EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 35, No. 1, 23-29, February 2003

Acetyl-CoA carboxylase β expression mediated by MyoD and muscle regulatory factor 4 is differentially affected by retinoic acid receptor and retinoid X receptor

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Accepted 10 January 2003

Abbreviations: ACC, acetyl-CoA carboxylase; CPT-1, carnitine palmitoyl-CoA transferase-1; MRF, muscle regulatory factors; RAR, retinoic acid receptor; RXR, retinoid X receptor

Abstract

Mammals have two major isoforms of acetyl-CoA carboxyase (ACC). The 275 kDa β-form (ACCβ) is predominantly in heart and skeletal muscle while the 265 kDa α-form (ACCα) is the major isoform in lipogenic tissues such as liver and adipose tissue. ACCβ is thought to control fatty acid oxidation by means of the ability of malonyl-CoA to inhibit carnitine palmitoyl-CoA transferase-1 (CPT-1), which is a rate-limiting enzyme of fatty acid oxidation in mitochondria. Previously, it was reported that MyoD and other muscle regulating factors (MRFs) up-regulate the expression of ACCβ by interactions between these factors and several *cis*-elements of ACCβ promoter. We described here that ACCβ expression mediated by MRFs is regulated by retinoic acids. Endogenous expression of ACCβ in differentiated H9C2 myotube was significantly increased by retinoic acid treatment. However, on transient transfection assay in H9C2 myoblast, ACCβ promoter activity was suppressed by RXRα and more severely by RARα. These effects on ACCβ expression in myoblasts and myotubes by RXRα and RARα seem to be mediated by their interactions with MRFs because no consensus sequence for RXRα and RARα has been found in ACCβ promoter and retinoic acid receptors did not affect this promoter activities by itself. In transient transfection in NIH3T3 fibroblast, the activation of ACCβ promoter by MyoD, main MRF in myoblast, was significantly suppressed by RARα and to a less extent by RXRα while the RXRα drastically augmented the activation by MRF4, major MRF in myotube. These results explained that retinoic acids differentially affected the action of MRFs according to their types and RXRα specially elevates the expression of muscle specific genes by stimulating the action of MRF4.

Keywords: acetyl-CoA carboxylase; muscles; MyoD protein; receptors, retinoic acid; retinoids; transcription factors

Introduction

Acetyl-CoA carboxylase (ACC) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA. Mammals have two major isoforms of ACC. ACCα is expressed in lipogenic tissues such as adipose tissue and liver (Lopez-casillas *et al.*, 1988). Its product malonyl-CoA is two carbon donor for fatty acid synthesis and its availability is rate limiting factor in fatty acid synthesis. In contrast, ACCβ is predominantly expressed in skeletal muscle and heart (Thampy, 1989; Bianchi *et al.*, 1990). ACCβ is known to control fatty acid oxidation by regulating malonyl-CoA levels around mitochondria. Compared with α-isoform, ACCβ has additional hydrophobic N-terminus which contributes to the interaction with outer membrane of mitochondria. Moreover, there are many specific serine residues to be phosphorylated in response to various hormonal signals. Malonyl-CoA plays a critical role in controlling the activities of carnitine palmitoyl-CoA transferase 1 (CPT-1), the rate-limiting enzyme in fatty acid uptake and oxidation by mitochondria (Cook, 1984; Mcgarry *et al.*, 1989; Winz *et al.*, 1994). This function of ACCβ in muscle contributes to maintaining adult muscle phenotype and controlling energy homeosatsis.

Myogenesis is regulated by a family of four muscle regulatory transcription factors such as MyoD (Davis *et al.*, 1987), myogenin (Wright *et al.*, 1989), Myf-5 (Braun *et al.*, 1989), and muscle regulatory factor 4 (MRF4) (Braun *et al.*, 1990; Jeffrey *et al.*, 1990) that share a common dimerization motif, DNA-binding domain and the basic helix-loop-helix motif (Lassar *et al.*, 1989). Muscle regulatory transcription factors bind to a consensus sequence, E-box, located in target gene promoter and activate the transcription of the

target genes. They affect muscle gene transcriptions in forms of either homodimer or heterodimer with E-protein (Murre *et al.*, 1989; Lassar *et al.*, 1991). Although these factors contain the common motif, each of these factors activates the specific target genes inducing normal muscle development and maintaining normal muscle phenotype. Therefore, the ordered expression of these factors during myogenesis controls the temporal and spatial expression patterns of muscle specific genes, properly. MyoD and Myf5 can be considered as determining factor of myogenic lineages. Cultured myoblast cell lines express MyoD mRNA, Myf-5 mRNA (Ott *et al.*, 1991) whereas myogenin mRNA is expressed upon myotube fusion and MRF4 plays major role in high expression of muscle gene in fully differentiated myotube (Bober *et al.*, 1991; Smith *et al.*, 1994; Megency and Rudnicki,1995). Previously, it has been shown that MyoD and MRF4 are able to enhance promoter activity of ACCβ (Lee *et al.*, 2001). It was also previously reported that induction of ACCβ accompanies the differentiation of H9C2 cells into myocytes and the antisense RNA to ACCβ mRNA inhibits muscle differentiation (Lee and Kim, 1999).

Retinoic acids have been known as potential inducer of cell growth arrest and differentiation (De Luca, 1991). These receptors act as transcriptional activators by binding as heterodimer or homodimer to specific nucleotide sequence of target genes. It was reported to interact physically muscle b-HLH protein and retinoic acid receptors (Solanes *et al.*, 2000). The interaction of retinoic acid receptors and myogenic b-HLH proteins is important for the execution of retinoid induced myogenic differentiation (Froeschle *et al.*, 1998).

In this study, we introduced that the transcriptional activities of MyoD or MRF4 which regulates ACCβ promoter activity might be regulated by retinoic acids during muscle differentiation.

Materials and Methods

Materials

9-*cis*-Retinoic acid and all-*trans*-retinoic acid was purchased from Sigma Aldrich (St. Louis, MO). The concentrations of their stock solutions were adjusted to 1 mM in dimethyl sulfoxide. H9C2 and NIH3T3 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). DMEM, DMEM-F12 media, FBS, horse serum, antibiotics, and LipofectAMINE Plus kit were purchased from Invitrogen (Int. Antwoordnummer, VT Groningen, Nederland). Polyvinylidene fluoride (PVDF) membrane and enhanced chemiluminescence (ECL plus) system was purchased from Amershame (Uppsala, Sweden). Luciferase assay kit and reporter lysis buffer were purchased from Promega (Medison, WI).

Cell culture

H9C2 Cells were maintained in DMEM-F12 medium supplemented with 10% (v/v) FBS and 100 unit/ml penicillin G sodium, 100 mg/ml streptomycin sulfate and 250 ng/ml amphotericin B in 5% CO_2 at 37°C. The differentiation medium for H9C2 cell differentiation was DMEM-F12 supplemented with 2% (v/v) horse serum and 100 unit/ml penicillin G sodium, 100 mg/ml streptomycin sulfate and 250 ng/ml amphotericin B. H9C2 cells at confluent state were differentiated into myotubes by cultivation in differentiation medium for 6 days. NIH3T3 cells were maintained in DMEM medium with 10% (v/v) fetal bovine serum and 100 unit/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 250 ng/ml amphotericin B.

Transient transfection

Cells used in this experiment were maintained as monolayer culture and grown in appropriate media. Plasmid DNAs were prepared using plasmid midi kits (Qiagen, Hilden, Germany). Concentration of all plasmids was adjusted to 100 ng/μl and confirmed in 1% agarose gel before transfection. Cells were plated in six-well tissue culture plate at a density of 2×10^5 cells/well in 2 ml of media. After 24 h attachment period of plated cells, transfections were performed with LipofectAMINE and Plus reagent. According to the manufacturer's protocol, 500 ng of each construct of pPIIβ-1317/+17-Luc, pE-box-tk-Luc, and p6X(+8/+27)-tk-Luc, 100 ng of pCMV β-gal, 200 ng of pcDNA3-MyoD or pcDNA3-MRF4, and 100 ng of pCMX-RXRα or pCMX-RARα were mixed. 4 μl of plus reagent diluted in 100 μl of media lacking serum and antibiotics was mixed, and then these Plus reagent mixtures were added to DNA mixture. After 15 min, LipofectAMINE mixtures which were prepared by mix 2 μl of LipofectAMINE and 100 μl of media lacking serum and antibiotics were added to Plus reagent mixture following further incubation for 15 min. Also, cells plated in six-well tissue culture plate were washed by phosphate buffered saline (PBS) and replaced by 800 μl media lacking serum and antibiotics. After 15 min, the mixtures of DNA, LipofectAMINE and Plus reagent were added in each well. After 3 h, the medium containing the DNA, LipofectAMINE and Plus reagent complex was removed and replaced by appropriated media containing serum and antibiotics. Cells were then further cultured for 24 h and treated by 9-*cis*-retinoic acid or all-*trans*-retinoic acid of concentration of 1 μM for 48 h.

Luciferase and β-galactosidase assay

Transfected cells were harvested in 200 μl of reporter lysis buffer. The lysed cells were centrifuged to remove cell debris and supernatant was collected. Luciferase activities in lysates were measured, using Luciferase assay kit. For β-galactosidase assay, 20 μl of cell lysates were mixed with 180 μl of assay reagent composed of 3.2 mg of 0-nitrophenol-β-D-galactopyranoside, 4 ml of 0.1 M sodium phosphate buffer adjusting pH 7.5, 40 μl of 100× Mg solution (0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol) and absorbance change 420 nm for 1 min at 37°C was measured in microplate spectrometer. Luciferase activities were expressed as the values normalized by β-galactosidase activity. Each experiment was performed in triplicate.

Western blotting

Cells in 10-cm plates were washed twice with cold PBS. 400 μl of digitonin buffer 1 M Tris-HCl (pH 7.5), 0.5 M EDTA (pH 8.0), 0.25% sucrose, 1.5 mM PMSF, 100 mg/ml digitonin, 10 mM sodium fluoride were directly added to culture dish on ice. The diffusate was collected and cell debris was removed by centrifugation. The proteins in diffusate were separated on a 5% SDS-PAGE and transferred to a PVDF membrane. ACCβ protein band was detected using streptavidine-HRP conjugate and by ECL plus.

Statistical analysis

All transfection studies were performed in three to five separate experiments, where triplicate dishes were used. The data were represented as mean±SD. Statistical analysis was carried out using Excel (Microsoft, Redmond, WA).

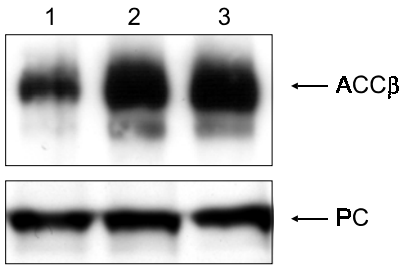

Figure 1. Increased expression of ACCβ by retinoids in H9C2 myotubes. H9C2 cells were almost completely differentiated into myotubes by incubation in differentiation medium for 6 days as described in Materials and Methods. Differentiated myotubes were treated with dimethyl sulfoxide (lane 1), 1 μM all-*trans*-retinoic acid (lane 2), or 1 μM 9-*cis*-retinoic acid (lane 3) for 24 h. Thirty μg of protein of cellular diffusate were subjected to Western blot anaylsis using streptavidin-HRP conjugate. The bands for ACCβ and pyruvate carboxylase (PC) were indicated as arrows.

Results

ACCβ induction by retinoids in H9C2 myotubes

H9C2 cells are rat heart myoblast cells. H9C2 cells do not express ACCβ protein in state of myoblast. But when H9C2 myoblast is fully differentiated into myotube, ACCβ is actively expressed (Figure 1, lane 1). When we treated all-*trans* or 9-*cis*-retinoic acid to completely differentiated H9C2 myotubes, ACCβ expressions were remarkably increased (Figure 1, lane 2 and 3), whereas the levels of pyruvate carboxylase were not changed. These results imply that retinoic acids enhance ACCβ expression in myotubes.

Suppression of ACCβ promoter by retinoid receptors

Even if ACCβ is not expressed, ACCβ promoter activity is very high in H9C2 myoblast when compared with those in fibroblast or hepatoma cell lines. This is probably due to the presence of MRFs in H9C2 myoblasts. We tested how the retinoid receptors affect the promoter activity in H9C2 myoblasts. Unexpectedly, transient transfection assay shows that ACCβ promoter activities were suppressed 38% by RXRα and more severely 65% by RARα (Figure 2). These results displayed the disagreement of retinoic acid

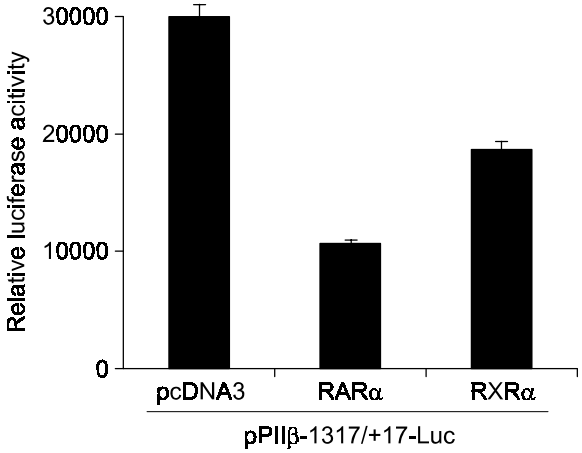

Figure 2. RXRα or RARα effects on ACCβ promoter in H9C2 cells. ACCβ promoter, pPIIβ-1317/+17-Luc, was transfected into H9C2 myoblast, accompanying with RXRα or RARα-overexpressing construct. The amount of constructs transfected per each well were as followings: 0.5 μg of pPIIβ-1317/+17-Luc, 0.1 μg of pCMV β-galactosidase and 0.1 μg of overexpression vectors. Twenty four hours after transfection, the media were replaced containing 1 μM of the respective ligand. The luciferase activities were assayed 24 h after ligand treatment and expressed as the values standardized with β-galactosidase activities.

effects on ACCβ promoter in myoblast and myotube.

Effects of retinoids on the induction of ACCβ promoter by MRFs

Sequence analysis could not reveal any retinoid responsive element in ACCβ promoter. Overexpresion of RARα or RXRα with the treatment of their ligands in NIH3T3 fibroblasts did not significantly change the ACCβ promoter activities (Figure 3A), suggesting that retinoid receptors affect the ACCβ promoter not by their direct DNA-binding but probably by the interactions with MRFs existing in myoblasts or myotubes. The fact that the retinoids and their receptors differentially affected the ACCβ promoter activities in myoblasts and myotubes which express different kinds of MRFs, led to test the retinoid actions on transcriptional activities of MyoD and MRF4 which are primary MRF in myoblasts and myotubes, respectively. The activation of ACCβ promoter by MyoD in NIH3T3 cells was significantly suppressed 56% by RARα but was not affected by RXRα. The overexpression of both RARα and RXRα also suppressed the MyoD activities to a similar extent as RARα alone. However, the effects of retinoid receptors were completely different on MRF4-mediated activation of ACCβ promoter. The activation of ACCβ promoter by MRF4 was significantly increased 332% by RXRα, 95% by RARα, and 268% by both RARα and RXRα. The RXRα activated MRF4 activities most effectively. These data conclude that the activities of myogenic determinant MyoD was inhibited by RARα whereas those RARα and RXRα markedly increase the activities of MRF4 being expressed in terminally differentiated myotubes.

Retinoid effects on heterologous promoters containing E-boxes or noble MRF-binding elements

It was previously reported that the myogenic regulatory factor-mediated activation of ACCβ promoter depends on E-box region from -493 to -403 and one novel *cis*-element from +17 to +24. MRFs are generally known to bind to E-boxes in a form of heterodimer with E-protein whereas the proximal novel sequence of ACCβ promoter was bound by MRF homodimer. To test how the retinoid receptors affect the activation by different quaternary forms of MRF, we used the heterologous promoter containing E-box region or six copy of novel *cis*-element of ACCβ promoter at upstream of tk minimal promoter. Our data showed that the activation of heterologous promoter through noble *cis*-element by MyoD is suppressed by RARα and activation of heterologous promoter containing E-box region by MRF4 is enhanced by RXRα (Figure 4). However, RARα did not inhibit activation of E-box containing promoter by MyoD and

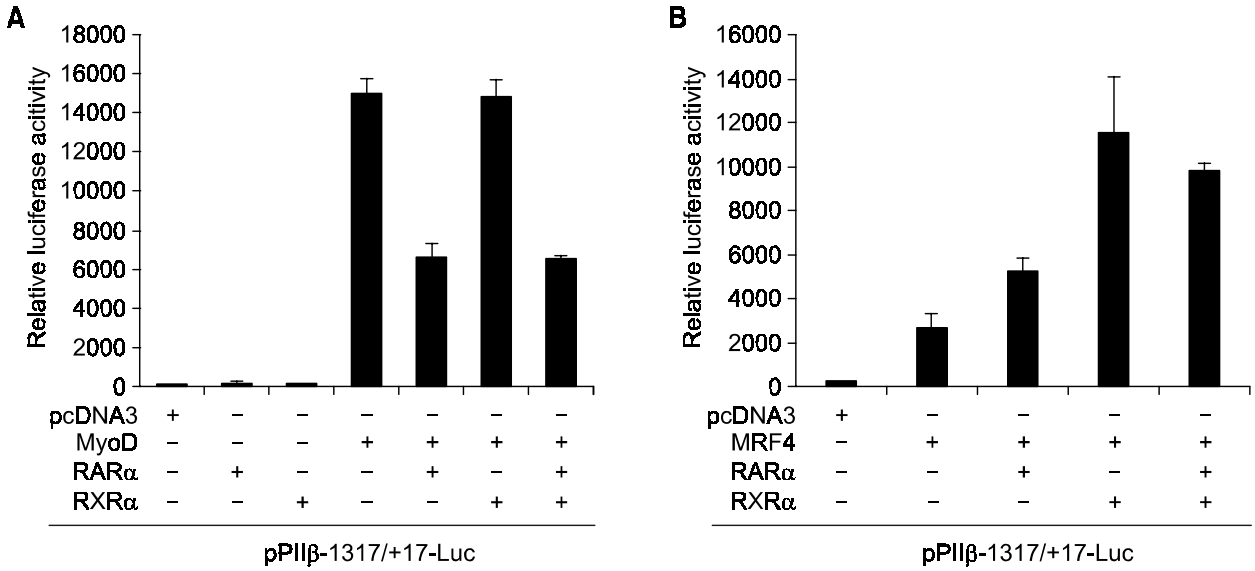

Figure 3. Interaction of retinoic acid receptors and muscle specific regulatory factors on ACCβ promoter. ACCβ promoter construct, pPIIβ-1317/+17-Luc, was transfected with the constructs expressing β-galactosidase, retinoid receptors, MyoD (A), or MRF4 (B) into NIH3T3 fibroblasts as indicated. Total DNA amounts for transfection were adjusted to 0.9 μg per each well with pcDNA3.0, and the amount of each plasmid is as follows: 0.5 μg of pPIIβ-1317/+17-Luc construct, 0.1 μg of pCMV β-galactosidase, 0.1 μg of pCMX-RARα or pCMX-RXRα and 0.2 μg of pcDNA3-MyoD or pcDNA3-MRF4. At 24 h after transfection, the media were replaced, containing 1 μM of the respective ligand. At 48 h after transfection, the luciferase activities were measured and shown as the values.

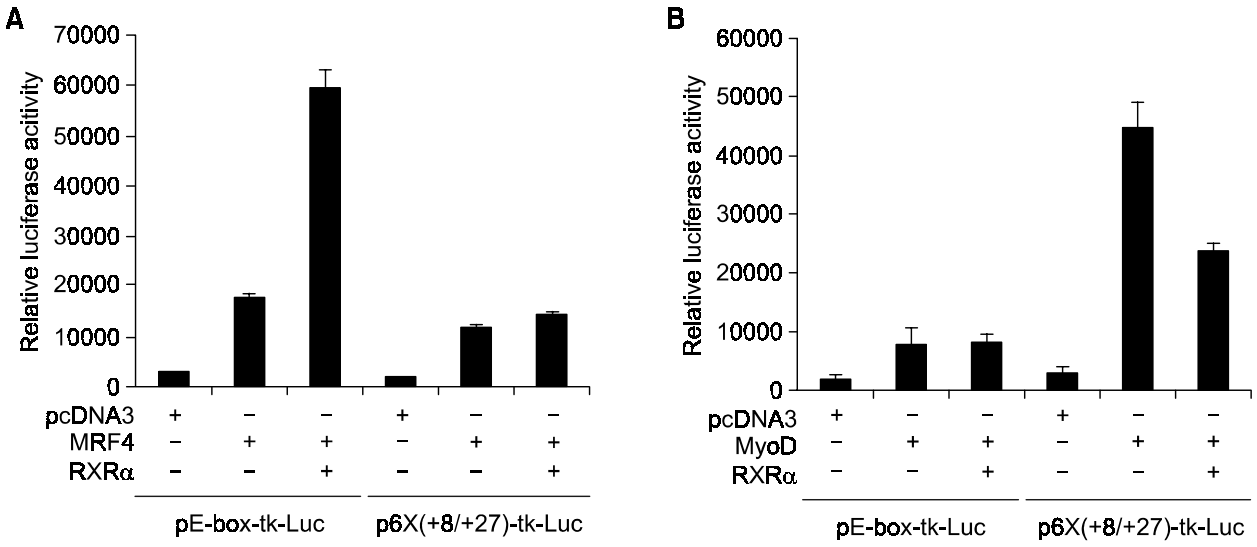

Figure 4. RXRα enhanced MRF4 effect on E-box region of ACCβ and RARα suppressed MyoD effect on noble *cis*-element. Reporter constructs, such as pE-box-tk-Luc containing E-box regions (-493/-403) and p6X(+8/+27)-tk-Luc containing six copies of noble *cis*-element, were transfected with overexpression vectors as indicated into NIH3T3 fibroblasts. Total amounts of DNA for transfection were adjusted to 0.9 μg with pcDNA3.0 and the amount of each plasmid used in transfection is as followings: 0.5 μg of the pE-box-tk-Luc or the p6X(+8/+27)-tk-Luc, 0.1 μg of the pCMV β-galactosidase, 0.2 μg of the pcDNA3-MRF4 or the pcDNA3-MyoD and 0.1 μg of the pCMX-RXRα or the pCMX-RARα. Next day transfection, the cells were treated with 1 μM of the respective ligand for 24 h. The luciferase activities were standardized with β-galactosidase activities.

RXRα did not increase transcriptional activation by MRF4 through noble *cis*-element.

Discussion

Myogenesis is regulated by muscle specific regulating factors such as MyoD, myogenin, Myf5 and MRF4. These regulatory proteins are capable of directly activating expression of skeletal muscle specific genes such as those encoding the contractile proteins. Expression of either Myf5 or MyoD is required for skeletal muscle lineage determination and MRF4 is expressed at high level in adult muscle. MyoD is primary MRF expressed in myoblast stage (Choi *et al.*, 1990; Rudnicki *et al.*, 1993; Atchley *et al.*, 1994), while MRF4 plays a major role in maintaining the high levels of skeletal muscle gene expressions (Hinterberger *et al.*, 1991). Retinoic acids are known as inducer of cell growth arrest and differentiation (De Luca, 1991). Their actions are mediated by two families of ligand-inducible transcriptional factors, the retinoic acid receptor (RARα, β and γ) and retinoid X receptor (RXRα, β and γ) which are members of the nuclear receptor superfamily (Giguere,1994; Mangelsdorf and Evans, 1995; Leid *et al.*, 1992). RARs are bound and activated by *all-trans* retinoic acid (Giguere *et al.*, 1987; Petkovich *et al.*, 1987) whereas RXRs by *9-cis* retinoic acid (Heyman *et al.*, 1992; Levin *et al.*, 1992). These retinoic acid receptors also show the different expressional patterns according to muscle differentiation stages. RARα is plentiful and RXRα is scarce in myoblast stage while, in contrast, RXRα is abundant and RARα is rare in myotube stage (Downes *et al.*, 1994). In this study, we demonstrated that ACCβ transcriptional induction by muscle specific regulating factors is differentially regulated by retinoic acids. When completely differentiated H9C2 myotubes were treated with retinoids, the levels of ACCβ were significantly increased (Figure 1). However, transient transfection assays in myoblasts, where the ACCβ promoter is active probably due to the presence of MyoD, showed that ACCβ promoter activities were suppressed by RXRα and more severely by RARα (Figure 2). These data suggested the possibility that retinoid receptors might differentially affect each MRF expressed in stage-specific manner during myogenesis. To check this possibility, the transient transfection assay was performed using NIH3T3 fibroblasts, which do not express any MRF. As expected, the data in NIH3T3 showed that RARα suppressed MyoD-mediated activation of ACCβ promoter, but in contrast RXRα rather significantly enhanced MRF4 activities (Figure 3). These effects of retinoid receptors on ACCβ promoter were not mediated by their direct interaction with ACCβ promoter sequence, because any conserved responsive element for retinoid receptor can not be found in ACCβ promoter and retinoic acid receptors by themselves did not activate ACCβ promoter at all. The endo-

genous ACCβ expression is not observed in myoblast cells endogenously expressing high levels of MyoD, until myoblasts differentiate into myotubes. These facts indicate the presence of the accessory factors involved in regulating MRF-mediated activations of muscle genes and suggest that retinoid receptors might probably have the roles, in part, as one of these accessory factors. Moreover, it has been reported that retinoic acid receptors and basic helix-loop-helix proteins directly interact each other and synergistically affect muscle target genes (Froeschle *et al.*, 1998). In general, the regulations of eukaryotic transcription occur by forming the complexes of several transcription activators and repressors on the promoter. MRF-mediated activation of ACCβ promoter is controlled by two important *cis*-elements, E-box region (-493/-403) and noble sequence (+17/+24). MRFs bound to these elements have different quaternary structures. Heterodimers of MRF and E-protein is known to generally bind to E-box region, whereas the novel *cis*-element of ACCβ promoter has strong affinity to homodimers of MRFs and its MRF-binding is suppressed by heterodimer formation with E-protein (Lee *et al.*, 2001) To exclude the effects of other several transcription factors bound to ACCβ promoter and to check the effects of retinoid receptors on the activities of MRFs containing the different quaternary structures, the transient transfection assays were performed using the heterologous promoters containing E-box region of ACCβ or six copies of novel *cis*-element at upstream of tk minimal promoter. These attempts showed that RXRα enhances MRF4 action on E-box regions but not on noble *cis*-element. RARα suppresses the activities of MyoD acting on noble *cis*-element but not on E-box regions (Figure 4). These data suggest that RXRα preferentially enhances the activities of heterodimer of MRF4 and E-protein, and RARα is likely to inhibit the activities of MyoD homodimer.

In summary, the data in present study conclude that RARα suppressed the transcriptional activities of MyoD homodimer in myoblast, and RXRα elevated the actions of MRF4-E-protein heterodimer in terminally differentiated myotubes.

Acknowledgement

This work was supported by Korea Reasearch Foundation Grant (KRF-2001-015-FS0003).

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