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Ankrd1 is a transcriptional repressor for the androgen receptor that is downregulated by testosterone

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ABSTRACT

The ankryn repeat domain proteins, Ankrd1 and Ankrd2, are expressed at the highest levels in skeletal muscle and heart where they are localized to the I band of the sarcomere through binding to titin and myopaladin. Ankrd1 and Ankrd2 migrate from the sarcomere to the nucleus when muscle is stressed, and act as coregulators for a growing number of transcription factors. Expression of Ankrd1 is altered by castration suggesting a link to androgen action. This investigation explored the effects of testosterone on Ankrd1 and Ankrd2 expression and determined whether Ankrd1 or Ankrd2 binds to or regulates the transcriptional activity of the androgen receptor (AR). Incubation of rat L6 myoblasts expressing the human AR (L6.AR) with testosterone reduced mRNA levels for Ankrd1 by approximately 50% and increased those for Ankrd2 by 20-fold. In reporter gene assays conducted with CHO cells co-transfected with an ARE-Luc reporter gene, Ankrd1 blocked the ability of testosterone to increase reporter gene activity while Ankrd2 had no effect. The effect of Ankrd1 and Ankrd2 on repression of the MAFbx promoter by testosterone was also tested in C2C12 cells using an MAFbx-Luc reporter gene (pMAF400-Luc); Ankrd1 blocked repression of pMAF400-Luc by testosterone while Ankrd2 did not. Co-immunoprecipitation studies revealed that Ankrd1 bound to the AR whereas Ankrd2 did not. The effect of Ankrd1 or Ankrd2 on changes in gene expression induced by testosterone in L6.AR cells was also evaluated. Incubation of L6.AR cells with testosterone modestly reduced myogenin mRNA levels but did not significantly alter those for mdm2, MEF2d, TnnI1, TnnI2, or p21. When cells were transfected with Ankrd1, testosterone markedly reduced mRNA levels for MEF2d, myogenin, p21 and TnnI1, increased those for TnnI2, but did not alter those for mdm2. When cells were transfected with Ankrd2, testosterone increased MEF2d and myogenin mRNA levels, having the opposite effect to cells transfected with Ankrd1; Ankrd2 did not change the effects of testosterone on TnnI1, TnnI2, p21, or mdm2 mRNA levels. In conclusion, testosterone regulates the expression of Ankrd1 and Ankrd2; Ankrd1 binds to and directly regulates the transcriptional activity of the AR whereas Ankrd2 does not; expression levels of both Ankrd1 and Ankrd2 modulate effects of testosterone on gene expression in cultured myoblasts.

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1. Introduction

Ankryn repeat domain 1 (Ankrd1, also known as CARP) is one of three muscle ankryn repeat proteins (MARPs). The MARPS are a family of proteins that also includes Ankrd2 (also known as ARRP) and diabetes-associated ankryn repeat protein (DARP). Ankrd1 in is found in the nucleus in myoblasts, and is redistributed with myogenic differentiation to the cytoplasm [1] where, in skeletal and cardiac muscle, it and other MARPs bind preferentially to titin at the I band of the sarcomere [2]. MARPs have been proposed to transmit signals from the sarcomere to the nucleus, particularly in response to muscle stress; for example, Ankrd2 has been shown to migrate from myofibrils to the nucleus of myofibers after muscle injury by injection of cardiotoxin [3]. Although MARPs have been most intensively studied for their role in skeletal and cardiac muscle function, they appear to serve important functions in skin, vasculature and other tissues. Ankrd1 was first identified in endothelial cells [4] but subsequently also identified in skeletal muscle, heart, ovarian cancers [5], renal podocytes [6], healing wounds [7], and mouse mammary epithelial cells [8]. Expression of Anrd1 is highest in heart under normal physiological conditions [9], while that of Ankrd2 is greatest in skeletal muscle [9]. Ankrd1 expression is elevated in skeletal muscle by paralysis such as occurs after nerve transection [10]. Ankrd2 expression has been reported to be elevated in denervated skeletal muscle [11] and stretched skeletal muscle [12].

MARPs are not essential for life, and appear to serve functions in muscle that are at least partially redundant [13]; instead, MARPs

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appear to fine tune muscle properties. MARPs are thought to transmit signals from the sarcomere to the nucleus where they modulate gene expression. Ankrd1 downregulates gene expression in the heart [14] and binds to and regulates the transcriptional activity of p53 [15]. Silencing of Ankrd2 altered the expression of genes for multiple pathways that included TGF-ß, Wnt signaling and p53 [16]. Ankrd2 has also been shown to bind proteins with PDZ and SH3 domains, and multiple transcription factors including YB-1, p53, PAX6, LHX2, NHIL3 and MECP2 [16,17].

Expression levels of Ankrd1 were recently reported to be affected by castration in bulls [18] suggesting that this gene was regulated by testosterone thereby raising the possibility that Ankrd1 might bind to and modify transcriptional regulation by the receptor for testosterone, the androgen receptor (AR). Classically, the AR binds DNA at androgen response elements leading to transcriptional activation of target genes. Transcriptional repression by the AR has also been reported through tethering to other transcription factors, such as Oct1 when it is bound to the promoter of the muscle atrophy F-Box (MAFbx) gene [19]. The goals of the present study were to determine whether testosterone regulated expression of Ankrd1 or Ankrd2, if these proteins bound the AR, and whether they modified transcriptional activity of the AR.

2. Materials and methods

2.1. Reagents and plasmids

Testosterone powder was from Paddock Laboratories Inc. (Minneapolis, MN). A pCMV-Sport 6 expression vector encoding rat Ankrd1 was obtained from the Harvard Cancer Center DNA Resource (Plasmid ID MmCD00317032). A plasmid expressing mouse Ankrd2 was obtained from OriGene Technologies (Rockville, MD, Cat# MC210080). The plasmid pREP4-hAR expressing full length human AR and pCMV.Sport.ß-gal, expressing ß-galactosidase under a CMV promoter were as described previously [20]. MAF400-luc is an MAFbox luciferase expression vector described in prior studies [19]. The plasmid pTk-Renilla expresses renilla luciferase and was from Promega (Madison, WI). pARE-Luc expresses firefly under the control of an mouse mammary tumor virus androgen response element (ARE) as described [21].

2.2. Cell lines

CHO-K1 cells were obtained from American Tissue Culture Collection (ATCC, Manassas, VA) and were maintained in F12K medium with 10% FBS and 1% penicillin/streptomycin solution (final concentrations 100 U/ml and 100 μg/ml, respectively). L6 rat myoblasts were from ATCC; L6.AR myoblast cells stably express the human AR (L6.AR cells) under a retroviral transgene and have been described elsewhere [22]. L6 and L6.AR cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution (growth media). To initiate differentiation, media was replaced with DMEM supplemented with 1% penicillin/streptomycin solution and either 2% horse serum (HS, differentiation media) or 2% charcoal-dextran stripped horse serum (CDS-HS, CDS-differentiation media). C2C12 cells were obtained from ATCC and were maintained as described for L6.AR cells.

2.3. Effects of Ankrd1 or Ankrd2 on reporter gene activities

For studies with pARE-Luc, CHO cells were seeded into 12-well plates (3×10^4 cells per well) and incubated overnight. Cells were transfected with pREP4-hAR, pTk-Renilla, pARE-Luc, and vectors expressing one of the following: ß-gal, Ankrd1 or Ankrd2. Transfection was accomplished using Lipofectamine 2000 Reagent (Life Technologies, Grand Island, NY) and the manufacturers recommended procedures. After 4 h, fresh media was added as above, except that charcoal-dextran-stripped FBS was used. Media was supplemented with testosterone or vehicle (ethanol) and incubations were continued for 20 h. Luciferase activity was determined using the Dual Luciferase Reporter Assay (Promega) following the manufacturers recommended procedures.

In experiments with pMAF400-Luc, C2C12 cells were seeded into wells and transfected as above except that pMAF400-Luc was included rather than pARE-Luc. After incubation with the transfection mixture for 4 h, this media was removed and replaced with differentiation media and incubation was continued for 48 h. Media was removed and replaced with fresh differentiation media containing CDS-HS and supplemented with testosterone or ethanol. After incubation overnight, luciferase activities were determined.

2.4. Binding of AR and Ankrd1 or Ankrd2

CHO cells were seeded into 6 well plates and incubated overnight then transfected with pREP4-hAR and a vector encoding either Ankrd1 or Ankrd2. After 4 h incubation with the transfection mixture, the media was removed and replaced with fresh media followed by overnight incubation. Cells were lysed with 1X RIPA Lysis Buffer (Cell Signaling Technology, Inc., Danvers, MA). Lysates were cleared by centrifugation at 13,000 RPM in a microcentrifuge at 4 °C. Protein concentrations were determined with the Biorad protein assay using bovine serum albumin as a standard. Aliquots of lysates containing 500 μg of protein were incubated with primary antibody (anti-androgen receptor (N-20): sc-816, Santa Cruz Biotechnology, Inc., Dallas, TX) overnight at 4 °C. The samples were then mixed with Protein A-agarose beads (Millipore, Billerica, MA) for 4 h at 4 °C. After washing beads three times with PBS, proteins wereeluted with SDS–PAGE sample buffer (BioRad, Hercules, CA).

For Western blotting, samples were denatured by incubation in boiling water in SDS–PAGE sample buffer containing 2-mercaptoethanol, subjected to SDS–PAGE, and transferred to PVDF membranes. After blocking with blocking buffer (5% bovine serum albumin in Tris-buffererd saline containing 0.1% Tween 20 [TTBS]), membranes were probed with anti-AR (1:400, Cat# N20:sc816 Santa Cruz), anti-Ankrd1 (1:500; Cat# H-120:sc30181 Santa Cruz), Anti-Ankrd2 (1: 500; Cat# A-14:sc138110 Santa Cruz) or anti-ß-tubulin (Cat# ab6046 Abcam, Inc., Cambridge, MA) in TTBS supplemented with 2.5% bovine serum albumin, washed with TTBS and probed with horseradish peroxidase conjugated secondary antibodies. Immunostaining was visualized by enhanced chemiluminescence and captured on photographic film.

2.5. Effects of Ankrd1 or Ankrd2 on testosterone-induced gene expression changes

L6.AR cells (3×10^4/well) were seeded into 24 well plates and incubated overnight with growth medium then transfected with vectors expressing Ankrd1 or Ankrd2. After 4 h incubation with DNA-Lipofectamine complexes media was removed and replaced with differentiation media and cells were incubated an additional 48 h. Media was removed and fresh CDS-differentiation media was added and supplemented with either testosterone or vehicle. After overnight incubation RNA was extracted for qPCR.

2.6. Real-time PCR for mRNA expression

mRNA was extracted using RNeasy mini Kits and further enriched and treated with DNAse I to digest genomic DNA using the RNeasy miniElute Cleanup Kit (QIAGEN). RNA quantity and quality were measured using the Agilent RNA 6000 nano kit in

an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Synthesis of cDNA libraries and real time PCR were performed as described [22] using probes were from Applied Biosystems Assays on Demand inventories (Life Technologies, Inc.) as follows: Ankrd1 (Rn00566329), Ankrd2 (Rn01410782), MEF2d (Rn00578329), Myogenin (Rn00567481), Mdm2 (Rn01502814), p21 (Rn00589996), TnnI1 (Rn00567843), TnnI2 (Rn00437157).

3. Statistics

Data are expressed as means ± SEM. The significance of differences between pairs of means was determined with a unpaired Student's *t*-test. Statistical calculations were performed with Prism 6.0 (GraphPad).

4. Results

The possibility that testosterone regulated expression of Ankrd1 or Ankrd2 was explored in rat L6 myoblasts or L6 cells expressing the hAR (L6.AR cells). At concentrations of 1 or 50 nM, testosterone reduced mRNA levels for Ankrd1 in both cells types, but significantly more so in L6.AR cells (Fig. 1A). Testosterone increased mRNA levels for Ankrd2 in L6.AR but not L6 cells (Fig. 1B). To test whether these effects on mRNA levels involved binding of hormone to the AR, several additional experiments were performed. We next evaluated the effects of the non-aromatizable androgen (DHT) on Ankrd1 and Ankrd2 mRNA levels in L6 and L6.AR cells. There was no significant change in levels of mRNA for either gene in L6 cells treated with DHT, whereas in L6.AR cells DHT reduced Ankrd1 levels but increased Ankrd2 levels (Fig. 1C and D). We next tested whether the androgen receptor antagonist bicalutamide reduced these actions of DHT. Bicalutamide reduced the effects of DHT on mRNA levels for both Ankrd1 and Ankrd2 but did not completely block DHT actions. These results indicate that signaling through the AR reduces expression levels of Ankrd1 and increases those for Ankrd2.

To test whether Ankrd1 altered transcriptional activity of the AR, CHO cells were co-transfected with plasmids expressing the AR and either Ankrd1, Ankrd2 or ß-galactosidase as well as one of two reporter genes: pARE-Luc or pMAF400-Luc. pARE-Luc contains well characterize androgen response elements from the MMTV promoter and is upregulated by binding of the AR whereas pMAF400-Luc is repressed by the AR in mouse C2C12 myoblasts [19]. Ankrd1 blocked transcriptional activation of pARE-Luc by testosterone in CHO cells while ß-gal or Ankrd2 did not (Fig. 2A). Similarly, whereas the expected repression of the pMAF400-Luc reporter was observed in cells expressing ß–gal or Ankrd2 and incubated with Ts, Ts did not repress pMAF400-Luc reporter activity in cells expressing Ankrd1 (Fig. 2B). Western blotting of cell lysates confirmed that transfection greatly increased expression levels of both Ankrd1 and Ankrd2 in CHO and C2C12 cells

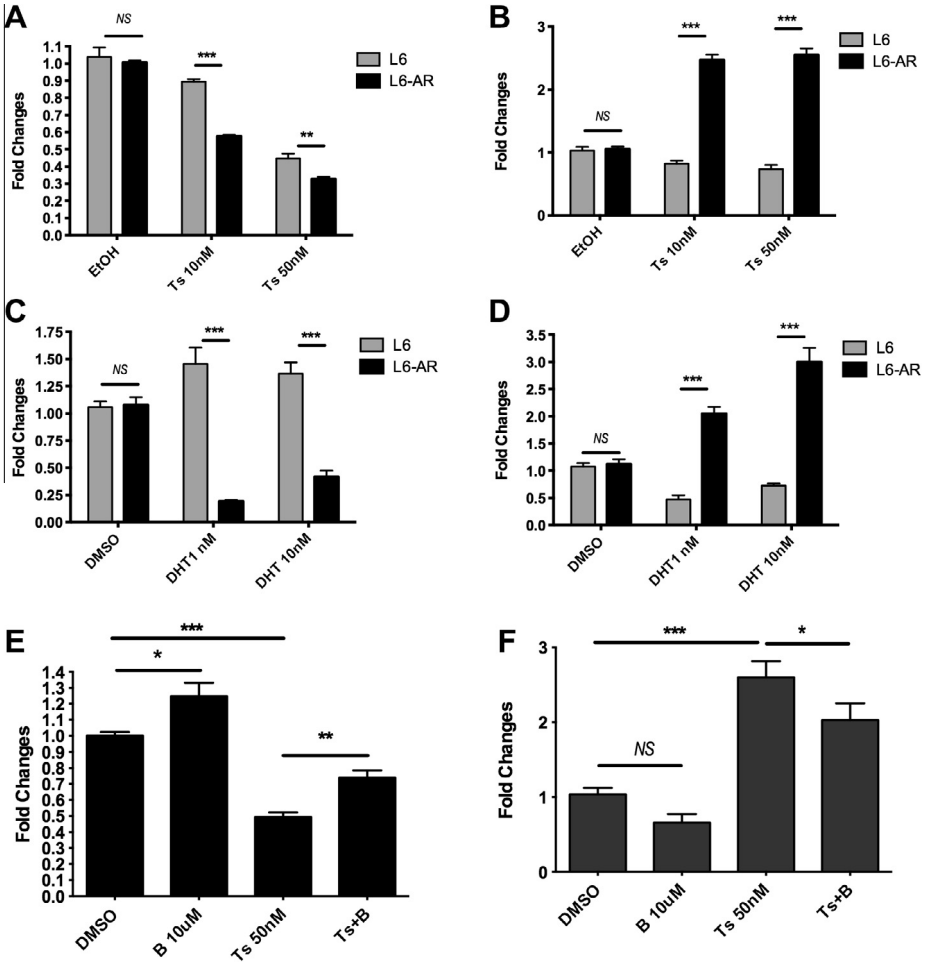

Fig. 1. Androgens regulate levels of Ankrd1 and Ankrd2 mRNA. C2C2 cells were incubated in differentiating medium for 48 h after which fresh medium supplemented with CDS-HS was added and supplemented with androgens (Ts, testosterone; DHT, dihydrotestosterone) or vehicle as indicated. After incubation overnight mRNA levels for Ankrd1 (panels A, C and E) or Ankrd2 (panels B, D and F) were determined by real time PCR. Data are expressed as means ± SEM for 6 separate determinations from two separate experiments. mRNA levels are expressed as fold-change relative to vehicle treated cells. NS, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. $N = 6–9$.

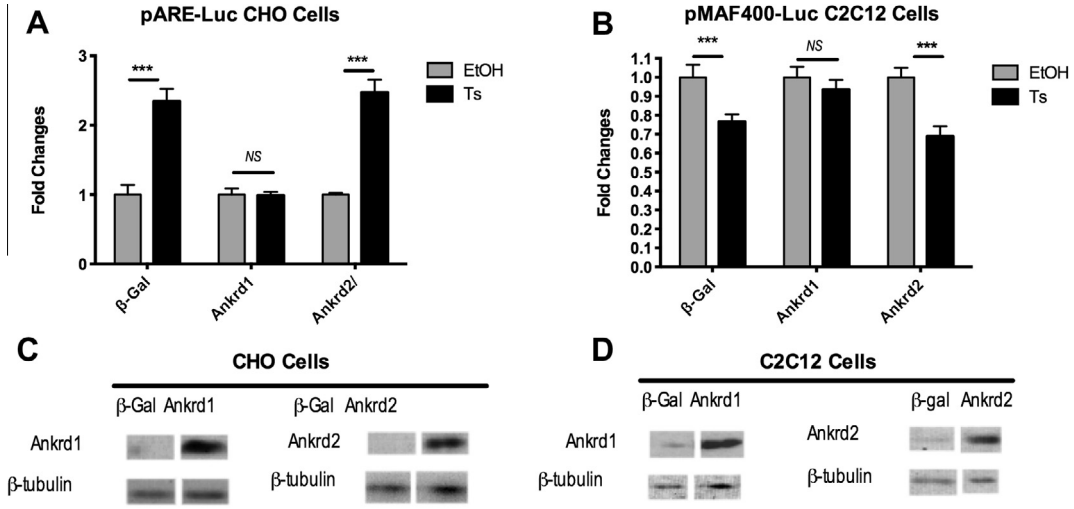

Fig. 2. Ankrd1 but not Ankrd2 reduces AR transcriptional activity. (A) CHO cells were transfected with pREP4.AR, pMAF400, and either ß-gal, Ankrd1, or Ankrd2 for 4 h. Complete medium was added with/without testosterone (Ts, 100 nM) or ethanol (EtOH). Renilla activity in cell lysates was determined 20 h later. (B) C2C12 cells were co-transfected with pREP4.AR, pMAF400, and either ß-gal, Ankrd1, or Ankrd2 for 4 h. Media was replaced with that containing 2% HS and incubations were continued for 72 h. Media was replaced with that containing 2% CDS-HS with either Ts (100 nM) or EtOH. Incubations were continued overnight at which time luciferase activity was determined. Data are expressed as mean values ± SEM; $N = 8$. (C and D) Western blotting for the indicated proteins of lysates from cells transfected as in A and B.

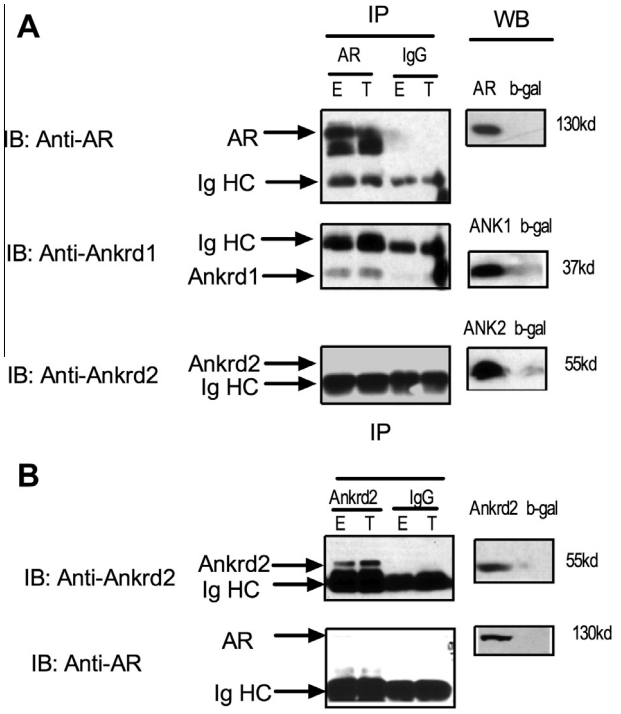

Fig. 3. The AR binds Ankrd1 but not Ankrd2. (A) Lysates from CHO cells transfected with vectors expressing the AR and either Ankrd1 or Ankrd2 were subjected to immunoprecipitation with antibodies against AR followed by Western blotting as indicated. (B) As in panel A, except that immunoprecipitation was performed using antibodies against Ankrd2. Images are representative of Western blots for two separate experiments.

(Fig. 2C and D). We next tested whether Ankrd1 or Ankrd2 bound to the AR in CHO cells. In pull-downs of protein complexes formed with the AR using anti-AR antibodies, Western blots confirmed the presence in immunoprecipitated proteins of the AR and Ankrd1, but not Ankrd2 (Fig. 3A). Similarly, when pull-downs were performed with antibodies against Ankrd2, no AR was detected in the immunoprecipitated protein by Western blotting (Fig. 3B). Thus, the AR associates with Ankrd1 but not Ankrd2, and Ankrd1 reduces transcriptional regulation by the AR.

To test whether Ankrd1 or Ankrd2 alter gene expression responses elicited by testosterone on L6.AR myoblasts, mRNA levels for genes related to myogenesis (myogenin, MEF2, p21), myofibrils (TnnI1, TnnI2), and a ubiquitin ligase (mdm2) [23] were examined in L6.AR cells transfected with ß-gal, Ankrd1, or Ankrd2 then incubated with or without testosterone. Incubation of L6.AR cells with testosterone modestly reduced myogenin mRNA levels but did not significantly alter mdm2, MEF2d, TnnI1, TnnI2 or p21 mRNA levels (Fig. 4). When cells were transfected with Ankrd1, testosterone markedly reduced MEF2d, myogenin, p21 and TnnI1, increased TnnI2, and did not alter mdm2 mRNA levels (Fig. 4). When cells were transfected with Ankrd2, testosterone increased MEF2d and myogenin mRNA levels, having the opposite effect to cells transfected with Ankrd1. Ankrd2 did not change effects of testosterone on TnnI1, TnnI2, p21, or mdm2 mRNA levels (see Fig. 4).

5. Discussion

Our data indicate that Ankrd1, but not Ankrd2, binds the AR and represses its transcriptional activity in reporter gene assays. Specifically, Ankrd1 blocks both activation of transcription by the AR at an androgen response element in pARE-Luc and repression of transcription by the AR in pMAF400-Luc, which occurs through tethering of the AR to Oct1 [19]. Thus, Ankrd1 appears to be a transcriptional repressor for the AR, adding this nuclear hormone receptor to the list of transcriptional regulators with which Ankrd1 interacts in its role of transmitting signals from the contractile apparatus to the nucleus. Ankrd2 did not bind the AR or alter testosterone-induced changes in reporter gene activity indicating that it is not a direct transcriptional effector for the AR. Other transcription factors known to interact with Ankrd1 include p53 and YB-1 [15,17]. Ankrd2 binds proteins with PDZ and SH3 domains, and a growing list of transcription factors that includes YB-1, p53, PAX6, LHX2, NHIL3 and MECP2 [16,17]. Thus, Ankrd1 and Ankrd2 bind distinct but overlapping groups of transcription factors. An interesting though unanswered question is what structural features of Ankrd1 and Ankrd2 explain this difference. Another open question is what other receptors in the steroid hormone receptor family bind Ankrd1.

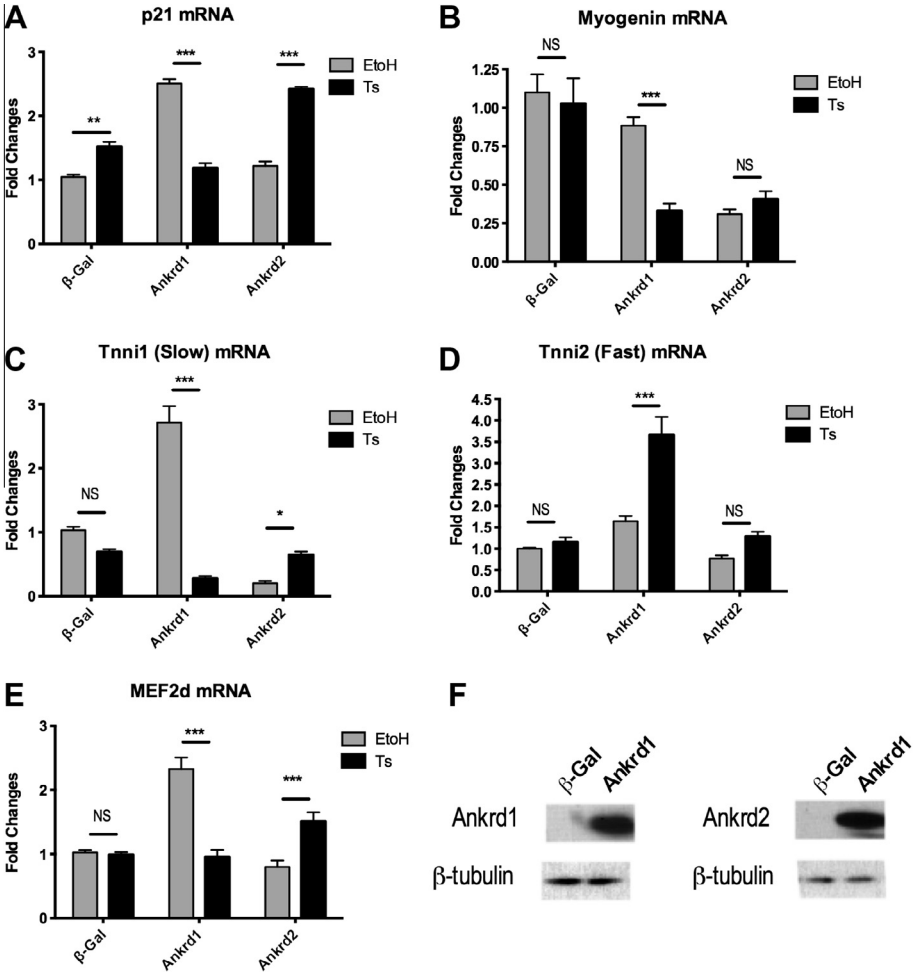

Fig. 4. Effects of Ankrd1 and Ankrd2 on gene expression in the presence or absence of testosterone (Ts). (A–E) L6.AR cells were transfected with plasmids expressing the indicated proteins and differentiated then incubated with testosterone (Ts) or ethanol (EtOH) as in Fig. 2. mRNA levels were determined by qPCR and expressed relative to cells transfected with a vector expressing ß-gal and incubated with EtOH. (F) Western blotting of cell lysates from L6.AR cells transfected with vectors expressing ß-Gal, Ankrd1 or Ankrd2.

Our results demonstrated that both Ankrd1 and Ankrd2 altered the gene expression changes stimulated by testosterone in cultured L6.AR myoblasts. The effects of Ankrd2 on testosterone-regulated gene expression are presumably indirect because this protein did not bind the AR or modify testosterone responses of androgen-responsive reporter genes. By contrast, at least some of the effects of Ankrd1 on gene expression changes elicited by testosterone are likely to be due to direct interactions of Ankrd1 with the AR although indirect interactions are not excluded by our findings. The genes affected and direction of change are distinct forAnkrd1 and Ankrd2, supporting the conclusion that Ankrd1 and Ankrd2 have unique and perhaps complimentary roles in specifying the effects of androgens on skeletal muscle.

Variations in the levels of Ankrd1 and Ankrd2 may fine-tune the effect of androgens on gene expression in health and disease. Expression levels of Ankrd1 and Ankrd2 are modulated in skeletal muscle by multiple stimuli and stresses. Ankrd1 expression is elevated in skeletal muscle by nerve transection, amyotropic lateral sclerosis [24], spinal muscular atrophy [25], and muscular dystrophies [25,26]. Ankrd2 expression has been reported to be elevated in denervated or stretched skeletal muscle [11,12]. Changes in Anrd1 and Ankrd2 mRNA expression levels are associated with differences in effects of androgens on gene expression in denervated gastrocnemius muscle across time after denervation [27,28]. For example, microarray analysis has shown that gene expression changes observed in denervated gastrocnemius muscle induced by treatment of animals with nandrolone, a synthetic ligand for the AR, are very different at 7 versus 35 days after nerve transection [27]. We have proposed that one or more transcriptional effectors are greatly up or downregulated after denervation and that expression levels of the effectors change across time after denervation [27]. It may be that the changes over time in the increased levels of Ankrd1 and Ankrd2 observed after denervation [10,11] explain in part the large differences in gene expression changes upon initiating administration of nandrolone at different times after denervation. Preliminary studies of the expression of Ankrd1 and Ankrd2 in denervated skeletal muscle at times between 7 and 56 days support this possibility (C Ruggiero, unpublished observations).

It has been reported that Ankrd1 expression levels differ between bulls and steers [18]. Our findings further support the conclusion that Ankrd1 expression is regulated by testosterone and suggest that testosterone also regulates the expression of Ankrd2. An interesting question that has not been explored is whether there is sexual dimorphism in the expression levels of Ankrd1 and Ankrd2 in various tissues.

Acknowledgments

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