Physical and Functional Interactions between the Transcriptional Inhibitors Id3 and ITF-2b

EVIDENCE TOWARD A NOVEL MECHANISM REGULATING MUSCLE-SPECIFIC GENE EXPRESSION

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We have used an interaction cloning strategy to identify an inhibitory isoform of the ITF-2 transcription factor, ITF-2b, that interacts with the transcriptional inhibitor Id3/HLH462. The interaction was confirmed in vitro, and inside intact myogenic C2C12 cells. As expected, overexpression of either Id3/HLH462 or ITF-2b effectively inhibited the activation of the muscle-specific creatine kinase promoter by the myogenic transcription factor MyoD. However, when overexpressed simultaneously, ITF-2b and Id3/HLH462 counteracted each other's inhibitory effect to produce a reduced overall inhibition. Moreover, while ITF-2b inhibited the creatine kinase promoter, it acted as a weak transactivator on an artificial promoter consisting of three tandem copies of the consensus myogenenic factor DNA binding site. Further investigation indicated that the ITF-2b/MyoD heterodimer bound to its specific DNA binding site in vitro, and the DNA binding was effectively blocked by Id3/HLH462. Additional analysis revealed the presence of transcripts for both the activating (ITF-2a) and inhibitory (ITF-2b) isoforms in differentiating C2C12 cultures, suggesting that both isoforms might participate in regulating the differentiation process. Taken together, this study reveals a more complex pattern of regulatory interactions involving the helix-loop-helix proteins than was previously anticipated.

The basic helix-loop-helix (bHLH) family of transcription factors have been shown to play an important role in regulating muscle-specific gene expression (1, 2). Myogenic bHLH factors heterodimerize with ubiquitously expressed bHLH proteins (referred to as E proteins) and transactivate gene expression through binding to consensus DNA binding sites (E boxes) in the promoter region of various muscle-specific genes (3, 4). In contrast, the Id family proteins, which contain an HLH domain but lack the DNA-binding basic region (5–8), interact preferentially with the E protein family members (8) and sequester them from dimerization with the tissue-specific bHLH factors (5–8), thereby inhibiting gene expression (5). More recent studies have also demonstrated the existence of a new group of bHLH proteins that contain the basic and the HLH domain but nevertheless function as transcriptional inhibitors. One of these inhibitory bHLH proteins, beta3, interacts with E proteins to form heterodimers that are incapable of DNA binding (9). Recently, an alternatively spliced variant of the E protein ITF-2, ITF-2b, was identified and shown to inhibit the MyoD-mediated transactivation of the cardiac alpha-actin promoter (10), but the exact mechanism responsible for this inhibition has not yet been directly tested. Here we reported the independent and accidental cloning by the yeast two-hybrid system of a full-length ITF-2b cDNA while we were searching for protein factors that interact with the Id protein, Id3/HLH462. We have confirmed that Id3/HLH462 and full-length ITF-2b physically interacted with each other in vitro and complemented each other in a mammalian two-hybrid assay in the myogenic C2C12 cell line. Interestingly, although ITF-2b exhibited a dose-dependent inhibitory effect on the muscle creatine kinase (MCK) promoter in C2C12 cells, it functioned as a transactivator on an artificial promoter containing three tandem E box sites. Moreover, when both Id3/HLH462 and ITF-2b were co-expressed, their combined inhibitory effect on the MCK promoter was attenuated. Gel shift studies revealed that the ITF-2b/MyoD heterodimer formed a strong DNA binding complex comparable with that formed by the MyoD/E47 heterodimer, but the formation of both heterodimer-DNA complexes was effectively inhibited by Id3/HLH462. Analysis of ITF-2 and Id3/HLH462 gene expression revealed that transcripts of both activating (ITF-2a) and inhibitory (ITF-2b) isoforms were present initially in C2C12 cells placed under differentiation-promoting conditions, while the expression of Id3 was high in proliferating cells and declined as the culture differentiated. The potential implication of the expression pattern of ITF-2b and its interaction with Id3/HLH462 in the regulation of terminal myogenesis was discussed.

MATERIALS AND METHODS

Cloning of ITF-2b by Yeast Two-hybrid System—An Id3/HLH462 cDNA containing the complete open reading frame was removed from the pHHL462 plasmid (ATCC, Rockville, MD) with EcoRI/XhoI digest and subcloned into the EcoRI/SalI site of the GAL4 DNA binding vector, pGBT9 (Clontech, Palo Alto, CA) to form pGTHLH. An 11-day mouse embryo cDNA library in pGAD10 vector (Clontech) was screened according to the manufacturer’s protocol to select for genes whose products interact with Id3/HLH462.

Cell Culture—Mouse myogenic C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and gentamycin as described previously (11). Cell differentiation was induced by changing to DMEM + 5% horse serum.

Transient Transfection—A cDNA containing the complete open reading frame of ITF-2b, corresponding to nucleotides 506-2613 of the published sequence (10) was removed from pGAD10 by EcoRI digestion and

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subcloned into the pEMSV mammalian expression vector (12). An XhoI fragment of Id3/HLH462 from pHLH462 was blunt-end-ligated into the blunt EcoRI site of pEMSV vector to form the Id3 expression plasmid. The pEMSV/CAT reporter construct was made by inserting three copies of the E box DNA binding site (5) into the BglII site of pCMV-Control plasmid (Promega, Madison, WI) with the 3' SV40 enhancer sequence removed by HincII digest. The MCKCAT reporter gene construct contained a 3300-base pair promoter region of the muscle creatine kinase gene (13). Various amounts of constructs as described in the individual figure legend was transfected into C2C12 cells cultured on 100-mm dishes by calcium phosphate (14), and the cells were incubated for 3 days in differentiation-permissive medium (DMEM + 5% horse serum) after transfection before harvesting. Chloramphenicol acetyltransferase (CAT) assay was done essentially as described (14).

**Co-immunoprecipitation Assay in Vitro**—The ITF-2b cDNA containing the complete open reading frame was removed as a SalI restriction fragment from the pGAD10 vector and subcloned into the XhoI site of pBlueScript (Strategene, La Jolla, CA) to form pBSITF-2b. The FLAGHLH462 plasmid was made by polymerase chain reaction to insert an antigenic FLAG epitope (15) to the N-terminal of the full-length Id3/HLH462 protein. FLAGHLH462 was made by removal of a PstI/NotI fragment from the HLH462 plasmid to partially delete the second helix domain. All proteins were made using the TNT®-coupled *in vitro* transcription/translation reticulocyte lysate system (Promega) and labeled with [3H]leucine. Immunoprecipitation was carried out in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.2% Triton X-100, using the M2 anti-FLAG antibody (Eastman Kodak Co.).

**Interactions of ITF-2b with Id3/HLH462 in the Mammalian Two-hybrid System**—A fragment containing the complete open reading frame of ITF-2b was removed from the pGAD10 vector and cloned into the XhoI site of pBlueScript (Strategene, La Jolla, CA) to form pBSITF-2b. The FLAGHLH462 plasmid was made by polymerase chain reaction to insert an antigenic FLAG epitope (15) to the N-terminal of the full-length Id3/HLH462 protein. FLAGHLH462 was made by removal of a PstI/NotI fragment from the HLH462 plasmid to partially delete the second helix domain. All proteins were made using the TNT®-coupled *in vitro* transcription/translation reticulocyte lysate system (Promega) and labeled with [3H]leucine. Immunoprecipitation was carried out in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.2% Triton X-100, using the M2 anti-FLAG antibody (Eastman Kodak Co.).

**gel Shift Assay**—The assay was carried out as described (5) using *in vitro* translated proteins produced by TNT®-coupled transcription and translation. The oligonucleotide used in the assay corresponds to the consensus MEF2 binding site (13).

**RT-PCR Analysis of mRNA Expression of ITF-2a, ITF-2b, and Id3/HLH462**—Total RNAs were isolated from differentiated C2C12 cells at day 0, 2, 4, 6, 8, and 10 by the guanidinium isothiocyanate-phenol-chloroform extraction method (16). First strand cDNA was made by reverse transcription at 37°C for 2 h and used for PCR amplification using primers that are specific to ITF-2a, ITF-2b, Id3, or myogenin. Amplification was first performed with β-actin primers to normalize the amount of cDNA present in each preparation, and the normalized values were used to adjust for the amount of templates used in the PCR reaction with the gene specific primers. The primer sets specific to each gene for PCR were listed as follows.

- **ITF-2a**: 5'-GGGACACGCGATTGTGAATTAAGGA-3', 5'-GTCCCTCATAATGGGAAGACGAGC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CGACATGAACCACTGCTACTC-3', 5'-GTCCTTCATAATTGGGAGATGACG-3', 5'-GGTCAGTGGCTAAAGCTCCTC-3'.

- **ITF-2b**: 5'-GGGACACGCGATTGTGAATTAAGGA-3', 5'-GTCCCTCATAATGGGAAGACGAGC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CGACATGAACCACTGCTACTC-3', 5'-GTCCTTCATAATTGGGAGATGACG-3', 5'-GGTCAGTGGCTAAAGCTCCTC-3'.

- **Id3/HLH462**: 5'-GGGACACGCGATTGTGAATTAAGGA-3', 5'-GTCCCTCATAATGGGAAGACGAGC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CGACATGAACCACTGCTACTC-3', 5'-GTCCTTCATAATTGGGAGATGACG-3', 5'-GGTCAGTGGCTAAAGCTCCTC-3'.

- **Myogenin**: 5'-GGGACACGCGATTGTGAATTAAGGA-3', 5'-GTCCCTCATAATGGGAAGACGAGC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CGACATGAACCACTGCTACTC-3', 5'-GTCCTTCATAATTGGGAGATGACG-3', 5'-GGTCAGTGGCTAAAGCTCCTC-3'.

- **β-Actin**: 5'-GGGACACGCGATTGTGAATTAAGGA-3', 5'-GTCCCTCATAATGGGAAGACGAGC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CTCCCTCAGAGAGTATGAGCC-3', 5'-CGACATGAACCACTGCTACTC-3', 5'-GTCCTTCATAATTGGGAGATGACG-3', 5'-GGTCAGTGGCTAAAGCTCCTC-3'.

A typical PCR profile used was: 94°C 30 s, 59°C 30 s, 72°C 1 min for 27 cycles for ITF-2a and ITF-2b, 25 cycles for Id3/HLH462 and myogenin, and 20 cycles for β-actin, followed by extension at 72°C for additional 10 min.

**RESULTS**

We have used the yeast two-hybrid interaction cloning strategy to identify gene whose products can interact with Id3/HLH462. Out of about 4 × 10⁶ yeast clones co-transformed with GBTHLH and pGAD10 mouse embryonic cDNA library, 95 clones grew in the absence of histidine, among which 51 clones showed β-galactosidase activity. Further analysis confirmed 32 clones that showed Id3/HLH462-dependent galactosidase expression. Sequence analysis revealed that all are members of the E protein family (Table I). One of these clones appeared to correspond to a novel splicing variant of ITF-2b, ITF-2b, recently isolated by the Mcburney’s laboratory and reported to exhibit the unusual property of being able to inhibit the MyoD-mediated transactivation of α-cardiac actin gene expression (10). The clone that we isolated contains the complete reading frame of ITF-2b fused to the GAL4 transactivation domain at nucleotide position 506 of the published sequence and ends in the 3'-untranslated region at position 2613 (10).

Since the apparent interaction of two putative inhibitory HLH proteins was unexpected, we wanted to confirm the association of the two proteins under other assay conditions. First, we examined by co-immunoprecipitation experiment to see if *in vitro* translated Id3/HLH462 and ITF-2b proteins could interact with each other without the attached GAL4 DNA binding and transactivation domains used in the yeast two-hybrid assay. Using an antibody directed against the FLAG antigenic epitope, we showed that ITF-2b was co-precipitated with the FLAG-tagged Id3/HLH462 protein. A second helix deletion mutant of Id3 (FLAGHLH462), which exhibited a dramatically reduced inhibitory effect on MCK gene expression, co-precipitated ITF-2b to a much lower extent (Fig. 1A). An unrelated protein, luciferase, was not precipitated at all by the anti-FLAG antibody. The result indicates that the interaction of ITF-2b and Id3 is specific and may be dependent on an intact HLH domain in Id3. The relatively small amount of ITF-2b co-precipitated in comparison with the amount added may reflect the inefficiency of the *in vitro* assay system or steric hindrance by the precipitating antibody.

As a further confirmation of the ability of ITF-2b to associate with Id3, we also analyzed the interaction between these two proteins using the mammalian two-hybrid systems. As shown in Fig. 1B, full-length ITF-2b fused to the VP16 transactivation domain was able to complement an Id3/GAL4 DNA binding domain fusion protein to transactivate a GAL4-regulated promoter in transfected C2C12 cells. The resulting CAT activity was 50–200-fold higher than what was obtained with expression vectors lacking one or the other of the interacting proteins and reached about 20% the level obtained with the covalently linked Gal4-VP16 fusion protein. Thus, the two inhibitory proteins could associate inside mammalian cells with affinity that is consistent with a specific noncovalent interaction.

Next we want to confirm that ITF-2b could indeed inhibit muscle-specific gene expression as reported previously (10) and to examine the potential consequence when two mutually interacting inhibitory HLH proteins were co-expressed in the same cell. We first demonstrated that both Id3/HLH462 and ITF-2b could inhibit the ability of MyoD to transactivate the expression of a muscle-specific creatine kinase (MCK) promoter-regulated CAT reporter gene in a dose-dependent manner.
Interestingly, when ITF-2b and Id3/HLH462 were co-expressed, the inhibitory effect on MCK gene expression was attenuated relative to what was observed when each protein was expressed by itself. This apparent mutual interference of inhibitory activity presumably was due to the formation of Id3/HLH462 and ITF-2b complexes that were no longer capable of inhibiting MCK gene expression.

In order to determine the mechanism through which ITF-2b might inhibit MCK promoter activity, we examined whether ITF-2b protein could form homo- or heterodimers that bind to the consensus MyoD/E protein binding site (E box). As shown in Fig. 3, only a faint DNA-protein complex was detectable when the labeled oligonucleotide was incubated with in vitro translated ITF-2b protein alone, suggesting that ITF-2b either could not homodimerize efficiently or that the homodimer could not bind DNA well. When ITF-2b was co-incubated with the activating E protein, E47, the mobility of the shifted complexes that were formed resembled those formed by ITF-2b and E47 heterodimers, suggesting that the ITF-2b/E47 heterodimer might bind DNA with relatively low affinity. That ITF-2b/E47 heterodimers were formed was supported by the fact that the presence of the ITF-2 protein resulted in a much reduced level of apparent E47 homodimers/DNA complexes as compared with that seen with E47 alone. In contrast to these weak interactions, ITF-2b/MyoD heterodimers formed a very strong DNA complex comparable to the E47/MyoD heterodimer DNA complex, but the formation of both complexes was still efficiently blocked by Id3/HLH462. Taken together, our data indicate that ITF-2b did not inhibit MyoD function because of failure to bind DNA.

Unlike ITF-2b, the human ITF-2 protein (also called E2-2) with the first 49 amino acid missing from its N-terminal was reported to cooperate with MyoD to transactivate an artificial reporter gene, (MEF1)4CAT, containing four tandem MyoD binding sites.

**Fig. 1. Interaction of ITF-2b with Id3/HLH462 in vitro and in a mammalian two-hybrid system.**

*A*, reticulocyte lysates containing the indicated combinations of in vitro-translated [3H]Leu-labeled ITF-2b, and FLAG epitope-tagged Id3/HLH462 wild-type or mutant proteins were used in co-immunoprecipitation studies using an anti-FLAG antibody. In vitro translated luciferase was used as a negative control for nonspecific trapping. Immunoprecipitates were dissolved in 2× SDS sample buffer and analyzed on 15% SDS-polyacrylamide gel electrophoresis gel (first through the fourth lanes). For comparison, ITF-2b- and luciferase-containing reticulocyte lysates equal to the amounts used for immunoprecipitation were also analyzed (fifth and sixth lanes). The gel was fixed, soaked in fluorographic enhancing solution (Amersham Corp.), and exposed to Kodak x-ray film.

*B*, ITF-2b and Id3/HLH462 were subcloned into appropriate mammalian expression vectors to fuse in-frame with the VP16 transactivation domain and the GAL4 DNA binding domain, respectively, to produce the expression constructs pVPITF-2b and pMHLH462. 5 μg of each resulting construct were co-transfected into C2C12 cells with 4 μg of CMVβgal and 2 μg of the reporter construct G5CAT which contains the GAL4 DNA binding sites in its promoter. Negative control cultures were transfected with either the pMHLH462 or pVPITF-2b construct and the reciprocal empty expression vectors pVP16 or pM, respectively, as indicated. Cells transfected with 5 μg of a construct encoding covalently linked VP16 and GAL4 domains were included as a positive control to determine the maximal activation that could be achieved in the test system. The interaction between ITF-2b and Id3/HLH462 was analyzed by CAT assay after normalization for β-galactosidase activity. The data presented was from one experiment with triplicate determinations (average ± S.E.). Similar results were obtained in two other experiments.

**Fig. 2. Effect of ITF-2b and Id3/HLH462 on MCKCAT expression in C2C12 cells.** The indicated amounts of ITF-2b and Id3/HLH462 expression constructs (in pEMSV plasmid) were transfected into C2C12 cells by the calcium phosphate method, together with 4 μg of CMVβgal and 2 μg of a MyoD expression plasmid (pEMC11) and 2 μg of a MCKCAT reporter plasmid. The total amount of plasmid DNA used in each transfection was equalized by adding different amounts of the empty pEMSV vector. The transfection efficiency was normalized according to the β-galactosidase activity in cell extract and the CAT activity was expressed as percent of the activity obtained with pEMCE11 alone without either ITF-2b or Id3 (control). The data represent averages and ranges of duplicate determinations and similar results were observed in three independent experiments.
binding MEF1 sites (4). Since the inhibitory activity of ITF-2b appeared to reside in the N-terminal first 83 amino acids (10), it was assumed that the differential activity of the two proteins was due to the absence of the 49 amino acids. To test this assumption, we also examined the effect of the full-length ITF-2b protein on the MyoD-mediated transactivation of a reporter gene containing three copies of the MEF1 sites (3ESV-CAT). Surprisingly, whereas Id3/HLH462 remained fully inhibitory on this reporter gene, the full-length ITF-2b protein was no longer inhibitory but instead enhanced the transcriptional activity of MyoD (Fig. 4). It thus appeared that ITF-2b-MyoD heterodimerization did not always result in a transcriptionally silent DNA binding complex.

While the preceding data provided clear evidence for the biochemical function of the ITF-2b protein, the biological relevance of this protein to the muscle differentiation process could not be inferred from these results alone. To explore this question further, we have analyzed the mRNA expression patterns of both the activating and "inhibitory" ITF-2 isoforms (ITF-2a and ITF-2b) by RT-PCR during the in vitro differentiation of the myogenic C2C12 cells. Results of this assay indicated that both transcripts were absent in proliferating cells but became detectable as the cells were placed into differentiation-permissive conditions (Fig. 5). In contrast, the Id3 gene was expressed in proliferating C2C12 cells and down-regulated as the cultures were allowed to differentiate. Whether both Id3 and ITF-2b proteins are co-expressed in the same cells at some point during the differentiation process is an interesting question that would need to be confirmed when appropriate antibodies become available.

**DISCUSSION**

We reported in this manuscript the cloning of an inhibitory form of the murine ITF-2 protein, ITF-2b, by virtue of its interaction with another transcriptional inhibitory protein Id3. The interaction was confirmed by in vitro co-immunoprecipitation analysis and DNA binding assay, by functional complementation in the mammalian two-hybrid system and by mu-
Id3 Interaction with ITF2b

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