A Novel Muscle-specific β1 Integrin Binding Protein (MIBP) that Modulates Myogenic Differentiation

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Abstract. Myogenesis is regulated by cell adhesion receptors, including integrins of the β1 family. We report the identification of a novel muscle-specific β1 integrin binding protein (MIBP). MIBP binds to the membrane-proximal cytoplasmic region shared by β1A and β1D integrins, and the binding occurs in vivo as well as in vitro. Furthermore, we show that MIBP is abundantly expressed by C2C12 myogenic cells before fusion, and the expression of MIBP is dramatically downregulated during subsequent differentiation. Finally, we show that overexpression of MIBP in C2C12 cells resulted in a suppression of fusion and terminal differentiation, suggesting that MIBP may play a key role in controlling the progression of muscle differentiation.

Key words: integrin binding protein • muscle • myogenic differentiation • signal transduction • myogenin
Membrane-proximal cytoplasmic region shared by β1A and β1D. Furthermore, MIBP expression was dramatically downregulated during C2C12 myogenic differentiation, and overexpression of MIBP in myogenic cells resulted in a suppression of myogenesis. The results suggest that MIBP may play an important role in controlling muscle differentiation.

Materials and Methods

Cells, Antibodies, and Other Reagents

Mouse myoblast cells C2C12 and A frican green monkey COS-7 cells were from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Rabbit anti-β1 integrin antibody M2C21 was kindly provided by Dr. John A. McDonald (Mayo Clinic, Scottsdale, AZ). Mouse monoclonal antimyogenin F5D was obtained from the Developmental Studies Hybridoma Bank.

Yeast Two-Hybrid Assays

A cDNA fragment encoding the cytoplasmic domain of human integrin β1D (residues 749-801) was amplified by PCR and inserted into the E.coli/hol site in the pLexA vector (pLexA/β1D). The bait construct was introduced into EGY48 (pLexA-lacZ) yeast cells by transformation. The transformants were used to screen a human heart MATCHMAKER LexA cDNA library (>3 × 10^6 independent clones) as described previously (Tu et al., 1999). To analyze protein-protein interaction between MIBP and β1 mutants, yeast cells were cotransformed with pB42AD and pLexA expression vectors encoding MIBP and the β1 sequences. The transformants were plated and grown of blue colonies in the lacZ-deficient medium indicates a positive interaction (Tu et al., 1999).

Northern Blot

A MIBP cDNA probe was prepared by labeling the full-length human MIBP cDNA using an AlkPhos-direct labeling-detection system (Amer- sham Pharmacia Biotech). A blot containing equal amounts of poly(A) RNA (2 μg/lane) from different human tissues (Clontech Laboratories, Inc.) was hybridized with the MIBP probe. The hybridized mRNA bands were detected with CDP-Star Detection System (Amer sham Pharmacia Biotech).

Immunoblotting of Human Tissues for MIBP

Human fetal tissues were washed twice with PBS, homogenized in lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA. 0.2 mM 4-(2-aminoethyl)benzenesulfonylfluoride, HCl, 10 μg/ml aprotinin, 3 μg/ml pepstatin A and 5 μg/ml leupeptin), and analyzed by immunoblotting with monoclonal anti-MIBP antibody 5B4.7.

Expression and Purification of Recombinant Maltose Binding Protein, Glutathione S-transferase-, and His-tagged Fusion Proteins

DNA constructs encoding maltose binding protein (MBP)-MIBP (pMAL-C2/MBP), glutathione S-transferase (GST)-β1D (pGEX-5x-1/β1D), and His-MIBP (pET-15b/MIBP) fusion proteins were generated by inserting cDNAs encoding full-length or partial sequences of human MIBP and β1D integrin into the corresponding vectors. The recombinant vectors were used to transform Escherichia coli cells, and the recombinant proteins were purified with glutathione-Sepharose 4B beads, amylose-agarose beads, and Ni-NTA Resin (Novagen), respectively.

Production and Characterization of a Monoclonal Anti-MIBP Antibody

Mouse monoclonal anti-MIBP antibody was generated using purified His-MIBP recombinant protein as an antigen (Tu et al., 1999). Hybridoma supernatants were screened for anti-MIBP antibody activity by ELISA and immunoblotting. One mAb (clone 5B4.7) recognized MBP-MIBP and His-MIBP but not MBP or irrelevant His-tagged proteins.

Coprecipitation Assays

For direct binding assays, glutathione-Sepharose 4B beads were preincubated with affinity-purified GST fusion protein containing the β1D cytoplasmic domain (GST-β1D) or GST as a control (5 μg/30 μl beads), and then mixed with His-MIBP (5 μg) and incubated at 4°C for 1 h. After washing, His-MIBP coprecipitated with GST-β1D was detected by immunoblotting with anti-MIBP antibody 5B4.7. To perform GST fusion protein pull down assays using cell lysates, C2C12 cells were washed once with cold PBS and lysed using the lysis buffer (PBS, 1% Triton X-100, 0.2 mM 4-(2-aminoethyl)benzenesulfonylfluoride, HCl, 10 μg/ml aprotinin, 3 μg/ml pepstatin A and 5 μg/ml leupeptin). The cell lysates (500 μg) were incubated with equal amounts (10 μg) of GST-β1D, or GST alone as a negative control, and the GST fusion proteins were precipitated with glutathione-Sepharose 4B beads. MIBP in the precipitates was detected by immunoblotting with anti-MIBP antibody 5B4.7.

Coimmunoprecipitation Assays

The full-length MIBP cDNA was inserted into the HindIII/Xal site of pFLAG-CMV2 vector (Kodak). COS-7 cells were transfected with pFLAG-MIBP, or pFLAG-G-CMV2 as a control, using Lipofectamine Plus (Life Technologies, Inc.). 48 h after transfection, the cells were lysed using lysis buffer. Cell lysates (500 μg protein) were incubated with agarose beads conjugated with mouse monoclonal anti-FLAG antibody M2 (50 μl) or protein A-agarose beads coupled with an irrelevant mouse IgG (50 μl) at 4°C for 1 h. The beads were washed and FLA-G-MIBP and β1 integrin were detected in precipitates by immunoblotting with anti-FLAG antibody M5 and anti-β1 integrin antibody M2C21, respectively.

Myogenic Differentiation

C2C12 cells were cotransfected with pFLAG-MIBP (or FLAG-CMV2 as a control) and a vector containing a neomycin-resistant marker pEGFP-c2, Clontech Laboratories Inc., using Lipofectamine Plus. The transfectants were selected with 0.5 mg/ml G418 and cloned. Five clones (E3.11, D9.8, B3, C4, and D4) that stably express MIBP were obtained. The expression of FLAG-MIBP by the transfectants was analyzed by immunofluorescence staining and immunoblotting with anti-FLAG antibody M5. To analyze the effect of MIBP overexpression on myogenic differentiation, C2C12 cells stably expressing FLAG-MIBP, FLAG control transfectants, and the parental C2C12 cells were grown in DMEM containing 10% FBS in 24-well collagen-coated plates until confluence was reached. Myogenic differentiation was induced by switching the medium to DMEM containing 2% horse serum. Myogenin was detected by immunoblotting with monoclonal antimyogenin antibody FSD.

Results

Identification and Cloning of a Novel MIBP

We have used yeast two-hybrid screens to identify β1 integrin cytoplasmic binding proteins. A bait construct (pLexA/β1D) encoding the β1D integrin cytoplasmic domain (residues 749-801) was used to screen a human heart LexA cDNA library (>3 × 10^6 independent clones). 15 positive clones were obtained. DNA sequencing showed that plasmids from 3 out of the 15 positive clones contained an open reading frame encoding a novel protein that we termed muscle integrin binding protein or MIBP (Fig. 1A). The binding of MIBP to the β1D cytoplasmic domain was confirmed by yeast two-hybrid binding assays using purified pET-15b encoding MIBP (Table I). In control experiments, elimination of either the β1D or MIBP sequence failed to activate the reporter genes. In addition, replacement of the integrin cytoplasmic sequences with those of irrelevant proteins (e.g., lamin C) abolished the interaction (Table I), further confirming the specificity of MIBP.
the interaction. We also found that MIBP interacts with the β1A cytoplasmic domain (Table I). Northern blot analysis of human tissues revealed that MIBP mRNA is predominantly expressed in skeletal and cardiac muscle (Fig. 1 B). No expression was detected in brain, placenta, lung, liver, kidney, or pancreas (Fig. 1 B).

### Table I. Interactions of MIBP with Cytoplasmic Domains of β1 Integrins in Yeast Two-Hybrid Binding Assay

| Reporter gene | pB42AD construct | pLexA construct |
|---------------|------------------|-----------------|
| LEU2         | pB42AD-MIBP      | pLexA-β1D       |
| LacZ         | +                | +               |
| pB42AD-MIBP  | -                | -               |
| pLexA        | -                | -               |
| pLexA-β1A    | -                | -               |
| pLexA-lamin C| -                | -               |
| pLexA-β1D    | -                | -               |
| pLexA-β1A    | -                | -               |

Interactions between proteins encoded by the pLexA and pB42AD constructs were determined by the activation of the reporter genes (LEU2 and LacZ). pLexA-β1D contains residues 749–801 of the β1D integrin cytoplasmic domain. pLexA-β1A contains residues 749–798 of the β1A integrin cytoplasmic domain.

**MIBP Binds to β1 Integrins In Vitro and In Vivo**

To test whether MIBP can directly bind to the β1 integrin cytoplasmic domain, we expressed and purified a GST fusion protein containing the β1D cytoplasmic domain. GST–β1D (Fig. 3 A, lane 2), but not GST (Fig. 3 A, lane 1), interacted with the purified recombinant His-tagged MIBP, indicating that the two proteins can directly interact with each other in the absence of other proteins. In addition, mammalian MIBP protein expressed by the C2C12 myoblast lysates was coprecipitated with GST–β1D fusion protein (Fig. 3 B, lane 3) but not GST (Fig. 3 B, lane 2). Thus, both mammalian and recombinant MIBP proteins interact with the β1 integrin cytoplasmic domain in vitro. To test whether MIBP associates with β1 integrins in vivo, we expressed a FLAG-tagged MIBP in mammalian cells (Fig. 3 D, lane 1). Coimmunoprecipitation experiments with a monoclonal anti-FLAG antibody showed that the β1 integrins (Fig. 3 C, lane 2) were specifically co-precipitated with FLAG–MIBP (Fig. 3 D, lane 2) from the lysate of the FLAG–MIBP transfectants, but not from that
of the control transfectants (Fig. 3 C, lane 5). In additional control experiments, no β1 integrins were precipitated from the FLAG-MIBP lysates with a control mouse IgG (Fig. 3, C and D, lane 3). Thus, MIBP forms a complex with the β1 integrins in mammalian cells as well as in vitro.

The Membrane-proximal Region of the β1 Integrin Cytoplasmic Domains Mediates the Interaction with MIBP

The cytoplasmic domains of β1D and β1A share a common membrane-proximal region. Since MIBP binds to both β1D and β1A cytoplasmic domains (Table I), it most likely recognizes a site located within this region. To test this, we generated a series of β1D/β1A mutants and analyzed their ability to interact with MIBP in yeast two-hybrid binding assays. The results showed that MIBP specifically interacts with the membrane-proximal region of the β1D or β1A cytoplasmic domain (Table II).

The Expression of MIBP Is Downregulated during Myoblast Differentiation

Table II. MIBP Binds to the Membrane-proximal Region of the β1 Integrin Cytoplasmic Domain

| β1D integrin cytoplasmic domain deletion mutants | β1D integrin sequence | MIBP binding |
|-----------------------------------------------|----------------------|--------------|
| Int β1D (749–801)                             | LIWKLLMIIHRRFEAFKFEKKMNAKW DTQENPYKSP1NFKPNPYGRKAGL | +            |
| ΔBDMT1 (749–795)                              | LIWKLLMIIHRRFEAFKFEKKMNAKW DTQENPYKSP1NFKPNPY | +            |
| ΔBDMT2 (749–785)                              | LIWKLLMIIHRRFEAFKFEKKMNAKW DTQENPYK | +            |
| ΔBDMT3 (749–777)                              | LIWKLLMIIHRRFEAFKFEKKMNAKW DT | +            |
| ΔBDMT4 (773–801)                              | ------------------------AKW | -            |

Interactions between proteins encoded by the pLexA and pB42AD constructs were determined by the activation of the reporter genes LEU2 and LacZ.

To begin to investigate the role of MIBP in myogenic differentiation, we analyzed MIBP expression during myogenic differentiation using the mouse C2C12 myoblast line as a model system. The results showed that abundant MIBP protein is expressed before terminal differentiation of C2C12 myoblasts (Fig. 4 A, lane 1). Myogenic differentiation was induced by switching the culture medium to DME containing 2% horse serum. Myotubes were observed within the first 2 d of induction, and >80% of the cells were fused into multinucleated myotubes on day 4. The MIBP expression level was decreased upon induction of myogenic differentiation (Fig. 4 A, lanes 2–8). Less than 10% of MIBP was expressed 4 d after the induction of differentiation (Fig. 4 A, compare lanes 2 and 6), and the expression of MIBP was further decreased beyond detection after day 5. In control experiments, the same membrane was stripped and reprobed with an anti-β1 integrin antibody (MC231, which recognizes both β1A and β1D integrins). The β1 integrins (β1A and/or β1D) were detected at all stages of C2C12 differentiation (Fig. 4 B, lanes 1–8).
Overexpression of MIBP in Myoblasts Inhibits Myogenic Differentiation

The striking downregulation of MIBP during myogenic differentiation suggests that a higher MIBP expression level may prevent myoblasts from undergoing terminal differentiation. To test this, we overexpressed FLAG-tagged MIBP in C2C12 myoblasts. The expression of FLAG-tagged MIBP in the FLAG-MIBP transfectants, but not C2C12 cells transfected with a vector lacking the MIBP sequence, was confirmed by immunoblotting with an anti-FLAG antibody (Fig. 5 A, lanes 1 and 2) and the anti-MIBP antibody 5B4.7 (Fig. 5 A, lanes 3 and 4). Two independently isolated C2C12 clones (E3.11 and D9.8) that express FLAG-MIBP at a level comparable to that of endogenous MIBP in the proliferating myoblasts (Fig. 5 B) were selected for further analysis. As expected, myogenin (a biochemical marker for myogenic differentiation; Andrés and Walsh, 1996) (Fig. 5 C) and abundant multinucleated myotubes (Fig. 5 E) were detected in both the parental C2C12 cells and the vector control transfectants after induction of myoblast differentiation. In contrast, no multinucleated myotubes were detected in E3.11 and D9.8 cells overexpressing FLAG-MIBP under identical experimental conditions (Fig. 5 E). Similar results were obtained with all other FLAG-MIBP-overexpressing C2C12 clones that were analyzed (Fig. 5 E). Consistent with the suppression of myotube formation, no myogenin was detected in the cells overexpressing FLAG-MIBP (Fig. 5 C, lanes 6 and 8). The expression of FLAG-MIBP in the FLAG-MIBP transfectants but not the parental C2C12 or the vector control transfectants, before and after the induction of myoblast differentiation was confirmed by immunoblotting with an anti-FLAG antibody (Fig. 5 D). We conclude from these experi-
ments that overexpression of FLAG-tagged MIBP in C2C12 myoblasts suppresses terminal myogenic differentiation.

Discussion

In this study, we have identified and cloned a novel muscle β1 integrin binding protein, MIBP, and provided strong evidence that overexpression of FLAG-tagged MIBP in C2C12 myoblasts suppresses terminal myogenic differentiation.

Figure 4. Downregulation of MIBP expression during myogenesis. (A) Mouse C2C12 cells were induced to differentiate by switching the medium to DME containing 2% horse serum at day 0. Cells were harvested from 1 d before induction to 6 d after induction. Equal amounts (5 μg) of the cell extracts were loaded onto each lane. MIBP was detected by immunoblotting with mAb 5B4.7. (B) Expression of β1 integrins during C2C12 differentiation. The membrane in A was stripped and reprobed with antibody MC231 that recognizes both β1A and β1D integrins.

Figure 5. Overexpression of FLAG-MIBP inhibits C2C12 myogenesis. (A) Lysates (5 μg/lane) of the FLAG-MIBP (lanes 2 and 4) and the vector only control (lanes 1 and 3) transfectants were immunoblotted with anti-FLAG antibody M5 (lanes 1 and 2) or anti-MIBP antibody 5B4.7 (lanes 3 and 4). (B) Lysates (5 μg/lane) of parental C2C12 cells (lane 1), the vector-only control (lane 2), FLAG-MIBP-expressing E3.11 (lane 3), and D9.8 (lane 4) clones were immunoblotted with anti-MIBP antibody 5B4.7. (C and D) Cells were harvested immediately before induction of differentiation or 4 d after the induction as indicated in the figure. The expression of myogenin (C) and FLAG-MIBP (D) was analyzed by immunoblotting with monoclonal antimyogenin antibody F5D and anti-FLAG antibody M5, respectively. Each lane was loaded with 10 μg of the cell extracts. E shows the morphology of proliferating myoblasts (panels a, c, e, g, i, k, and m) and myoblasts that were induced to differentiate for 4 d (panels b, d, f, h, j, l, and n). Panels a and b are parental C2C12 cells, c and d are control-transfected cells, e–n are FLAG-MIBP transfected clones E 3.11 (panels e and f), D 9.8 (panels g and h), B 3 (panels i and j), C 4 (panels k and l), and D 4 (panels m and n). Bar, 200 μm.
evidence for an important role of MIBP in the regulation of terminal myogenesis. Using C2C12 myogenic cells as a model system, we show that MIBP is abundantly expressed in proliferating myogenic cells. The expression level of MIBP decreases upon induction of terminal myogenenic differentiation, and becomes undetectable after the majority of the myoblasts have fused to form multinucleated myotubes. This striking downregulation of MIBP suggests that the amount of MIBP may be a crucial element in the decision-making process of fusion versus proliferation during myogenic differentiation. In support of this, overexpression of an epitope-tagged MIBP under a promoter that is not subject to regulation in muscle cells resulted in a complete suppression of the terminal differentiation of C2C12 cells.

MIBP is shown to bind to the $\beta_1$ integrin cytoplasmic domain, which is known to play a key role in controlling myoblast proliferation and differentiation (Sastry et al., 1999). In addition to suppression of myogenic differentiation, our preliminary results indicate that after switching to differentiation medium, expression of FLAG-MIBP in C2C12 myoblasts enhances cell proliferation (Li, J., R. Mayne, and C. Wu, unpublished observations). Taken together, our results suggest that MIBP most likely functions in the regulation of myogenesis via its interaction with the $\beta_1$ integrin cytoplasmic domain. In this regard, it is particularly interesting to note that the MIBP-binding site is located within the membrane-proximal region of the $\beta_1$ integrin cytoplasmic domain, a region likely to play an important role in integrin activation (Hughes et al., 1996). It has been shown that the ligand binding affinity of $\alpha$5$\beta_1$ integrin in myoblasts is downregulated during myogenesis (Boettiger et al., 1995). Moreover, this inactivation of $\alpha$5$\beta_1$ integrin is functionally important to myogenesis (Boettiger et al., 1995) and could potentially contribute to the matrix switch (from a fibronectin-rich matrix to a laminin-rich matrix) that accompanies myogenic differentiation (Gullberg et al., 1998). Thus, one mechanism whereby MIBP potentially functions is by regulating integrin activation, and consequently, matrix deposition and cell adhesion. In addition, MIBP could modulate signal transduction from integrins to other downstream targets such as focal adhesion kinase and paxillin (Sastry et al., 1999), and thereby influence the decision of myoblasts to fuse and undergo terminal differentiation.

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