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The triiodothyronine nuclear receptor c-ErbAα1 inhibits avian MyoD transcriptional activity in myoblasts

Laetitia Daury, Muriel Busson, François Casas, Isabelle Cassar-Malek, Chantal Wrutniak-Cabello, Gérard Cabello*

Unité d'Endocrinologie Cellulaire, UMR Différenciation Cellulaire et Croissance (INRA, Université Montpellier II, ENSAM), Institut National de la Recherche Agronomique (INRA), 2 place Viala, 34060 Montpellier Cedex 1, France

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Abstract Thyroid hormone stimulates myoblast differentiation, through an inhibition of AP-1 activity occurring at the onset of differentiation. In this study we found that the T3 nuclear receptor c-ErbAα1 (T3Rα1) is involved in a mechanism preserving the duration of myoblast proliferation. Independently of the hormone presence, T3Rα1 represses avian MyoD transcriptional activity. Using several mutants of T3Rα1, we found that the hinge region plays a crucial role in the inhibition of MyoD activity. In particular, mutations of two small basic sequences included in α helices abrogate the T3Rα1/MyoD functional interaction. Similarly, the T3 receptor also represses myogenin transcriptional activity. Therefore, despite stimulating avian myoblast differentiation by a T3-dependent pathway not involving myogenic factors, T3Rα1 contributes to maintain an optimal myoblast proliferation period by inhibiting MyoD and myogenin activity.

Key words: T3 nuclear receptor; MyoD; Myogenin; Myoblast; Differentiation

1. Introduction

Thyroid hormone is a major regulator of muscle development. In vivo, this hormone not only increases the number [1] and the diameter of myofibers [2], but also influences their metabolic and contractile features [3,4]. In addition, triiodothyronine (T3) promotes fetal to neonatal myosin isoform transition [5]. In vitro studies of our team have provided first evidence that the T3 myogenic influence includes an increased myoblast withdrawal rate from the cell cycle leading to a stimulation of terminal differentiation [6,7]. Moreover, overexpression experiments established that the T3 nuclear receptor c-ErbAα1 (T3Rα1) is involved in the myogenic activity of the hormone [8]. However, these data raised a contradiction between in vivo and in vitro experiments, by the observation that T3 stimulates muscle development despite it reduces the duration of myoblast proliferation.

To conciliate these data, we searched for other mechanisms leading to a preservation of the proliferation period. Interestingly, a crucial mechanism involved in the T3 myogenic influence, repression of AP-1 activity (Jun/Fos transcriptional activity) by liganded T3Rα1, is only functional at a particular stage of myoblast progression in the myogenic program characterized by RXR expression [9,10]. In the same line, as MyoD is involved in the induction of myoblast withdrawal from the cell cycle [11,12], we have studied the possibility that a T3Rα1-dependent mechanism could influence the activity of this myogenic factor. In the present work, we bring evidence that independently of the T3 presence, T3Rα1 inhibits CMD1 (avian MyoD) transcriptional activity, through a functional interaction involving the hinge domain of the receptor.

2. Materials and methods

2.1. Cell cultures

Quail myoblasts of the QM7 cell line [13] were seeded at a plating density of 7000 cells/cm^2. They were grown in Earle 199 medium supplemented with tryptose phosphate broth (0.2%), L-glutamine (2 mM), gentamicin (50 μg/ml), and fetal calf serum (10%). Serum was T3 depleted according to [14]. After hormonal depletion, T3 and T4 levels measured by radioimmunoassay were always lower than the detection limit of the assay.

2.2. Plasmids and reporter genes

The myogenin-CAT reporter plasmid contains the −131/+40 fragment of the chicken myogenin promoter upstream the chloramphenicol acetyltransferase (CAT) coding sequence [15]. The expression vectors for chicken c-ErbAα1, c-ErbAα1 Δ1–36 and MyoD (pRSV c-erbAα1, pSG5-Δ1 and pRSV CMD1) have previously been described [16–18]. The expression vector encoding murine c-ErbAα1 (pSG5 c-erbAα1) was constructed by insertion of a 1.2 kb fragment encoding murine c-ErbAα1, cloned by PCR, in pSG5 vector. The expression vector encoding murine c-ErbAα1 Δ1–256 (pSG5 α1t) has been described elsewhere [19]. The expression vector for rat Gal4/c-ErbAα1 (pSVGal4α1) has been provided by Dr F. Flamant (ENS Lyon, France). The expression vectors for rat c-ErbAα1-D, c-ErbAα1-1, c-ErbAα1-2, and c-ErbAα1-3 mutants (pMT2 c-ErbAα1-D, pMT2 c-ErbAα1-1, pMT2 c-ErbAα1-2, and pMT2 c-ErbAα1-3) have been constructed by Lee and Mahdavi [20]. Mutant and wild-type c-ErbAα1 proteins used in this study are presented in Fig. 1. The expression vector encoding N-CoR (pCEP4 N-CoR) has previously been described [21].

2.3. Transient transfections and CAT assays

Transient transfections were performed using the calcium phosphate co-precipitation procedure [9]. 1 μg of pCMV β-galactosidase expression vector was cotransfected to provide an internal control of transfection efficiency. After cell exposure to precipitates for 24 h, the DNA-containing medium was replaced with fresh medium containing T3 (10^{-8} M) when indicated, and the cells were grown for a further 24 h. β-Galactosidase activity was measured as previously described [22]. CAT enzymatic activity was measured by following the kinetics of

*Corresponding author. Fax: (33)-4-67 54 56 94.
E-mail address: cabello@ensam.inra.fr (G. Cabello).

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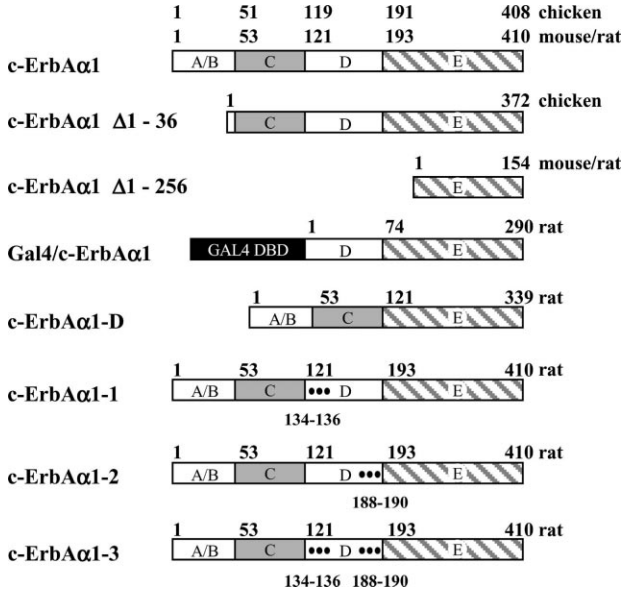

Fig. 1. Wild-type and mutants of the c-ErbAα1 receptor used in this study. Schematic representation of c-ErbAα1, c-ErbAα1 Δ1–36, c-ErbAα1 Δ1–256, Gal4/c-ErbAα1, c-ErbAα1-D, c-ErbAα1-1, c-ErbAα1-2 and c-ErbAα1-3 proteins. A/B: T3-independent transactivation domain. C: DNA binding domain. D: Hinge domain. E: Hormone binding domain.

chloramphenicol acetylation [9]. Results are expressed as percentage of control values after β-galactosidase normalization.

In parallel experiments, c-ErbAα wild-type and mutant expression, as well as localization of the proteins, was assessed in cytoimmunofluorescence experiments using RHTII antibody raised against the COOH-terminus of the protein [23], according to the procedure described by Wrutniak et al. [23].

2.4. Statistical analysis

Statistical analyses were performed using the paired *t*-test [24].

3. Results

3.1. c-ErbAα1 expression inhibits CMD1 transcriptional activity in myoblasts

The influence of avian c-ErbAα1 expression on CMD1 transcriptional activity was studied in transient transfection experiments using the QM7 avian myoblast line, with a reporter gene driven by a minimal myogenin promoter responding to CMD1 [15]. Whereas CMD1 induced an 11-fold stimulation of myogenin promoter basal activity ($P < 0.001$), this influence was fully abrogated by coexpression of avian c-ErbAα1 (11-fold inhibition relative to CMD1 alone; $P < 0.001$, Fig. 2A). Moreover, addition of 10^{-8} M T3 in the culture medium did not significantly influence CMD1 transcriptional activity, and did not affect the inhibitory activity of T3Rα1. Similar data were obtained using another promoter regulated by MyoD (β-tropomyosin promoter, data not shown).

3.2. The hinge domain of c-ErbAα1 is involved in the inhibition of CMD1 transcriptional activity

To identify the T3Rα1 functional domains involved in the inhibition of CMD1 transcriptional activity, we assessed the activity of a truncated form of the receptor [25] harboring a deletion of the NH_2-terminus, c-ErbAα1 Δ1–36. This T3Rα1 mutant displayed a significant inhibitory influence on CMD1 transcriptional activity (2-fold inhibition of CAT activity relatively to CMD1 alone, $P < 0.025$, Fig. 2B). When corrected for the frequency of cells displaying a c-ErbA staining in the nucleus assessed in cytoimmunofluorescence experiments, the recorded inhibition was of the same magnitude than observed for the integral receptor (Table 1). These data suggest that the amino-terminal part of c-ErbAα1 including amino acids 1–36 is not essential for the functional interaction with CMD1.

In additional experiments, we used several mutants of murine or rat c-ErbAα1. This approach was justified by the very high conservation degree of the amino acid sequence of the T3

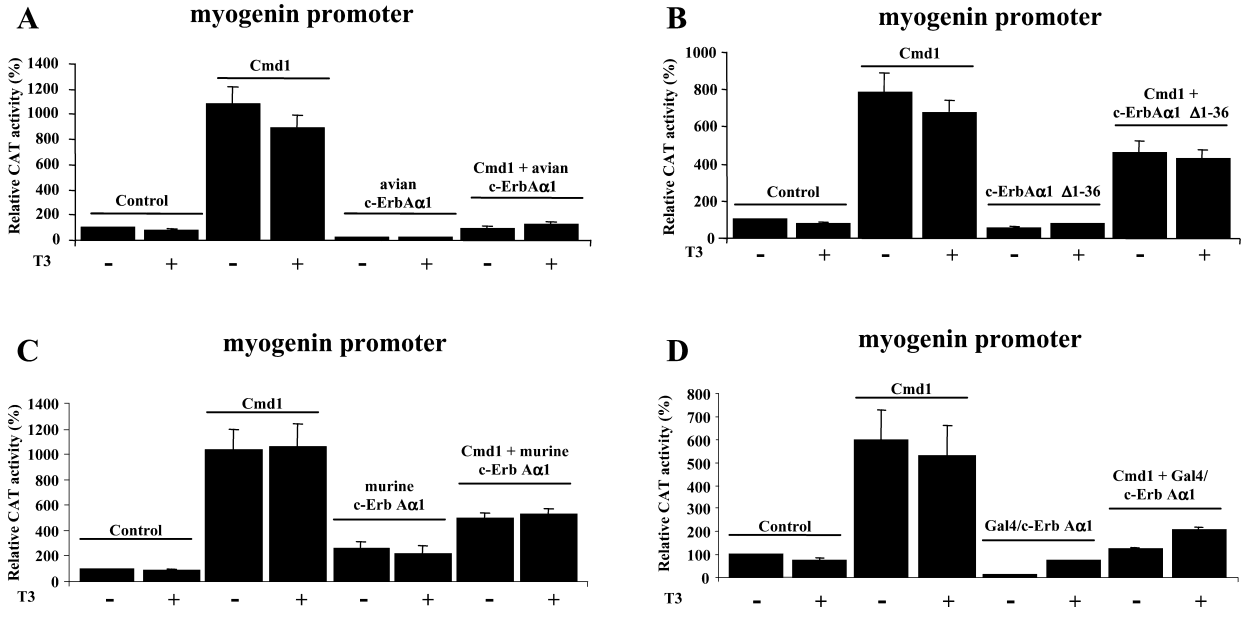

Fig. 2. c-ErbAα1 inhibits CMD1 transcriptional activity. Cells were transfected with 1 μg/dish of the myogenin-CAT reporter gene and, when indicated, 2 μg of CMD1, avian c-ErbAα1 (A), c-ErbAα1 Δ1–36 (B), murine c-ErbAα1 (C) and Gal4/c-ErbAα1 (D) expression vectors. Results are expressed as percentages of CAT activity in control cells, after β-galactosidase normalization. Data are presented as the means ± S.E.M. of five (A), four (B) or three (C and D) separate transfection experiments.

Table 1
Comparison of the inhibition of CMD1 transcriptional activity by c-ErbA wild-type and mutants corrected for transfection efficiency

	−T3	+T3
Avian c-ErbAα1	13.9	13.1
c-ErbAα1 Δ1–36	16.8	14.9
Murine c-ErbAα1	17.0	16.2
Gal4/c-ErbAα1	17.6	13.3
c-ErbAα1 Δ1–256	5.0	1.6
c-ErbAα1-D	2.1	2.3
c-ErbAα1-1	1.2	0
c-ErbAα1-2	0	0
c-ErbAα1-3	2.1	0

The inhibition corrected for transfection efficiency (CI) has been calculated according to the following equation: CI = % inhibition/FOC where % inhibition = 100−[(CAT activity CMD1+c-ErbA) × 100/(CAT activity CMD1)], FOC (frequency of overexpressing cells) = % of cells with overexpressed c-ErbA located in the nucleus. This frequency has been established by counting the number of nuclei stained with an antibody raised against c-ErbA (cytoimmunofluorescence experiments) in three independent transfection experiments.

receptor among species [26]. As expected, like its avian orthologue, murine T3Rα1 displayed also a repressive influence on CMD1 transcriptional activity, by inducing a 2.1-fold inhibition ($P < 0.025$, Fig. 2C). A same influence was recorded using murine MyoD (data not shown). Moreover, a rat c-ErbAα1 mutant, Gal4/c-ErbAα1, in which the NH_2-terminal domain including the first 120 amino acids have been replaced by the DNA binding domain of the yeast Gal4 transcription factor, also inhibited CMD1 transcriptional activity (4.6-fold inhibition relative to CMD1 alone; $P < 0.001$, Fig. 2D). Therefore, despite minor changes in their amino acid sequence, all T3Rα1 tested shared the ability to repress CMD1 transcriptional activity. After correction for transfection efficiency, it also appeared that their inhibitory potential was quite similar (Table 1). Therefore, these data brought confirmation that the NH_2-terminus sequence of the receptor is not involved in this functional interaction.

Interestingly, deletion of amino acids 1–256 (amino-terminal sequence, hinge region and a small part of the ligand binding domain) abolished the ability of T3Rα1 to inhibit CMD1 activity, thus ruling out the hypothesis that the carboxy-terminal domain of the receptor plays a significant role in T3Rα1/CMD1 functional interactions (Fig. 3A).

This set of data suggested that the region including amino acids 120–256, corresponding to the hinge domain and the beginning of the ligand binding domain of T3Rα1, is involved

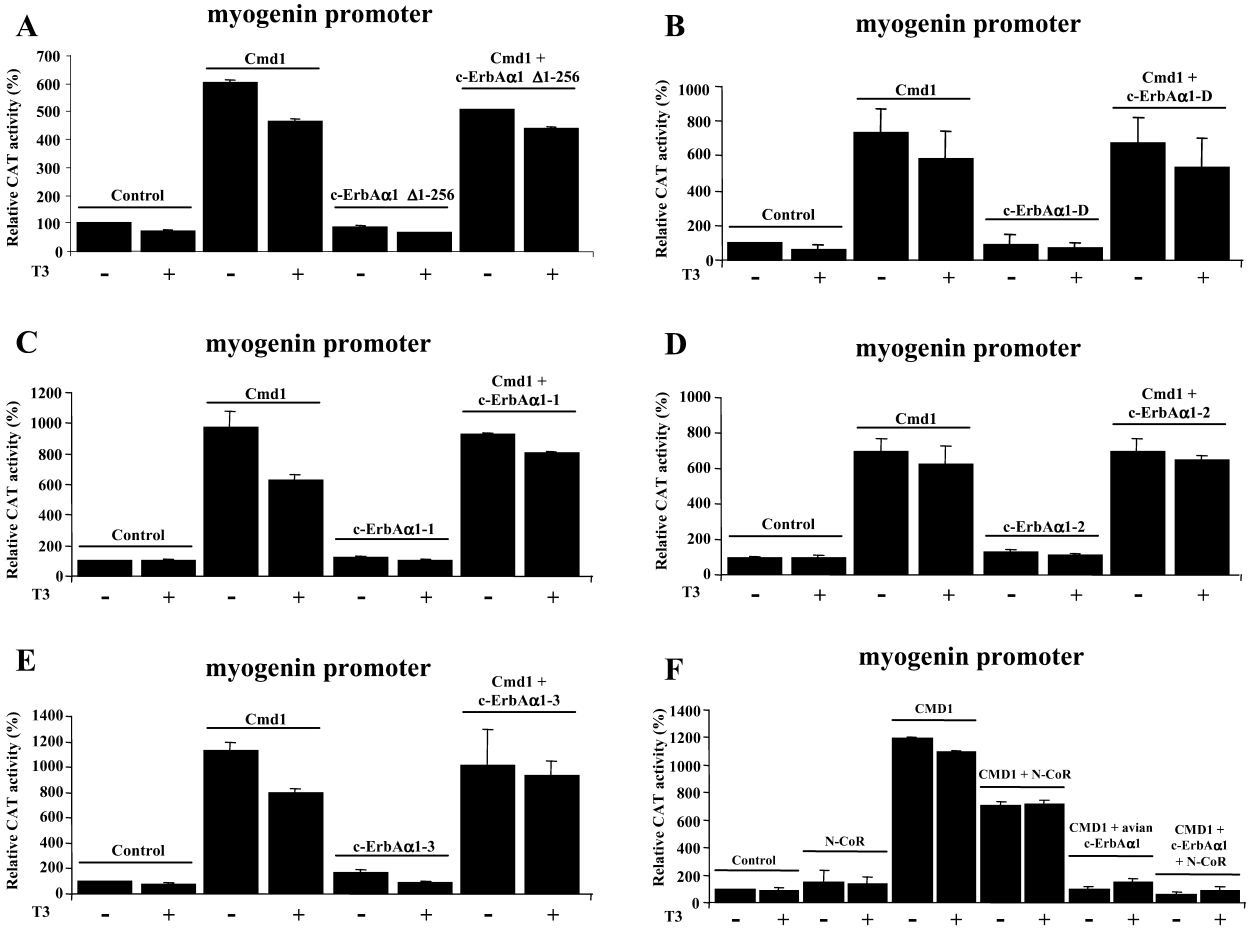

Fig. 3. Mutation in the hinge domain abrogates the repression of CMD1 transcriptional activity by c-ErbAα1. Cells were transfected with 1 μg/dish of the myogenin-CAT reporter gene and, when indicated, 2 μg of CMD1, c-ErbAα1 Δ1–256 (A), c-ErbAα1-D (B), c-ErbAα1-1 (C), c-ErbAα1-2 (D) and c-ErbAα1-3 (E) expression vectors. F: Influence of N-CoR on the inhibition of CMD1 transcriptional activity by c-ErbAα1. Cells were transfected with 1 μg/dish of the myogenin-CAT reporter gene, and when indicated 2 μg of CMD1, 2 μg of avian c-ErbAα1 and 4 μg of N-CoR expression vectors. Results are expressed as percentages of CAT activity in control cells, after β-galactosidase normalization. Data are presented as the means ± S.E.M. of three (B, C, D, E) or four (A, F) separate transfection experiments.

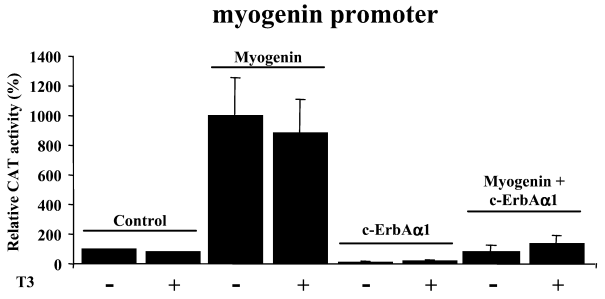

Fig. 4. c-ErbAα1 inhibits myogenin transcriptional activity. Cells were transfected with 1 μg/dish of the myogenin-CAT reporter gene, 2 μg of myogenin and/or c-ErbAα1 expression vectors. Results are expressed as percentages of CAT activity in control cells, after β-galactosidase normalization. Data are presented as the means ± S.E.M. of three separate transfection experiments.

in the inhibition of CMD1 transcriptional activity. To define more accurately the sequence involved in the T3Rα1/CMD1 functional interaction, we studied the influence of a specific deletion of the hinge domain, by coexpressing c-ErbAα1-D and CMD1. In agreement with our previous data, this T3Rα1 mutant did not inhibit CMD1 transcriptional activity (Fig. 3B). In additional experiments, we overexpressed a T3 receptor mutant (c-ErbAα1-3) in which the basic $K^{134}RK$ and $R^{188}RK$ sequences were substituted by the neutral residues TIT [20]. These mutations fully abrogated the ability of the receptor to inhibit CMD1 transcriptional activity (Fig. 3C). In addition, T3Rα1 mutants bearing a similar mutation in only one of the $K^{134}RK$ and $R^{188}RK$ sequences, also failed to inhibit CMD1 transcriptional activity (Fig. 3D and E). These results demonstrated that these two sequences are greatly involved in the T3Rα1/CMD1 functional interaction.

The hinge region of T3Rα1 includes nuclear co-repressor interaction domains close to the $R^{188}RK$ basic sequence. We observed that overexpression of the nuclear hormone co-repressor, N-CoR [21], significantly decreased CMD1 transcriptional activity ($P < 0.01$), as already demonstrated by Bailey et al. [27]. However, it did not influence the repression induced by T3Rα1 (Fig. 3F). These data clearly suggest that the functional interaction between the T3 receptor and the myogenic factor did not involve competition for the recruitment of this co-repressor.

3.3. *Myogenin transcriptional activity is also inhibited by c-ErbAα1*

In order to extend our study to another myogenic factor, we studied the influence of T3Rα1 on the transcriptional activity of myogenin, which is essentially involved in the induction of myoblast terminal differentiation. Whereas myogenin expression induced a 10-fold stimulation of the reporter basal activity, this influence was fully abrogated by coexpression of avian c-ErbAα1 (13-fold inhibition relatively to myogenin alone, $P < 0.001$; Fig. 4). These data indicate that T3Rα1 functional interaction with myogenic factors is not restricted to CMD1, and that, independently of the hormone, the T3 receptor could be also involved in mechanisms leading to a negative control of myoblast differentiation.

4. Discussion

In the present study, we brought evidence that c-ErbAα1 represses CMD1 transcriptional activity by a T3-independent mechanism. As hormone binding induces conformational changes in the COOH-terminus of the receptor leading to crucial modifications in its functionality, this result is consistent with the observation that this part of the receptor does not play any significant role in this unexpected activity. In agreement with these data, the product of the v-ErbA oncogene, lacking the AF2 sequence located in the ligand binding domain of the receptor, also inhibits CMD1 transcriptional activity (data not shown). Also interesting is the observation that this activity of the T3 receptor is not restricted to the avian protein and appears to be extended to mammalian receptors: mouse and rat T3Rα1 repress avian and mammalian MyoD activity (data not shown).

In search for the functional domains of the receptor involved in this functional interaction by using several T3Rα1 mutants, we concluded that the NH_2-terminal sequence (A/B domains) and the DNA binding domain (C domain) are not needed to promote the inhibition of CMD1 activity. However, deletion of almost all the A/B domain (c-ErbAα1 Δ1–36) significantly reduced this influence, in agreement with previous data indicating that in transient transfection experiments, this protein displays a dual cytoplasmic and nuclear localization [28], thus altering the efficiency of this protein at the nuclear level. This possibility is well supported by the observation that after correction for the frequency of nuclear localization of this mutant, its inhibitory activity is not different of that recorded for the integral receptor (Table 1).

This set of data underlines the importance of the T3Rα1 hinge region (D domain). As expected from these results, all mutants deleted from this functional domain are unable to inhibit CMD1 transcriptional activity (c-ErbAα1 Δ1–256, c-ErbAα1-D). Interestingly, the D domain harbors several short basic sequences highly conserved in all species [26], suggesting a particular importance for the receptor function. In addition, similar highly conserved sequences have been described in the basic DNA binding domain of myogenic factors [29]. Moreover, several reports have established that such domains are involved in protein–protein interactions with various transcription factors, including HLH and zinc finger-containing proteins [30–32]. In particular, the MyoD basic domain has been shown to physically interact with the basic domain of Twist [33]. Therefore, this negative functional interaction between CMD1 and c-ErbAα1 could involve the basic helices of the two proteins. In agreement with this possibility, we found that mutations of one or two of these short basic sequences in T3Rα1 ($K^{134}RK$ in the A helix at the beginning of the D domain and $R^{188}RK$ at the end of an α helix of the D domain terminus [34,35]), fully abrogated the ability of the T3 receptor to inhibit CMD1 transcriptional activity.

Several data argue in favor of a direct T3Rα1/CMD1 functional interaction. First, overexpression of N-CoR, a co-repressor interacting with nuclear receptors and MyoD [21,27] is without influence on the inhibition of CMD1 activity by the T3 receptor. Second, in addition to co-repressors, as this influence occurs independently of the T3 presence, the ligand-dependent binding of co-activators to c-ErbA is clearly not involved in the T3Rα1/CMD1 functional interaction. These observations rule out the possibility that competition between the receptor and the myogenic factor for the recruitment of a common co-regulator could be involved in the regulation described in this study. Moreover, a direct interaction is also

substantiated by a previous study describing a direct MyoD/retinoid receptor interaction [36]. Preliminary studies performed in the laboratory also suggest the occurrence of such a direct interaction, but probably involving a third protein partner.

In conclusion, we have previously shown that inhibition of AP-1 activity (c-Jun/c-Fos) by the hormone occurring since RXR expression [9], leading to the expression of BTG1, an antiproliferative protein inducing myoblast differentiation [37] is probably a crucial pathway involved in the myogenic T3 influence. In addition, this study suggests the occurrence of a new mechanism possibly involved in a subtle control of myoblast terminal differentiation by the thyroid hormone apparatus. By inhibiting MyoD activity, T3Rα1 could contribute to maintain an optimal myoblast proliferation period, whereas the induction of RXR expression at the onset of terminal differentiation [10] could allow the liganded T3 receptor to inhibit AP-1 activity, a major myogenic repressor [38].

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