Analysis of the inhibition of MyoD activity by ITF-2B and full length E12/E47

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Running title: Full length E12 and E47 inhibit MyoD

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SUMMARY

MyoD heterodimerizes with E type factors (E12/E47 and ITF-2A/ITF-2B) and binds E box sequences within promoters of muscle-specific genes. In transient transfection assays, MyoD activates transcription in the presence of ITF-2A but not ITF-2B, which contains a 182 aa N-terminal extension. The first 83 aa of the inhibitory N-terminus of ITF-2B show high sequence homology to the N-terminus of full length E12/E47. Previous studies that showed activation of MyoD by E12 used an artificially N-terminally truncated form. Here we show that the full length form of E12 inhibits MyoD function. A conserved α-helix motif, capable of interacting with the transcriptional machinery, was not essential for inhibition. Further, the fusion of N-terminal ITF-2B sequences or non-inhibiting ITF-2A sequences to truncated E12 was sufficient in converting the activator into an inhibitor. Overexpression of ITF-2B did not inhibit C2C12 myogenesis or affect levels of endogenous muscle genes, consistent with the finding that inhibitory E type proteins are present in muscle. Further, we found that MyoD co-transfected with either ITF-2B or ITF-2A converted fibroblasts into myoblasts with the same frequency. Our findings suggest that the ability of E type proteins to inhibit MyoD activity is dependent on the context of the E box.
INTRODUCTION

Skeletal muscle development is known to be dependent on a family of basic helix-loop-helix (bHLH) transcription factors, termed the myogenic regulatory factors (MRFs). The family includes MyoD, myf5, myogenin and myf6/MRF4/herculin (1-7). Ectopic expression of any family member in a wide range of non-muscle cells results in the conversion of these cells to the skeletal muscle lineage (8,9). The myogenic regulatory factors regulate muscle-specific gene expression by heterodimerizing with the E type family of ubiquitous bHLH transcription factors via the HLH domains and binding the E box consensus sequence (CANNTG) within muscle-specific promoters via the basic domains (10,11). Homodimers of MyoD are inactive while heterodimers between MyoD and E type proteins can activate E box-containing promoters. Therefore, heterodimerization with the E type transcription factors is necessary for activity of the myogenic regulatory factors.

The E type family of bHLH transcription factors includes E12/E47 (12-14), ITF-2 (15-17), and HEB (18). The E type proteins contain two activation domains, the AD1 domain and the AD2 or LH domain (19,20). Two alternatively spliced forms of mouse ITF-2, termed ITF-2A and ITF-2B, were shown to differentially regulate MyoD activation. In transient transfection assays, ITF-2B was found to inhibit MyoD activation of the cardiac α-actin muscle-specific promoter (21) and the muscle-specific creatine kinase (MCK) promoter (22). Inhibition was shown to occur in both P19 cells and C2C12 cells. The activator, ITF-2A, and the inhibitor, ITF-2B, are co-expressed in several cell types including skeletal muscle cells (21,22).
The activity of the myogenic regulatory factors is also inhibited by the Id gene family (23). These factors contain a HLH domain but lack a basic domain. They are thought to inhibit the activity of the myogenic factors by forming inactive heterodimers with the E type proteins, thus sequestering them from myogenic factor binding (23,24). Several studies suggest this is not the primary mechanism by which inhibition by ITF-2B occurs. Inhibition by Id can be reversed by the addition of excess E type activator, while inhibition by ITF-2B cannot (21). Further, MyoD/ITF-2B heterodimers bind DNA (22). Together, these results suggest that ITF-2B can form a stable heterodimer with MyoD, which can then bind the E box consensus, but this complex is unable to activate transcription.

Basic helix-loop-helix transcription factors have also been shown to inhibit MyoD activity. Twist inhibits MyoD activity by mechanisms involving the titration and sequestration of the E types, by inhibition of the MyoD co-factor MEF2 and direct inhibition via interaction with the basic domain of MyoD (25-27). MyoR has also been shown to inhibit MyoD activity by mechanisms including competition for DNA binding sites, active repression through a repressor domain and titration of the E type proteins (28). More recently, the bHLH protein OUT, has been shown to inhibit MyoD activity by E type titration and by preventing E12-MyoD heterodimers from binding DNA (29).

Here we set out to examine the role of the N-terminal domain of ITF-2B in the inhibition of MyoD activity. We found that the full length E12/E47 proteins contain a domain that is highly similar to the N-terminal inhibitory region of ITF-2B. Like ITF-2B, both full length E12 and E47 proteins were shown to inhibit transactivation of the exogenous cardiac α-actin promoter by MyoD.
EXPERIMENTAL PROCEDURES

Plasmid Constructs - The cDNAs utilized in these experiments were all driven by the mouse pgk-1 promoter (30). Expression constructs of mouse ITF-2A and ITF-2B, human ΔE12 (partial E12 cDNA), MyoD, CAT, puromycin and LacZ as well as the cardiac α-actin promoter driving the LacZ gene (CA-LacZ) have been previously described (21). The promoter construct contained 440 bp of the human cardiac α-actin promoter (31), with one E box that is essential for expression during skeletal myogenesis (32). The human full length E12 and E47 cDNAs were kindly provided by C. Murre (33). The PGK-vector plasmid contains the pgk-1 (phosphoglycerate kinase) promoter alone.

The mutant ITF-2B plasmid, which corresponds to Mutant 2 (34) was constructed utilizing the Muta-gene™ Phagemid in vitro mutagenesis kit, according to manufacturer’s instructions (BioRad, Richmond, CA). The plasmid used was a 728 bp Sal1-Sst1 fragment of ITF-2B subcloned into pBluescript KS+ (Stratagene Cloning Systems, La Jolla, CA). The mutagenic oligonucleotide used was AAACATCGCAGCTGATCTAGACGATCACTCAGCTCT (altered nucleotides in bold).

The chimeric proteins were constructed by PCR amplification. The amino terminus of ITF-2B (initiator methionine underlined) was amplified with the 5’oligonucleotide sequence AAAGGTAACATGCATCAACACACAGCGA and the following 3’ oligonucleotide sequences: AAAGGTAACCACATGAAGACGGCAAACCC (aa 1-182); AAAGGTAACCATTCTGGAATTGACAAAA (aa 1-99); AAAGGTAACCACA
TTTGAGCCAGTGAAA (aa 1-49); AAAGGTACCATCGCACTGAAAATCCAGT (aa 1-24).
The fragment from aa 11-28 was amplified using the following oligonucleotides:
AAAGGTACCATGGGACGGACAAAGAGCTG and AAAGGTACCAGAGGCGAAACATCGCA.
The fragment encompassing aa 25-49 was amplified using the following oligonucleotides: AAAGGTACCATTTTCGCTCCTGTA and
AAAGGTACCACATTGAGCCAGTGAAA. The first 182 aa of ITF-2A were amplified using the following oligonucleotides: AAAGGTACCATGTCTGCGCATTACACC and AAAGGTACCAGAGGAAAAGCTGTTGTTTCC. PCR was performed utilizing 10 ng of ITF-2A or ITF-2B cDNA, 0.25 µM of each oligonucleotide and 2.5 units of Taq polymerase (Life Technologies, Inc., BRL, Burlington, ON) in 100 µl. Standard PCR conditions were employed with annealing temperatures between 55-68°C. Each fragment was then inserted into a Kpn1 site (boldface sequences in oligonucleotides) upstream of and in frame with ΔE12.

Cell Culture and Transfections - P19 embryonal carcinoma cells were cultured as previously described (35). Transfections were carried out by the calcium phosphate method (36). Briefly, 7.5x10⁵ cells in 5 ml of medium were exposed to DNA precipitate for 6-9 hrs in a 60-mm tissue culture dish. The DNA precipitates consisted of 4 µg of CA-LacZ and 1 µg of PGK-CAT in the absence or presence of 2 µg of PGK-MyoD and 5 µg of the various E type genes. Each transfection was brought up to 12 µg total DNA with PGK-vector plasmid. Cultures were harvested 24 hr after transfection.

β-galactosidase and chloramphenicol acetyltransferase (CAT) assays were performed as previously described (37,38). β-galactosidase activities for each culture were normalized for transfection efficiency against CAT activity as well as to the activity
of MyoD alone. The background activity of the promoter alone was subtracted from each sample within an experiment.

C2C12 myoblasts were cultured under growth conditions in 15% 1:1 cosmic calf:fetal bovine serum (Cansera, Rexdale, ON, Canada and Hyclone, Logan, Utah, respectively) in α-MEM. Cell lines which stably expressed ITF-2A, ITF-2B, ΔE12 or PGK-vector were created by transfecting 10 μg of PGK-ITF-2A, PGK-ITF-2B or PGK-vector plasmid, 1 μg of PGK-puromycin and 1 μg of PGK-LacZ by the calcium phosphate method. Myoblasts were plated into 60 mm dishes, selected for puromycin resistance for one week, and then transferred into differentiation media (2% horse serum) for 4 days.

Transient transfections of C2C12 myoblasts were carried out utilizing FuGENE™ 6 transfection reagent as per the manufacturers instructions (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, 11 μg of PGK-ITF-2B or PGK-vector and 1 μg of pEGFP-N1 (CLONETECH Laboratories, Inc., Palo Alto, CA) and FuGENE™ 6 reagent mixtures were added to C2C12 cells in 100mm dishes. GFP fluorescence was utilized to assess transfection efficiency. Myoblasts were transferred from growth media to differentiation media 24 hours later.

Whole-mount in situ hybridization of micromass cultures - For in situ hybridization, C2C12 stable cell lines were differentiated as above and fixed with 4% paraformaldehyde overnight at 4°C. The cells were rehydrated and in situ hybridizations were carried out as previously described (39). A total of about 80 colonies were present on each plate and the extent of myogenesis was calculated by
counting colonies containing multinucleated myotubes that expressed the transfected gene product. This experiment was repeated twice with similar results.

DIG-labeled riboprobes were transcribed from single-stranded DNA templates according to the manufacturer’s instructions (Boehringer Mannheim) and quantitated as previously described using an anti-DIG antibody conjugated to alkaline phosphatase (39). A 165 bp Sst1-Acc1 fragment, which contained 5’untranslated and coding regions of ITF-2A was utilized to create the ITF-2A-specific probe. The ITF-2B-specific probe consisted of a PCR fragment containing the N-terminal 99 amino acids of ITF-2B. The ΔE12 specific probe consisted of a 429 bp Sst1-Xho1 fragment within the coding region of ΔE12. Images were captured using Sony DXC-9503 3CCD colour video camera and analyzed using Northern Eclipse image analysis software (Empix Imaging, Inc.).

**Western blot analysis** - Myoblast cells expressing PGK-ITF-2A, PGK-ITF-2B and PGK-vector were differentiated as above, and myosin was harvested by the Burridge and Bray method (40), omitting the second dialysis step. Protein concentration of the myosin extracts was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Two micrograms of protein were electrophoresed by 7% SDS-PAGE and transferred to nitrocellulose overnight at 30V at 4°C. Blots were probed with anti-MHC mouse monoclonal antibody, MF20 (1:1000 dilution) as a control for total myosin (41), and with anti-MHC IIB antibody BF:F3 (undiluted) against Type IIB myosin (42). Anti-mouse Ig, horseradish peroxidase linked whole antibody (Amersham Life Sciences, Oakville, ON, Canada) secondary antibody was used at a 1:5000 dilution. The reaction was visualized using the ECL SuperSignal® Substrate (Pierce, Rockford,
IL) for western blotting and autoradiography. Quantitation was carried out utilizing NIH Image 1.58.

**Northern Blot Analysis** – The lithium chloride/urea extraction method was used to isolate total RNA, and 6 μg were examined by Northern blot analysis as previously described (43). The ITF-2B probe used was a Ssp1-Not1 fragment of ITF-2B, which encodes the full length cDNA. This probe detected transfected ITF-2B and endogenous forms of ITF-2. Other probes utilized were a 600 base pair EcoRI fragment of rat myosin light chain (MLC) 1/3 and a 600 base pair Pst1 fragment from the last exon of human cardiac α-actin. Northern blots were visualized by autoradiography and quantitated by NIH Image 1.58.

**Myogenic conversion of 10T1/2 fibroblasts** - 10T1/2 fibroblasts were cultured under growth conditions in 10% 1:1 cosmic calf:fetal bovine serum in α-MEM. Cells on gelatin-coated coverslips were transfected in the absence or presence of 1 μg PGK-MyoD, 2.5 μg of the various E type genes, and 0.5 μg of pEGFP-N1 (CLONETECH Laboratories, Inc. Palo Alto, CA) utilizing the FuGENE™ 6 transfection reagent. Total DNA in each transfection was brought up to 4 μg with PGK-vector plasmid. Following 24 hours, cells were transferred to differentiation media, containing 2% horse serum, for 6 days. Transfection efficiency for each culture was scored utilizing GFP fluorescence and total number of cells was estimated by counting Hoechst-stained nuclei. To identify myogenic conversion, cells were fixed with methanol at -20°C and stained for myosin heavy chain (41) as described (43). Immunofluorescence was visualized with a Zeiss Axioskop microscope.
RESULTS

Full length E12 and E47 inhibit MyoD activity - Previous results have shown that the amino-terminal 83 amino acids of ITF-2B were required for ITF-2B to inhibit MyoD activity (21). Consequently, we set out to determine if other E type proteins contained similar N-terminal sequences and possibly, similar inhibitory activity. An alignment of the N-terminal 83 amino acids with the N-termini of full length E12 and full length E47 (33) revealed that this domain is 51% identical among the three E type proteins (Fig 1). This region has been shown to contain a conserved \( \alpha \)-helix that functions as a transactivation domain when fused to the Gal4 DNA binding domain (34). The E12 and E47 proteins, termed \( \Delta \)E12 and \( \Delta \)E47, utilized in activation studies with MyoD, are fragments of the full length proteins and contain a synthetic initiator methionine (12). These fragments are missing the first 216 aa compared to full length E12 and E47 (33). Subsequently, the partial proteins are missing the region that is similar to the N-terminal region of the inhibitor ITF-2B (summarized in Fig. 2A). To our knowledge, the full length E12 and E47 proteins have not been tested with respect to their effect on MyoD activity.

In order to determine if the presence of the conserved N-terminal region (represented in Fig. 1) modified the activity of E12 and E47, we examined the effect of full length E12 and E47 proteins on MyoD. This involved determining the ability of MyoD to transactivate the cardiac \( \alpha \)-actin promoter in the presence of each E type protein after transient transfection into P19 cells. In agreement with previously published results, MyoD alone transactivated the cardiac \( \alpha \)-actin promoter indicating the presence of endogenous activating E type proteins in P19 cells (21)(Fig. 2B).
Furthermore, MyoD was active in the presence of ΔE12 and ITF-2A but not in the presence of ITF-2B. In the presence of full length E12 and full length E47, MyoD activity was decreased 3 and 5-fold, respectively, in multiple repetitive assays. Therefore, full length E12 and E47 proteins inhibited MyoD activity as effectively as ITF-2B.

*Mutation of the conserved α-helix does not affect inhibition* - A conserved α-helix has been identified from aa 11-28 of E12, E47, and ITF-2B that functions as a transcriptional activator when fused to the Gal4 DNA binding domain (34). Since this region lies within the inhibitory domain of ITF-2B and can interact with components of the transcriptional machinery, we set out to determine if these sequences were required for inhibition. A mutant was generated in which 2 conserved, non-polar residues were replaced with arginine (Fig. 3A). These changes were shown previously to disrupt the transactivation domain (34). Here we show that this mutant still functions as an inhibitor, decreasing MyoD activity 4-fold compared to MyoD alone (Fig. 3B).

*Lengthening the N-terminus of ΔE12 converts the activator into an inhibitor* - In order to identify the amino acids of ITF-2B that are sufficient for inhibition, various portions of the N-terminal domain of ITF-2B were fused onto the N-terminus of the activating ΔE12 protein (Fig.4A). To ensure the inhibition was specific to the inhibitory region of ITF-2B, the N-terminal 182 amino acids of the activator, ITF-2A, were also fused to the N-terminus of ΔE12. In transient co-transfection experiments, we observed that the addition of various fragments of the ITF-2B N-terminal sequences as well as the N-terminus of ITF-2A were all sufficient in converting ΔE12 into an inhibitor.
of MyoD activity (Fig. 4B). The fusion proteins inhibited MyoD activity between 2 and 5-fold. This range of inhibition may be due to small variations in conformation or expression levels between the different fusion proteins. This conversion did not appear to be sequence dependent since both non-inhibitory sequences from ITF-2A and inhibitory sequences from ITF-2B were capable of converting ΔE12 into an inhibitor. Consequently, as few as 18 amino acids are sufficient to convert ΔE12 from an activator into an inhibitor, in a sequence independent manner.

Overexpression of ITF-2B has no effect on myogenesis or fibre type generation - To study the physiological relevance of ITF-2B inhibition of MyoD activity, ITF-2B was stably and transiently overexpressed in C2C12 myoblasts. Cells stably expressing ITF-2B, ITF-2A or ΔE12 were differentiated to determine if overexpression of ITF-2B was sufficient to inhibit the process of myogenesis (Fig. 5). The differentiation of C2C12 myoblasts was monitored by counting colonies that contained multinucleated myotubes and overexpression of the E type proteins was determined by in situ hybridization. Myotubes were found to express high levels of ITF-2B (Fig. 5A), compared to endogenous staining (Fig. 5B). The total number of colonies containing myotubes and expressing ITF-2A, ΔE12, or lacZ (average of 24 colonies), was not substantially different from the number of colonies containing myotubes and expressing the inhibitor ITF-2B (22 colonies). C2C12 cell lines stably expressing ITF-2A and ITF-2B to levels equivalent to endogenous levels of ITF-2 transcripts were found to express similar amounts of cardiac α-actin and differentiate as efficiently as control cell lines (data not shown). Therefore, C2C12 cells stably overexpressing ITF-
2B were found to differentiate with equal efficiency as cells stably overexpressing ITF-2A, ΔE12, or control transfected cells.

The differences we observed in the ability of ITF-2B to inhibit MyoD activity may be due to the examination of exogenous versus endogenous promoters or to the transient versus stable expression levels of factors. To distinguish these two possibilities, C2C12 myoblasts were transiently transfected with ITF-2B, differentiated into muscle and examined by Northern blot analysis (Fig. 6). We found that levels of exogenous ITF-2B are high early during differentiation of transiently transfected cells (data not shown). ITF-2B expression remained at an average of 3-fold over the level of endogenous forms of ITF-2 (Fig. 6A, compare lanes 1 and 2 to 3 and 4) on day 4 of differentiation. Expression levels were normalized against 18S. The level of expression of cardiac α-actin and MLC 1/3 was not altered in cultures expressing ITF-2B compared with control cells. Therefore, the transient overexpression of ITF-2B was not sufficient to inhibit myogenesis, or to affect MRF activity on the endogenous cardiac α-actin promoter.

To further examine the activity of transiently transfected ITF-2B on endogenous muscle-gene promoters, we tested the ability of MyoD to convert fibroblasts to myoblasts in the presence of various E type proteins (Fig. 7). We found that MyoD could convert 10T1/2 fibroblasts to the myogenic lineage with similar efficiency when co-transfected with ITF-2A or ITF-2B. Similarly, full length E12 could convert cells as
efficiently as ΔE12 in the presence of MyoD. Therefore, expression of the inhibitory forms of the E type proteins was not sufficient to inhibit myogenic conversion by MyoD.

In addition, we tested the possibility that E type factors may play a role in the ability of the MRFs to generate different fibre types by activating the endogenous expression of different MHC isoforms. Myoblasts expressing ITF-2A or ITF-2B were differentiated to determine if overexpression of either form affects MHC IIB protein expression when compared to control transfected cells. We observed no difference in the amount of MHC IIB protein present in ITF-2A or ITF-2B expressing cells compared with control myoblast cells by western blot analysis (data not shown).
DISCUSSION

We have shown that full length E12/E47 contains an N-terminal domain homologous to the N-terminal inhibitory domain of ITF-2B. In transient transfection assays, full length E12/E47 inhibits MyoD function. Therefore, similar to ITF-2, E12/E47 can either activate or inhibit MyoD activity depending on which domains of E12/E47 are present in the MyoD/E12/E47 heterodimer. The inhibitory activity of ITF-2B did not require a conserved α-helix, shown to be necessary for transactivation by Gal4-E2-2 fusion proteins (34). This indicates that the inhibitory activity within the N-terminal region is separable from the activating function seen by others (34).

Consequently, the ability of ITF-2B to inhibit MyoD does not require interactions specifically between the α-helical domain and the transcriptional apparatus. Finally, the overexpression of ITF-2B in C2C12 myoblasts or MyoD-transfected fibroblasts did not result in the inhibition of myogenesis. Therefore, while the presence of the inhibitory domain inhibits MyoD transactivation of exogenous promoters, it does not inhibit MyoD transactivation of endogenous muscle promoters.

The fusion of activating sequences from ITF-2A or inhibitory sequences from ITF-2B onto the N-terminus of ΔE12 converted ΔE12 from an activator into an inhibitor. Since the inhibition was sequence-independent, it is possible that the fusion of ectopic sequences may change the conformation of the amino terminus of E12, transforming it from an activator into an inhibitor. This conformational change may simulate the mechanism by which ITF-2B inhibits MyoD activity via its N-terminal domain.
Alternatively, the creation of a fusion protein with ∆E12 may prevent the MyoD/∆E12 fusion heterodimers from binding DNA.

Since ITF-2B forms stable heterodimers with MyoD that are capable of binding DNA (21,22), one hypothesis is that the conformation adopted by the N-terminal extension of ITF-2B may sterically hinder a domain of MyoD which is necessary for transactivation. Alternatively, ITF-2B may hinder the binding of the E type/MRF heterodimer to transcription factors bound to other elements within muscle specific promoters (22). It is possible that other sites are required for inhibition since ITF-2B has been shown to inhibit the activity of muscle promoters including cardiac α-actin (21) and MCK, but not the activation of an artificial promoter containing E boxes (22).

Overexpression of ITF-2B did not result in the inhibition of myogenesis in C2C12 cells and did not modulate the expression of myosin heavy chain (type IIB), myosin light chain 1/3, or cardiac α-actin. Further, when compared to the activators, ITF-2A and ∆E12, ITF-2B and full length E12 did not inhibit the ability of MyoD to convert fibroblasts to the myogenic lineage. These findings suggest that ITF-2B expression is not sufficient to inhibit MyoD activation of endogenous muscle-gene promoters. Therefore, ITF-2B is unlikely to play a role in inhibiting myogenesis in vivo, consistent with the presence of the inhibitor ITF-2B in skeletal myocytes and adult skeletal muscle (21,22). In contrast, Twist and MyoR, which are not expressed in skeletal muscle cells and do inhibit differentiation when overexpressed in myoblasts, are more likely bHLH physiological inhibitors of myogenesis (28,44,45).
The differential inhibition of MyoD by ITF-2B is similar to the finding that Id will inhibit MRF transactivation of exogenous promoters, but will not block myogenesis in C2C12 cells 2 days after the onset of differentiation (24). Further, mice null for Id1, Id2, Id3, or Id1/Id3 (46-48) and E2A, HEB, or E2-2 gene products do not show a muscle phenotype (49,50). The analysis of these knock-out mice is complicated by functional redundancy, demonstrated by the ability of HEB to replace E2A in supporting B-lymphocyte development (51).

Many studies have shown that the function and requirement for specific DNA elements differs in transient transfection studies compared to transgenic mouse models (52,53). Furthermore, transient transfection studies have shown that the activity of cardiac α-actin and MCK varies with the size of the promoter fragment used (54,55). These studies suggest that the regulation of an endogenous promoter is dependent on a complex set of factors and promoter elements. Active MyoD-ITF-2B heterodimers may require association with factors bound to sites within endogenous promoters, which are not present in the exogenous promoters tested. Alternatively, other factors bound to the endogenous promoter may abrogate the inhibition of MyoD activity by ITF-2B. Finally, the inhibition seen on an exogenous promoter may be negligible compared to the full activity of an endogenous promoter.

Other differences in the regulation of exogenous versus endogenous promoters may be due to differences in their chromatin
structure (56). Coactivators such as p300/CBP and PCAF (57-59) and the corepressor N-CoR (60) are known to play a critical role in regulating MyoD activity. The activity of such factors may abrogate the inhibitory function of ITF-2B on an endogenous promoter, whereas chromatin remodelling may not be involved in the activation of an exogenous promoter.

Although it seems unlikely, the presence of endogenous activating E type proteins could have a compensating effect on the activity of myogenic regulatory factors in C2C12 and 10T1/2 cells, resulting in the lack of inhibition of myogenesis observed. Furthermore, the activity of the E type proteins may be limited by post-translational regulation or by degradation. It is possible that the inhibitory activity is acting in a subtle manner to regulate MRF function. For example, overexpression of ITF-2B may decrease levels of muscle genes we have not tested. Several studies indicate that the myogenic regulatory factors have specific target gene specificities (61-65). The ability of the E type dimerization partners to differentially regulate the activity of the myogenic regulatory factors may be one mechanism regulating the fine tuning of muscle-specific gene expression. A more detailed examination of mice null for the various E type factors may reveal a more complex role.

In summary, we have shown that E type proteins containing a conserved N-terminal domain, such as E12/E47 and ITF-2B, have the ability to inhibit MyoD activity in transient transfection assays. In
contrast, these inhibitors had no effect on myogenesis or myogenic conversion of fibroblasts. Therefore, the N-terminal region of E-type proteins seems critical for regulating MyoD activity on exogenous but not endogenous promoters. The presence of E-type inhibitory proteins does not appear to play a physiological role in the on/off regulation of myogenesis.

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REFERENCES


FIGURE LEGENDS

FIG. 1. The N-terminal 83 amino acids of ITF-2B, full length E12 and full length E47 are conserved. The N-terminal 83 amino acid domain of the inhibitor, ITF-2B, is 51% identical to the N-termini of the full length E12 and full length E47 proteins. The conserved amino acids are boxed.

FIG. 2. Full length E12 and E47 proteins inhibit MyoD activity compared to endogenous E type proteins present in P19 cells. Panel A. comparison of the length and domain structure of E type bHLH proteins and their ability to inhibit MyoD activity. The conserved N-terminal 83 amino acids are shown as hatched boxes, the bHLH domains are shown as black boxes and the lengths of the various domains are indicated. Panel B, the ability of MyoD to transactivate the cardiac α-actin (CA) promoter in the presence of each E type protein was examined. P19 cells were transfected with 4 μg of the reporter construct CA-LacZ and 1 μg of the standardization reporter PGK-CAT, as well as 5 μg of the various E type expression constructs and 2 μg of MyoD. Error bars represent standard error with n=4 or 5.

FIG. 3. Mutation of amino acids within the conserved α-helix, which are crucial for activation by the ADI domain, does not affect inhibition. Panel A, two amino acids within the conserved α-helix, which have been shown to be important for transactivation by the ADI domain, were substituted by site-directed mutagenesis. Panel B, to test the effect of the mutation on the ability of ITF-2B to inhibit
MyoD activity, 1.5 μg of the mutated ITF-2B, wild-type ITF-2B or ΔE12 were cotransfected with 4 μg of CA-LacZ, 1.5 μg of PGK-MyoD and 1 μg of PGK-CAT. Error bars represent standard error from 7 different experiments.

FIG. 4. **The N-terminal 182 amino acids of ITF-2A and various sizes of the ITF-2B N-terminus are sufficient to transform ΔE12 from an activator to an inhibitor of MyoD.** Panel A, the N-terminal 182 amino acids of ITF-2A and ITF-2B, as well as varying lengths of the ITF-2B N-terminus, were fused in frame to the N-terminus of ΔE12. The fused domains and the bHLH domain of each chimeric protein are represented by the hatched and black boxes, respectively, and MyoD activity with each is summarized. Panel B, to test the effect of each chimeric protein on the activity of MyoD, 5 μg of each was co-transfected with 4 μg of CA-LacZ, 2 μg of PGK-MyoD and 1 μg of PGK-CAT. The activity of MyoD in the presence of each chimeric protein was compared to that in the presence of ΔE12 as shown (* indicates values are significantly different from ΔE12 by student t-test). Error bars represent standard error between 4 and 14 different experiments.

FIG. 5. **The stable expression of ITF-2B does not inhibit the differentiation of C2C12 myoblasts.** Myoblasts were transfected with ITF-2B, selected for stable expression, and differentiated in media containing 2% horse serum. In situ hybridization was used to identify differentiated colonies overexpressing ITF-2B (Panel A) compared to colonies expressing endogenous ITF-2B levels (Panel B). The bar represents 0.2mm.
FIG. 6. **Transient overexpression of ITF-2B does not affect the expression of endogenous cardiac α-actin in differentiated C2C12 myoblasts.** C2C12 cells were transfected with ITF-2B and transferred to differentiation media the next day. Total RNA from differentiated cultures was harvested 4 days later. Northern blots containing 6 μg of RNA were probed with ITF-2B, MLC1/3, cardiac α-actin and 18s as indicated.

FIG. 7. **ITF-2A and ITF-2B convert fibroblasts to myoblasts with the same efficiency when co-transfected with MyoD.** 10T1/2 fibroblasts were transfected with 1 μg of PGK-MyoD, 2.5 μg of the various E type genes and 0.5 μg of GFP. Number of cells expressing MHC following 6 days in differentiation media was determined by immunofluorescence. The percentage of MHC positive cells was normalized against transfection efficiency and MyoD conversion was set to 100. *Error bars* represent standard error between 3 different experiments.
A

 contin  ADII-LH  bHLH  MyoD Activity

ΛE12

ITF-2A

ADI

ITF-2B

Full length E12 & E47

B

Cotransfectant  Fold inhibition of MyoD

-  

ΛE12

ITF-2A

ITF-2B

Full length E12

Full length E47

Normalized β-galactosidase activity
A

Conserved α-helix

Mutation

GTDKELSDDLDFSAMFS

GTDKELSDRLDRSAMFS

B

Co-transfectant

Fold inhibition of MyoD activity

- E12

ITF-2B

Mutated ITF-2B

Normalized β-galactosidase activity

3x

4x
Analysis of the inhibition of MyoD activity by ITF-2B and full length E12/E47
Helen Petropoulos and Ilona S. Skerjanc

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