The muscle integrin binding protein (MIBP) interacts with \( \alpha 7\beta 1 \) integrin and regulates cell adhesion and laminin matrix deposition

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Received for publication 20 December 2002, revised 18 March 2003, accepted 12 May 2003

Abstract

Integrins are \( \alpha \beta \) transmembrane receptors that function in key cellular processes, including cell adhesion, differentiation, and extracellular matrix deposition through interactions with extracellular, membrane, and cytoplasmic proteins. We previously identified and cloned a muscle \( \beta 1 \) integrin cytoplasmic binding protein termed MIBP and found that the expression level of MIBP is critical in the decision-making process of terminal myogenic differentiation. We report here that MIBP interacts with the \( \alpha 7\beta 1 \) integrin but not the \( \alpha 5\beta 1 \) integrin in C2C12 myoblasts, suggesting an important role of integrin \( \alpha \) chains in the regulation of the \( \beta 1\)-MIBP interaction. Furthermore, consistent with its selective binding activity toward the \( \alpha 7\beta 1 \) laminin receptor, we have found that overexpression of MIBP in C2C12 myoblasts resulted in a significant reduction of cell adhesion to laminin and inhibition of laminin matrix deposition. By contrast, neither cell adhesion to fibronectin nor fibronectin matrix deposition was significantly altered in cells overexpressing MIBP. Finally, we show that both the protein level and tyrosine phosphorylation of paxillin, a key signaling molecule involved in the cellular control of myogenic differentiation, are increased by MIBP. These results suggest that MIBP functions in the control of myogenic differentiation by regulating \( \alpha 7\beta 1 \) integrin-mediated cell interactions with laminin matrix and intracellular signaling through paxillin.

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Keywords: MIBP; Integrin; Laminin; Cell adhesion; Signaling

Introduction

Myogenic differentiation is a highly regulated process that is controlled to a large extent by cellular interactions with extracellular matrix (Song et al., 1992; Sastry and Horwitz, 1996; Gullberg et al., 1998; Burkin and Kaufman, 1999). The specific response of myogenic cells depends on, among other things, the composition of the extracellular matrix. For example, laminin promotes myogenic differentiation (Foster et al., 1987), whereas fibronectin inhibits it (Podleski et al., 1979; von der Mark and Ocalan, 1989). The importance of laminin in muscle development and function has been underscored by a number of clinical and experimental studies showing that defects in the structure, expression, or deposition of laminin are closely associated with, or even are the causal factors, in the pathogenesis of congenital muscular dystrophies. For example, null- or truncation-mutations in the laminin \( \alpha 2 \)-chain gene result in congenital muscular dystrophies in humans as well as in mice (Helbling-Leclerc et al., 1995; Hillaire et al., 1994; Kuang et al., 1998; Miyagoe et al., 1997; Sunada et al., 1994, 1995; Wewer and Engvall, 1996; Xu et al., 1994). Reduction of other laminin chains, including \( \beta 1 \), has also been associated with a number of cases of congenital muscular dystrophies (Hayashi et al., 1993; Higuchi et al., 1994; Li et al., 1997; Sewry et al., 1997; Wewer et al., 1995).

Cell adhesion to extracellular matrix is mediated by integrins and other cell surface receptors (Hynes, 1992; Sastry and Horwitz, 1996; Schwartz et al., 1995; Song et al., 1992). A number of integrins, including laminin receptor \( \alpha 7\beta 1 \) integrin and fibronectin receptor \( \alpha 5\beta 1 \) integrin, are expressed by myoblasts (Burkin and Kaufman, 1999; Gullberg et al., 1998). The expression level, subtype, and acti-
vation state of the integrins are precisely regulated during myogenesis (Song et al., 1992; Boettiger et al., 1995; Sastry and Horwitz, 1996; Gullberg et al., 1998; Burkin et al., 1999). For example, three developmentally regulated α7 cytoplasmic domains (A, B, and C) resulting from alternative RNA splicing have been identified (Collo et al., 1993; Song et al., 1993; Zipper et al., 1993, Hodges and Kaufman, 1996). The α7B is expressed in myoblasts as well as in muscle fibers. In contrast, α7A and C isoforms are expressed only after the initiation of terminal myogenic differentiation. The α7Bβ1 integrin plays important roles in myogenesis (Kaufman et al., 1985; Burkin and Kaufman, 1999; Gullberg et al., 1998). It mediates cell adhesion to laminin and participates in a number of cellular processes in response to laminin (Burkin and Kaufman, 1999; Crawley et al., 1997; Yao et al., 1996a). The α7Bβ1 integrin also participates in the formation of the postsynaptic membrane (Burkin et al., 1998, 2000), integrin-mediated calcium signaling (Kwon et al., 2000), and skeletal muscle regeneration (Kaariainen et al., 2002). Mutations in human integrin α7 gene have been identified in patients with congenital myopathies (Hayashi et al., 1998). Diminished α7 expression has also been found in laminin-related congenital muscular dystrophies (Hodges et al., 1997; Vachon et al., 1997) and in muscular dystrophies of unknown etiology (Pegoraro et al., 2002). In the mouse, inactivation of the integrin α7 gene also results in a form of muscular dystrophy (Mayer et al., 1997). In contrast, increasing levels of the α7Bβ1 integrin in dystrophic mice ameliorates the development of muscular dystrophy and prolongs their lifespan (Burkin et al., 2001). These clinical and experimental studies have provided strong evidence for crucial roles of the α7Bβ1 integrin in the pathogenesis of muscle diseases.

In addition to mediating cell adhesion to extracellular matrix, integrins participate in myogenic differentiation by mediating signal transduction (Sastry et al., 1996, 1999; Kwon et al., 2000). One of the key downstream targets of integrin signaling in myogenic differentiation is paxillin. Increases in the protein level and tyrosine phosphorylation of paxillin effectively inhibited terminal myogenic differentiation (Sastry et al., 1999).

We recently identified a novel muscle protein termed as MIBP that specifically interacts with the β1 integrin cytoplasmic domain (Li et al., 1999). The expression of MIBP was dramatically downregulated during myogenic differentiation. Furthermore, overexpression of MIBP effectively suppressed myogenic differentiation (Li et al., 1999), suggesting that the level of MIBP is crucial to the initiation of myogenic differentiation. The particular β1 integrin recognized by MIBP, however, was not known. In this study, we show that MIBP associates with the laminin receptor α7Bβ1 integrin but not the fibronectin receptor α5β1 integrin. Furthermore, we have found that MIBP is functionally involved in the regulation of cell adhesion to laminin and laminin matrix deposition. Finally, we show that both the protein level and tyrosine phosphorylation of paxillin are upregulated by MIBP. These results shed light on the mechanism by which MIBP functions in the regulation of myogenic differentiation.

Materials and methods

Cells, antibodies, and other reagents

Mouse C2C12 myoblasts were from American Type Culture Collection (Rockville, MD). Rabbit polyclonal anti-α7 cytoplasmic domain antibodies (α7A2 and α7B2) have been previously described (Song et al., 1993; Martin et al., 1996). Rabbit polyclonal anti-α5 integrin antibody (Ab33) and anti-β1 integrin antibody (MC231) were kindly provided by Dr. John A. McDonald (Mayo Clinic, Scottsdale, AZ). Rabbit anti-laminin antibody was purchased from Chemicon (Temecula, CA). Rabbit anti-entactin antibody was kindly provided by Dr. Albert Chung (University of Pittsburgh). Monoclonal anti-paxillin antibody and horseradish peroxidase (HRP)-conjugated anti-phospho-tyrosine antibody RC-20H were purchased from BD Transduction Laboratories (San Diego, CA). Rabbit polyclonal anti-FAK antibody (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and myogenic differentiation

C2C12 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. C2C12 cells transfected with a FLAG vector encoding MIBP or the control FLAG vector lacking the MIBP sequence were grown in the same medium supplemented with 0.5 mg/ml G418. For myogenic differentiation assays, the cells were seeded in DMEM containing 2% horse serum as previously described (Li et al., 1999).

Production of CMV-α7BX2-FLAG

Rat α7BX2 cDNA (Burkin et al., 1998) was subcloned in frame into p3XFLAG-CMV14 vector (Sigma). The construct was verified by DNA sequencing. C2C12 cells were grown in Dulbecco’s medium (low glucose) containing 20% FCS, 0.5% chick embryo extract, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml kanamycin. Cells were transfected with 5 μg of linearized CMV-α7BX2-FLAG plasmid using Superfect Reagent (QIAGEN Inc., Valencia, CA) following company directions. Transfected cells were grown in medium containing 500 μg/ml G418 (Invitrogen Life Technology). Synthesis of FLAG-tagged integrin α7 was confirmed by Western blotting.
Cell adhesion assay

Cell adhesion assays were performed as described (Braut-Boucher et al., 1995). Briefly, cells (1 × 10^6) were labeled with 10 mM Calcein-AM (Molecular Probes, Eugene, OR) in OPTI-MEM medium. After incubation at 37°C in a CO₂ incubator for 30 min, the cells were washed twice with OPTI-MEM medium and seeded (6 × 10^4 cells/well) in fibronectin- or laminin-coated 96-well plates (Becton Dickinson). Cells were incubated at 37°C in a CO₂ incubator for 1 h and then the wells were washed three times with OPTI-MEM medium. The total cells (before washing) and the adhered cells (after washing) were quantified by using a CytoFluor Multi-well Plate Reader (Perseptive Biosystems, excitation wavelength = 485 nm; emission wavelength = 530 nm).

Immunoprecipitation assays

Cells (as specified in each experiment) were cultured in DMEM containing 10% FBS and harvested with a cell scraper. For immunoprecipitation with monoclonal anti-FLAG antibodies, cells were lysed in the lysis buffer (1% Triton X-100 in phosphate-buffered saline containing 0.2 mM AEBSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, and 5 μg/ml leupeptin). The lysates were clarified by centrifugation at 10,000 g for 15 min and preincubated with 20 μl protein A beads. The precleared cell lysates (700 μg) were mixed with 20 μl anti-FLAG antibody-conjugated beads (M2 beads; Sigma) or the control beads (protein A–agarose beads coupled with an irrelevant mouse IgG) and incubated at 4°C for 1 h. The beads were washed three times with phosphate-buffered saline. The immunoprecipitates were analyzed by immunoblotting with anti-FLAG antibody M5, anti-β1 integrin antibody MC231, anti-α7B integrin antibody, or anti-α5 integrin Ab33 as specified in each experiment.

For immunoprecipitation with anti-paxillin and anti-FAK antibodies, cells were lysed with the RIPA buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1.0% Triton X-100, 0.25% sodium deoxycholate, 30 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate and protease inhibitors). The lysates (300 μg) were incubated with 2 μg monoclonal anti-paxillin antibody or 2 μg polyclonal anti-FAK antibody (C-20) at 4°C for 1 h, and then mixed with protein G beads (20 μl) and incubated at 4°C for another hour. The beads were pelleted by centrifugation and washed with the RIPA buffer three times. The immunoprecipitates were analyzed by immunoblotting with monoclonal anti-paxillin antibody, anti-FAK C-20 antibody, or HRP conjugated anti-phosphotyrosine antibody RC20H as specified in each experiment.

Isolation of extracellular matrix proteins

Extracellular matrix proteins were isolated as previously described (Wu et al., 1993, 1995). Briefly, cell monolayers were harvested with a cell scraper and pelleted by centrifugation. The extracellular matrix fraction was isolated by sequential extraction of the cells with the following: first, 3% Triton X-100 in 10 mM Tris buffer (pH 8.0) containing 150 mM NaCl and protease inhibitors; second, DNase I (100 μg/ml) in 50 mM Tris–HCl (pH 7.4) containing 10 mM MnCl₂ and 1 M NaCl; third, 2% sodium deoxycholate in 50 mM Tris–HCl (pH 8.8) containing 10 mM EDTA. The deoxycholate-insoluble extracellular matrix fractions were analyzed by immunoblotting with antibodies recognizing laminin, fibronectin, and entactin, respectively.

Northern blotting

RNA was isolated from C2C12 cells by using an RNAeasy mini kit (Qiagen) following the manufacturer’s protocol. The isolated RNA (5 μg) was separated on a 1% agarose gel containing formaldehyde. RNA was transferred to Hybond N+ nylon membrane (Amersham Pharmacia). A 708-bp paxillin cDNA fragment encoding paxillin residues 322–557 was prepared as previously described (Tu et al., 1999). The paxillin cDNA fragment (25 ng) was labeled with 32P-CTP using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN). The blot was prehybridized with Express Hyb Hybridization solution (CLONTECH) at 68°C for 1 h and then hybridized with the 32P-labeled paxillin probe for 3 h at 68°C. Messenger RNA bands hybridized with the 32P-labeled paxillin probe were visualized by autoradiography. In control experiments, the same membrane was probed with a 32P-labeled β-actin cDNA probe to confirm the equal loading.

Results

MIBP interacts with α7β1 integrin but not α5β1 integrin in C2C12 myoblasts

We previously reported that MIBP specifically interacts with the sequence LIWKLLMIIHDRREFAKFEKEKMNAKDWT in the membrane-proximal region of the β1 cytoplasmic domain (Li et al., 1999). In this study, we tested the ability of MIBP to associate with β1 integrin chains paired either with α7B or with α5 that are expressed by myoblasts and that are known to play important roles in myogenesis. To facilitate immunoprecipitation of MIBP, we expressed an epitope-tagged MIBP (FLAG-MIBP) in C2C12 cells (Fig. 1C, lane 4). FLAG-MIBP was precipitated from the cell lysates with a monoclonal anti-FLAG antibody M2 (Fig. 1C, lane 6) but not with an irrelevant mouse IgG (Fig. 1C, lane 5). Immunoblotting analyses of the immunoprecipitates with antibodies specific for β1 (Fig. 1D, lane 6) and α7B (Fig. 1A, lane 6) revealed that a fraction of α7Bβ1 integrin was coprecipitated with FLAG-MIBP. In contrast, despite the presence of α5 integrin in the cell lysates that could be readily detected by anti-α5 anti-
bodies (Fig. 1B, lane 4), no α5 was detected in the FLAG-MIBP immunoprecipitate (Fig. 1B, lane 6). In control experiments, none of the integrins were detected in the precipitates obtained with the irrelevant mouse IgG (lanes 2 and 4). Immunoprecipitation was performed as described in Materials and methods. Proteins in the immunoprecipitates were resolved in the reducing SDS-PAGE and analyzed by immunoblotting with an antibody specific for the cytoplasmic domain of α7B integrin (A), an antibody specific for the cytoplasmic domain of α5 integrin (B), an anti-FLAG antibody (C), and an anti-β1 integrin antibody (D), respectively. Lanes 1 and 4 were loaded with cell lysates (10 μg/lane) as indicated in the figure. Full-length α7B and α5 integrins and their cleavage products containing α7B or α5 cytoplasmic domain, FLAG-MIBP, and β1 integrin are marked. Unecleaved α7B and α5 migrate at approximately 120 and 130 kDa, respectively. (E) Lysates of C2C12 cells transfected with a FLAG-α7BX2 expression vector (lane 2) or the parental C2C12 cells (lane 1) were immunoprecipitated with mouse monoclonal anti-FLAG antibody M2 and probed with anti-MIBP antibody. The immunoprecipitates from the lysates of the parental C2C12 cells (lane 3), the FLAG-α7BX2 transfectants (lane 4), or a control transfectants lacking FLAG-α7BX2 (lane 5), and the supernatants from the parental C2C12 cells (lane 6) and the FLAG-α7BX2 transfectants (lane 7) were separated on SDS-PAGE. Note that MIBP was detected in the FLAG-α7 immunoprecipitates (lane 4) but not the control precipitates (lanes 3 and 5), and the amount of MIBP that remained in the supernatant of the FLAG-α7BX2 transfectants (lane 7) was reduced compared with that of the control (lane 6), suggesting that a fraction of MIBP forms a complex with the α7BX2β1 integrin.

Fig. 1. MIBP associates with the α7Bβ1 integrin but not the α5β1 integrin. (A–D) Lysates of the C2C12 cells transfected with a FLAG-MIBP expression vector (lanes 4–6) or a mock control vector (lanes 1–3) were incubated with beads conjugated with mouse monoclonal anti-FLAG antibody M2 (lanes 3 and 6) or an irrelevant mouse IgG (lanes 2 and 4). Immunoprecipitation was performed as described in Materials and methods. Proteins in the immunoprecipitates were resolved in the reducing SDS-PAGE and analyzed by immunoblotting with an antibody specific for the cytoplasmic domain of α7B integrin (A), an antibody specific for the cytoplasmic domain of α5 integrin (B), an anti-FLAG antibody (C), and an anti-β1 integrin antibody (D), respectively. Lanes 1 and 4 were loaded with cell lysates (10 μg/lane) as indicated in the figure. Full-length α7B and α5 integrins and their cleavage products containing α7B or α5 cytoplasmic domain, FLAG-MIBP, and β1 integrin are marked. Unecleaved α7B and α5 migrate at approximately 120 and 130 kDa, respectively. (E) Lysates of C2C12 cells transfected with a FLAG-α7BX2 expression vector (lane 2) or the parental C2C12 cells (lane 1) were immunoprecipitated with mouse monoclonal anti-FLAG antibody M2 and probed with anti-MIBP antibody. The immunoprecipitates from the lysates of the parental C2C12 cells (lane 3), the FLAG-α7BX2 transfectants (lane 4), or a control transfectants lacking FLAG-α7BX2 (lane 5), and the supernatants from the parental C2C12 cells (lane 6) and the FLAG-α7BX2 transfectants (lane 7) were separated on SDS-PAGE. Note that MIBP was detected in the FLAG-α7 immunoprecipitates (lane 4) but not the control precipitates (lanes 3 and 5), and the amount of MIBP that remained in the supernatant of the FLAG-α7BX2 transfectants (lane 7) was reduced compared with that of the control (lane 6), suggesting that a fraction of MIBP forms a complex with the α7BX2β1 integrin.
MIBP regulates cell adhesion to laminin but not to fibronectin

The adhesion of myoblasts to laminin is mediated to a large extent by the α7β1 integrin (Crawley et al., 1997; Song et al., 1993; Yao et al., 1996a). The observation that MIBP recognizes the α7β1 integrin (Fig. 1) prompted us to test whether MIBP plays a role in the regulation of cell adhesion to laminin. The parental C2C12 cells, as shown in previous studies (Yao et al., 1996a), adhered to laminin (Fig. 2A). Overexpression of MIBP by transfecting the C2C12 cells with a FLAG-MIBP expression vector significantly reduced cell adhesion to laminin (Fig. 2A). By contrast, no reduction of adhesion to laminin was observed in C2C12 cells transfected with a control FLAG vector lacking the MIBP sequence (Fig. 2A). To test whether the overexpression of MIBP globally reduces the adhesiveness of the cells, we measured the adhesion of the cells expressing different levels of MIBP to fibronectin. The results showed that cell adhesion to fibronectin was not altered by the overexpression of FLAG-MIBP of FLAG-MIBP (Fig. 2B). Thus, consistent with its selective binding activity toward the laminin receptor α7β1 integrin, MIBP is involved in the regulation of cell adhesion to laminin but not to fibronectin.

MIBP regulates the deposition of laminin, but not fibronectin, into extracellular matrix

Recent studies have demonstrated that integrins not only mediate cell adhesion to matrix proteins but also participate in the deposition of extracellular matrix (Colognato et al., 1999; Sasaki et al., 1998; Schwartz et al., 1995; Schwarzbauer, 1999; Stephens et al., 1995; Wu, 1997). Because MIBP interacts with the laminin receptor α7β1 integrin and inhibits cell adhesion to laminin, we sought to determine whether MIBP exerts an effect on the deposition of laminin into extracellular matrix. To do this, we isolated extracellular matrices from C2C12 cells that overexpress MIBP as well as the parental C2C12 and the FLAG vector control cells. Immunoblotting analyses of the extracellular matrices with antibodies recognizing laminin β1 chain showed that approximately fivefold less laminin was incorporated into the extracellular matrix in the C2C12 cells overexpressing FLAG-MIBP (Fig. 3A and D). Probing the same samples with an anti-fibronectin antibody or an anti-entactin antibody showed that the amounts of fibronectin (Fig. 3B and E) or entactin (Fig. 3C) in the extracellular matrix were not significantly altered, indicating that the decrease of laminin in the extracellular matrix is not caused by a general reduction of extracellular matrix. In further experiments, we analyzed the total cellular level of laminin in C2C12 cells expressing different levels of MIBP. The level of cellular laminin was slightly reduced in cells overexpressing MIBP (Fig. 4A and B). The reduction of the total cellular level of laminin, however, was much smaller than the reduction of laminin matrix resulting from overexpression of MIBP (Fig. 3D).

MIBP suppresses the expression of α7A integrin

We previously showed that MIBP is abundantly expressed in myoblasts and that MIBP expression is dramatically downregulated after initiation of terminal myogenic differentiation (Li et al., 1999). By contrast, although α7β1 integrin, to which MIBP binds (Fig. 1), is expressed throughout myogenic differentiation, the α7αβ1 integrin is expressed only after the initiation of myogenic differentiation (Collo et al., 1993; Song et al., 1993; Ziober et al., 1993). To test whether the downregulation of MIBP and the induction of the α7A integrin during myogenic differentiation are tightly coupled to each other, we analyzed the expression of α7A integrin in C2C12 cells overexpressing FLAG-MIBP by immunoblotting with an antibody specifically recognizing the α7A cytoplasmic domain. As expected, α7A was not expressed in the parental C2C12 cells or the C2C12 cells overexpressing FLAG-MIBP that were cultured in the growth medium (Fig. 5A, lanes 1, 5, and 9). The expression of α7A in the parental C2C12 cells was markedly increased within several days after switching the medium to the differentiation medium (Fig. 5A, lanes 3 and 4). By contrast, no α7A integrin was detected in C2C12 cells overexpressing FLAG-MIBP under the identical experimental conditions (Fig. 5A, lanes 7, 8, 11, and 12). Thus, overexpression of MIBP inhibits the expression of the α7A integrin, suggesting that the downregulation of MIBP during myogenic differentiation is indeed necessary for myogenic differentiation and the induction of α7A integrin.
The α7A and B isoforms are synthesized from the same α7 gene from transcripts generated by alternative RNA splicing (Collo et al., 1993; Song et al., 1993; Ziober et al., 1993). To test whether the expression of the α7B integrin is also regulated by MIBP during myogenic differentiation, we analyzed the expression of α7B in the MIBP-overexpressing C2C12 cells that were cultured in growth medium and in differentiation medium, respectively. The results showed that, unlike the expression of the α7A integrin, the expression of the α7B integrin was not altered in the C2C12 cells overexpressing FLAG-MIBP (Fig. 5B). The inhibition of α7A expression in cells overexpressing MIBP most likely reflects MIBP-induced inhibition of myogenic differentiation and the absence of developmentally regulated alternative splicing essential to generate the α7A transcripts.

MIBP regulates both the protein level and tyrosine phosphorylation of paxillin

We next sought to identify intracellular signaling intermediates that are regulated by MIBP. Previous studies by Sastry et al. demonstrated that both the expression level and
tyrosine phosphorylation of paxillin are critical to the decision making process of myogenic differentiation (Sastry et al., 1999). To begin to test whether paxillin is regulated by MIBP, we compared the level of paxillin in C2C12 cells that express different levels of MIBP. Immunoblotting analyses of the total cell lysates showed that cells overexpressing FLAG-MIBP (Fig. 6A, lanes 2–5) expressed a higher level of paxillin than the control transfectants (Fig. 6A, lane 1) and the parental C2C12 cells (not shown). Consistent with the immunoblotting results, more paxillin was immunoprecipitated from the cells overexpressing FLAG-MIBP (Fig. 7A). To estimate the relative level of paxillin in cells overexpressing FLAG-MIBP, we adjusted the amounts of samples loaded onto each lane. The results showed that overexpression of FLAG-MIBP resulted in an approximate threefold increase of the protein level of paxillin (Fig. 7B). In marked contrast to paxillin, the level of FAK was not significantly altered by overexpression of FLAG-MIBP (Fig. 6B). These results suggest that paxillin is a specific target regulated by MIBP. To further analyze this, we compared paxillin mRNA levels in cells that express different levels of MIBP. Northern blotting analyses showed that the amount of the paxillin transcript was not significantly increased by overexpression of MIBP (Fig. 8), suggesting that the elevation in paxillin protein is caused primarily by an upregulation at the translation or posttranslational level.

To test whether MIBP plays a role in the modulation of tyrosine phosphorylation of paxillin, we analyzed paxillin immunoprecipitates from cells expressing different levels of MIBP. Tyrosine-phosphorylated paxillin was detected by immunoblotting with an anti-phosphotyrosine antibody. The results show that the level of tyrosine phosphorylation of paxillin is significantly increased in the cells overexpressing FLAG-MIBP (Fig. 7C). Higher levels of tyrosine phosphorylation of paxillin are critical to the decision making process of myogenic differentiation (Sastry et al., 1999). To begin to test whether paxillin is regulated by MIBP, we compared the level of paxillin in C2C12 cells that express different levels of MIBP. Immunoblotting analyses of the total cell lysates showed that cells overexpressing FLAG-MIBP (Fig. 6A, lanes 2–5) expressed a higher level of paxillin than the control transfectants (Fig. 6A, lane 1) and the parental C2C12 cells (not shown). Consistent with the immunoblotting results, more paxillin was immunoprecipitated from the cells overexpressing FLAG-MIBP (Fig. 7A). To estimate the relative level of paxillin in cells overexpressing FLAG-MIBP, we adjusted the amounts of samples loaded onto each lane. The results showed that overexpression of FLAG-MIBP resulted in an approximate threefold increase of the protein level of paxillin (Fig. 7B). In marked contrast to paxillin, the level of FAK was not significantly altered by overexpression of FLAG-MIBP (Fig. 6B). These results suggest that paxillin is a specific target regulated by MIBP. To further analyze this, we compared paxillin mRNA levels in cells that express different levels of MIBP. Northern blotting analyses showed that the amount of the paxillin transcript was not significantly increased by overexpression of MIBP (Fig. 8), suggesting that the elevation in paxillin protein is caused primarily by an upregulation at the translation or posttranslational level.

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lation were detected even after adjusting the loading amount of the samples (Fig. 7D). In parallel experiments, we found that overexpression of MIBP did not alter the tyrosine phosphorylation of FAK (Fig. 9). We conclude from these experiments that both the protein level and the tyrosine phosphorylation of paxillin, but not FAK, are upregulated by MIBP.

Discussion

Myogenic differentiation is a highly regulated process that is controlled by multiple factors, including extracellular matrix, transmembrane receptors, and intracellular signaling molecules (Sastry and Horwitz, 1996; Gullberg et al., 1998; Burkin and Kaufman, 1999). In a previous study, we found that the expression of MIBP is dramatically decreased during terminal myogenic differentiation (Li et al., 1999). The downregulation of MIBP expression is critical to the progression of terminal myogenic differentiation, as expression of an epitope-tagged MIBP under a promoter that is not subject to regulation in muscle cells suppresses terminal myogenic differentiation. Although the results from previous studies have indicated that the expression level of MIBP is a crucial element in the decision-making process of terminal myogenic differentiation, it was not known how an elevation in the expression of MIBP inhibits this stage of muscle development. In this study, we have addressed several questions that are important to the understanding of the mechanism by which MIBP regulates terminal myogenic differentiation.

The first question is whether the interaction between MIBP and the β1 integrin is regulated by α subunits that are associated with the β1 integrin. This is important because β1 integrin chains pair with different α subunits and these heterodimers have different and even contrasting roles in the regulation of myogenic differentiation (Sastry et al., 1996, 1999). We have focused on the α7/β1 and the α5/β1 integrin in this study, as they are the predominant integrin receptors for laminin and fibronectin, respectively, in C2C12 myoblasts (Yao et al., 1996b; Ziober and Kramer, 1996). Our results show that MIBP associates with the α7/β1 integrin but not with α5/β1 integrin. Thus, although the MIBP recognition sequence LIWKLLMIIHDRREFAKFEKEKMNAKWDT (Li et al., 1999), which is localized in the membrane-proximal region of the β1 cytoplasmic domain, is present in both the α7/β1 and the α5/β1 integrins, only the one in the α7/β1 integrin is available for MIBP binding. How the presence of α5 inhibits the MIBP-β1 interaction is currently not known. Because there is evidence suggesting that the cytoplasmic domains of the α and β subunits can directly interact with each other (Calderwood et al., 2000; Haas and Plow, 1996; Muir et al., 1994), one interesting possibility is that α5 inhibits the MIBP-β1 association through a direct interaction with the β1 cytoplasmic domain. Alternatively, the α5 cytoplasmic domain could influence the MIBP-β1 interaction through an...
is that MIBP inhibits the deposition of laminin but not in future studies.

The second question we addressed is whether MIBP plays a role in the regulation of cell–matrix adhesion. The results from this study show that a high expression level of MIBP reduces cell adhesion to laminin but not to fibronectin. Because the adhesion of myoblasts to laminin promotes myogenic differentiation, the reduction in the cell adhesion to laminin is likely responsible, at least in part, for the suppression of myogenic differentiation induced by the overexpression of MIBP. Previous experiments have shown that α7β1, and α6β1 to a lesser extent, mediate the adhesion of myoblasts to laminin (Crawley et al., 1997; Yao et al., 1996a). While α7 integrin was coimmunoprecipitated with FLAG-MIBP (Fig. 1), we have failed to detect α6 integrin in the FLAG-MIBP immunoprecipitates (J.L. and C.W, unpublished observations). Thus, the simplest explanation for the inhibitory effect of MIBP on cell adhesion to laminin is that it results from a specific binding of MIBP to the α7β1 integrin. This is consistent with the observation that overexpression of MIBP reduced but did not eliminate cell adhesion to laminin (Fig. 2A). One consequence of this might be to alter integrin-mediated regulation of intracellular calcium levels (Kwon et al., 2000). However, we cannot rule out at this time the possibility that the inhibition of cell adhesion to laminin is mediated by other (yet to be identified) activities of MIBP. We are currently mapping the site of MIBP that is involved in the interaction with the α7β1 integrin, which should help us to test this hypothesis in future studies.

Another important and perhaps related finding of this study is that MIBP inhibits the deposition of laminin but not fibronectin into the extracellular matrix, suggesting that the deposition of different constituents of extracellular matrix can be selectively regulated. Given the opposing effects of laminin and fibronectin on terminal myogenic differentiation, this would allow the cells to precisely control the progression of the terminal myogenic differentiation. The deposition of laminin into the extracellular matrix is regulated by, among other things, cell surface α7β1 integrin (Colognato et al., 1999).

Thus, one plausible mechanism by which MIBP regulates laminin matrix deposition is through its interaction with the α7β1 integrin. This is further supported, although not proven, by the finding that fibronectin matrix deposition, a process that is mediated primarily by cell surface α5β1 integrin (Akiyama et al., 1989; Darribere et al., 1990; Fogerty et al., 1990; Wu et al., 1993) (to which MIBP does not bind), is not affected by MIBP. Reduction of laminin in the extracellular matrix has been implicated in the pathogenesis of specific forms of congenital muscular dystrophies. In certain cases of congenital muscular dystrophies, the reduction is caused by mutations in the laminin genes (Colognato et al., 1999; Helbling-Leclerc et al., 1995; Hillaire et al., 1994; Kuang et al., 1998; Miyagoe et al., 1997; Sunada et al., 1994, 1995; Wewer and Engvall, 1996; Xu et al., 1994). However, in some other cases of congenital muscular dystrophies, this is caused by mutations in other uncharacterized genes (Hayashi et al., 1993; Higuchi et al., 1994; Li et al., 1997; Sewry et al., 1997; Wewer et al., 1995), which likely affect laminin synthesis or matrix deposition. The finding that the deposition of laminin matrix is modulated by MIBP raises an interesting possibility that alteration of MIBP expression could contribute to the pathogenesis of certain forms of muscular dystrophies. In this regard, it is interesting to note that the reduced level of α7 integrin has also been associated with laminin-related muscular dystrophies (Hodges et al., 1997; Vachon et al., 1997).

Several intracellular signaling proteins including FAK and paxillin have been identified as key components of the integrin-signaling pathway that regulates myogenic

![Fig. 8. Northern blotting analyses of paxillin transcript expressed by C2C12 cells expressing different levels of MIBP. Equal amounts of total RNA (5 μg/lane) isolated from the FLAG vector control cells (lane 1) and FLAG-MIBP-overexpressing cells E3.11 (lane 2) and B3 (lane 3) were separated on a 1% denaturing gel and transferred to nylon membrane. (A) The membrane was probed with a 32P-labeled paxillin cDNA probe as described in Materials and methods. (B) The same membrane was reprobed with a 3P-labeled actin cDNA probe to confirm the equal loading. Transcripts hybridized with the 32P-labeled cDNA probes were visualized by autoradiography.](Image 65x571 to 275x722)

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![Fig. 9. Overexpression of MIBP does not alter tyrosine phosphorylation of FAK FAK was immunoprecipitated from equal amounts of cell lysates derived from the FLAG vector control cells (lane 1) or the FLAG-MIBP-overexpressing cells E3.11 (lane 2) and B3 (lane 3) as described in Materials and methods. Equal amounts of the immunoprecipitates were loaded to each lane and analyzed by immunoblotting with anti-FAK antibody (A) and anti-phosphotyrosine antibody (B), respectively.](Image 329x132 to 539x254)

Fig. 9. Overexpression of MIBP does not alter tyrosine phosphorylation of FAK. FAK was immunoprecipitated from equal amounts of cell lysates derived from the FLAG vector control cells (lane 1) or the FLAG-MIBP-overexpressing cells E3.11 (lane 2) and B3 (lane 3) as described in Materials and methods. Equal amounts of the immunoprecipitates were loaded to each lane and analyzed by immunoblotting with anti-FAK antibody (A) and anti-phosphotyrosine antibody (B), respectively.
differentiation (Sastry et al., 1999). An elevation of the protein level or tyrosine phosphorylation of either protein shifts the balance from differentiation to proliferation. The third question we addressed in this study is whether MIBP plays a role in the regulation of the level or tyrosine phosphorylation of FAK and paxillin. Our results indicate that both the protein level and tyrosine phosphorylation of paxillin are regulated by MIBP. In contrast, neither the protein level nor tyrosine phosphorylation of FAK is altered by MIBP. Thus, paxillin, but not FAK, functions as an intracellular target regulated by MIBP. It is important to note that our results do not rule out a role of FAK that is upstream, or in parallel, of MIBP in the regulation of myogenic differentiation. Nevertheless, our results do suggest that the expression and tyrosine phosphorylation of paxillin and FAK can be separately regulated, a phenomenon that has also been observed in integrin-induced FAK and paxillin signaling in quail myoblasts (Sastry et al., 1999).

In summary, the results presented in this report provide new evidence that MIBP has an important role in the cellular regulation of myogenic differentiation. Furthermore, they shed light on the mechanism by which MIBP functions in this process. Because the level of MIBP expression itself is regulated by soluble factors in the serum (Li et al., 1999), the effects of MIBP on cell–matrix interactions and signaling described in this report suggest that MIBP may be part of a pathway that coordinates signals from soluble growth factors and the extracellular matrix during myogenic differentiation.

Acknowledgments

This work was supported by grants from the Muscular Dystrophy Association and the NIH (GM65188 and AR47716) to C.W., and grants from the Muscular Dystrophy Association and the NIH (AG14632) to S.J.K.

References


