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Research Article

Muscle LIM protein promotes expression of the acetylcholine receptor γ-subunit gene cooperatively with the myogenin-E12 complex

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Abstract. Muscle LIM protein (MLP, also referred to as CRP3) is a muscle-specific LIM-only protein, which consists of two LIM motifs. MLP functions as a positive regulator during myogenesis. Here we report that MLP serves as a cofactor regulating the expression of the nicotinic acetylcholine receptor (AChR) γ-subunit gene in skeletal muscle cells. We found that MLP promoted the expression of the AChR γ-subunit gene in C2C12 myotubes, but not in C2C12 myoblasts or NIH3T3 fibroblasts. Furthermore, we showed that MLP interacted with myogenin in vivo and enhanced the binding ability of the myogenin-E12 heterodimer to the E boxes in the AChR γ-subunit gene promoter. Together, these results suggest that MLP promotes the specific expression of the AChR γ-subunit gene cooperatively with the myogenin-E12 complex during myogenesis.

Key words. MLP/CRP3; myogenin; E12; AChR γ-subunit gene; regulation of gene expression.

Muscle LIM protein (MLP, also referred to as CRP3) belongs to the LIM-only class of the LIM domain protein family. MLP itself consists of two LIM motifs, each followed by a glycine-rich region [1]. The LIM motif is a protein-binding interface found in a diverse group of proteins that include LIM kinases and LIM homeodomain proteins. This motif is frequently found in proteins involved in cell differentiation and cell fate determination, suggesting that LIM-based protein interactions may mediate specific regulatory processes in the cell [2–4]. MLP is a positive regulator of myogenesis. Overexpression of MLP in C2C12 cells leads to an enhancement of muscle differentiation, whereas expression of antisense MLP dramatically inhibits myogenesis [1]. In vitro protein-binding assays demonstrated that MLP binds MyoD, MRF4 and myogenin in vitro. In vivo experiments showed that MLP interacts with the MyoD-E47 complex and enhances the DNA-binding activity associated with this complex. Therefore, MLP is proposed to promote myogenesis by enhancing the activity of MyoD [5]. During myogenesis, the appearance of MLP coincides with differentiation of muscle cells. MLP accumulates during muscle fiber formation and is down-regulated in adult skeletal muscle. At the beginning of muscle differentiation, MLP is restricted to the nucleus. As differentiation proceeds, it also accumulates in the cytoplasm [1]. The dual localization of MLP suggests that MLP may play multiple roles during myogenesis.

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The skeletal muscle acetylcholine receptor (AChR) is a ligand-gated channel composed of four types of structurally related subunits, which assemble into a heteropentamer ($\alpha_2\beta\gamma\delta$) [6]. Although the four AChR subunit genes map to at least three different loci [7], their expression is tissue-specific and coordinately regulated during skeletal muscle development. The four subunit mRNAs found in fetal muscle are down-regulated by nerve-evoked muscle activity, and all four mRNAs are more highly concentrated in the region of the sarcoplasm nearest the neuromuscular junction and are much less abundant in extrasynaptic areas of adult innervated muscle [8–11]. Nuclear run-on experiments have shown that the increase in mRNA levels is caused by transcriptional activation in differentiating myocytes [12] and in muscle tissue after denervation [13]. Thus, the coordinated changes of these four mRNAs are apparently regulated at the transcriptional level.

E boxes (CANNTG) are extensively present in the promoters of the four AChR subunit genes, and they are required for the full transcriptional activities of these genes [14–20]. E boxes are targeted by myogenic regulatory factors (MRFs), including MyoD, myogenin, MRF4 and Myf5 [21, 22]. Among MRFs, myogenin is the most likely to be involved in the control of the four AChR subunit genes, because in contrast to mRNAs coding for MyoD, Myf5, and MRF4, the myogenin mRNA is up-regulated by 200 to 400-fold following denervation [23]. Structural and functional analyses of the chick AChR γ-subunit gene upstream sequence have demonstrated that the interaction of mygenin and E-boxes in the AChR γ-subunit gene promoter plays an important role in the transcriptional activity of this gene [24]. Here, we investigate the effect of MLP on the expression of the AChR γ-subunit gene during myogenesis. We find that MLP enhances the expression of the AChR γ-subunit gene in differentiating C2C12 muscle cells. Furthermore, we also show that this effect is performed through the interaction of MLP and the myogenin-E12 complex.

Materials and methods

Plasmids

Total RNA was isolated from rat denervated adult skeletal muscle with TRIzol reagent (GIBCO BRL) as described by the manufacturer, and rat MLP cDNA was amplified using RT-PCR. The sense primer and antisense primer were 5′-tcaggatccGTCTTCACCATGCCGAACTG-3′ and 5′-atcgaattcTAGCAGGGCTGTGAGAAAGC-3′, which contain the *Bam*HI or *Eco*RI site, respectively. The resulting 640-bp cDNA product, containing the entire open reading frame of MLP, was cloned into pUC18 at *Bam*HI-*Eco*RI sites and sequenced. The MLP cDNA was then subcloned into pcDNA3 (Invitrogen) to generate the MLP expression plasmid pcDNA3-MLP. The HA-tagged MLP expression plasmid pCMV-HA-MLP, containing an HA epitope fused to the N terminus of MLP, was constructed by ligating the full-length MLP cDNA in frame into the expression plasmid pCMV-HA (Clontech). The expression plasmid pBind-MLP, which expresses a fusion protein Gal4-MLP, was constructed by ligating the full-length MLP cDNA in frame into the expression plasmid pBind (Promega). The 960-bp upstream sequence of the chick AChR γ-subunit gene was subcloned from pBLCAT2-γ [24] into pGL3-Basic (Promega) to construct the luciferase reporter gene expression plasmid pGL3-γ. pPGK and pPGK-myogenin [25] were kindly provided by Dr. I. Skerjanc (Department of Biochemistry, Western Ontario University, Canada). pYN3218 and pYN3218-E12 [26] were gifts from Dr. G. Kato (Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, Md.).

Cell culture, transfection and luciferase assays

C2C12 myoblasts and NIH3T3 fibroblasts (a gift from Dr. J. Schmidt, SUNY at Stony Brook, Stony Brook, N. Y.) were cultured as described previously [24]. When C2C12 myoblasts were to be tested, bromodeoxyuridine was added to the medium (5 µg/ml) to suppress differentiation [27]. DNA was transfected into all cells by calcium phosphate coprecipitation [28]. DNA calcium phosphate precipitates containing 3 µg pGL3-γ, 0.3 µg pRL-TK (Promega, internal control for transfection efficiency) and 3 µg pcDNA3 or pcDNA3-MLP, were added to 35-mm-diameter dishes containing 10^5 cells. After 4 h, cells were subjected to a 2-min osmotic shock and then fed growth medium containing 15% FBS. All NIH3T3 cells and part of the C2C12 cells were switched to differentiation medium (DMEM, 2% horse serum) on the following day and allowed to differentiate. The other C2C12 cells were kept in growth medium. After 48 h, cells were harvested and firefly luciferase activities as well as *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter System (Promega) according to the manufacturer's protocol. Firefly luciferase activities were normalized to *Renilla* luciferase activities. A minimum of three independent transfections were performed for each experimental group.

RT-PCR

C2C12 cells were transfected with pcDNA3 or pcDNA3-MLP as described above. After 48 h, total RNA was isolated with TRIzol reagent (GIBCO BRL). RT-PCR was performed with a SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacturer's instructions. For the AChR γ-subunit gene, oligonucleotides 5′-TTGCTTGCCTCATTCCACAGC-3′ and 5′-TTTGTCCTCCTTTCGAGCCAC-3′ were used

as the primers. For the GAPDH gene (as internal control), oligonucleotides 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ were used. PCR conditions were: for the AChR γ-subunit gene, 23 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s; for the GAPDH gene, 21 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s. The PCR products were analyzed by 1% agarose gel with ethidium bromide staining. Expression levels of the AChR γ-subunit gene were normalized against GAPDH mRNA.

Coimmunoprecipitation assays

NIH3T3 cells were transfected with control plasmids pPGK and pCMV-HA or expression plasmids pPGK-myogenin and pCMV-HA-MLP as described above. After 48 h, cells were rinsed in Tris-buffered saline, scraped, and centrifuged at 6000 rpm for 5 min. The cell pellet was resuspended in nuclear lysis buffer [50 mM HEPES (pH 7.2), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate, 0.1% Triton X-100, 2 μg/μl aprotinin, 2 μg/μl pepstatin, 2 μg/μl leupeptin] and gently rocked at 4 °C for 1 h as described previously [29]. Extracts were centrifuged at 10,000 rpm for 5 min, and the supernatant was aliquoted and frozen at –80 °C for storage. Myogenin monoclonal antibody (5 μg; Santa Cruz Biotechnology) was added to each group containing 300 μg protein in 10 mM HEPES (pH 7.6), 250 mM NaCl, 0.25% Nonidet P-40 (NP-40), and 5 mM EDTA, and the mixture was incubated at 4 °C for 1 h. A 50% slurry (25 μl) of protein A-agarose beads (Roche Applied Science) was subsequently added to each sample, and the samples were incubated for an additional 1 h at 4 °C. After being rinsed with nuclear lysis buffer, the final bead pellets were resuspended in SDS loading buffer and subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer onto a nitrocellulose membrane. Western blot analysis was carried out by using HA polyclonal antibodies (Clontech) to detect the HA-MLP fusion protein.

Mammalian two-hybrid assays

CheckMate Mammalian Two-Hybrid System (Promega) was used for mammalian two-hybrid assays. NIH3T3 cells were cotransfected with 3 μg of pBind or pBind-MLP and 3 μg of pG5*luc* along with 3 μg of pPGK-myogenin, pYN3218-E12 [26], or both. Cells were harvested after 48 h, and firefly luciferase activities as well as *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter System (Promega) according to the manufacturer's recommendations. Firefly luciferase activities were normalized to *Renilla* luciferase activities. A minimum of three independent transfections were performed for each experimental group.

GST fusion proteins and electrophoretic mobility shift assays

The full-length MLP cDNA was ligated in-frame with GST into the *Bam*HI-*Eco*RI sites of pGEX-2TK (Phamacia). The resulting construct produced GST-MLP fusion protein. The full-length E12 cDNA was ligated in frame with GST into the *Eco*RI-*Sal*I sites of pGEX-4T-2 (Phamacia). The resulting construct produced GST-E12 fusion protein. The GST-myogenin fusion protein expression vector has been described previously [24]. The GST fusion proteins were expressed in *Escherichia coli* BL21 and purified with glutathione Sepharose 4B (Amersham) according to the procedure recommended by the manufacturer. The quality of the purified proteins was checked using 10% SDS-PAGE. The probes used in electrophoretic mobility shift assays were generated by labeling the *Hin*dIII-*Bgl*II-digested –324/+36 fragment of the chick AChR γ-subunit gene promoter with [α-^{32}P] dATP using the Klenow large fragment. Electrophoretic mobility shift assays were conducted as described previously [30]. Labeled probes (5000 cpm) and purified GST-myogenin (0 or 5 ng), GST-E12 (0 or 10 ng), and GST-MLP (0, 5, 10 or 20 ng) were used for each 20-μl reaction in a buffer containing 50 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 10 μg/ml BSA, 10% glycerol, 20 mM HEPES (pH 7.6), and 5 μg/ml poly-(dI-dC)·(dI-dC)·(Sigma). The mixture was incubated at 37 °C for 20 min, and then protein-DNA complexes were separated by native PAGE and visualized by autoradiography.

Results

MLP promotes the expression of the AChR γ-subunit gene

Given the role of nuclear MLP in development, we set out to investigate whether MLP promotes the expression of the AChR γ-subunit gene in differentiating muscle cells (myotubes). To explore this possibility, C2C12 and NIH3T3 cells were transfected with pGL3-γ and pcDNA3 or pcDNA3-MLP, and the luciferase activities of the cell extracts were measured. As shown in figure 1A, the luciferase activity in myotubes cotransfected with pGL3-γ and pcDNA3-MLP was more than threefold higher than that in myotubes cotransfected with pcDNA3 and pGL3-γ. In contrast, only basal luciferase activities were found in myoblasts and fibroblasts cotransfected with pGL3-γ and pcDNA3-MLP. These results show that MLP increases the cell type-specific transcription of the AChR γ-subunit gene.

To further confirm that MLP promotes the expression of the AChR γ-subunit gene during myogenesis, C2C12 and NIH3T3 cells were cotransfected with pcDNA3 or pcDNA3-MLP, and mRNA levels of the AChR γ-subunit gene were evaluated using semiquantitative RT-PCR. As

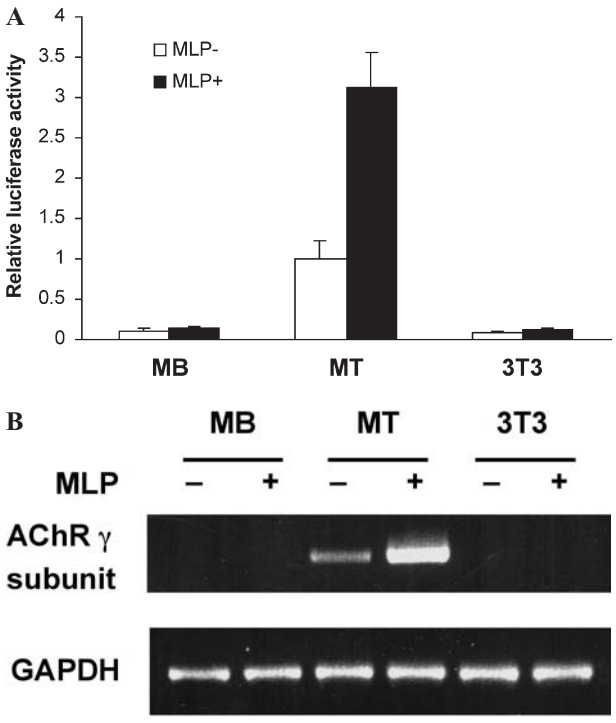

Figure 1. Effect of MLP on the expression of the AChR γ-subunit gene during myogenesis. MB, C2C12 myoblasts; MT, C2C12 myotubes; 3T3, NIH3T3. (*A*) C2C12 and NIH3T3 cells were cotransfected with pGL3-γ along with control expression plasmid or with expression plasmid encoding MLP. After 48 h, cells were harvested and luciferase activities were measured with the Dual-Luciferase Reporter System (Promega). (*B*) C2C12 and NIH3T3 cells were cotransfected with pcDNA3 or pcDNA3-MLP. After 48 h, total RNAs were isolated and mRNA levels of the AChR γ-subunit gene were evaluated using semiquantitative RT-PCR. GAPDH mRNA was used as an internal control.

shown in figure 1B, AChR γ-subunit mRNA was expressed in myotubes, but not in myoblasts and NIH3T3 cells, and there was an evident increase in AChR γ-subunit mRNA level in myotubes transfected with pcDNA3-MLP compared with that in myotubes transfected with pcDNA3. These results further demonstrate that MLP promotes the expression of the AChR γ-subunit gene during myogenesis.

MLP and myogenin interact in vivo

Since MLP does not contain a transcription activation domain (TAD) [5], MLP is unlikely to promote the expression of the AChR γ-subunit gene by itself in differentiating myocytes. The heterodimer of myogenin-E12 activates the expression of the AChR subunit genes during myogenesis, so MLP may promote gene expression by interacting with the myogenin-E12 complex. To examine if MLP and myogenin interact in vivo, two approaches, coimmunoprecipitation assays and mammalian two-hybrid assays, were performed. For the coimmunoprecipitation assays, NIH3T3 cells were transfected with control expression plasmids or with expression plasmids encoding myogenin, HA-tagged MLP, or both. Following immunoprecipitation with an anti-myogenin monoclonal antibody, the precipitates were immunoblotted using an anti-HA polyclonal antibody. Western blotting revealed that HA-MLP was detected only in myogenin precipitate from the cells coexpressing HA-MLP and myogenin (fig. 2), showing that MLP associates with myogenin in the nuclear compartment.

This in vivo interaction was further confirmed by mammalian two-hybrid assays. For these studies, expression plasmids encoding a Gal4-MLP fusion protein plus myogenin or E12 were cotransfected into NIH3T3 cells along with the luciferase reporter gene pG5*luc*. As predicted, the fusion protein Gal4-MLP did not activate the reporter gene because it lacks a functional TAD. When an E12 expression plasmid was coexpressed with Gal4-MLP, only a basal level of luciferase activity was detected. Conversely, when myogenin was coexpressed with Gal4-MLP, enzyme activity was induced approximately fourfold, suggesting that myogenin binds to MLP and activates reporter gene expression via the myogenin TAD. Coexpression of both myogenin and E12 with Gal4-MLP gave rise to a significant increase in the reporter gene as a result of the heterodimer formation between myogenin and E12, indicating that MLP is also capable of interacting with the myogenin-E12 heterodimer complex in vivo (fig. 3B).

MLP enhances the binding activity of the myogenin-E12 complex to the AChR γ-gene promoter

Given that MLP interacts with myogenin-E12 heterodimers in vivo, we next decided to investigate the possibility that MLP influences the DNA-binding properties associated with this heterodimer complex. To address this question, we first expressed GST-myogenin, GST-E12, and GST-MLP fusion proteins in *Escherichia coli* BL21

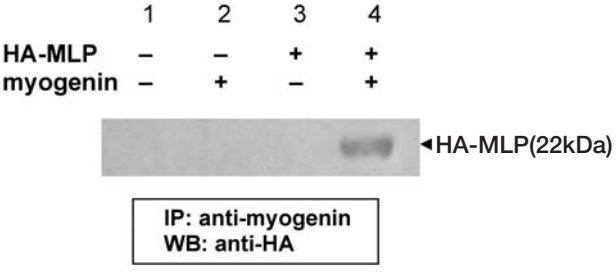

Figure 2. Coimmunoprecipitation assays of MLP and myogenin in NIH3T3 cells. NIH3T3 cells were transfected with control expression plasmids or with expression plasmids encoding myogenin, HA-tagged MLP, or both. After 48 h in differentiation medium, cells were harvested and subjected first to myogenin immunoprecipitation and then to Western blot analysis using HA polyclonal antibodies (WB). The 22-kDa HA-MLP is detected only in cells coexpressing myogenin and MLP, demonstrating that myogenin and MLP associate with one another within the nuclear compartment.

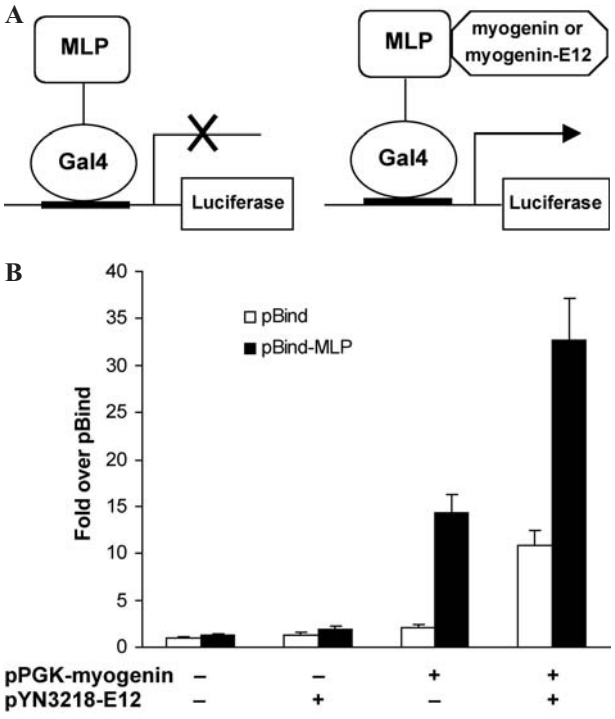

Figure 3. Mammalian two-hybrid analyses of MLP and myogenin interactions in NIH3T3 cells. (*A*) Schematic diagram of the mammalian two-hybrid of MLP with myogenin or myogenin-E12 in NIH3T3 cells. Myogenin intrinsically contains an activation domain, so either myogenin or myogenin-E12 heterodimer could promote the transcription of the reporter gene by binding with MLP. (*B*) NIH3T3 cells were cotransfected with pBind or pBind-MLP, and the pG5*luc* reporter gene along with control expression plasmids or with expression plasmids encoding myogenin, E12, or both. After 48 h, luciferase activities of the cell extracts were tested with the Dual-Luciferase Reporter System (Promega).

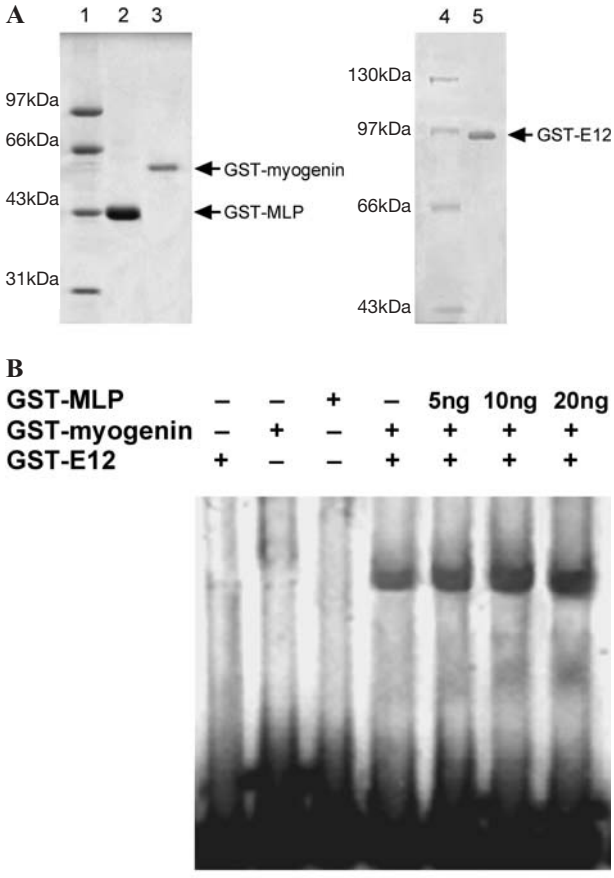

Figure 4. Electrophoretic mobility shift assays using purified GST-MLP, GST-myogenin, and GST-E12. (*A*) The quality of the purified proteins is checked using 10% SDS-PAGE. Lanes 1, 4, protein molecular-weight standards; lanes 2, 3, 5, purified GST-MLP, GST-myogenin, and GST-E12. (*B*) ^{32}P-labeled probes of the –324/+36 fragment of the AChR γ-subunit gene promoter were incubated with the purified GST fusion proteins. The DNA-protein complexes were separated by nondenaturing PAGE and then visualized by autoradiography.

and purified them with glutathione Sepharose 4B (fig. 4 A). GST-myogenin, GST-E12, and GST-MLP fusion proteins were incubated with a ^{32}P-labeled –324/+36 fragment of the AChR γ-subunit gene promoter, which contains three E boxes [23]. The generated protein-DNA complexes were separated by native PAGE and visualized by autoradiography. As shown in figure 4 B, purified GST-MLP did not bind to the probe. As expected, only a weak shifted band was detected when the binding reaction was performed with GST-myogenin or GST-E12 alone. However, when both GST-myogenin and GST-E12 were present, a shifted band of high intensity was detected, illustrating that heterodimer formation is required for strong interaction under these assay conditions. When GST-MLP was added to the reactions, about twofold to threefold enhancement in the bound myogenin-E12 complex was observed. Interestingly, no super-shifted bands were found when binding reactions were performed with GST-myogenin, GST-E12 and GST-MLP in the electrophoretic mobility shift assays, indicating that GST-MLP does not reside in the DNA-protein complexes. Thus, although MLP enhances myogenin-E12 binding to the E-boxes in the AChR γ-subunit gene promoter, MLP likely dissociates from these complexes once the DNA-protein complexes enter the gel.

Discussion

The stage- and tissue-specific expression of the AChR genes is controlled primarily by the interaction of myogenic factors and E boxes [14–20]. The chick AChR γ-subunit gene promoter contains three E boxes within the –324/+36 fragment [24]. Binding and activating analyses demonstrate that the activation of the AChR γ-subunit gene promoter is controlled primarily by E boxes, which are activated by myogenin in vivo [24].

In this study, we found that MLP promotes the expression of the AChR γ-subunit gene in differentiating muscle

cells, but not in myoblasts or fibroblasts. This finding suggests that MLP contributes to muscle-specific expression of the AChR γ-subunit gene. Our coimmunoprecipitation and mammalian two-hybrid assays revealed that MLP interacts with myogenin (fig. 2) or myogenin-E12 heterodimers, but not E12 alone (fig. 3B), indicating that MLP and E12 may interact with myogenin at different positions. These results are in agreement with the finding of Kong et al. [5]. The interaction of MLP and the myogenin-E12 complex enhances the binding of the myogenin-E12 complex to the AChR γ-subunit gene promoter, suggesting that MLP promotes the expression of the AChR γ-subunit gene by facilitating the binding of myogenin-E12 heterodimers to the E boxes in the AChR γ-subunit gene promoter.

MLP is an essential positive regulator of myogenic differentiation and appears to participate in regulatory processes controlling muscle-specific gene expression [1, 5]. The recent studies demonstrated that MLP interacts with T-cap, a 19-kDa muscle-specific protein that was isolated as a titin-binding protein at the Z disc. MLP is required for the stabilization of T-cap with the titin complex through the independent association with α-actinin at the Z disc [31]. T-cap may block the secretion of myostatin, a key negative regulator of skeletal muscle growth [32]. These data suggest that MLP is a crucial factor for regulation of growth and differentiation of muscle cells by interacting with various proteins.

In our study, we did not detect MLP in the myogenin-E12-DNA complex by electrophoretic mobility shift assay. However, the inability seems to be a feature shared by other DNA binding-enhancing proteins such as Rb, pX, and AP1, which increase the DNA-binding activity of LSP, CREB, and JunD, respectively, without generating a ternary complex in electrophoretic mobility shift assays [33–35]. The enhancement in DNA binding of the myogenin-E12 complex induced by MLP can be explained by the following possibilities. One is that MLP enhances formation of the myogenin-E12 heterodimer. The other possibility is that MLP may affect the conformation of the myogenin-E12 heterodimer complex, thereby promoting DNA sequence recognition.

MLP exhibits a dual subcellular localization, initially present exclusively in nuclei of early differentiated muscle cells and later accumulating at high levels in the cytoplasm [1]. Nuclear MLP appears to be a positive regulatory factor of myogenesis by interacting with MyoD through its first LIM domain [5]. Cytoplasmic MLP is preferentially associated with actin filaments and the Z disc region, suggesting that the function of cytoplasmic MLP is associated with the architecture and contraction of muscle cells. MLP was recently reported to serve as a bridging molecule between α-actinin and spectrin, thus providing a link between the myofibril and the membrane cytoskeleton [36]. Future studies might investigate the mechanisms by which MLP translocates and distributes in striated muscle cells.

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