hMusTRD1α1 Represses MEF2 Activation of the Troponin I Slow Enhancer

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The novel transcription factor hMusTRD1α1 (human muscle TFI-I repeat domain-containing protein 1α1; previously named MusTRD1; O’Mahoney, J. V., Guven, K. L., Lin, J., Joya, J. E., Robinson, C. S., Wade, R. P., and Hardeman, E. C. (1998) Mol. Cell. Biol. 18, 6641–6652) was identified in a yeast one-hybrid screen as a protein that binds within an upstream enhancer-containing region of the skeletal muscle-specific gene, TNNI1 (human troponin I slow; hTnIslow). It has been proposed that hMusTRD1α1 may play an important role in fiber-specific muscle gene expression by virtue of its ability to bind to an Inr-like element (nucleotides -977 to -960) within the hTnIslow upstream enhancer-containing region that is necessary for slow fiber-specific expression. In this study we demonstrate that both MEF2C, a known regulator of slow fiber-specific genes, and hMusTRD1α1 regulate hTnIslow through the Inr-like element. Co-transfection assays in C2C12 cells and Cos-7 cells demonstrate that hMusTRD1α1 represses hTnIslow transcription and prevents MEF2C-mediated activation of hTnIslow transcription. Gel shift analysis shows that hMusTRD1α1 can abrogate MEF2C binding to its cognate site in the hTnIslow enhancer. Glutathione S-transferase pull-down assays demonstrate that hMusTRD1α1 can interact with both MEF2C and the nuclear receptor co-repressor. The data support the role of hMusTRD1α1 as a repressor of slow fiber-specific transcription through mechanisms involving direct interactions with MEF2C and the nuclear receptor co-repressor.

Skeletal muscle formation and maturation requires the coordinated expression of genes resulting in the diversification of skeletal muscle cells into either slow or fast twitch myofibers (2–4). The establishment of fiber types is due in part to properties intrinsic to myoblasts (5, 6) and to the responsiveness of the myofiber to physiological cues such as functional innervation and biomechanical load (7–10). The molecular mechanisms that dictate these functionally specialized myofiber types at a transcriptional level are less well understood (11–13). Deletion mapping of fast and slow contractile protein genes has yielded information on potential factors that might be involved in fiber-specific expression (14–18). Recently, diverse signaling pathways and transcription factors have been described that influence slow myofiber type specification, including the calcium/calmodulin-dependent phosphatase calcineurin, the Ras mitogen-activated protein kinase pathway, and the transcriptional co-activator peroxisome proliferator-activated receptor-γ co-activator-1 (PGC-1α) (19, 30). Members of the MEF2 gene family are key regulatory factors common to two of these pathways. Paradigms that promote slow fiber specification in mice such as endurance activity and low frequency tonic pacing of the motoneuron result in an increase in MEF2 levels and the induction of a MEF2-responsiv transgene (31, 32). This occurs concomitant with the induction of slow/oxidative marker genes such as troponin I slow and myoglobin that have been shown to be regulated by MEF2. It is proposed that this regulation occurs through the calcineurin-mediated hypophosphorylation of MEF2 that enhances the function of the MEF2 transcriptional activation domain. Recently, forced expression of PGC-1α in fast fiber muscles in mice was shown to be capable of eliciting morphological features and gene expression patterns characteristic of slow fibers (30). It is possible that this regulatory capability involves calcineurin and MEF2 because both factors potentiate the PGC-1α-mediated activation of the slow fiber-specific promoters of troponin I slow (TnIslow) and myoglobin.

TnIslow has been used as a model gene to study mechanisms of slow fiber-specific expression. TnIslow is the major isoform of the TnI gene family expressed in all newly formed fibers but is down-regulated in future fast and up-regulated in future slow myofibers during fetal development (33, 34). The responsiveness of TnIslow to physiological cues that elicit slow fiber gene expression such as innervation and biomechanical load is well documented (7, 9, 35, 36). The regions of the human and rat TnIslow genes that direct slow fiber-specific expression have been defined using somatic cell injection and transgenic analysis (1, 14, 16, 36). These analyses defined a 157-bp upstream enhancer (USE1) region of the human and a similar 128-bp region of the rat TnIslow gene (14, 37) that are critical in directing slow myofiber-specific expression. Sequence motif and electrophoretic mobility shift assay (EMSA) analysis of the 157-bp hTnIslow USE identified potential transcription factor-binding sites within this region including a MEF2 consensus element and USE B1 that contains an initiator of transcription or Inr-like element (1). The Inr-like element was shown to be

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The abbreviations used are: USE, upstream enhancer (of the TnIslow gene); aa, amino acid(s); DBD, DNA-binding domain; NCoR, nuclear receptor co-activator; TF, transcription factor; TnIslow, troponin I slow; GST, glutathione S-transferase; PGC-1α, peroxisome proliferator-activated receptor-γ co-activator-1; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; HDAC, histone deacetylase.
essential for high level, slow fiber-specific expression. In addition, transgenic analysis has demonstrated that the MEPF2-binding site contributes to expression in the soleus muscle (31). Using the yeast one-hybrid assay, a novel 944-aa DNA-binding protein, MusTRD1, was isolated that binds to the Inr-like element within the USE B1 region (1). Mutation of the Inr-like core sequence (GATTAA) within the USE-B1 region (nucleotides -977 to -960) to USE-B1b (GATAc) abrogated both slow fiber-specific expression from the USE and DNA binding by MusTRD1. MusTRD1 contains five repeat domains that share ~70% homology to the six repeat domains of the Inr-like binding protein TFI-I (38). Two MusTRD1 homologues, GTF3 and CREM (consisting of 959 aa), from humans and three mouse orthologues, BEN 1, 2, and 3, have been isolated (39–43). We have discovered multiple MusTRD isoforms in mice that arise from alternative splicing of a single gene, Gtf2ird1, on mouse chromosome 5 (44). In keeping with the nomenclature that we have devised for the mouse isoforms, we have changed the name of human MusTRD1 to hMusTRD1 (human muscle TFI-I repeat domain-containing protein 1α). The name of the isoform is based on the peptide sequence. The first number indicates the unique combination of C-terminal repeat domains present. Isoforms contain 1 of 2 possible C-terminal exons that are designated “α” (exon 31) or “β” (exon 30). The final number indicates which of the seven possible specific combinations of unique C-terminal exons is present.

In this study, we show that hMusTRD1α acts as a repressor of hTnlslow transcription and that repression occurs through the hTnlslow USE. We show that MEPF2 (myocyte enhancer factor 2C) transcriptional activation via the USE is dependent upon an intact Inr-like element and is blocked by hMusTRD1α. hMusTRD1α binds to the Inr-like element via two DNA-binding domains (DBDs) located between aa 351 and 458 and aa 544 and 944, respectively. Transient transfection analysis of a deletion series of hMusTRD1α revealed that repression can occur in the absence of DNA binding. hMusTα1α is a potent repressor by itself, and GST pull-down analysis revealed that it can interact directly with nuclear receptor corepressor (NCOR). This report demonstrates that hMusTRD1α interacts directly with MEPF2 and can prevent MEPF2 from binding to its cognate binding site. The data support a role for hMusTRD1α as a repressor of slow fiber-specific genes by preventing MEPF2-mediated transcriptional activation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The hTnlslow USEB1tk luc and hTnlslow USEB1btk luc reporter constructs contain the human Tnlslow USE (−1031 to −874) and a mutated B1b version (1) linked to the thymidine kinase promoter. The B1btk and B1btk luc reporter constructs contain three copies of the B1 elements located between aa 351 and 458 and aa 544 and 944, respectively. Transient transfection analysis of a deletion series of hMusTRD1α revealed that repression can occur in the absence of DNA binding. hMusTα1α is a potent repressor by itself, and GST pull-down analysis revealed that it can interact directly with nuclear receptor corepressor (NCOR). This report demonstrates that hMusTRD1α interacts directly with MEPF2 and can prevent MEPF2 from binding to its cognate binding site. The data support a role for hMusTRD1α as a repressor of slow fiber-specific genes by preventing MEPF2-mediated transcriptional activation.

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GST or GST fusion proteins with in vitro translated 35S-labeled hMusTRD1/H92511350aa or MEF2C initially for 30 min at 30 °C and for a further 40 min at room temperature in PPI buffer (20 mM Hepes, pH 7.9, 200 mM KCl, 1 mM EDTA, 4 mM MgCl2, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 10% glycerol). Bound proteins were resolved by 10% SDS-PAGE. The gels were dried and exposed to a phosphorimaging screen, and the bands were quantified using a Molecular Dynamics Storm 860 reader and ImageQuant software (Molecular Dynamics).

RESULTS

MEF2C Activates and hMusTRD1a1 Represses Transcription through the hTnIslow USE—The transcriptional regulation of the hTnIslow USE via MEF2C and hMusTRD1a1 was investigated initially in C2C12 muscle cultures. Expression vectors encoding hMusTRD1a1 or MEF2C were co-transfected into C2C12 cells with luciferase reporter gene constructs driven by the hTnIslow USE (Fig. 1). MEF2C elicited an approximate 2-fold increase in hTnIslow USE transcriptional activity in differentiated C2C12 cultures (Fig. 1B, left panel). In contrast, hMusTRD1a1 repressed the basal activity mediated by the hTnIslow USE by ~2-fold. To assess the transcriptional effects of hMusTRD1a1 on MEF2C-mediated transactivation, hMusTRD1a1 and MEF2C expression constructs were co-transfected together with the hTnIslow USE. hMusTRD1a1 repressed the MEF2C-mediated transcriptional activation of the hTnIslow USE by ~3-fold. The specific involvement of the Inr-like element in this regulation was examined using USE-B1b that contains a mutation in this element that prevents hMusTRD1/H92511 binding (1) (Fig. 1A). There is a 36-fold reduction in activity upon mutation of the GATTAA core sequence demonstrating that basal transcriptional activity is mediated by the B1 region (Fig. 1B). Furthermore, mutation of this core sequence resulted in a 68-fold reduction of MEF2C-mediated induction of the hTnIslow USE, demonstrating that this site is necessary for MEF2C-mediated transcriptional activation. These results also suggest that an unidentified factor binding

FIG. 1. hMusTRD1a1 represses MEF2C activation through the B1 region in the hTnIslow USE. Transient co-transfections of C2C12 cells were performed using expression constructs for hMusTRD1a1 (1α1g44aa) or MEF2C and (A) reporter constructs containing the intact hTnIslow USE or mutated USE B1b site linked to luciferase (B, C2C12). Transient co-transfections of Cos-7 cells were performed using the expression construct for hMusTRD1a1 (1α1g44aa) and (A) reporter constructs containing the trimerized B1 or B1b regions linked to luciferase (B, Cos-7). Activation or repression is expressed as fold induction of basal (empty) expression vector activity that was set at 1. The columns represent the mean values of triplicates; the bars indicate S.E. C, reverse transcription-PCR was performed on total RNA isolated from undifferentiated C2C12 myoblasts (Mb), differentiated C2C12 myotubes (Mt), Cos-7 cells and embryonic day 13.5 mouse hindlimbs (ED 13.5). PCR was performed on 5 ng of pcDNA3.1hMusTRD1α1g44myc/his (pcDNA) as a positive control.

GST or GST fusion proteins with in vitro translated 35S-labeled hMusTRD1α1g44aa or MEF2C initially for 30 min at 30 °C and for a further 40 min at room temperature in PPI buffer (20 mM Hepes, pH 7.9, 200 mM KCl, 1 mM EDTA, 4 mM MgCl2, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 10% glycerol). Bound proteins were resolved by 10% SDS-PAGE. The gels were dried and exposed to a phosphorimaging screen, and the bands were quantified using a Molecular Dynamics Storm 860 reader and ImageQuant software (Molecular Dynamics).
to B1 is needed for basal activity and activation mediated by MEF2C and that hMusTRD1α1 competes with the same DNA site to mediate repression.

hMusTRD1α1-mediated repression of the USE could be due to an intrinsic property of the protein. Alternatively, it could repress by modifying other myogenic factors present in C2C12 cells, or it could recruit other MusTRD isoforms because these cells express a number of MusTRD isoforms, including the mouse orthologue of hMusTRD1α1 (44) (Fig. 1C). To discriminate these possibilities, expression studies were conducted in Cos-7 cells that do not express myogenic regulatory factors and express negligible amounts of MusTRD transcripts. In addition, the B1 region of the USE was used so that the Inr-like element was present but not the MEF2-binding site. The B1 region was trimerized and linked to a heterologous promoter driving luciferase to achieve a sufficient level of expression in Cos-7 cells (Fig. 1A). hMusTRD1α1 was co-transfected together with a construct bearing either the B1 or B1b region. hMusTRD1α1 repressed basal transcriptional activity by ~3-fold (Fig. 1B). Mutation of the core GATTAA sequence in the B1b version resulted in a 4-fold loss of basal transcriptional activity in Cos-7 cells. These data demonstrate that hMusTRD1α1-mediated repression can occur in the absence of MEF2C and its binding site.

hMusTRD1α1 Contains Two DNA-binding Domains—To locate the region(s) of hMusTRD1α1 responsible for DNA binding, we examined the functional capabilities of a deletion series of hMusTRD1α1 cDNAs. As a first step in this process, we demonstrated that in vitro translated full-length hMusTRD1α1 (hMusTRD1α11,944aa) binds to the Inr-like element within the B1 region of the hTnI slow USE (Fig. 2A). Oligonucleotides containing either a trimerized B1 or B1b region were incubated with in vitro translated hMusTRD1α11,944aa protein. A protein-DNA complex formed when the B1 region was used as a probe but did not form with the mutated oligonucleotide. The presence of hMusTRD1α1 in the slowest migrating complex was demonstrated using an α-MusTRD1α11,204aa antibody directed against the first 20 aa of hMusTRD1α1. The specificity of the antibody was shown by Western immunoblotting (Fig. 2B). A supershift of the upper complex occurred in the presence of the antibody, demonstrating that it contains hMusTRD1α1 binding to the B1 region.

Truncated versions of hMusTRD1α1 were generated by progressive C-terminal deletion of putative regulatory regions as well as an N-terminally deleted peptide lacking repeat domains 1 and 2 (ΔN444aa) (Fig. 3A). PCR products were subcloned into the pcDNA3.1/myc/his expression vector for in vitro translation of proteins that were 350, 458, 564, 689, 786, and 500 aa in length (Fig. 3B). These proteins and plasmids were used in subsequent EMSA and transfection assays, respectively (Figs. 3C and 4). hMusTRD1α11,350aa containing repeat domain 1 only, was unable to bind the B1 region (Fig. 3C). In contrast, hMusTRD1α11,458aa clearly binds DNA, demonstrating that a DNA-binding domain (DBD1) is located in the N terminus between aa 351 and 458. The presence of a second DNA-binding domain (DBD2) between aa 544 and 944 was demonstrated by the binding of hMusTRD1α11,458aa to the B1 region most avidly as indicated by a band of greater intensity in comparison with the other deletions. This suggests that hMusTRD1α11,786aa may contain both DBDs and that DBD2 may exist in aa 544–786. The binding activities of hMusTRD1α11,458aa, hMusTRD1α11,564aa, hMusTRD1α11,786aa and hMusTRD1α11,944aa were lost upon mutation of the B1 region, further indicating that the GATTAA site is the core area for interaction for both DBD1 and DBD2. These data demonstrate that hMusTRD1α1 contains two DBDs.

hMusTRD1α1 Can Repress in the Absence of DNA Binding—To determine the functional significance of the two DBDs in hMusTRD1α1-mediated repression, the hMusTRD1α1 C-terminal truncation and N-terminal deleted constructs were co-transfected into Cos-7 cells along with a luciferase construct driven by the trimerized B1 region. All constructs repressed activity (Fig. 4). These data demonstrate that hMusTRD1α1-mediated repression is a dominant feature of this protein and that repression capability resides in at least one (aa 1–350) and possibly more regions of the protein (Fig. 3A). In addition, because hMusTRD1α11,458aa does not contain a DBD but can repress, repression capability does not rely on interaction of the protein with its cognate DNA-binding site.

Mammalian one-hybrid assays in Cos-7 cells were used to confirm that hMusTRD1α1 can repress in the absence of binding to its cognate DNA-binding site. Fusion proteins containing the DBD of GAL4 linked to hMusTRD1α11,944aa were constitutively expressed in Cos-7 cells in combination with a luciferase reporter gene construct driven by a trimerized GAL4 DNA-binding site (Fig. 5A). Reporter gene activity was determined and expressed as fold repression over basal GAL4 DBD activity (Fig. 5B). hMusTRD1α11,944aa was able to repress the basal activity by ~3.5-fold by utilizing the GAL4 DBD. The repressive capability of hMusTRD1α11,944aa was tested against the repression domains present in the N-terminal portion of the potent co-repressor NCOR (45). Repression was similar with both factors. Taken together, these results validate the co-transfection results shown in Fig. 4 and demonstrate that hMusTRD1α11,944aa can repress.
without binding to its cognate DNA-binding site and therefore must contain intrinsic repression capabilities. In addition, these results show that the repressive activity of hMusTRD1α1 is comparable with a known repressor molecule, NCoR, that functions without directly binding DNA.

**hMusTRD1α1 and NCoR Can Physically Interact in Vivo and in Vitro**—The ability of hMusTRD1α1 to interact directly with the common co-repressor NCoR was determined using co-immunoprecipitation assays. hMusTRD1α1(350aa) was used because it is the smallest peptide that can repress in the absence of DNA binding. An antibody that recognizes the C-terminal region of NCoR was used to successfully co-immunoprecipitate a protein complex containing full-length NCoR and hMusTRD1α1(350aa) from Cos-7 cells (Fig. 6A). GST pull-down assays were used to demonstrate in vitro interactions between 35S-labeled hMusTRD1α1(350aa) and the region of NCoR that contains the nuclear receptor interaction domains responsible for interaction with other proteins, GST-NCoR1648–2453 (45) (Fig. 6B). These protein-protein interaction data confirmed that hMusTRD1α1 can interact with NCoR via a mechanism that is independent of DNA binding. Taken together, these findings suggest that NCoR and hMusTRD1α1 could cooperate to mediate the transcriptional repression of the hTnIslow gene. hMusTRD1α1 Abrogates MEF2C Binding to the hTnIslow USE MEF2 Site through Direct Interaction—We have shown that hMusTRD1α1 is capable of repressing MEF2C-mediated activation of the USE. The binding of MEF2C to its recognition site within the USE in the presence of hMusTRD1α1 was examined by EMSA in an effort to understand the mechanism of this repression. Oligonucleotides corresponding to the functional MEF2 binding site within the USE (hTnIslow USE) bound efficiently to in vitro translated MEF2C. However, oligonucleotides containing the same sequence except for four altered bases in the core recognition sequence (mutMEF2)
failed to bind, thus demonstrating the specificity of the interaction (Fig. 7A). The addition of increasing amounts of hMusTRD1α blocked the interaction between MEF2C protein and the oligonucleotide containing its binding site in a dose-dependent manner (Fig. 7A).

This finding suggests that hMusTRD1α1-mediated repression could occur through a direct interaction between hMusTRD1α and MEF2C. To test this further, GST pull-down assays were performed using GST-hMusTRD1α1 fusion pro-
MEF2C. It could prevent the proper interaction of MEF2C with the enhancing factor either by sequestering MEF2C in conjunction with NCoR or by occupying the Inr-like element (Fig. 8).

A growing number of signaling pathways have been found to converge on the MEF2 proteins, thereby regulating their essential role in transcriptional control of muscle-specific genes (reviewed in Ref. 47). MEF2C and MEF2A are substrates for p38 mitogen-activated protein kinase (48, 49), and MEF2C is a substrate for BMK1/ERK5 (50). Other pathways involve calcium-sensitive proteins including calcineurin, which activates MEF2 by direct dephosphorylation (32) and Ca\textsuperscript{2+}-calmodulin-dependent protein kinase, which activates MEF2 by alleviating the repression imposed by the histone deacetylases (HDACs) (51–53). Signaling via Ca\textsuperscript{2+}-dependent pathways has recently been proposed as a potential means of fiber-type adaptation because sustained patterns of nerve stimulation elevate intracellular calcium levels (20), and recent experiments in mice support this hypothesis (31, 54). Additionally, the transcriptional co-activator PGC-1\textalpha has been found to elicit slow fiber-specific gene expression, as well as mitochondrial biogenesis, through interaction with MEF2 (30). Therefore, MEF2 is a key factor involved in translating endurance activity and electrical activity-mediated changes in intracellular Ca\textsuperscript{2+} levels within muscle fibers into muscle gene transcriptional activity. It is clear that by disrupting the transcriptional activation capacity of MEF2C in the USE of \textit{hTnI\textsubscript{slow}}, as demonstrated in this study, hMusTRD\textsubscript{1a1} acts at a nodal point in the regulation pathway of slow fiber-specific genes.

hMusTRD\textsubscript{1a1} repression can occur through at least two distinct mechanisms. hMusTRD\textsubscript{1a1} can interact directly with the B1 region via two DBDs. Truncated versions of hMusTRD\textsubscript{1a1} containing either or both DBDs bind specifically to the B1 region of the USE through the core binding motif GATTAA defined by O’Mahoney \textit{et al.} (1). In all instances, mutation of this sequence to GATatc prevented binding. Furthermore, the
α-HuMusTRD1α1-200a antibody was capable of directly blocking HuMusTRD1α1 DNA binding activity, further verifying the specificity of this DNA-protein complex. The DBDs in hMusTRD1α1 are located within aa 351–458 and 544–944. Calvo et al. (42) recently reported the isolation of a series of truncated GTF3 proteins encoded by the mouse orthologue of the hMusTRD1α1-encoding gene. The GTF3 proteins were isolated by their ability to bind to the rat T-box slow upstream regulatory element, which acts similarly to the human USE. On the basis of the truncations, it was concluded that the DBD was located in the C-terminal region, which would be consistent with DBD2 demonstrated here.

The association of hMusTRD1α1 with the nuclear receptor co-repressor N-CoR demonstrates a second mode of repression. N-CoR functions as a co-repressor not only for nuclear hormone receptors but also for multiple classes of transcription factors (55, 56). The multiple, N-terminal repression domains mediate interactions via mSin3 with large complexes containing class I HDACs (57) or, by direct association, with class II HDACs (58), thereby modifying chromatin structure through histone hypoacetylation. Co-immunoprecipitation studies recently revealed an association between hMusTRD1α1 and the class I histone deacetylase HDAC3 (59). It is possible that this interaction is mediated by an N-CoR-dependent mechanism. In support of this claim, Wen et al. (59) found that in addition to HDAC3, eight other proteins including N-CoR and TFII-I, a protein which acts similarly to the human USE. On the basis of the truncations, it was concluded that the DBD was located in the C-terminal region, which would be consistent with DBD2 demonstrated here.

Recent reports have demonstrated that the mouse MusTRD homologues GTF3 and PEBP2 also act as transcriptional repressors in other experimental systems (40, 42). BEN was shown to repress TFII-I-mediated activation of the c-fos promoter despite its apparent inability to bind either to the c-fos promoter or to TFII-I (43). It was concluded that the repression occurs through competition for nuclear shuttling components and transcriptional co-factors. On the basis of current knowledge, it would seem that the MusTRDs are complex multifunctional proteins distinctly different from the basic helix-loop-helix transcription factor family. The discovery of multiple splice isoforms of MusTRD, which modify regions of the C-terminal half (44) and the potential to be viewed as multiple functional complexes. On the other hand, the functional consequences of these modifications may provide an entry point for understanding the diverse mechanisms by which the MusTRDs regulate transcription.

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