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Muscle LIM Protein Promotes Myogenesis by Enhancing the Activity of MyoD

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The muscle LIM protein (MLP) is a muscle-specific LIM-only factor that exhibits a dual subcellular localization, being present in both the nucleus and in the cytoplasm. Overexpression of MLP in C2C12 myoblasts enhances skeletal myogenesis, whereas inhibition of MLP activity blocks terminal differentiation. Thus, MLP functions as a positive developmental regulator, although the mechanism through which MLP promotes terminal differentiation events remains unknown. While examining the distinct roles associated with the nuclear and cytoplasmic forms of MLP, we found that nuclear MLP functions through a physical interaction with the muscle basic helix-loop-helix (bHLH) transcription factors MyoD, MRF4, and myogenin. This interaction is highly specific since MLP does not associate with nonmuscle bHLH proteins E12 or E47 or with the myocyte enhancer factor-2 (MEF2) protein, which acts cooperatively with the myogenic bHLH proteins to promote myogenesis. The first LIM motif in MLP and the highly conserved bHLH region of MyoD are responsible for mediating the association between these muscle-specific factors. MLP also interacts with MyoD-E47 heterodimers, leading to an increase in the DNA-binding activity associated with this active bHLH complex. Although MLP lacks a functional transcription activation domain, we propose that it serves as a cofactor for the myogenic bHLH proteins by increasing their interaction with specific DNA regulatory elements. Thus, the functional complex of MLP-MyoD-E protein reveals a novel mechanism for both initiating and maintaining the myogenic program and suggests a global strategy for how LIM-only proteins may control a variety of developmental pathways.

Skeletal myogenesis involves a series of well-orchestrated developmental events in which embryonic stem cells become committed to the myogenic cell lineage, producing muscle precursor cells known as myoblasts (reviewed in reference 49). Myoblasts subsequently withdraw from the cell cycle, fuse into multinucleate syncytia, and synthesize a large number of muscle-specific genes. The onset and progression of the entire myogenic program are controlled through a complex set of signaling pathways that are mediated by environmental cues (reviewed in references 34, 40, and 43). Many of these control points directly affect transcriptional events, often impinging on several muscle-specific transcription factors.

Of the many known transcription factor families that regulate myogenesis, the MyoD family stands out as a key regulator. This family of proteins, which includes MyoD, myogenin, Myf-5, and MRF4, shares the remarkable property of inducing myogenic terminal differentiation when expressed in a variety of nonmuscle cells (reviewed in reference 40). The muscle regulatory factors (MRFs) are proteins containing a basic helix-loop-helix (bHLH) motif, with the basic region responsible for DNA binding and the HLH domain mediating protein-protein interactions. The MRFs heterodimerize with widely expressed bHLH E proteins, such as E12 and E47, and activate muscle-specific gene expression by binding to E-box consensus sequences (CANNTG) which are present in the control regions of most muscle-specific genes (reviewed in references 10, 21, 29, 49, and 62). Although over 70 different bHLH factors have been identified, the MRFs remain unique in that each contains a conserved muscle recognition motif (MRM) within the basic domain that is necessary for the activation of muscle-specific genes (9, 17, 27). The requirement of the MRM for initiating myogenesis has led to the hypothesis that this region may interact with specific cofactors to activate the myogenic program (9, 16, 17).

A second family of proteins that has been implicated as a key regulator of myogenesis is the myocyte enhancer factor-2 (MEF2) family. The MEF2 proteins belong to a large group of transcription factors containing a highly conserved MADS domain (reviewed in reference 48). MEF2 activates several muscle-specific structural genes (37, 41) as well as the expression of the MRF genes *myogenin*, *MRF4*, and *Xenopus MyoD* (19, 35, 46). Similarly, expression of the MRFs in fibroblasts induces MEF2 DNA-binding activity (14), suggesting that MEF2 and the MRFs are involved in a reciprocal regulatory circuit that ensures high expression levels of each factor in skeletal muscle cells. In addition, MEF2 and the MRFs interact through their respective MADS and bHLH motifs to synergistically activate muscle-specific gene expression. Thus, MEF2 and the MRFs may function as cofactors for one another in generating a myogenic phenotype (32, 45, 46).

Since MRF activity is controlled through both positive and negative regulatory pathways, additional factors also are likely to be involved in many of the complex transcription circuits that control myogenesis. One potential candidate is the muscle LIM protein (MLP) (also referred to as CRP3), which has been implicated as a positive regulator of myogenesis (1). MLP belongs to the LIM superfamily of proteins, which plays important roles in a variety of cellular functions. MLP itself consists of two LIM motifs, each followed by a glycine-rich

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region. The LIM motif forms a cysteine-rich, zinc-binding domain that initially was identified in the gene products of *Lin-11*, *Isl-1*, and *Mec-3* (23, 31, 61). The LIM motif utilizes the consensus sequence $CX_2CX_{17-19}HX_2CX_2CX_2CX_{16-20}CX_2$ (C/H/D) to generate two tandem zinc finger structures, which have been shown to mediate protein-protein interactions (4, 7, 22, 42). *MLP* transcripts are detected exclusively in skeletal and cardiac muscle, appearing during the early stages of terminal differentiation (1). Overexpression of *MLP* in C2C12 myoblasts promotes muscle differentiation, whereas antisense *MLP* prevents myogenesis (1). Similarly, mice homozygous null for *MLP* exhibit defects in both striated and cardiac muscle structure and function (3). However, the molecular mechanism by which MLP influences muscle development remains unknown and likely is complex since MLP exhibits a dual subcellular localization, with the protein accumulating in nuclei at the beginning of muscle differentiation and later in the cytoplasm as differentiation proceeds (1). The functional roles associated with the nuclear and cytoplasmic MLP proteins have not been established.

As a first step towards identifying the molecular pathway by which MLP enhances myogenesis, we examined how nuclear MLP influences muscle differentiation. Our results suggest that MLP plays an essential role in the nucleus where it physically interacts in vivo with the bHLH myogenic regulatory factors MyoD, myogenin, and MRF4. The association between these muscle-specific proteins is mediated by the first LIM motif of MLP and by the bHLH region of the MRFs. Although MLP lacks a functional transcription activation domain (TAD), it interacts with the MyoD–E-protein heterodimer, enhancing the DNA-binding activity of this transcription complex. We propose that MLP, like MEF2, functions as a cofactor for the myogenic bHLH proteins and that formation of an MLP–MyoD–E-protein complex may be crucial for muscle differentiation. Our studies also suggest a global strategy for how LIM-only proteins may control a variety of developmental pathways.

MATERIALS AND METHODS

Gene constructs and expression plasmids. Rat *MLP* cDNA was cloned by using reverse transcriptase PCR, with adult skeletal muscle poly(A)$^+$ RNA as the starting material. The resulting 0.6-kb cDNA product, containing the entire open reading frame, was cloned into pGEM-T (Promega). Hemagglutinin (HA)-tagged MLP expression vector pDCR-MLP, containing an HA epitope fused to the N terminus of MLP, was constructed by ligating the full-length *MLP* cDNA into the HA-pDCR expression plasmid (64). To generate a nucleus-localized MLP (*nls*-MLP), an oligonucleotide containing the simian virus 40 nuclear localization signal (KKKRRVE) (30) was ligated in-frame between the HA epitope and the MLP start codon in plasmid pDCR-MLP. The MLP expression plasmids pcDNA-MLP and pcDNA-*nls*-MLP were similarly constructed by ligating a PCR-generated HA-MLP (from pDCR-MLP) or HA-*nls*-MLP (from pDCR-*nls*-MLP) insert into the pcDNA3 expression plasmid (Invitrogen). All constructs were verified by DNA sequencing.

MLP inserts for Gal4-MLP comprising MLP amino acids 1 to 66 [Gal4-MLP (1-66)], Gal4-MLP (1-119), Gal4-MLP (1-171), Gal4-MLP (1-194), and Gal4-MLP (110-194) were generated by PCR amplification with complementary primers to the MLP template. Each 5′ and 3′ primer contained an *Eco*RI restriction site and an *Xba*I restriction site, respectively. The generated PCR products were digested with *Eco*RI and *Xba*I and cloned in-frame into the Gal4 (1-147) vector (38) containing the Gal4 DNA-binding domain (DB). The *Hin*dIII-*Xba*I fragment from each Gal4-MLP construct (containing the Gal4-DB and MLP deletion fragments) was subsequently subcloned into pcDNA3 to generate pcDNA-Gal4-MLP constructs.

Cell culture, DNA transfections, and mammalian two-hybrid assays. C3H10T1/2 fibroblasts were cultured as described previously (33). C2C12 myoblasts were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 15% fetal bovine serum (FBS). For stable DNA transfections, C2C12 cells were first treated with 0.1 μM chloroquine (Sigma) for 2 h, and then DNA calcium phosphate precipitates consisting of 2 μg of pDCR, pDCR-MLP, or pDCR-*nls*-MLP were added to each 100-mm-diameter dish containing 10^6 cells. After 5 h, the precipitates were removed and the cultures were fed fresh growth medium. On the following day, the cells were divided among six 100-mm-diameter dishes into which growth medium containing 400 μg of G418 (GIBCO) per ml was placed. After 10 days, equivalent numbers (~200) of G418-resistant colonies were pooled for each experimental group. The pooled cells were plated at 10^5 cells per 100-mm-diameter dish and switched to differentiation medium (containing low-glucose DMEM plus 2% horse serum) on the following day. After 3 to 4 days, cultures were harvested for immunocytochemistry or Western analysis.

For transient DNA transfections, DNA calcium phosphate precipitates consisting of 2 μg of pEM-MyoD (15) and 2 μg of pcDNA, pcDNA-MLP, or pcDNA-*nls*-MLP were added to 100-mm-diameter dishes containing 1.6×10^6 C3H10T1/2 cells in basal Eagle medium (GIBCO) supplemented with 10% FBS. After 4 h, cells were subjected to a 2-min osmotic shock and then fed growth medium containing 15% FBS. Cells were switched to differentiation medium (low-glucose DMEM, 2% horse serum) on the following day and allowed to differentiate. After 48 h, cultures were harvested for immunocytochemistry or Western analysis. For Gal4 assays, DNA precipitates were added to 35-mm-diameter dishes containing 10^5 cells. The precipitates contained 1 μg of luciferase reporter plasmid pG5E1b-luc (11) and 0.5 μg of Gal4 (1-147), Gal4-MLP (1-66), Gal4-MLP (1-119), Gal4-MLP (1-171), or Gal4-MLP (1-194). After 4 h, the precipitates were removed and cultures were fed growth medium containing 10% FBS. Two days later, cell extracts were harvested and luciferase activities were assayed with a Promega luciferase kit in accordance with the manufacturer's recommendations.

For mammalian two-hybrid assays, 3 μg of Gal4 (1-147), Gal4-MLP (1-119), or Gal4-MLP (110-194) expression plasmids was cotransfected with 5 μg of $(Gal4)_5$-CAT (38) along with 3 μg of pEM-MyoD, pECE-E47 (47), or both. In some instances 3 μg of pECE-MyoD~E47 (47) or pMT2-MEF2 (66) also was tested. Cell extracts were harvested after 36 h in differentiation medium, and chloramphenicol acetyltransferase (CAT) assays were performed as described by Gorman et al. (26). The amount of extract used for each assay was normalized to the protein content of each sample. A minimum of three independent transfections were performed for each experimental group.

Immunocytochemistry and Western analysis. For myosin heavy-chain staining, cultures were rinsed with phosphate-buffered saline (PBS) and fixed in 90% methanol at −20°C for 10 min. After incubation with the antimyosin monoclonal antibody MF-20 (5), a fluorescein-conjugated or biotin-conjugated antimouse secondary antibody was added and immunoreactivity was visualized by fluorescence microscopy or by using a Vectastain ABC reagent (Vector Laboratories, Inc.), respectively. For HA monoclonal antibody staining, cultures were fixed in 1% formaldehyde in PBS for 20 min at room temperature. After permeabilization with 0.5% Triton X-100 for 20 min, cultures were incubated with a blocking solution containing 5% horse serum plus 2% bovine serum albumin for 30 min at 37°C. Cultures then were incubated with the HA antibody (12CA5; Boehringer Mannheim) at a concentration of 5 μg/ml in blocking solution containing 0.1% Triton X-100 overnight at 4°C, followed by incubation with a fluorescein-conjugated antimouse secondary antibody for 1 h at room temperature. Immunoreactivity was visualized by fluorescence microscopy.

For Western analysis, cell extracts representing equal cell numbers were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to a nitrocellulose filter. After being blocked with 5% nonfat dry milk in Tris-buffered saline (10 mM Tris [pH 8.0], 150 mM NaCl), the filter was incubated with the MF-20 antibody; this was followed by the addition of a horseradish peroxidase-conjugated secondary antibody. The antigen-antibody complex was visualized by enhanced chemiluminescence (Amersham). The amount of myosin heavy chain was quantified by densitometry.

In vivo coimmunoprecipitation assays. In an effort to examine MLP and MyoD interactions in vivo, C3H10T1/2 cells were transfected with control expression plasmids or with expression plasmids containing MyoD and HA-MLP as described above. Following 48 h in differentiation medium, cells were rinsed in Tris-buffered saline, scraped, and centrifuged at $1,500 \times g$ for 5 min. The cell pellet was resuspended in nuclear lysis buffer and gently rocked at 4°C for 1 h as described previously (33). Extracts were centrifuged at 10,000 rpm for 5 min, and the supernatant was aliquoted and frozen at −80°C for storage. A MyoD rabbit polyclonal antibody (33) was added to each group in 10 mM HEPES (pH 7.6)–250 mM NaCl–0.25% Nonidet P-40 (NP-40)–5 mM EDTA, and the mixture was incubated at 4°C for 1 h. A 50% slurry (25 μl) of protein A-agarose beads subsequently was added to each sample, and the sample was incubated for an additional 1 h at 4°C. After being rinsed with nuclear lysis buffer, the final bead pellet was resuspended in SDS loading buffer and subjected to protein gel electrophoresis, followed by transfer to a nylon membrane. Western analysis was carried out as described above by using an HA monoclonal antibody to detect the presence of the MLP protein.

His-tagged proteins and in vitro protein binding assays. The full-length *MLP* cDNA was ligated in-frame with six histidine residues into the *Sal*I-*Hin*dIII sites of pQE32 (Qiagen, Inc.). The resulting construct produced His-tagged MLP (His-MLP). His-MLP containing only the first LIM motif (His-LIM1) or only the second LIM motif (His-LIM2) was similarly generated by ligating MLP (1-119) or MLP (110-194) in-frame with the His-tagged sequence present in the pQE31 vector at the *Bam*HI-*Sma*I sites (Qiagen, Inc.). His-MyoD and His-E47 have been described previously (28). The CRP cDNA was isolated from pGEXKG-CRP via a *Bam*HI/*Hin*dIII digest and cloned in frame to pQE30 (Quiagen, Inc.). The His-tagged proteins were purified according to the procedure recommended

by the manufacturer (Qiagen, Inc.). Purified proteins were analyzed by SDS-PAGE and Coomassie blue staining.

The following plasmids were used to generate in vitro-translated proteins: pBS-E12, pEM-MyoD, pEM-MyoDΔN, pEM-MyoDΔN, ΔC-VP16, pEM-MyoDE12 basic, pEM-E12MyoDbasic, and pEM-E12[NN→AT, D→K] (E12ATK) (16, 63); pEM-myogenin (20); pEM-MRF4 (50); pcDNA-MyoD~E47 and pcDNA-E47, which were subcloned from pECE-MyoD~E47 and pECE-E47, respectively (47); pMT2-MEF2 (66); and pcDNA-B-ATF-VP16 (18). The m^7GpppG-capped RNA was synthesized from each specific linearized DNA template by using T7 or T3 RNA polymerase and an in vitro transcription kit (Ambion). For in vitro translations, synthesized RNA was added to a rabbit reticulocyte lysate containing the appropriate amino acid mix and the mixture was incubated at 30°C for 1 h. Labeled protein products were obtained by supplementing the reactions with [^{35}S]methionine (Amersham).

For in vitro protein binding assays, 1 μg of His-MLP was incubated with 5 μl of ^{35}S-labeled in vitro-translated protein in 250 μl of binding buffer (200 mM NaCl, 10 mM Tris [pH 7.5], 50 μM $ZnCl_2$, 20 mM imidazole, 0.25% NP-40) for 2 h at 4°C. Ten microliters of a 50% Ni^{2+}-agarose bead slurry, previously equilibrated with the binding buffer, also was included in each binding reaction mixture. The bound proteins were subjected to three (1-ml) washes in binding buffer followed by two washes in binding buffer lacking NP-40. The protein complex was eluted from the beads by being heated to 95°C for 5 min and was subsequently analyzed by SDS-PAGE and autoradiography.

Electrophoretic mobility shift assays (EMSAs). Oligonucleotide probes were generated by labeling one strand with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled single-stranded DNA then was mixed with a 10-fold molar excess of the unlabeled complementary oligonucleotide strand. After denaturation by boiling for 5 min, the mixture was allowed to anneal at room temperature. Gel mobility shift assays were conducted as described previously (39). Purified His-MyoD (5 ng) and His-E47 (10 ng) were used for each 25-μl reaction mixture in a buffer containing 20 mM HEPES (pH 7.9), 5 mM dithiothreitol, 50 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 50 μM $ZnCl_2$, 10% glycerol, and 10 μg of bovine serum albumin. The mixture was incubated at 37°C for 15 min, and then 2 μg of poly(dI-dC) and ^{32}P-labeled E-box probes were added, followed by incubation at room temperature for 15 min. His-MLP (0, 5, 10, 20, or 40 ng) in 6 M urea was then added to the incubation mixture, and the mixture was allowed to incubate for an additional 15 min at room temperature. In all groups the urea concentration was kept constant. Protein-DNA complexes then were separated by PAGE, visualized by autoradiography, and quantified with a Molecular Dynamics PhosphorImager.

RESULTS

Nuclear MLP enhances myogenesis. MLP is a positive regulator of myogenesis that exhibits a dual nuclear and cytoplasmic localization (1). Cytoplasmic MLP has been shown to be associated with actin filaments and the Z line of sarcomeres (2, 3, 55), suggesting that it may be involved in the assembly of skeletal myofibrils. The function of nuclear MLP remains unknown, but likely is important to myogenesis since MLP is restricted to the nucleus at the beginning of muscle differentiation (1). Similarly, *Drosophila melanogaster* MLP is exclusively nuclear (2), suggesting that in this organism the cytoplasmic function of MLP is not utilized. Given the potential role of nuclear MLP in development, we set out to investigate whether MLP enhances vertebrate myogenesis primarily through nuclear events. As a first step in exploring this possibility, an HA-tagged MLP protein construct containing a simian virus 40 nuclear localization signal (*nls*-MLP) was generated. The cellular localization of MLP and *nls*-MLP then was analyzed by immunocytochemistry using a monoclonal antibody against the HA epitope. As shown in Fig. 1A, wild-type MLP exhibits the predicted dual subcellular localization, being present in both the cytoplasm and nucleus, whereas *nls*-MLP is predominantly nuclear. In order to compare how MLP and *nls*-MLP affect myogenic differentiation, C2C12 myoblasts were stably transfected with expression plasmids encoding each protein. Equivalent numbers of G418-resistant colonies were pooled and induced to differentiate. After 4 days in differentiation medium, cultures were fixed and stained for myosin heavy-chain expression. In agreement with Arber et al. (1), C2C12 cells overexpressing MLP (C2C12-MLP) differentiate with a higher efficiency than control C2C12 cells (Fig. 1B). Interestingly, cells overexpressing *nls*-MLP (C2C12-*nls*-MLP) exhibit an even greater differentiation efficiency, forming much larger myotubes than do C2C12-MLP cells (Fig. 1B). Western blot analysis confirmed that C2C12-*nls*-MLP cells accumulate approximately five times more myosin than C2C12-MLP cells and twenty times the skeletal myosin content of control C2C12 cells (data not shown). These results demonstrate that nuclear MLP functions as a potent activator of myogenic differentiation, which suggests that the wild-type MLP protein enhances myogenesis through specific nuclear events.

The ability of MLP and *nls*-MLP to enhance myogenesis also was compared in transient DNA transfection assays using C3H10T1/2 fibroblasts as the recipient cells. Overexpression of MLP produces no observable phenotypic alterations in the C3H10T1/2 cells, whereas expression of the muscle regulatory factor MyoD rapidly induces these cells to differentiate into myocytes (Fig. 2A). Cotransfection of MyoD with MLP produces a 20% increase in the number of myosin-positive cells compared to the number obtained with MyoD alone. In agreement with what was found for the C2C12 stable transfections, cotransfection of MyoD and *nls*-MLP doubles the number of myosin-positive cells generated in these studies. Similarly, *Drosophila* MLP, which accumulates exclusively in the nucleus (reference 2 and unpublished results), also dramatically enhances the number of MyoD-induced myosin-positive cells (Fig. 2A). Western blot analysis revealed that the level of myosin expression induced by MyoD increases 5- to 10-fold when *nls*-MLP is expressed in these cells (Fig. 2B), again demonstrating that nuclear MLP enhances myogenesis, possibly through a MyoD-dependent pathway.

MLP does not contain a TAD. Members of the LIM protein family are believed to function in transcription regulation during development (56). For example, the LIM homeobox genes, including *Lin-11*, *Mec-3*, and *Lim-1*, are involved in cell lineage specification (23, 52, 61). Similarly, the LIM-only genes, *RBTN1* and *RBTN2*, are associated with chromosomal translocations in T-cell leukemias, and *RBTN2* plays an essential role in erythropoiesis (58, 60). The observations that MLP functions in the nucleus and that *nls*-MLP enhances MyoD activity suggest that MLP also may act as a transcription activator. To test this idea, a Gal4 reporter gene system was employed to ascertain whether MLP has any inherent transcriptional activity. For these studies, N- and C-terminal truncations of MLP were generated and fused in-frame with the DNA-binding domain of Gal4 (38). Each Gal4 fusion protein expression plasmid was transfected into C3H10T1/2 cells along with a Gal4 luciferase reporter gene construct. Cells transfected with the reporter gene and a construct expressing only the Gal4 DNA-binding domain exhibit no luciferase activity, whereas Gal4-VP16 serves as a potent transcriptional activator (Fig. 3). When Gal4 chimeric genes containing the full-length MLP or any of the C- or N-terminal-truncated forms of MLP are tested, the fusion proteins are stably produced (data not shown) but only background levels of luciferase activity are observed, strongly suggesting that MLP lacks a functional TAD. Interestingly, MLP becomes a potent activator of MyoD-induced myogenesis when a VP16 TAD (*nls*-VP16-MLP) is added to the protein (data not shown). These results imply that MLP likely is involved in myogenic transcriptional regulation, even though MLP lacks a functional TAD.

MLP interacts with the bHLH MRFs. Since *nls*-MLP greatly enhances the myogenic activity of MyoD, we reasoned that MLP and MyoD may interact with each other to promote myogenesis. To test this hypothesis, in vitro protein binding assays were performed in which ^{35}S-labeled proteins were incubated with His-tagged MLP immobilized on Ni^{2+}-agarose beads. The protein complexes were washed extensively, and

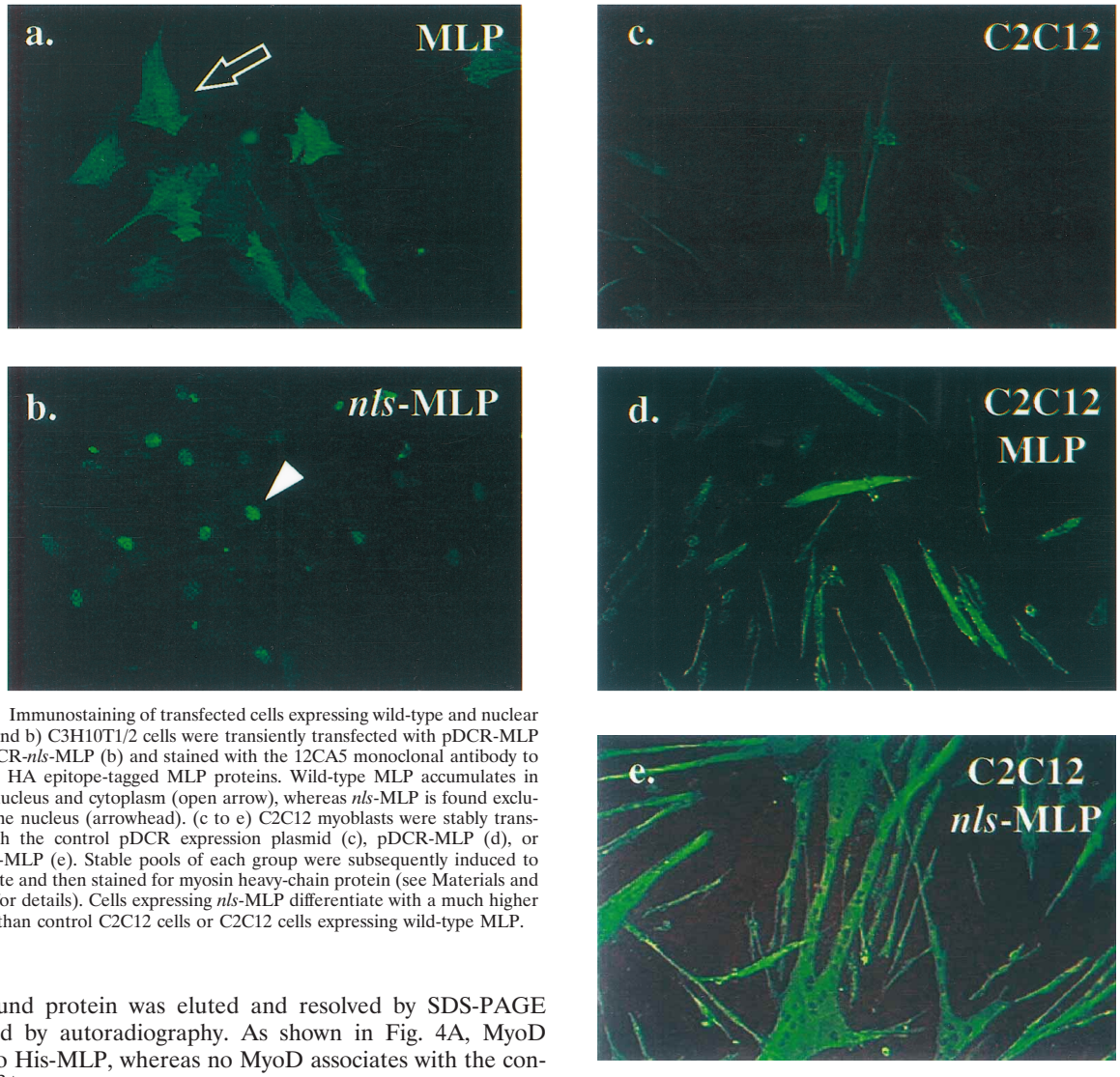

FIG. 1. Immunostaining of transfected cells expressing wild-type and nuclear MLP. (a and b) C3H10T1/2 cells were transiently transfected with pDCR-MLP (a) or pDCR-*nls*-MLP (b) and stained with the 12CA5 monoclonal antibody to detect the HA epitope-tagged MLP proteins. Wild-type MLP accumulates in both the nucleus and cytoplasm (open arrow), whereas *nls*-MLP is found exclusively in the nucleus (arrowhead). (c to e) C2C12 myoblasts were stably transfected with the control pDCR expression plasmid (c), pDCR-MLP (d), or pDCR-*nls*-MLP (e). Stable pools of each group were subsequently induced to differentiate and then stained for myosin heavy-chain protein (see Materials and Methods for details). Cells expressing *nls*-MLP differentiate with a much higher efficiency than control C2C12 cells or C2C12 cells expressing wild-type MLP.

the bound protein was eluted and resolved by SDS-PAGE followed by autoradiography. As shown in Fig. 4A, MyoD binds to His-MLP, whereas no MyoD associates with the control Ni^{2+}-agarose beads. In contrast, MEF2 and E47, which also play essential roles in the activation of muscle-specific genes (reviewed in reference 43), do not interact with MLP (Fig. 4A). Similarly, no interaction is detected between MLP and the control c-Fos protein (Fig. 4A) or between MLP and other nonmuscle bHLH factors such as Mist1 (36) (data not shown).

The interaction between MLP and additional MRFs also was tested to establish if MLP associates with other members of the MRF family. Again, in vitro protein binding assays were carried out with ^{35}S-labeled MRF4 and myogenin and His-MLP. As predicted, both MRF4 and myogenin interact with MLP, whereas widely expressed bHLH protein E12 does not bind to MLP (Fig. 4B), confirming that MLP interacts specifically with the MRFs. To test whether MLP also interacts with a MyoD-E47 heterodimer, we utilized a construct (MyoD~E47) which generates a MyoD protein tethered to the E47 protein, which in effect produces a "preformed" MyoD-E47 heterodimer (47). As shown in Fig. 4B, the tethered MyoD~E47 protein also retains the ability to interact with MLP, indicating that MLP may influence the functional properties of the MyoD–E-protein heterodimer. Thus, although MLP does not directly associate with MEF2 or with E proteins E12 and E47, MLP does interact with the bHLH MRFs even when complexed as a heterodimer with E47.

MyoD and MLP interactions require the basic domain of MyoD and the first LIM motif of MLP. The ability of MyoD, MRF4, and myogenin to associate with MLP suggests that the highly conserved bHLH region that is common to each of the MRFs may be involved in this interaction. To address this possibility, in vitro protein binding assays were performed with full-length MyoD and several MyoD proteins containing N- and C-terminal truncations. As predicted from our earlier studies, wild-type MyoD interacts with MLP under these experimental conditions (Fig. 4C). Similarly, the truncated MyoD protein MyoDΔN, which lacks the N-terminal TAD, retains the ability to bind to MLP, demonstrating that the MyoD TAD is not required for MLP interaction. MyoDΔN, ΔC-VP16, which contains only the bHLH region of MyoD (plus a VP16 TAD), again retains the ability to interact with MLP, whereas the control fusion protein B-ATF-VP16, which belongs to the basic leucine zipper protein family (18), does not bind to this LIM-only protein (Fig. 4C).

Previous studies have established that the basic region of each MRF contains a conserved MRM that is defined by the

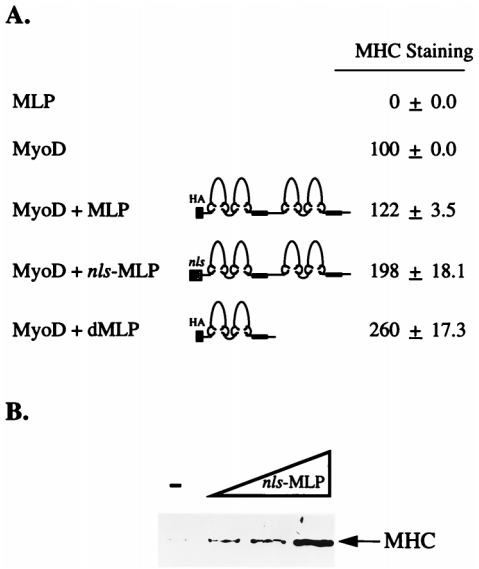

FIG. 2. The myogenic activity of MyoD is enhanced by *nls*-MLP. (A) C3H10T1/2 cells were transfected with expression plasmids encoding MLP, MyoD, MyoD plus MLP, MyoD plus *nls*-MLP, or MyoD plus *Drosophila* MLP (dMLP). Myosin heavy-chain immunofluorescence was performed to determine the numbers of differentiated cells in the cultures. The level of differentiation obtained with cells transfected with MyoD alone was set at 100. The results represent the averages (+/− standard errors of the means) from at least three independent transfections. (B) Western blot analysis of myosin heavy-chain (MHC) expression of cell extracts from C3H10T1/2 cells transiently cotransfected with 2 μg of MyoD and 0, 0.5, 1.0, or 1.5 μg of *nls*-MLP. The amount of myosin heavy-chain protein increases 5- to 10-fold in the presence of *nls*-MLP.

position of alanine and threonine residues (alanine 114 and threonine 115 in MyoD). The alanine and threonine positions within the MRM are not found in any other known bHLH proteins and are specific requirements for muscle gene activity (9, 16, 17). To further investigate whether the MRM region is essential to MLP interactions, a MyoD construct in which the basic region of E12 replaced the MyoD basic region (MyoD E12 basic) was tested. In contrast to our earlier results obtained with the wild-type MyoD protein, replacing the basic region of MyoD with that of E12 severely diminishes MLP interaction, again suggesting that the MyoD basic region is crucial for MLP binding (Fig. 4C). However, the basic domain of MyoD alone is not sufficient for MLP interaction since an E12 protein in which the basic domain has been replaced with the MyoD basic domain (E12 MyoD basic) also does not interact with MLP (Fig. 4D). Similarly, when E12ATK was tested (an E12 protein in which two amino acids within the basic domain and one in the helix 1 junction were changed to the corresponding MRM residues in MyoD), no interaction with MLP was observed. These results suggest that although the MyoD basic domain is required for MLP interaction, the basic domain alone is not sufficient to permit MLP binding. Interestingly, the ability of MyoD and MLP to interact with one another correlates with the relative myogenic activities associated with the altered proteins, since E12 and E12 MyoD basic do not generate a myogenic phenotype when expressed in C3H10T1/2 cells and E12ATK is only 5% as active as MyoD (16). Together, these data strongly suggest that MLP interacts solely with bHLH proteins belonging to the MRF family.

Given that MLP interacts with the basic domain of MyoD, we next set out to establish if MyoD interacts with other LIM proteins and whether the MyoD-MLP interaction occurs through the LIM1 motif, the LIM2 motif, or through both motifs. For these studies, we examined the ability of MyoD to associate with the related MLP protein, CRP (42). CRP is a particularly important candidate since it is closely related to MLP. However, unlike the *MLP* gene, the *CRP* gene exhibits a very broad expression pattern and the protein remains exclusively cytoplasmic. Interestingly, although CRP is structurally related to MLP, MyoD does not interact with this LIM protein (Fig. 5A), confirming the specificity of interaction associated with MLP and the MRFs. Finally, we also examined which MLP LIM motif is responsible for associating with MyoD by generating His-MLP proteins that consist of only the first LIM motif (His-LIM1) or the second LIM motif (His-LIM2). ^{35}S-labeled MyoD protein then was incubated with His-LIM1 or His-LIM2 as described above, and the resulting complexes were resolved by SDS-PAGE. As shown in Fig. 5B, His-LIM1 interacts with MyoD in a fashion similar to that of the full-length MLP protein. In contrast, no interaction is observed when MyoD is incubated with His-LIM2, suggesting that MyoD does not recognize the second LIM motif of MLP. Thus, the MLP LIM1 motif mediates the interaction with the MyoD basic domain.

In vivo interactions between MLP and MyoD. Although it is clear that MLP specifically binds to the myogenic bHLH factors in vitro, we wished to examine whether these factors also interact in vivo by using a variety of assay systems. Two approaches were taken; one involved coimmunoprecipitation and the second utilized mammalian two-hybrid assays. For the coimmunoprecipitation assay, C3H10T1/2 cells were transfected with control expression plasmids or with expression plasmids encoding MyoD, an HA-tagged *nls*-MLP, or both (see Materials and Methods for details). Following 48 h in differentiation medium, cells were harvested and subjected to MyoD immunoprecipitation with a MyoD-specific antibody. This material then was run on SDS-PAGE, followed by transfer to a nylon membrane. Western analysis using an HA monoclonal antibody revealed that the 22-kDa MLP protein is detected only in cells coexpressing MyoD and MLP (Fig. 6), demonstrating

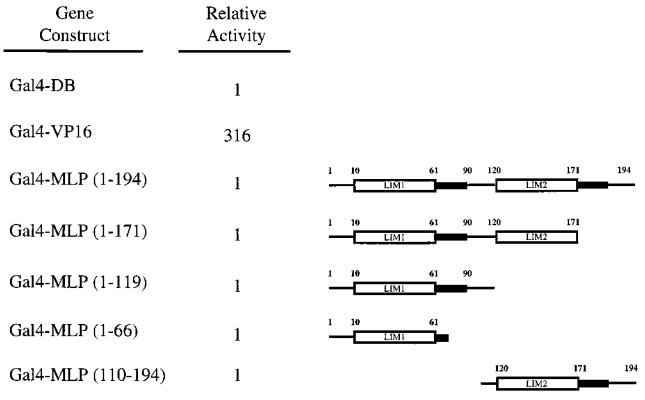

FIG. 3. MLP lacks a functional TAD. C3H10T1/2 cells were cotransfected with the pG5T-luciferase reporter gene and each of the indicated Gal4-MLP fusion protein expression plasmids (see Materials and Methods for details). The LIM1 and LIM2 domains are indicated as well as the glycine-rich region (solid box). The numbers above each gene construct indicate amino acid positions (1 to 194) of the MLP protein. The relative activity represents the fold increase in luciferase expression compared to the basal level detected with Gal4-DB. Gal4-VP16 was used as a positive control. A minimum of three independent transfections were performed for each experimental group. The range of luciferase activities obtained with Gal4-DB and each of the Gal4-MLP constructs was 234 to 285 relative light units (RLU)/10 μl of cell extract, whereas the Gal4-VP16 values ranged from 55,505 to 121,572 RLU/10 μl of cell extract.

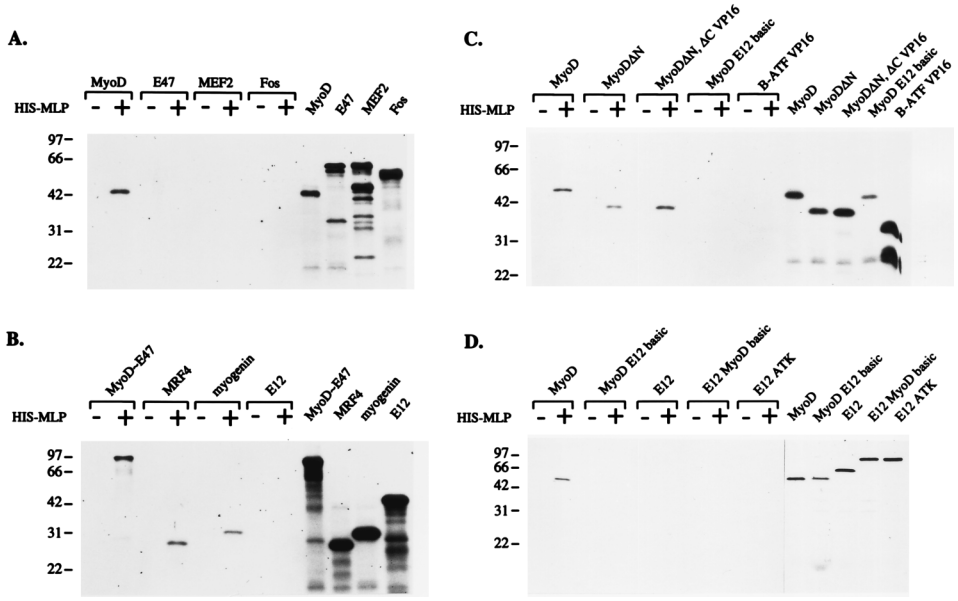

FIG. 4. In vitro protein binding assays using His-tagged MLP and in vitro-translated proteins. ^{35}S-labeled proteins were incubated with (+) or without (−) His-MLP on Ni^{2+}-agarose beads. Bound proteins were eluted and subjected to SDS-PAGE and autoradiography. (A) MLP specifically interacts with MyoD but not with E47, MEF2, or Fos. (B) MLP also interacts with MRF4 and myogenin as well as with the MyoD∼E47 tethered protein but not with E12. (C) MyoD containing an N-terminal TAD deletion (MyoDΔN) retains the ability to interact with MLP, as does MyoDΔN, ΔC-VP16, which only contains the bHLH region of MyoD plus a VP16 TAD. A negative control, the leucine zipper fusion protein B-ATF-VP16, also containing a VP16 TAD, does not interact with MLP. MyoD E12 basic also fails to interact with MLP. (D) The MyoD basic domain alone is not sufficient for MLP interaction. Although MLP interacts with MyoD, it fails to associate with E12 MyoD basic or with E12ATK, suggesting that the MyoD basic domain must remain in the context of the MyoD protein to allow MLP binding. None of the proteins interact with the Ni^{2+}-agarose beads alone (−). The last 4 or 5 lanes of each panel represent the ^{35}S-labeled input proteins tested with His-MLP.

that, in vivo, MLP and MyoD associate with one another in the nuclear compartment.

This in vivo association result was confirmed by using a mammalian two-hybrid system. For these studies, expression plasmids encoding a Gal4-MLP fusion protein plus MyoD or E47 were cotransfected into C3H10T1/2 cells along with the reporter gene $(Gal4)_5$-CAT (Fig. 7A). As predicted, the chimeric protein Gal4-MLP (LIM1), which contains the first MLP LIM motif fused in-frame with the DNA-binding domain of Gal4, does not activate the $(Gal4)_5$-CAT reporter gene because it lacks a functional TAD (Fig. 7A). When an E47 expression plasmid is coexpressed with Gal4-MLP (LIM1), only basal levels of CAT activity are detected, confirming our in vitro data that E47 does not interact with MLP. Conversely, when MyoD is coexpressed with Gal4-MLP (LIM1), CAT expression is induced approximately fourfold, suggesting that MyoD binds to MLP and activates CAT gene expression via the MyoD TAD. Coexpression of both MyoD and E47 with Gal4-MLP (LIM1) generates a much higher activation of the $(Gal4)_5$-CAT reporter gene as a result of the heterodimer formation between MyoD and E47, indicating that MLP also is capable of interacting with the MyoD–E-protein heterodimer complex in vivo (Fig. 7A). Similarly, MyoDΔN, ΔC-VP16, but not a MyoD protein containing an E12 basic domain (MyoD E12 basic), interacts with Gal4-MLP (LIM1) (data not shown), confirming that the basic region of MyoD serves as the functional target for MLP in these assays. Identical results also were obtained when MRF4 and myogenin were tested with these various gene constructs (data not shown). As predicted from our in vitro protein binding assays, when Gal4-MLP (LIM2), containing only the second LIM motif, is cotransfected with MyoD alone, E47 alone, or both MyoD and E47, no significant induction of $(Gal4)_5$-CAT expression is detected (Fig. 7A). The difference in activity associated with LIM1 and LIM2 is not due to differences in protein accumulation since Western blot analysis confirmed that equivalent amounts of Gal4-MLP (LIM1) and Gal4-MLP (LIM2) fusion proteins are produced in these transfected cultures (data not shown). We conclude that MLP and MyoD associate with each other in vivo and that this association is mediated through the first LIM motif of MLP and through the basic domain of MyoD.

MLP may provide a protein binding surface to facilitate the assembly of the MEF2–MyoD–E-protein complex. Previous studies have shown that MEF2 and the myogenic bHLH factors work cooperatively to activate muscle-specific gene expression through a physical interaction involving the bHLH domain of the MRFs and the MADS domain of MEF2 (32,

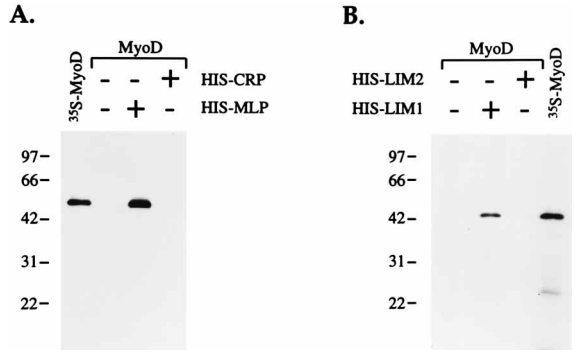

FIG. 5. In vitro protein binding assays using His-tagged LIM-containing proteins and in vitro-translated MyoD as described in the legend for Fig. 4. (A) MyoD associates with MLP but not with the related LIM protein CRP. (B) The LIM1 motif of MLP mediates the interaction with MyoD since His-LIM1 binds to MyoD, whereas His-LIM2 does not. None of the proteins interact with the Ni^{2+}-agarose beads alone (−).

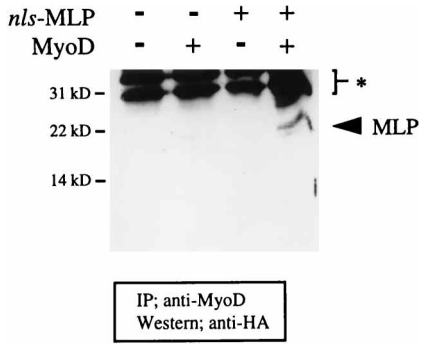

FIG. 6. MyoD and MLP associate with one another in vivo. C3H10T1/2 cells were transfected with control expression plasmids or with expression plasmids encoding MyoD, an HA-tagged *nls*-MLP, or both (see Materials and Methods for details). Following 48 h in differentiation medium, cells were harvested and subjected first to MyoD immunoprecipitation (IP) and then to Western analysis using an HA monoclonal antibody. The 22-kDa MLP protein is detected only in cells coexpressing MyoD and MLP, demonstrating that MLP and MyoD associate with one another within the nuclear compartment. Asterisk, nonspecific Ig light chains.

45). These results led to the suggestion that MEF2 may be a cofactor for the myogenic bHLH proteins. Our observation that MLP directly interacts with the MRF proteins through their bHLH motifs raises the possibility that MLP also may function as a cofactor in a regulatory circuit controlling skeletal myogenesis. To examine this possibility, mammalian trihybrid assays were performed to test whether MEF2 and MLP simultaneously associate with the MyoD–E-protein complex. Cotransfection of Gal4-MLP (LIM1) with the tethered MyoD~E47 expression plasmid produces the predicted fourfold induction in $(Gal4)_5$-CAT reporter gene expression (Fig. 7B). No significant induction in CAT expression is detected when MEF2 is cotransfected with Gal4-MLP (LIM1), confirming our earlier in vitro binding results (Fig. 4A). However, CAT expression is greatly enhanced when both MyoD~E47 and MEF2 are coexpressed along with Gal4-MLP (LIM1) (Fig. 7B), indicating that MEF2 interacts with MyoD~E47 in the presence of MLP. These results suggest that MLP may be involved in muscle-specific protein-protein interactions, possibly by providing a protein binding surface to promote formation of the MyoD–E-protein–MEF2 functional complex.

MLP promotes DNA binding of the MyoD–E-protein complex. We have shown that MLP enhances the myogenic activities associated with MyoD, possibly through a direct physical interaction. Given that MLP also interacts with MyoD–E-protein heterodimers in vitro and in vivo, we next decided to investigate the possibility that MLP influences the DNA-binding properties associated with this heterodimer complex. Purified His-MyoD, -E47, and -MLP proteins were incubated with ^{32}P-labeled E-box probes, and the generated protein-DNA complexes were resolved by native PAGE and autoradiography. As shown in Fig. 8A, purified His-MLP does not bind to the TnI E-box sequence. As expected, MyoD and E47, when tested alone, also do not interact with the E-box DNA. However, when both MyoD and E47 are present, a shifted band representing a MyoD-E47 heterodimer complex bound to DNA is detected (28). When MLP is added to the reactions, a further two- to threefold enhancement in the bound MyoD-E47 complex is observed (Fig. 8A). This increase in DNA binding is specific to MyoD since MLP has no effect on the binding of E47 homodimers to a μE5 E-box site (Fig. 8B). Similarly, MLP does not enhance the ability of E proteins to activate expression of a μE5 E-box reporter gene in vivo (data not shown). Identical results also have been obtained when purified glutathione *S*-transferase (GST)-MLP is substituted for His-MLP in these assays (data not shown). Interestingly, with either His-MLP or GST-MLP, we have never detected MLP in the MyoD-E47-DNA complex in these EMSA studies. Thus, although MLP increases MyoD-

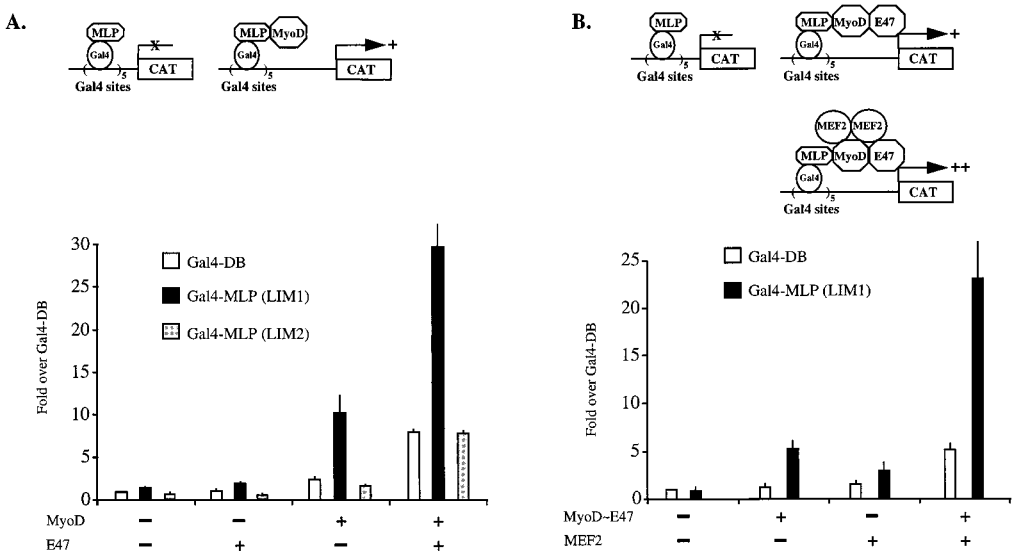

FIG. 7. Mammalian two-hybrid and trihybrid analyses of MLP and MyoD interactions in C3H10T1/2 cells. (A) C3H10T1/2 cells were cotransfected with Gal4-DB, Gal4-MLP (LIM1), or Gal4-MLP (LIM2) and the $(Gal4)_5$-CAT reporter gene along with MyoD, E47, or MyoD plus E47. Gal4-MLP (LIM1) does not activate CAT expression, whereas cotransfection of MyoD and Gal4-MLP (LIM1) produces a fourfold increase in CAT activity. Cotransfection of MyoD and E47 produces an even higher level of CAT expression. In all cases, Gal4-MLP (LIM2) generates only background levels of expression equivalent to the control Gal4-DB groups. (B) C3H10T1/2 cells were transiently cotransfected with Gal4-DB or Gal4-MLP (LIM1) and $(Gal4)_5$-CAT along with MyoD~E47, MEF2, or MyoD~E47 plus MEF2. Cotransfection of MyoD~E47 and Gal4-MLP (LIM1) generates a fourfold increase in CAT activity, whereas cotransfection of MEF2 and Gal4-MLP (LIM1) does not activate CAT expression. Cotransfection of MyoD~E47, MEF2, and Gal4-MLP (LIM1) produces a further increase in expression of the reporter gene. Error bars reflect the standard errors of the means from a minimum of three independent transfections.

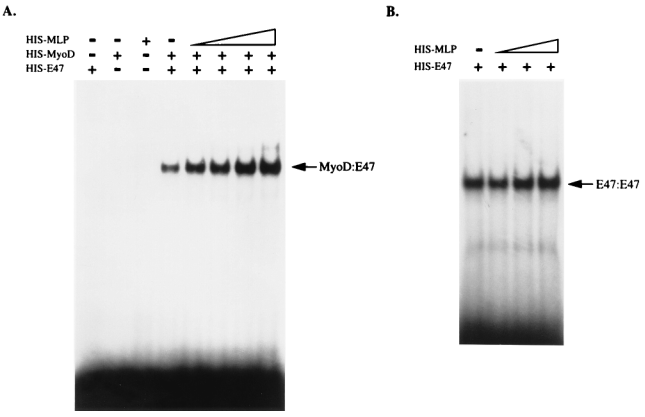

FIG. 8. EMSAs using purified His-MyoD, His-E47, and His-MLP proteins. ^{32}P-labeled E-box probes were incubated with purified His-tagged proteins, and the DNA-protein complexes were separated by nondenaturing PAGE. (A) E47 alone, MyoD alone, and MLP alone do not bind to the TnI E-box sequence (39). MyoD and E47 bind to DNA as a heterodimer complex. The DNA-binding ability of MyoD-E47 is enhanced in the presence of increasing amounts of His-MLP. (B) ^{32}P-labeled μE5 E-box probes were incubated with His-E47 in the absence or presence of His-MLP. E47 binds to the μE5 E box as a homodimer (28). The DNA-binding activity of the E47 homodimer is not affected by His-MLP.

E47 binding to the E-box target, MLP likely dissociates from these proteins once the bHLH-DNA complex enters the gel (see Discussion).

DISCUSSION

Two families of transcription factors that are known to play pivotal roles in muscle development are the bHLH MRFs and the MADS box MEF2 proteins. MEF2 and the MRFs act synergistically to activate the myogenic program through direct physical interactions involving the MADS box and the bHLH domains of the respective proteins (32, 45, 46). In addition, both the MRFs and MEF2 gene products are capable of activating expression of each gene family, forming an active regulatory circuit which ensures that high levels of *MEF2* and *MRF* gene expression are maintained during myogenesis (reviewed in references 40 and 44). Although each factor clearly binds to specific and different DNA target sequences, only one factor needs to be bound to DNA to obtain a synergistic response (45, 46). Interestingly, MEF2, which is present in a variety of cell types, including neurons (8), cooperates with the neuronal bHLH protein MASH1 to synergistically activate gene transcription through MASH1 binding sites (6). Thus, MEF2 also controls gene expression patterns outside of the myogenic lineage. Whether the MRFs utilize true muscle-specific cofactors to enhance their activities has not been established.

Recent studies suggest that additional protein families also may be crucial for myogenesis. The muscle LIM protein (MLP), which is found exclusively in striated muscle, positively influences skeletal muscle differentiation (1). MLP belongs to the LIM-only protein family, which plays essential roles in a wide variety of cellular functions including cell fate determination and terminal differentiation. An interesting feature of MLP is that it exhibits a dual subcellular localization, being present in the nucleus at the beginning of muscle differentiation and accumulating in the cytoplasm at later stages (1). How and where MLP functions to promote myogenesis is an important problem that needs to be solved to fully characterize the intricate regulatory pathways that establish and maintain a normal muscle phenotype. In this study, we found that nuclear MLP collaborates with the myogenic bHLH proteins to promote muscle differentiation. The collaboration between MLP and the MRFs is mediated through the basic region and first LIM motif of the respective proteins. The interaction of MLP and the MRFs enhances the DNA-binding activity associated with the MyoD-E47 heterodimer complex, suggesting that MLP promotes myogenic differentiation by recruiting or stabilizing MyoD to the correct DNA target. Understanding these molecular mechanisms is crucial to elucidating the regulatory networks that are operative during myogenesis.

Given that *nls*-MLP is a more potent activator of myogenic differentiation than wild-type MLP and that *Drosophila* MLP (which contains only a single LIM motif) accumulates predominantly in the nucleus (reference 2 and unpublished results; also see reference 55 for conflicting data), we believe that nuclear localization is essential for the myogenic enhancement properties associated with MLP, whereas the cytoplasmic function may have developed a specialized role during vertebrate evolution. Arber and Caroni (2) and Stronach et al. (55) have shown that cytoplasmic MLP associates with actin filaments and therefore may be involved in facilitating the assembly of the myofibril apparatus during muscle maturation. Indeed, recent studies have shown that MLP localizes to the Z line of sarcomeres in the adult organism (3). Similar studies have shown that the related LIM-only protein CRP also associates with the actin cytoskeleton (13). Interestingly, an MLP protein containing two linked second LIM motifs (M2-M2) accumulates exclusively along cytoplasmic actin filaments (2), suggesting that LIM2 is responsible for the cytosolic localization of MLP. In agreement with this finding, we have confirmed that LIM2 specifically interacts with cytoskeletal proteins (22a), whereas LIM1 interacts with MyoD. Thus, LIM1 appears to play a role in nuclear localization, interacting with the MRFs and enhancing the formation of MRF-DNA complexes, whereas LIM2 primarily interacts with cytoplasmic proteins involved in maintaining the cellular architecture (see Fig. 9). This dual localization suggests that MLP may provide a regulatory checkpoint for insuring appropriate transcriptional activity to support sufficient numbers of functional sarcomeres.

MLP appears to influence the myogenic activities of the MRFs through a direct physical interaction. This interaction is highly specific, as MLP does not complex with non-MRF bHLH proteins or with MEF2. However, MLP is capable of interacting with MyoD–E-protein heterodimers, indicating

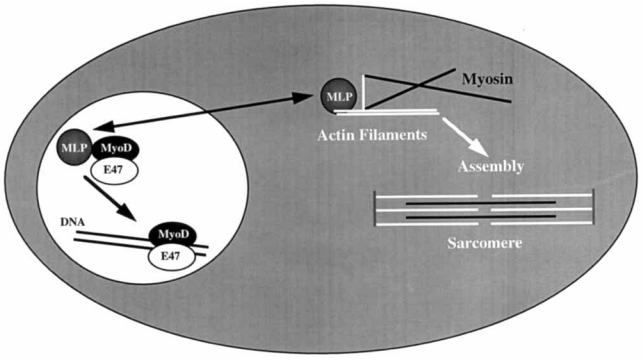

FIG. 9. Hypothetical model outlining potential roles for nuclear and cytoplasmic MLP in muscle development. MLP may serve a dual function in myogenesis by promoting DNA interaction of the MyoD-E47 heterodimer complex as well as by stabilizing the assembly of functional sarcomeres through interaction with skeletal α-actin filaments. See text for details.

that MLP and E proteins interact with MyoD at different positions. Indeed, the interaction between E proteins and MyoD depends on the HLH region (17), whereas the basic region plays a critical role in MLP and MyoD interactions (Fig. 4). In addition, a complex containing MLP, MyoD-E protein, and MEF2 is detected through mammalian trihybrid assays, indicating that MLP also may be involved in the formation of MyoD–E-protein–MEF2 complexes which ultimately generate a greater enhancement in muscle differentiation.

Additional mechanisms for the function of MLP may involve affecting the transcriptional activities of the MRFs. Although MLP does not contain a TAD per se, it is involved in muscle-specific transcription since MLP enhances the DNA-binding capabilities of the MyoD–E-protein complex, possibly by stabilizing the MRF protein complex on the DNA. Enhancement in DNA binding associated with the MyoD–E-protein heterodimer may account for the twofold increase in myosin-positive cells observed in transient C3H10T1/2 transfections. For instance, it is possible that a threshold level of activated MyoD is required to initiate myogenesis in these cells. MLP would, in effect, lower the threshold of MyoD that is needed since the DNA-binding properties of the MyoD–E-protein complex are enhanced in the presence of MLP. Therefore, cells that express low levels of MyoD (which normally are not converted into muscle cells) enter the myogenic program in the presence of *nls*-MLP. The enhancement of MyoD DNA binding associated with MLP may be of great importance to skeletal myogenesis given that differentiation of C2C12 myoblasts is blocked by the absence of MLP protein (1). Since the endogenous cellular level of MyoD is much lower than that of transiently transfected cells, it is possible that the low level of endogenous MyoD fails to bind DNA without the assistance of MLP.

The enhancement in DNA binding of the MyoD-E47 complex induced by MLP can be explained by the following possibilities. One is that MLP enhances formation of the MyoD-E47 heterodimer. The other possibility, although not mutually exclusive, is that MLP may affect the conformation of the MyoD–E-protein heterodimer complex (especially in the bHLH region), thereby promoting DNA sequence recognition. Our observation that *nls*-MLP enhances the myogenic activity of a MyoD~E47-tethered protein supports the latter possibility (unpublished results). Surprisingly, we have been unable to detect MLP in the MyoD-E47-DNA complex by gel mobility shift assays. However, the inability to detect MLP in the MyoD-E47-DNA complex appears to be a common theme among other DNA-binding-enhancing proteins such as Rb, pX, TAP-1, and Tax, which increase the DNA-binding activity of LAP, CREB, Jun, and ATF2, respectively, without generating a ternary complex in these assays (12, 24, 59, 65). Nonetheless, it remains formally possible that MLP is part of the MyoD-E47 complex once bound to DNA. The mammalian two-hybrid results support the idea that a stable MLP-MyoD-E47-DNA complex forms. In addition, we have found that *nls*-MLP-VP16, which contains a VP16 TAD, promotes MyoD-induced muscle differentiation more efficiently than *nls*-MLP (unpublished results), again suggesting that MLP is present in the actual RNA polymerase II transcription complex. Further studies will be required to conclusively demonstrate this potential interaction.

MLP is expressed exclusively in skeletal and cardiac muscle. Although the timing of *MLP* expression during embryogenesis has not been studied in detail in vertebrate systems, the *Drosophila MLP* gene is expressed in developing muscles subsequent to the formation of muscle precursor cells (1, 55). However, in chicken and rodent muscle cells, MLP is not detected in proliferating myoblasts but is up-regulated during terminal differentiation (1). These observations indicate that MLP may be involved in the later stages of muscle development, perhaps in initiating and maintaining the differentiated muscle phenotype. The function of MLP in cardiac cells has not been characterized. However, the pattern of subcellular localization of MLP observed in cardiac muscle cells is similar to that observed in skeletal muscle cells. In cardiac primary cell cultures, MLP initially localizes to the nucleus and later is found along actin filaments in the cytoplasm (2). Thus, it is possible that nuclear MLP interacts with cardiac muscle-specific bHLH factors, such as eHAND and dHAND, which play critical roles in early cardiac development (54). Future studies will be aimed at addressing the role of MLP in heart formation.

Many LIM proteins exert their influence over developmental events by affecting gene expression (23, 31, 61). LIM proteins that lack a DNA-binding homeodomain (i.e. RBTN1 and RBTN2) have been postulated to function in regulating cellular differentiation. RBTN1 and RBTN2 are proto-oncogenes that are activated in T-cell leukemia (51). Mice containing null mutations of the *Rbtn2* gene die at an early stage of development due to a complete absence of erythroid precursors, suggesting that RBTN2 is essential for normal erythroid development (60). TAL1, a bHLH transcription factor essential for erythroid development, is coexpressed with RBTN2 in cells of erythroid origin (53, 57). Significantly, RBTN1 and RBTN2 physically interact with TAL1 (58) through their respective LIM and bHLH domains. Similar results have been reported for pancreatic cells, where the LIM protein lmx-1 and the bHLH protein Pan-1 (E47) interact to synergistically activate expression of the insulin gene (25). These findings are in agreement with our studies showing that MLP interacts with muscle-specific bHLH proteins. Thus, the interactions between tissue-specific bHLH factors and LIM proteins may represent a common mechanism that is utilized by many different developmental systems to enhance transcriptional activity. Altering MLP activity in developing vertebrate and invertebrate models should provide additional information regarding how this factor functions during embryogenesis.

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