rapid communication

bHLH transcription factor MyoD affects myosin heavy chain expression pattern in a muscle-specific fashion

DAVID J. SEWARD, JOHN C. HANEY, MICHAEL A. RUDNICKI, AND STEVEN J. SWOAP.

A strong correlative pattern between MyoD gene expression and myosin heavy chain IIB (MHC IIB) gene expression exists. To test whether this correlative relationship is causative, MHC gene expression in muscles from MyoD(−/−) mice was analyzed. The MHC IIB gene was not detectable in the MyoD(−/−) diaphragm, whereas the MHC IIB protein made up 10.0 ± 1.7% of the MHC protein pool in the wild-type (WT) mouse diaphragm. Furthermore, the MHC IIA protein was not detectable in the MyoD(−/−) biceps brachii, and the MHC IIB protein was overexpressed in the masseter. To examine whether MyoD is required for the upregulation of the MHC IIB gene within slow muscle after disuse, MyoD(−/−) and WT hindlimb musculature was unweighted. MyoD(−/−) exhibited a diminished response in the upregulation of the MHC IIB mRNA within the soleus muscle as a result of the hindlimb unweighting. Collectively, these data suggest that MyoD plays a role in the MHC profile in a muscle-specific fashion.

NUMEROUS SIGNALING PATHWAYS and molecular mechanisms are currently being examined as candidates in governing differential gene expression in skeletal muscle. Three possibilities recently advanced, which may not be mutually exclusive, are the calcium-calcineurin pathway, a pathway involving Ras, and the pathway that invokes members of the myogenic regulatory factor family. First, calcium signals have been proposed to regulate slow fiber gene expression (reviewed in 16). Elevated calcium in chronically active muscle, such as slow muscle, activates both the calcium-dependent phosphatase, calcineurin, and calmodulin-dependent protein kinase (CaMK). Activated calcineurin dephosphorylates any number of cytosolic proteins, such as the nuclear factor of activated T cells (NFAT) family members, generating an intracellular signal that may induce slow fiber-specific gene expression (3). CaMK may modify MEF2 family members to invoke the slow fiber-type pathway (26). However, recent evidence suggests that the calcineurin-NFAT pathway may not signal fiber type-specific gene expression (20). A second pathway that may regulate differential gene expression is one involving the oncogene ras (15). Injection of a constitutively active Ras into denervated regenerating soleus muscle increases the number of type I fibers, possibly through activation of mitogen-activated protein kinase (15). The third pathway, tested herein, involves the expression of the members of the myogenic regulatory factor (MRF) family. MyoD and myogenin are expressed at high levels in fast and slow muscle, respectively (7, 8, 23, 25), whereas MRF4 is expressed at high levels in some slow fibers that exist in fast muscle but not in slow fibers of slow muscle (24). Expression of members of this family is induced in activated satellite cells but not in satellite cells that remain quiescent in adult skeletal muscle (4, 27). The correlation between MyoD and fast-muscle gene expression patterns holds under numerous experimental conditions that alter muscle fiber type and maintain satellite cells in their quiescent state, including altered thyroid hormone state (8, 11), muscle unweighting (14, 25), and murine myotonia (6). The relationship between MRF and myosin heavy chain (MHC) gene expression does not hold under models that can cause muscle degeneration and/or regeneration (9, 11, 13, 23). However, it may be that the upregulation of the MRF genes occurs in the activated satellite cells in these models, and not in myonuclei of mature fibers (10).

Because we previously showed a strong correlation between MyoD and MHC IIB gene expression patterns and have evidence from in vitro and cell culture exper-
imments suggesting that MyoD activates the MHC IIB gene (25), we tested the following two hypotheses: 1) skeletal muscles from animals lacking the expression of MyoD [MyoD(−/−)] would not express the MHC IIB gene, and 2) the soleus muscle from MyoD(−/−) mice would not activate the MHC IIB gene after a period of unweighting.

**MATERIALS AND METHODS**

**Animals.** The generation of MyoD(−/−) mice used in this study has been previously described (18). All other mice used were purchased from Harlan. Three-month-old male and female mice were used. All animal procedures were approved by the Williams College Animal Care and Use Committee.

**Muscle isolation and MHC protein analysis.** Animals were anesthetized with a cocktail containing the following (in mg/kg body wt): 25 ketamine, 1 acepromazine, and 5 xylazine. Animals were killed under anesthesia by cervical dislocation. Muscles (n = 4–8 for each muscle) were removed, weighed, immediately frozen in liquid nitrogen, and stored at −70°C until used. Muscles were homogenized in a dounce homogenizer in 175 mM KCl and 10 mM Tris, pH 6.8. An equal volume of the following solution was added to the muscle homogenate: 28% glycerol, 2.8% sodium dodecyl sulfate, 1 M β-mercaptoethanol, 0.002% bromophenol blue, and 62.5 mM Tris, pH 6.8 (final concentrations). The MHC proteins were separated using SDS-gel electrophoresis (22). Unpublished observations from our laboratory show that the MHC profile of a muscle as determined from a whole muscle homogenate is identical to that of the MHC profile of purified myofibrils.

**Hindlimb suspension in mice.** Wild-type (WT) mice were randomly assigned to one of two groups, control (n = 6) and hindlimb suspended (n = 6). MyoD(−/−) mice were also assigned to these groups (control, n = 7 and suspended, n = 5). The hindlimbs of mice were suspended for 2 wk. A 20-in. piece of string was coiled around the distal two-thirds of the animal’s tail and set into place using fast-setting plaster bandage. The string was run through a swivel attached to a small key ring that was attached to the top of the cage. The animals were lifted via the string so that the hindlimbs were just off of the cage floor. After 2 wk, animals were killed under anesthesia, and the soleus muscles were removed, weighed, and immediately frozen in liquid nitrogen. Muscles were stored at −80°C until use.

**RNA isolation and Northern blotting of muscle.** The soleus muscle from both legs were combined before RNA isolation. RNA was isolated as described previously (20, 25). RNA concentration was determined spectrophotometrically. Seven micrograms of RNA from each sample were heated at 65°C for 10 min in a solution of 50% formamide, 40 mM MOPS, 10 mM NaOAc, 1 mM EDTA, pH 7, and 2.2 M formaldehyde. The denatured RNA was run on a 1× MOPS gel in 1× MOPS-run buffer for 10 h at 22 V. The RNA was transferred to a Hybond-N membrane (Amersham) using capillary transfer. The membrane was ultraviolet-cross-linked and stored in a sealed bag at 4°C until use. Membranes were prehybridized in 6× sodium chloride-sodium phosphate-EDTA (SSPE) (0.9 M NaCl, 31 mM Na2HPO4, 1 mM NaH2PO4, 10 mM EDTA). 10× Denhardt’s solution, 0.1% SDS, and 150 µg/ml salmon sperm DNA at 42°C for 1 h. P32 end-labeled oligonucleotides specific for the mouse MHC IIB mRNA (5′-CACATTGTGTGATTTTCTCCTGCAC-3′) or the mouse 28S rRNA (5′-GGTCTAACCAGCTACGCCGTTCC-3′) were added to 6× SSPE and 0.1% SDS and then hybridized to the membrane overnight at 60 or 42°C, respectively. The membranes were washed extensively, with a final wash in 0.1× sodium chloride-sodium citrate (SSC) and 0.1% SDS at room temperature for 30 min. Blots were exposed to a PhosphorImager screen (Bio-Rad) and exposed either overnight (MHC IIB) or for 5 min (28S rRNA). For reprobing, we stripped the blots by pouring boiling 0.1× SSC with 0.1% SDS over the blot and allowing them to cool to room temperature.

**Statistics.** Data for the variables studied are reported as means ± SE. Statistically significant differences were determined using Dunn’s test following an ANOVA. All statistical analyses were performed using a computer software package (SigmaStat). The 0.05 level of confidence was accepted for statistical significance.

**Table 1. MHC isofrom distribution in selected mouse skeletal muscles**

<table>
<thead>
<tr>
<th></th>
<th>Type I MyHC</th>
<th>Type IIA MyHC</th>
<th>Type IIX MyHC</th>
<th>Type IIB MyHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT diaphragm</td>
<td>7.0 ± 2.1</td>
<td>39.0 ± 3.0</td>
<td>40.0 ± 2.5</td>
<td>10.0 ± 1.7</td>
</tr>
<tr>
<td>MyoD(−/−) diaphragm</td>
<td>10.0 ± 1.5</td>
<td>48.0 ± 5.0*</td>
<td>42.0 ± 6.0</td>
<td>ND*</td>
</tr>
<tr>
<td>WT biceps brachii</td>
<td>ND</td>
<td>19.1 ± 5.0</td>
<td>35.9 ± 0.4</td>
<td>45.0 ± 4.7</td>
</tr>
<tr>
<td>MyoD(−/−) biceps brachii</td>
<td>ND</td>
<td>56.6 ± 1.8*</td>
<td>43.4 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>WT masseter</td>
<td>ND</td>
<td>ND</td>
<td>93.2 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td>MyoD(−/−) masseter</td>
<td>ND</td>
<td>ND</td>
<td>78.1 ± 2.1*</td>
<td>21.9 ± 2.1*</td>
</tr>
<tr>
<td>MyoD(−/−) pectineus</td>
<td>2.1 ± 2.1</td>
<td>12.7 ± 2.4</td>
<td>46.6 ± 4.0</td>
<td>38.6 ± 7.0</td>
</tr>
<tr>
<td>MyoD(−/−) adductor longus</td>
<td>0.6 ± 0.6</td>
<td>5.7 ± 1.6</td>
<td>34.8 ± 10.8</td>
<td>58.9 ± 10.7</td>
</tr>
<tr>
<td>MyoD(−/−) adductor magnus</td>
<td>ND</td>
<td>4.8 ± 2.2</td>
<td>18.3 ± 0.8</td>
<td>76.9 ± 2.1</td>
</tr>
<tr>
<td>MyoD(−/−) vastus lateralis</td>
<td>ND</td>
<td>4.5 ± 0.5</td>
<td>9.6 ± 1.6</td>
<td>85.9 ± 2.0</td>
</tr>
<tr>
<td>MyoD(−/−) vastus medialis</td>
<td>8.6 ± 1.5</td>
<td>ND</td>
<td>41.4 ± 2.3</td>
<td>40.0 ± 2.4</td>
</tr>
<tr>
<td>MyoD(−/−) gracilis</td>
<td>8.3 ± 2.3</td>
<td>ND</td>
<td>36.2 ± 9.4</td>
<td>55.4 ± 11.6</td>
</tr>
<tr>
<td>MyoD(−/−) extensor digitorum longus</td>
<td>ND</td>
<td>ND</td>
<td>34.8 ± 0.2</td>
<td>65.2 ± 0.2</td>
</tr>
<tr>
<td>MyoD(−/−) plantaris</td>
<td>2.0 ± 0.2</td>
<td>4.0 ± 1.0</td>
<td>26.0 ± 3.0</td>
<td>68.0 ± 4.0</td>
</tr>
<tr>
<td>MyoD(−/−) soleus</td>
<td>42.0 ± 2.0</td>
<td>50.0 ± 2.0</td>
<td>8.0 ± 2.0</td>
<td>ND</td>
</tr>
<tr>
<td>MyoD(−/−) tibialis anterior</td>
<td>ND</td>
<td>ND</td>
<td>38.0 ± 2.0</td>
<td>62.0 ± 2.0</td>
</tr>
<tr>
<td>MyoD(−/−) pectoralis</td>
<td>ND</td>
<td>ND</td>
<td>40.1 ± 1.7</td>
<td>59.9 ± 1.7</td>
</tr>
<tr>
<td>MyoD(−/−) triceps</td>
<td>ND</td>
<td>ND</td>
<td>26.0 ± 2.4</td>
<td>74.0 ± 2.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. In those muscles with no differences in the myosin heavy chain (MHC) pool, only MyoD(−/−) values are shown for clarity. ND, not detected; *P < 0.05 vs. wild-type (WT) muscle.
RESULTS

To test directly the hypothesis that MyoD is required for MHC IIB gene expression, we examined MHC protein expression in a number of skeletal muscles of MyoD(-/-) mice (Table 1). The MHC IIB protein was not detected within the MyoD(-/-) diaphragm muscle, whereas this protein made up 10.0 ± 1.7% of the MHC isoform pool within the WT mouse diaphragm (Fig. 1A and Table 1). Lack of MHC IIB protein expression was mirrored at the mRNA level, where the MHC IIB mRNA was not detected by Northern analysis (Fig. 1B). Because the lack of MHC IIB expression in the MyoD(-/-) diaphragm was so striking, we examined the MHC pool within diaphragms of three other strains of mice (non-Swiss albino, National Institutes of Health Swiss, and C57Bl/6J), to determine whether strain-to-strain differences may exist in the MHC pool of the diaphragm. The diaphragm muscle from each strain examined expressed the MHC IIB protein at levels comparable to that of the WT mice (data not shown).

Two other muscles within the MyoD(-/-) mice contained a significantly different pool of the MHC isoforms. The biceps brachii did not express the MHC IIA protein (Fig. 2A and Table 1), whereas the masseter expressed the MHC IIB at elevated levels (Fig. 2B and Table 1). All of the other muscles examined displayed no significant difference in MHC isoform pool between MyoD(-/-) and WT. [For clarity, Table 1 shows data from only the MyoD(-/-) muscles.]

We further tested the hypothesis that MyoD is required for the activation of the MHC IIB gene in the soleus in response to unloading. Body and soleus weights and soleus-to-body weight ratios did not differ before or after 2 wk of unloading between WT and MyoD(-/-) mice. After 2 wk of unloading, body mass decreased ~3 g in both WT and MyoD(-/-) mice. Soleus-to-body weight ratios in both MyoD(-/-) and WT hindlimb-unweighted mice significantly decreased compared with normal controls (Fig. 3A). The unweighted WT soleus muscle expressed the MHC IIB mRNA at levels significantly higher than those found in the unweighted MyoD(-/-) soleus muscle (Fig. 3, B and C).

DISCUSSION

We have hypothesized that the correlative nature between MyoD and MHC IIB gene expression was causative because 1) MyoD activates the MHC IIB gene through what appears to be a myogenesis-independent pathway (25) and 2) the interaction of MyoD and an MHC IIB promoter E-box is critical for activation of this promoter both in cell culture and in vivo (25). However, the hypothesis that MyoD is required for MHC IIB gene expression is not fully supported.
here. We have shown that the lack of MyoD affects skeletal muscle phenotype in a remarkable muscle-specific pattern. We found that MyoD is required for expression of the MHC IIB gene in the diaphragm (Fig. 1). The lack of full activation of the MHC IIB in the MyoD(-/-) unweighted soleus shown herein (Fig. 3) suggests that MyoD also plays a critical role in the adaptive response to inactivity in the soleus muscle. This conclusion was suggested earlier after time-course analysis demonstrated that MyoD is activated within the unweighted soleus muscle, a model that keeps satellite cells quiescent (5), before the MHC IIB gene in rats (25) and in WT mice (data not shown). In contrast to the requirement of MyoD for MHC IIB expression in the diaphragm and unweighted soleus, the MyoD(-/-) masseter actually increased expression of the MHC IIB protein (Fig. 2B). Furthermore, the biceps brachii muscle also became “faster” in that the lack of the intermediate MHC IIA protein was compensated by an increase in the relative expression of the fast MHC IIX protein. Hence, MyoD by itself plays little role in determining fast muscle characteristics on a global scale. However, MyoD clearly is important in MHC IIB gene expression in a muscle-specific fashion.

The fact that MHC IIB is expressed in many of the skeletal muscles examined was not completely unexpected. MyoD(-/-) mice do not have the altered morphological phenotype in animals null for the MHC IIB (i.e., the MHC IIB knockout mouse; Ref. 1). Also, a previous investigation had histochemically identified “IIB fibers” at normal levels in MyoD(-/-) muscles in all but one muscle examined (7). One potential explanation for the lack of support from the MyoD(-/-) animals for this hypothesis is the functional redundancy observed within the MRF gene family. Elegant studies have demonstrated differing roles of each of the MRF genes in development and differentiation of the
myogenic lineage (19). However, both cell culture studies and MRF-null animals have demonstrated at least partial redundancy in the function of these genes. All MRF genes activate the MHC IIB gene in cell culture (12, 21, 25), presumably as a result of the myogenic nature of these genes. We found no difference in myogenin mRNA or MRF-4 mRNA levels (Myf-5 mRNA was not detectable by Northern analysis) in the diaphragm, tibialis anterior (TA), or gastrocnemius muscles between WT and MyoD(−/−) muscles (data not shown). These data suggest that the normal expression of the MHC IIB gene in the TA and gastrocnemius of the MyoD(−/−) mouse is not due to a compensatory increase in one of the MRF genes. However, we cannot rule out the possibility that translational and/or post-translational changes to the MRFs occur to activate the MHC IIB gene in the TA and not the diaphragm. Similarly, there may be compensatory changes in the Ras (15) or calcineurin (16) signaling pathways in MyoD(−/−) muscle that alter the expression of the MHC IIB gene.

The muscle-specific dependence of MHC gene expression on MyoD gene expression is perplexing. That is, why does the lack of MyoD expression blunt MHC IIB gene expression only in the diaphragm and the unweighted soleus muscle? Developmental processes are not likely responsible for the specific deficit in MHC IIB gene expression in the unweighted soleus and the control diaphragm. Both muscles develop from mesenchymal cells, as do the other muscles of the hindlimb musculature, which do express the MHC IIB protein at normal levels (Table 1). One potential explanation for the effect of lack of MyoD on the MHC profile of the diaphragm and soleus muscle is the recruitment pattern of these two muscles. The diaphragm is chronically recruited, as demonstrated by the respiration rate of the mouse, which ranges from 106 to 163 respirations/min (17). Both normal and unweighted soleus muscles are also chronically active, as demonstrated by electromyographic analysis (2). It may be that there exists a complex interaction among neural input, MyoD expression, and MHC IIB gene expression.

In conclusion, we have shown here that the lack of MyoD expression dramatically alters the MHC protein profile in selected skeletal muscles. Thus, it appears that the correlative relationship between MyoD expression and myofiber phenotype is causative in some musculature. Whether compensatory changes occur in muscles that exhibit a normal MHC phenotype such as the TA, or whether simply that MyoD is not required for a normal MHC phenotype in these muscles, is unclear and warrants further research.

This work was supported by National Science Foundation Grant IBN-9723351, National Institutes of Health Grant AR-46199-01, and in part by a Howard Hughes Medical Institute grant to Williams College.

REFERENCES


