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Inhibition of muscle-specific gene expression by Id3: requirement of the C-terminal region of the protein for stable expression and function

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ABSTRACT

We have examined the role of an Id-like protein, Id3 (also known as HLH462), in the regulation of muscle-specific gene expression. Id proteins are believed to block expression of muscle-specific genes by preventing the dimerization between ubiquitous bHLH proteins (E proteins) and myogenic bHLH proteins such as MyoD. Consistent with its putative role as an inhibitor of differentiation, Id3 mRNA was detected in proliferating skeletal muscle cells, was further induced by basic fibroblast growth factor (bFGF) and was down-regulated in differentiated muscle cultures. Overexpression of Id3 efficiently inhibited the MyoD-mediated activation of the muscle-specific creatine kinase (MCK) reporter gene. Deletion analysis indicated that the C-terminal 15 amino acids of Id3 are critical for the full inhibitory activity while deleting up to 42 residues from the C-terminus of the related protein, Id2, did not affect its ability to inhibit the MCK reporter gene. Chimeric protein containing the N-terminal region of Id3 and the C-terminus of Id2 was also non-functional in transfected cells. In contrast, wild-type Id3, the C-terminal mutants, and the Id3/Id2 chimera could all interact with the E-protein E47 *in vitro*. Additional studies indicated that truncation of the Id3 C-terminus might have adversely affected the expression level of the mutant proteins but the Id3/Id2 chimera was stably expressed. Taken together, our results revealed a more complex requirement for the expression and proper function of the Id family proteins than was hitherto expected.

INTRODUCTION

A large body of evidence has clearly documented the importance of the basic-helix–loop–helix (bHLH) family of transcription factors in the regulation of muscle-specific gene expression (1,2). Members of this protein family are distinguished by the presence of a helix–loop–helix (HLH) structural domain which mediates their dimerization (1), and a basic region adjacent to the HLH motif that is responsible for binding to the consensus DNA sequence (referred to as the E box) (3). Heterodimerization between the skeletal muscle-specific bHLH proteins, MyoD (4), Myogenin (5,6), Myf5 (7) and MRF4/herculin/myf6 (8–10), with more ubiquitously expressed bHLH proteins such as E12/E47 (11), E2-2 (12,13) and HEB(14), is believed to result in the formation of the functional transcription factor complex (15). Moreover, since the muscle-specific bHLH proteins homodimerized poorly and the E protein homodimers were unable to transactivate muscle-specific gene expression (11), heterodimerization between E proteins and myogenic bHLH proteins appeared to be critical for the activation of muscle-specific genes.

In addition to the E proteins and the myogenic families of bHLH proteins, a third group of HLH proteins lacking the basic region in front of the HLH motif has also been discovered (16–19). The first member of this group, Id1, was identified in myogenic cells, and was shown to bind preferentially to E proteins both *in vitro* (16,17) and in intact cells (20). Since Id1 could inhibit the binding of MyoD/E protein complex to E-box-containing oligonucleotides (16,17), and its mRNA level was down-regulated after muscle differentiation (20), the Id1 protein has been postulated to act as a dominant inhibitor of muscle differentiation. More recently, three other independent Id-family genes, Id2, Id3 (also known as HLH462) and Id4 have been isolated (17–22). Like Id1, all three proteins could interact with E proteins and inhibit its binding to the E-box motif *in vitro* (16–18,20). In particular, since Id3 was originally identified as a serum- and growth factor-inducible early response gene (18), induction of Id3 might contribute to the commonly observed inhibition of *in vitro* muscle differentiation by specific growth factors (23,24). Although Id3 exhibited a rather broad spectrum of expression in a variety of tissues (18,19), its expression in myogenic cells has never been directly documented.

Using the myogenic C2C12 cell line as a model system, we have examined in this study the expression level of Id3 mRNA, and analyzed the structure–function relationship of the Id3 protein as a potential inhibitor of muscle-specific gene expression. Our results indicated that Id3 transcripts were present in proliferating C2C12 cells, and that their expression level could be further induced by basic fibroblast growth factor (bFGF), a potent inhibitor of muscle differentiation (23,24). Moreover, our data showed that Id3 mRNA declined upon muscle cell differentiation and that overexpression of Id3 could efficiently inhibit the

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expression of a muscle creatine kinase (MCK) reporter gene. In contrast to a report which showed that the HLH domain of Id1 is necessary and nearly sufficient for its biological activity (25), our study revealed that the C-terminus of the Id3 protein was important for its full inhibitory effect and stable expression. Furthermore, a chimeric protein containing the Id3 N-terminal region and Id2 C-terminus was also inactive in transfected cells even though protein expression appeared to be normal. Interestingly, both the C-terminal truncated Id3 mutants and the Id3-Id2 chimeras retained their ability to interact with the E protein E47 *in vitro*, and the mutant Id3 proteins were able to inhibit specific DNA binding by E47 protein complexes, suggesting that the mutant and chimeric protein structures are not grossly altered. Taken together, these results indicated that structural domains in the various Id proteins are not freely interchangeable and that the biological action of Id3 might require additional functions that depends on its C-terminal residues.

MATERIALS AND METHODS

Plasmids

The eukaryotic expression vector pEMSV and the MyoD expression construct pEMC11 were both generous gifts from Drs Lassar and Weintraub. The reporter gene construct, pMCKCAT, containing 3300 bp of the 5′ region of the mouse muscle creatine kinase gene was produced by transferring the entire relevant region from –3300MCKCAT (a gift from Drs Buskin and Hauschka) into the pBluescript vector (Stratagene).

Expression construct of Id3 (pEMId3) was generated by ligating an *Xho*I fragment from the pBluescript-derived pHLH462 plasmid (American Type Culture Collection) containing the entire Id3 open reading frame into the blunt-ended unique *Eco*RI site of pEMSV. The various Id3 mutants were generated in the pBluescript vector by appropriate restriction digests. The N-terminal and C-terminal truncation mutants were designed to utilize existing in-frame initiation and termination codons in the vector or a three-frame termination codon sequence inserted downstream. The entire coding region of the mutants are then transferred into the pEMSV vector to generate the expression constructs. The extra amino acids that resulted from this and other manipulations are indicated in the legends to the figures containing schematic illustrations of the various constructs.

To generate the Id2 expression construct (pEMId2), an *Xho*I–*Eco*RI fragment containing the entire open reading frame of Id2 and part of the 3′ untranslated region was inserted into the pBluescript vector carrying the three-frame termination codons and an *Xho*I–*Bam*HI fragment from this construct was transferred into pEMSV. The C-terminal truncation mutants were then generated by appropriate restriction digest of pEMId2 followed by religation to make use of the downstream three-fame termination codons. The chimeric Id3/Id2 and Id2/Id3 constructs were generated by interchanging appropriate restriction fragments generated from the existing restriction sites. All mutant constructs were sequenced (26) to confirm the correct reading frame.

For *in vitro* studies, wild-type and mutant Id3 constructs tagged at their N-terminus with the FLAG epitope (27) were generated by polymerase chain reaction (PCR) which changes the ATG initiation codon to CTT. The PCR products were cloned into the pBluescript vector and the correct reading frame was confirmed by dideoxynucleotide sequencing. The biological activity of the wild-type FLAG-tagged Id3 protein was confirmed by its ability to inhibit MyoD mediated-transactivation of MCK reporter gene expression (data not shown). Subsequent to the completion of this study, we became aware of a single PCR generated mutation in all our FLAG-tagged constructs resulting in the change of Ala^{25} to Val. This mutation however appeared to have no effect on the *in vitro* protein interaction of either the wild-type or C-terminal truncated Id3 mutants (see below).

For immunoblot studies, the HA antigenic epitope tag was fused to the N-terminus of the Id3 protein by PCR using the pHLH462 plasmid as template and the synthetic oligonucleotide (CGC AAG CTT ACC ATG GGA TAC CCC TAC GAC GTC CCC GAC TAC GCC AAG GCG CTG AGC CCG) as the 5′ primer and the T7 sequencing primer as the 3′ primer. The PCR product was cloned into the *Hin*dIII and *Not*I digested pRC/CMV vector (Invitrogen) to produce the construct pHA-Id3. Plasmids expressing HAId3Δ15, HAId3Δ32, HAId3ΔIn and HAId3/Id2 were constructed by replacing the *Sac*II–*Xba*I fragment of pHA-Id3 with corresponding *Sac*II–*Xba*I fragments from plasmids containing Id3Δ15, Id3Δ32, Id3ΔIn and Id3/Id2 respectively.

Cell culture and transfection

C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Biocell) and (50 µg/ml) gentamycin (28). Each wild-type or mutant construct was transfected into C2C12 cells along with the MCK-CAT reporter gene, and the MyoD expression vector pEMC11 (16) by calcium phosphate precipitation (29). After culturing for one day in DMEM containing 10% FBS to allow for recovery, the transfected cells were rinsed three times with Hank's balanced salt solution (HBSS) and cultured in differentiation-permissive medium (DMEM containing 5% horse serum) for an additional 3 days before harvesting. The cell harvest and CAT assay were carried out as described (29). Products of the CAT assay were analyzed on thin layer chromatography (TLC) plate and visualized by autoradiography. The radioactive bands were excised and quantified by scintillation counting.

Northern blot analysis

Total RNAs were extracted by the guanidinium isothiocyanate-phenol-chloroform extraction method (30), fractionated by electrophoresis on 1% agarose-formaldehyde gel (31) and transferred onto nitrocellulose filters. The radioactive cDNA probe for Id3 was prepared by random-primed labeling of a fragment corresponding to the 3′ untranslated region (32). The cDNA probes for MCK and pCHOB were labeled in the same manner and hybridization was carried out as described previously (31). Following hybridization, the blots were washed twice with 1× SSC (0.15 M NaCl, 15 mM NaCitrate) and 0.1% SDS for 15 min at 50°C. The blots were then exposed to Kodak XAR film at –70°C.

In vitro transcription and translation

The FLAG-epitope-tagged Id3 mutants were produced using T3 polymerase and the TNT® transcription/translation coupled rabbit reticulocyte lysate system (Promega) in the presence of 0.5 µCi/µl ^{3}H-leucine (168 Ci/mmol) (Dupont) according to the manufacturer's protocol. E47 protein was made by coupled transcription and translation from a linearized pCITE construct (Novagen) carrying the E47 insert, using the T7 polymerase MyoD protein was produced from a pBluescript construct

containing the full length MyoD insert. The translation products were quantified by TCA precipitation according to the manufacturer's protocol.

Co-immunoprecipitation

Equivalent amounts of *in vitro* translated FLAG-tagged wild-type or mutant Id3 proteins were incubated with *in vitro* translated E47 or MyoD at 37°C for 20 min. Anti-FLAG M2 monoclonal antibody (IBI) (7 µg) was added and the sample volume was adjusted to 100 µl with immunoprecipitation buffer (10 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.2% Triton-X100). After 4 h at 4°C, 20 µl of protein G Sepharose beads (50% slurry) were added and the incubation was continued overnight with constant rocking. The beads were then washed four times with 500 µl immunoprecipitation buffer per wash followed by the addition of 20 µl SDS sample buffer (50 mM Tris–HCl pH 6.8, 12% glycerol, 4% SDS, 0.1 M DTT, 1 mM EDTA, 0.05% bromophenol blue). Samples were boiled for 10 min and centrifuged. The supernatant was loaded onto 15% SDS-polyacrylamide gel and fractionated by electrophoresis. The gel was fixed, incubated in Amplify® fluorographic enhancer (Amersham), dried and exposed to X ray film.

DNA binding assay

A 25 bp double-stranded oligonucleotide containing the MyoD binding site (MEF1) from the MCK enhancer was labeled with [γ-^{32}P]ATP (Dupont) by phosphorylation with polynucleotide kinase (16). *In vitro* translated proteins were mixed and preincubated together for 20 min at 37°C prior to the beginning of the assay. The volume of each sample was adjusted to 5µl with a mock-translation reaction mix containing no translated protein. A 15 µl aliquot of a DNA binding cocktail was then added and the final DNA binding assay mix, consisting of 20 mM HEPES pH 7.6, 50 mM KCl, 1 mM DTT, 5% glycerol, 1 µg of double-stranded poly(dI-dC) (Pharmacia), 0.5 ng γ-^{32}P-labeled probe (4×10^5 c.p.m.) and the *in vitro* translated proteins, was incubated at room temperature for another 15 min. The assay was terminated by loading the sample onto a 5% non-denaturing gel and electrophoresed using the Tris-borate–EDTA buffer system (16). Following electrophoresis, the gel was washed, dried and autoradiographed.

Immunoblot studies for protein expression

HA-tagged expression constructs were introduced into subconfluent cultures of COS-1 cells in 6-well cluster dishes by DEAE-Dextran- mediated transfection. Cells were incubated in 400 µl phosphate buffer saline (PBS) with 1 µg of plasmid and 10 µl DEAE-Dextran (20 mg/ml) at 37°C for 30 min, with gentle shaking every 5 min. An aliquot (4 ml) of DMEM+0.5%FBS containing 100 µM chloroquine (Sigma) was added and the incubation continued for 2.5 h. The cells were then osmotically shocked by treatment with 10% DMSO (Sigma) for 2.5 min at room temperature, and allowed to recover for 72 h in DMEM+10% FBS before harvest. Cells were rinsed twice with ice-cold PBS, scraped into TEN buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA), and pelleted by centrifugation. The pellets were solubilized in 2× Laemmli sample buffer and boiled for 15 min before loading onto a 15% SDS-polyacrylamide gel for electrophoresis. The proteins were electro-transferred onto nitrocellulose paper and immunoblotted with anti-HA monoclonal antibody using the enhanced chemiluminescence technique according to manufacturer's protocol (Dupont).

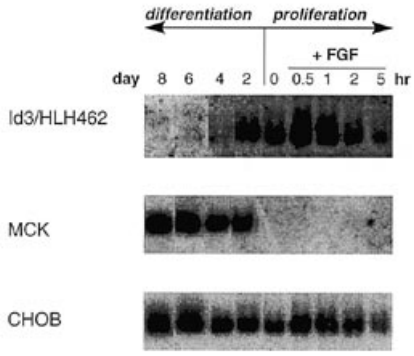

Figure 1. Id3 mRNA induction by bFGF and expression during *in vitro* differentiation of C2C12 cells. Proliferating C2C12 cells, grown in DMEM plus 10% FBS were either stimulated with 4 ng/ml bFGF for the times indicated (from 0 to 5 h) or rinsed and fed differentiation-permissive medium containing 5% horse serum and cultured for the indicated number of days. Total RNA was harvested and analyzed by northern blot hybridization to radioactive probes corresponding to 3′ untranslated region of Id3 (top panel), MCK, an indicator of myogenic differentiation (center panel), and pCHOB, which recognizes a constitutively expressed mRNA and serves a normalization control for RNA loading and transfer (bottom panel).

RESULTS

Expression of Id3 in C2C12 cells is regulated by fibroblast growth factor and by the physiological state of the culture

It was reported previously that the Id3 gene can be induced in mouse 3T3 fibroblasts by serum, platelet derived growth factors (PDGF), phorbol 12-myristate 13-acetate (TPA) and forskolin, and that the induced mRNA of Id3 has a short half-life characteristic of primary response genes (18). Here we show that the expression of Id3 could be detected in proliferating C2C12 mouse myoblasts and that its expression level was further induced in a transient manner by brief treatments with basic fibroblast growth factor (bFGF) (Fig. 1, top panel). Moreover, when C2C12 cells were induced to differentiate by exposure to a reduced-mitogen medium, the basal level of Id3 mRNA declined with time to almost undetectable levels in the differentiated cells. In contrast, expression of the muscle-specific creatine kinase (MCK) gene was undetectable in the proliferating cells but increased during exposure to the differentiation-permissive conditions. (Fig. 1, middle panel).

The effect of wild type Id3 and its mutants on MCK gene expression

Although the Id3 protein has been shown to inhibit *in vitro* binding of E protein homodimers and E protein/MyoD heterodimers to the E-box motif of the MCK enhancer (18), whether Id3 can inhibit E protein function in intact skeletal muscle cells has not been confirmed. We showed here that wild-type Id3 could indeed inhibit the expression of a MCK-CAT reporter gene when Id3 and MyoD expression vectors were cotransfected into C2C12 cells (Fig. 2). The inhibitory effect was dose-dependent in that cotransfection of 1–3 µg of Id3 expression vector with 2 µg of the

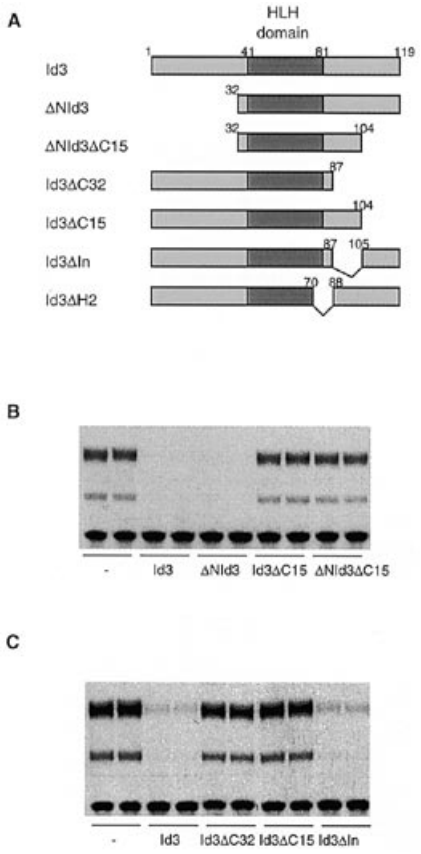

Figure 2. Effect of wild type and mutant Id3 proteins on MCK reporter gene expression. (**A**) A schematic diagram of the wild type and mutant Id3 constructs used in this assay. Deletion of the last 32 (Id3ΔC32) or 15 amino acids (Id3ΔC15) resulted in the addition of residues QFAL or PA respectively at the C-terminus of each protein. The N-terminal deletion constructs contain two additional residues (GR) at the N-terminus. (**B**) and (**C**) C2C12 cells were transfected with 10 μg of the indicated wild-type or mutant Id3 constructs together with 2 μg of MyoD expression construct, 2 μg of MCK-CAT reporter gene and 5 μg of CMVβgal. Cell extracts expressing equivalent amounts of β-galactosidase activity were assayed for CAT activity using ^{14}C-labeled chloramphenicol as substrate. The reaction products were analyzed by thin layer chromatography (TLC) and exposed to X-ray film overnight. The figure showed results of one representative experiment.

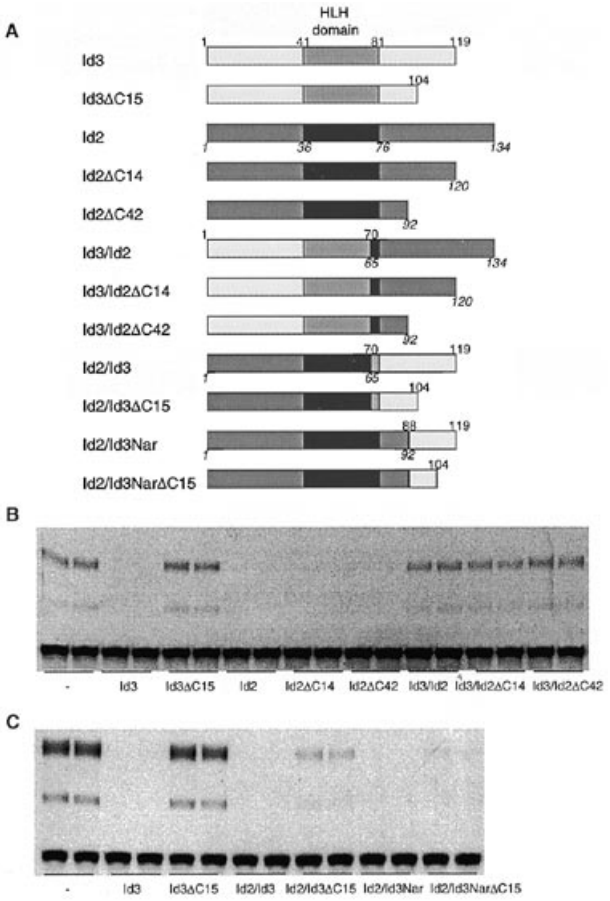

Figure 3. Effect of Id3-Id2 chimeric proteins on MCK reporter gene expression. (**A**) A schematic diagram of Id3, Id2 and various C-terminal deletion mutant and chimeric protein constructs used in this study. Deletion of the C-terminal 14 (Id2ΔC14) or 42 amino acids from Id2 (Id2ΔC42) resulted in the addition of residues LQPA and NSCSPLN respectively at their C-termini. (**B**) and (**C**) The indicated Id3, Id2 or chimeric Id3/Id2 constructs were transfected into C2C12 cells as described in Figure 2. MCK-CAT gene expression was determined by CAT assay and the TLC plate was exposed to X-ray film overnight. The result from a representative experiment is shown. (B) Effect of chimeric constructs with Id3 N-terminus and Id2 C-terminus. (C) Effect of the reciprocal chimera with Id2 N-terminus and Id3 C-terminus.

MyoD expression vector (pEMC11) gave rise to ~50% inhibition (data not shown), while near maximal (>90%) inhibition of the reporter gene activity was observed when 10 μg of wild-type Id3 expression vector was used. Interestingly, deletion of the last 15–32 amino acids from the Id3 C-terminal region (Id3ΔC15 and Id3ΔC32) drastically reduced its ability to inhibit the MCK-CAT reporter gene whereas deletion of an 'internal' region from residues 89 to 103 (Id3ΔIn) had little or no effect on the inhibitory activity (Fig. 2C). Deletion of much of the N-terminal region of Id3 (from residues 1 to 32, referred to as ΔNId3) also had no effect on the inhibitory activity of the wild-type protein and neither reduced nor augmented the effect of the C-terminal deletions (Fig. 2B). Similar results were observed with a reporter gene harboring three copies of the MCK E-box motif placed in front of the SV40 promoter (data not shown). Taken together, these results indicate that Id3 effectively inhibits gene expression mediated by the muscle-specific E-box, and that a region from residues 104 to the C-terminus of the Id3 protein may be critical for its transcriptional inhibitory activity.

The effect of Id3 and Id2 chimeras on MCK-CAT reporter gene expression

To determine if the requirement for the C-terminal region is unique to Id3 or is a common property shared by other members of the Id family proteins, we examined next the effect of wild-type and mutant Id2 proteins on MCK-reporter gene expression. As expected, wild-type Id2 also inhibited MCK reporter gene expression when overexpressed in C2C12 cells (Fig. 3B). However, in contrast to the Id3 C-terminal mutations, there was no reduction in the inhibition of MCK gene expression when either 14 or 42 amino acids were removed from the C-terminal region of Id2 (Fig. 3B). To further investigate the molecular basis for the differential action of Id3 and Id2 mutants on the MCK reporter gene, we made chimeric Id2-Id3 constructs, Id2/Id3 and Id2/Id3Nar, in which either the entire C-terminal region (starting at residue 117 in the second helix) or the

last 42 amino acids (residues ^{144}Arg to ^{186}Gly) respectively of Id2 was replaced by the corresponding C-terminal regions of Id3, and tested the ability of these constructs to inhibit MyoD-mediated MCK transactivation. We found that both chimeric proteins, Id2/Id3 and Id2/Id3Nar, exhibited full inhibitory activity similar to wild-type Id3 or Id2 (Fig. 3C). Deletion of 15 amino acids from the Id3 C-terminus of the Id2/Id3 chimera (Id2/Id3Δ15) resulted in only a slight loss of inhibitory activity (Fig. 3C), while deletion of 15 amino acids from the Id2/Id3Nar chimera (Id2/Id3NarΔC15) resulted in an even smaller loss of inhibitory activity. Thus, in the context of the Id2 protein, the last 15 amino acids at the C-terminal region of Id3 became much less important for full inhibition of MCK gene expression.

To address this issue further, the reciprocal chimeric constructs were generated by replacing the C-terminal region of Id3 starting from the middle of the second helix (residue 67) with the corresponding full-length or truncated Id2 C-terminus (Id3/Id2, Id3/Id2ΔC14 and Id3/Id2ΔC42). These chimeric constructs were tested for their ability to inhibit MCK gene expression under the same experimental conditions described above. Strikingly, both the full-length chimeric protein (Id3/Id2) and the C-terminal 14 amino acid-deletion mutant (Id3/Id2ΔC14) displayed dramatically reduced inhibitory activity when compared with either Id3 or Id2 and the mutant chimeric protein in which the last 42 amino acids of the Id2 C-terminal was deleted (Id3/Id2ΔC42) was essentially devoid of inhibitory activity (Fig. 3C). Results from these experiments are summarized in Table 1.

Table 1. Summary of the effect of wild type and mutant Id3 and Id2 constructs on MCK promoter activity in transfected cells

Name of construct	MCK activity (% control) (mean ± S.E.M.)	No. of replicates
EMSV (control)	100	23
Id3	7.2 ± 0.8	23
ΔNId3	6.7 ± 0.9	4
Id3ΔC32	84.5 ± 4.3	6
Id3ΔC15	92.9 ± 3.2	16
ΔNId3ΔC15	71.1 ± 4.3	4
Id3ΔIn	21.6 ± 4.4	6
Id3ΔH2	64.6 ± 2.4	4
Id2	9.0 ± 2.2	8
Id2ΔC14	5.5 ± 0.9	6
Id2ΔC42	6.0 ± 1.2	8
Id3/Id2	75.0 ± 3.4	8
Id3/Id2ΔC14	71.4 ± 4.9	8
Id3/Id2ΔC42	88.5 ± 4.2	8
Id2/Id3	3.6 ± 0.7	6
Id2/Id3ΔC15	28.8 ± 9. 7	6
Id2/Id3Nar	1.8 ± 0.1	4
Id2/Id3NarΔC15	5.0 ± 0.5	4

Expression levels of mutant and chimeric Id3 proteins

To address the reason for the lack of biological effect of the mutants and Id3/Id2 chimera, we transfected cytomegalovirus (CMV) promoter-regulated expression constructs of the various proteins tagged with an HA-antigenic epitope into Cos cells and analyzed the level of protein expression by immunoblotting using anti-HA antibody. As shown in Figure 4, whereas the wild type Id3 and the Id3ΔIn proteins were readily expressed under such conditions, neither of the C-terminal truncated mutants (Id3ΔC15 nor Id3ΔC32) was detectable. The apparent lack of expression is not simply due to a reduced transfection efficiency since the C-terminal truncated mutant proteins were still undetectable when wild-type and mutant constructs were co-transfected into the same culture. Interestingly, and in contrast to what was seen with the truncation mutants, the chimeric Id3/Id2 protein was expressed to levels comparable with the wild-type Id3 protein no matter whether it was transfected alone or cotransfected with the wild-type construct.

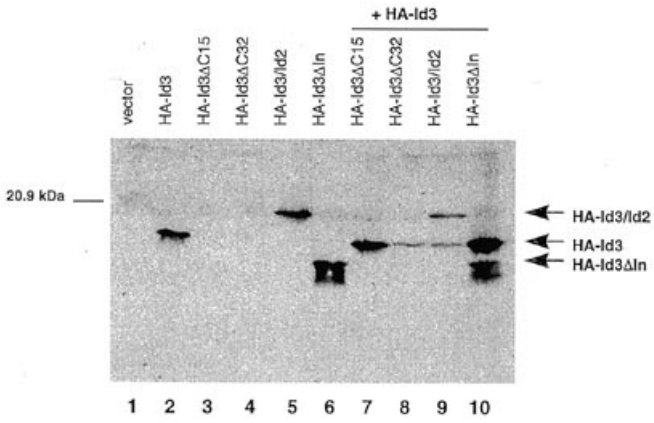

Figure 4. Expression of wild type and mutant HA-Id3 proteins and HA-Id3/Id2 chimera in transfected COS cells. 1 µg of each Id3 expression construct (as indicated) was either transfected alone (lanes 1–6) or co-transfected with 1 µg of the wild type construct (lanes 7–10) into COS cells by the DEAE-Dextran method. Cells were lysed 72 h post-transfection in 2× Laemmli sample buffer and analyzed by 15% SDS-PAGE. The samples were then electro-transferred to a nitrocellulose membrane, incubated with anti-HA monoclonal antibody and detected by ECL.

Interactions of wild type and mutant Id3 with the E proteins E47

A trivial reason for the lack of stable expression of the truncated Id3 proteins might be that the mutant proteins fail to fold properly and thus are rapidly degraded. Similarly, even though the chimeric Id3/Id2 protein was stably expressed, there is a remote possibility that the protein might still be improperly folded. One indirect way to see if these proteins are properly folded is to determine if they could still interact with E-proteins. To address this possibility, the binding of wild-type and mutant Id3 proteins to E47 protein was analyzed *in vitro* by co-immunoprecipitation. Wild-type and mutant Id3 proteins tagged with the FLAG antigenic epitope were produced *in vitro* in the presence of ^{3}H-leu, and immunoprecipitated with a monoclonal anti-FLAG antibody following incubation with ^{3}H-leu-labeled E47 protein made in a similar manner. As shown in Figure 5A, binding of the wild-type and all the C-terminal mutant Id3 proteins to E47 was almost indistinguishable. Moreover, neither the wild-type Id3 nor the C-terminal 15 amino acid-deletion mutant (Id3ΔC15) interacted with MyoD, suggesting the C-terminal truncation did not alter the dimerization specificity of the resulting protein (Fig. 5B). Similarly, the Id3/Id2 chimeric protein which was non-functional in intact cells still interacted with E47 protein *in vitro* (Fig. 5A) while an Id3 mutant in which the second helix domain was

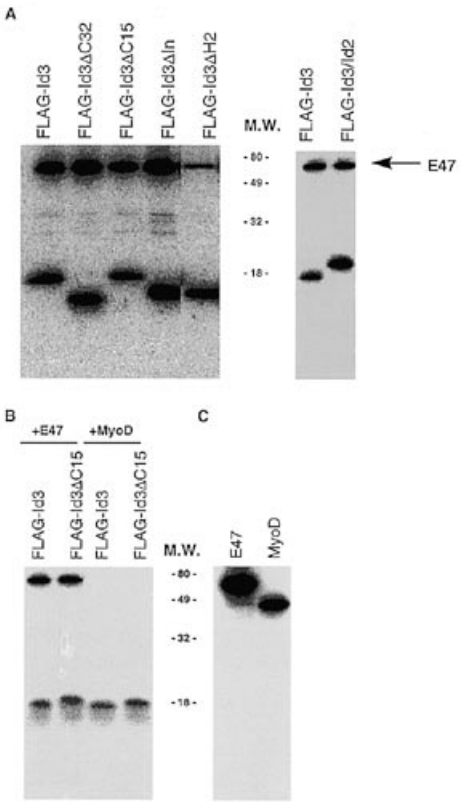

Figure 5. Co-immunoprecipitation of *in vitro*-translated wild type and mutant Id3, and chimeric Id3/Id2 proteins with E47. Wild-type, mutant and chimeric Id3 proteins tagged with FLAG-antigenic epitope at their N-termini were generated by *in vitro* transcription and translation in the presence of ^{3}H-Leu. E47 and MyoD proteins without the FLAG-tag were made similarly. (**A**) Various flagId3 proteins were mixed with E47 protein at a ratio of 3:1 and immunoprecipitated using a monoclonal antibody (M2) directed against the FLAG antigenic epitope. The co-immunoprecipitates were resolved on SDS-PAGE gel and autoradiographed. (**B**) A comparison of the ability of wild-type and truncated flagId3 proteins to immunoprecipitate E47 and MyoD protein. (**C**) 5 µl aliquots of *in vitro* translated E47 or MyoD protein used in the co-immunoprecipitation study.

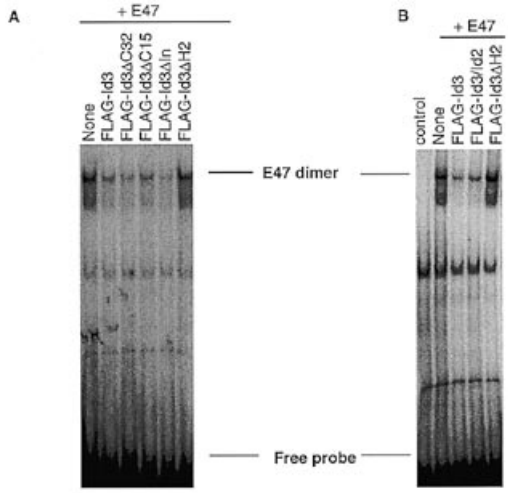

Figure 6. Effect of wild-type and mutant Id3, and chimeric Id3/Id2 proteins on the binding of E47 to the MCK E-box motif. *In vitro*-translated Id3 and E47 proteins were incubated at a ratio of 1:1 at 37°C for 20 min. The ^{32}P-labeled oligonucleotide probe corresponding to the MCK E-box motif was then added and the mixture was incubated for a further 15 min at room temperature before electrophoresis on a 5% non-denaturing acrylamide gel and autoradiographed. (**A**) Effect of wild-type and truncation mutants. (**B**) Effect of wild-type and Id3/Id2 chimeric proteins.

deleted (flagId3ΔH2) exhibited a markedly reduced ability to interact with E47. The small residual interaction with the ΔH2 mutant may be due to some non-specific binding under the condition of the assay or because interaction with E47 may in fact stabilize the truncated HLH domain of the mutant protein. Nevertheless, these results are consistent with the idea that the integrity of the helix–loop–helix structure is required for the heterodimerization of Id3 to E47, but that the C-terminal region is dispensable for such *in vitro* interactions. Moreover, the ability of the mutant and chimeric Id3 proteins to interact with E47 argues that these proteins are able to fold into the proper conformation, at least under the *in vitro* conditions. Finally, the result also argued that the unintentional Ala^{25}→Val mutation generated during PCR had not affected the specificity of the Id3 protein interaction.

Inhibition of DNA binding of E47 by wild type and mutant Id3 and by the Id3/Id2 chimera

To confirm that the truncated mutants and the chimeric Id3/Id2 protein were able to interact effectively with the E47 protein, we have also examined the ability of these proteins to interfere with E47 dimerization and binding to specific DNA sequences. As shown in Figure 6, both wild-type Id3, the C-terminal truncated mutant proteins, flagId3ΔC32 and flagId3ΔC15, and the chimeric Id3/Id2 protein efficiently inhibited the binding of E47 to an oligonucleotide fragment containing the E47 binding site. Similar results were obtained in the presence of *in vitro* translated MyoD suggesting that the mutant proteins could also interfere with the formation of E47/MyoD heterodimer and prevent their binding to DNA (data not shown). In contrast, deletion of the second helix, as would be expected, substantially reduced the ability of the mutant protein (flagId3ΔH2) to inhibit the binding of E47 to DNA. Our data therefore indicate that neither the C-terminal deletions nor the fusion between Id3 and Id2 domains reduced the ability of the mutant proteins to interact with E protein, thus arguing against any severe alteration of their overall protein conformation. In addition, our data indicate that an intact HLH domain is essential and indeed might be sufficient for Id3 to interact with E47 *in vitro*.

DISCUSSION

Although Id3 has been shown previously to interfere with the *in vitro* interaction between E protein and the MCK E-box enhancer (18), the physiological relevance of this interaction has never been confirmed. In this study, we show for the first time that Id3 mRNA was expressed in the C2C12 skeletal muscle cell line and that its expression could be further induced by basic fibroblast growth factor (bFGF), a potent inhibitor of the myogenic process (23,24). The expression level of Id3 was higher in proliferating myoblasts and was drastically reduced in differentiated muscle cultures (Fig. 1). These data are consistent with reports from other cell systems (18,21,33) and provide further support for the putative role of the Id family of transcription-inhibitors in regulating cell growth and differentiation (20,26,33). Although

expression of the MCK mRNA became detectable before the complete disappearance of the Id3 transcripts, Id3 expression might be regulated also at the protein level as had been reported for Id1 (20). Moreover, the complete disappearance of Id3 may not be a prerequisite for the onset of differentiation, since the differentiation process is known to be regulated by a delicate balance between positive and negative regulatory influences (20,29,34,35). Alternatively, a more tantalizing although speculative interpretation is that different Id family members might have been evolved to regulate different muscle-specific genes and that under physiological conditions, the Id3 protein might regulate genes that are expressed with slower kinetics than MCK.

Nevertheless, even though Id3 might not be the prime regulator of MCK under more physiological conditions, the apparent involvement of the Id3 protein in the muscle differentiation process was corroborated by our finding that overexpression of wild-type Id3 efficiently blocked the expression of an MCK reporter gene in transfected C2C12 cells (Fig. 2). Surprisingly, both the C-terminal region of Id3, as well as the HLH domain, were required for full inhibition of MCK reporter gene activity (Fig. 3), even though the C-terminal-truncated mutant Id3 proteins were able to co-immunoprecipitate the E47 protein (Fig. 5) and prevented the binding of E47 proteins to DNA *in vitro* (Fig. 6). Similar truncations of the Id2 protein did not significantly affect the biological activity of Id2 (Fig. 3B). Likewise, the HLH domain of Id1 was nearly sufficient by itself for *in vitro* interaction with E proteins and for the inhibition of MyoD-mediated transactivation in transfected cells (25). The requirement of the C-terminal residues is thus a property of Id3 not shared by other Id family proteins.

The apparent involvement of the C-terminal region in modulating the biological activity of Id family proteins is, however, not without precedent. An alternatively spliced isoform of Id1 (Id1.25) with an alternative C-terminus replacing the last 13 amino acids of Id1 has been shown to homodimerize *in vitro* while the original Id1 isoform only dimerized with E proteins and to a smaller extent with MyoD (36). Interestingly, both the Id2 gene (37) and the Id3 gene (unpublished observation) contain introns at roughly analogous positions in their C-terminal tails. If alternatively spliced transcripts were made, the resulting protein isoforms are likely to exhibit drastically different biological activity.

The molecular basis for the apparently unique requirement of the C-terminus is not completely evident. Immunoblot analysis (Fig. 4) suggests that truncating the C-terminal of Id3 might have adversely affected the expression level of the protein. This decrease in protein expression cannot be explained by the coincidental removal of the 3′UTR in our mutant constructs, since wild-type constructs completely lacking the entire 3′UTR functioned normally in transfected cells (unpublished observations). The apparent lack of expressed protein also cannot be readily explained by the destabilization of the mutant proteins due simply to a drastic reduction in protein size, since N-terminal truncated Id3 and C-terminal truncated Id2 proteins exhibited nearly normal biological activity, and the Id3ΔIn mutant with an internal deletion of 18 amino acid residues (89–105) at the C-terminus was also expressed normally and exhibited normal biological activity. The possibility that the C-terminal deletion had somehow affected proper overall folding of the mutant proteins inside the cell such that the misfolded protein was rapidly degraded also seems rather unlikely since the *in vitro* translated proteins functioned normally *in vitro*, arguing that the structural integrity

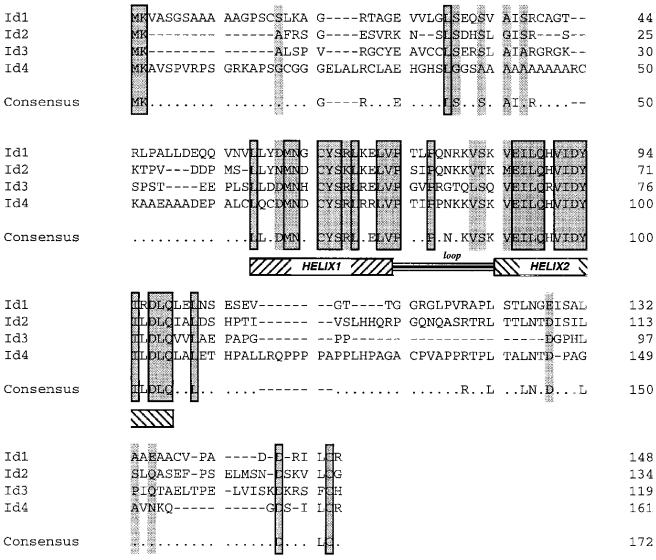

Figure 7. Alignment of Id3 with other members of the Id protein family. Computer generated alignment of the mouse Id family proteins that have been identified to date. The helix–loop–helix domain is indicated underneath the aligned sequences. Amino acids showing 100% identity in all four Id proteins are boxed and regions with conservative substitution are shaded. A consensus sequence for the Id family proteins is shown on the last line.

of these mutant proteins are not severely compromised. A more plausible explanation might be that a specific interaction between the C-terminal 15 amino acids and another region of the Id3 protein or other as yet unidentified proteins is necessary to maintain Id3 protein stability. Removal of this region would destroy this stabilizing interaction, causing the protein to be rapidly degraded. In this regard, others have reported that the Id1 protein level might be regulated at a post-transcriptional step during muscle differentiation (20). It would not be too surprising if biochemical mechanisms regulating the stability of Id3 protein also exist.

The importance of the C-terminal residues in mediating the stable expression and biological function of Id3 was addressed further using chimeric Id3/Id2 protein constructs. To our surprise, the chimeric protein Id3/Id2 (in which the N-terminal region of the Id3 was fused to the C-terminal end of Id2 at the middle of their second helices) could be expressed to a level virtually indistinguishable from that of wild-type Id3 (Fig. 4) but yet remained non-functional in the transfected cells (Fig. 3) This result argues, first of all, that either the replacement of the C-terminal portion of the Id3 by Id2 has abrogated the requirement for the C-terminal 15 amino acids or that the terminal tail of Id2 protein may have substituted for the critical Id3 C-terminus in enhancing the stability/expression of the chimeric protein.

Since the Id3/Id2 chimeric protein appeared to be stably expressed (Fig. 4), its inability to inhibit MCK reporter gene expression (Fig. 3) remained an enigma. The possibility that the chimeric protein was improperly folded seems very unlikely since the splicing of the two proteins should have resulted only in a single amino acid change (R72→H72) in the second helix. The integrity of the HLH domain was substantiated by our finding that the chimeric protein could interact well with E proteins *in vitro*, although we have not ruled out the possibility that the chimera might interact with slightly reduced affinity with E proteins that

remained undetected under our assay conditions. Alternatively, fusion of the two proteins could have affected the subcellular localization of the chimera so that it is no longer available for interaction with E proteins. Finally, a more unorthodox explanation of our finding might be that sequestration of E proteins is not the only mechanism involved in the inhibition of muscle-specific gene activation by the Id3 protein. Since maximal activation of muscle-specific genes requires additional transcriptional activators other than the MyoD/E protein families (12,38–40), a plausible, albeit speculative interpretation of our data is that the Id3 protein, either alone or in complex with E proteins, might interact with and inhibit the activity of these transcription factors and that the chimeric Id3/Id2 protein might be deficient in this interaction. In support of this possibility, we have observed that co-expression of E proteins only partially reversed the inhibitory effect of Id3, suggesting that other limiting component(s) might be involved (unpublished observation).

Regardless of the molecular basis, results from this study clearly demonstrated that the structural domains of the various Id proteins were not freely interchangeable. Except for a short stretch of residues at the very end of the proteins, the C-terminal regions of the various Id proteins are quite divergent. The HLH domains of the Id proteins are highly conserved (25,41,42) but there are several amino acids substitutions at the loop and second helical regions of Id3 that differs from other Id proteins (Fig. 7). Whether the presence of these atypical residues somehow accounts for the unique dependence of Id3 on its C-terminus for stable expression and biological function remains to be determined. As the evidence pointing to the importance of the Id family proteins in regulating cell growth (26,43–45) and differentiation (46–49) continues to mount, potential functional heterogeneity between various members of the Id protein family is also becoming increasingly apparent (50,51). A more complete understanding of the structural basis that underlies the functional heterogeneity will become an increasingly crucial issue that needs to be addressed.

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