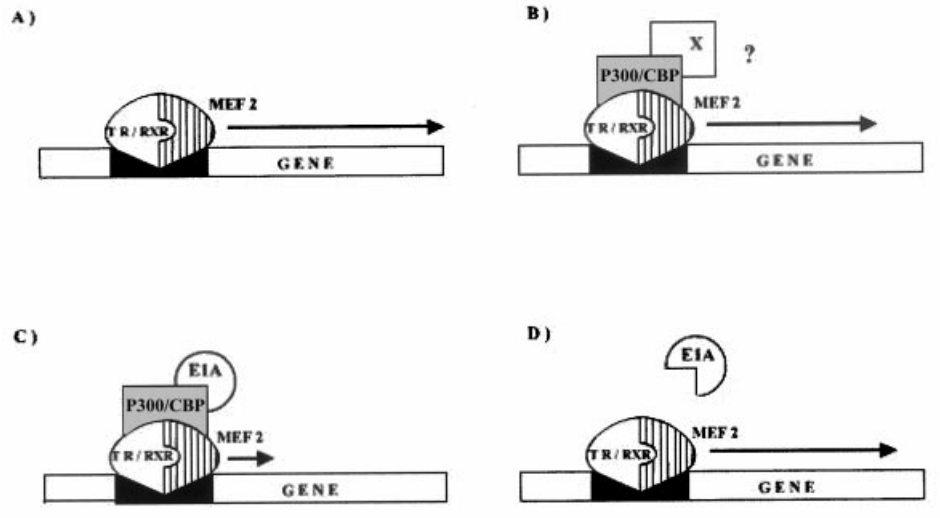

Figure 8 Model for α-myosin HC promoter regulation by TR–RxR–MEF2A–p300 complex

(**A**) Binding of TR–RxR–MEF2A complex to DNA-recognition sites on the α-myosin HC promoter region activates transcription of the reporter gene. (**B**) p300 is a co-activator that bridges the TR–RxR–MEF2A complex and components of the basal transcriptional machinery. p300 recruits a hypothetical transcriptional regulator on the α-myosin HC promoter. (**C**) E1A, by binding to p300, abrogates the p300-mediated TR–RxR–MEF2A transcriptional activity by acting as a negative regulator. (**D**) E1A alone is not able to abrogate the trancriptional activity induced by the TR–RxR–MEF2A complex.

activity of the α-myosin HC promoter suggests the hypothesis that p300 might be acting to bridge the TR–RxR–MEF2A complex and the cellular regulator/inhibitor of the basal transcriptional machinery. The model emerging from this study, illustrated schematically in Figure 8, suggests that p300 may play a key role in carrying and modulating the function of the TR–RxR–MEF2A complex.

Further work will attempt to identify this hypothetical transcriptional regulator and to elucidate the physical and functional relationships between the regulatory factors involved in the control of muscle-specific gene expression during myogenesis.

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