Differentiating Skeletal Muscle Cells Initially Express a Ventricular Myosin Heavy Chain.

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Abstract
Monoclonal antibodies (mAbs) to chicken myosin heavy chains were used to study the expression of myosins in differentiating muscle tissue. Using a monoclonal antibody (HV11) specific to an adult ventricular myosin heavy chain we demonstrate that this antibody reacts with differentiating muscle cells in the somites, stage 24 limb bud, developing heart, and regenerating fast and slow muscle in vivo. In addition, myotubes formed in cultures derived from myoblasts of the stage 26 limb bud and the 11 day embryonic pectoral muscle (PM) also react with this antibody. Immunoblots of myosin heavy chain peptide maps demonstrate that the same myosin isoform expressed in the adult chicken ventricle is also expressed in differentiating skeletal muscle cells. Other myosins were also detected in many newly differentiating muscle cells by our anti-myosin heavy chain mAbs, however, at the earliest stages of myogenesis only the myosin detected by HV11 was expressed in all differentiating muscle cells examined. Our observations suggest that expression of this myosin heavy chain isoform is a definitive marker for the activation of the myogenic program.

Key words: myosin, myosin heavy chain, myogenesis, muscle, muscle differentiation, monoclonal antibodies

Terminal differentiation of myogenic precursor cells results in the initial appearance of muscle-specific proteins, the most prominent of which is the heavy chain subunit of sarcomeric myosin. In avian and mammalian systems, myosin heavy chain (MHC) is encoded by a large and complex multigene family [29, 32], the members of which are differentially expressed during development and maturation of muscle fibers [2]. Our understanding of the diversity of MHC expression has continued to increase as new antibody and cDNA probes to specific MHCs are produced and characterized. A complete description of the regulation of MHC expression during development will be a key component in understanding the unfolding of the myogenic program.

Recent studies of MHC expression have focussed on the extrinsic factors that determine which MHC proteins accumulate in a skeletal muscle fiber. Innervation [13, 34], thyroid hormone [11, 16], pattern of muscle activity [9], muscle load [22, 23, 30], and injury or trauma [13,33] are examples of some of the external signals to which a muscle fiber responds by altering the subset of MHC proteins that are expressed. Differences in the inherent ATPase activity of some members of the myosin family [6] and a direct correlation between maximum velocity of muscle fiber shortening and MHC composition in some fibers [31] have provided an enzymatic basis for proposing that changes in functional demands on the muscle result in the expression of the MHCs which best fit the immediate need of the fiber.

Other studies have suggested that some of the diversity of MHC expression can be traced to differences in myogenic stem cells. Distinct myogenic lineages are characterized by the morphology of the myotubes they produce in culture [8], the culture media they require for growth and differentiation [39], and the subset of myosin heavy and light chains synthesized by the myotubes they produce [27,28]. Based on grouping avian MHCs into two classes with mAbs, Stockdale and Miller [37] have proposed a model for avian myogenesis in which discrete myogenic lineages, which are temporally and may be spatially segregated, are responsible for some of the diversity of MHC expression in developing muscle. However, in contrast to these results, another laboratory using a different set of mAbs has observed that all newly differentiated muscle fibers in the early chicken embryo have a common MHC phenotype and that diversity in MHC composition only occurred subsequent to terminal differentiation [38]. These workers have proposed that there is only a single type of myogenic stem cell that gives rise to a generic muscle cell and that MHC specialization is the result of extrinsic signals in the local environment.

In this report we use a mAb library to avian MHCs to examine the MHC composition of differentiating muscle cells in embryonic heart and somites, limb buds, regenerating fast and slow adult muscle, and cultured muscle cells derived from early limb buds and from fetal PM tissue. Similar to Sweeney et al. [38] we find that all newly differentiating muscle cells examined reacted with a mAb that we demonstrate to be specific to adult
ventricular MHC. Some cells appear to react only with the ventricular-specific antibody at the earliest stages of myogenesis while other cells co-express different isoforms. Our observations indicate that the expression of ventricular MHC is unalterably linked to terminal differentiation of muscle in the chicken. We suggest that irrespective of the number of myogenic lineages, the appearance of this MHC is a definitive marker for the activation of the myogenic program.

Materials and Methods

Monoclonal antibody production

HV11, EB165, NA1 and NA2 mAbs were produced as previously described [5, 13]. BALB/c mice were injected intraperitoneally with electrophoretically eluted myosin heavy chains [3, 6] from adult chicken ventricle (HV11), 14 day old embryonic PM (EB165) and neonatal anterior latissimus dorsi (ALD) muscle (NA1 and NA2). Hybridomas were prepared from the spleens of immunized mice [17]. All antibody dilutions were prepared from ascites fluids.

ELISA

ELISA were performed as previously described [3,4]. Myosins from the indicated muscles were plated on 96 well microtiter dishes at 0.5 μg per well, blocked with 2% horse serum in phosphate buffered saline (PBS), and incubated with HV11 mAb in a 1% horse serum-PBS solution for 30 minutes at 37°C. Wells were washed three times with PBS and bound antibody was detected with the Vectastain screening kit (Vector Laboratories).

Chickens and eggs

White leghorn chickens and eggs were used for these studies. Adult chickens were at least 6 months of age. Chicken embryos were staged as described by Hamburger and Hamilton [18].

Muscle cell cultures

Cell cultures were prepared as previously described [12] with some modifications. Pectoral muscles from 11 day old fetal chickens were removed, placed in Ham’s F-10 (Irvine Scientific) and finely minced. The media was carefully decanted and 10ml of a 0.1% trypsin solution at 37°C was added to the cells. After 4-5 minutes, trypsinization was stopped with the addition of 40 ml of 20% horse serum (Hazelton) in Ham’s F-10. Cells were pelleted by centrifugation at 500 × g, and the pellet was resuspended in media composed of 80% Ham’s F-10, 15% horse serum (Gibco), 1% CaCl₂ (100mM), 0.5% Glutamine (100 mM) and 3% embryo extract. The cells were filtered through two layers of nitex in a double swiney filter and plated at a density of approximately 3x10⁵ cells per 35 mm² dish. Cultures were also prepared from myogenic cells obtained from stage 26 hind limb buds [27]. Hind limb buds from embryos were excised and minced in Ham’s F-10. The remainder of the preparation was as stated above. Cultures were grown for 1-6 days and were fed every second day with media.

Cold injury and muscle regeneration

Regeneration was induced in adult chicken PM and ALD by cold injury [13]. The birds were anesthetized with sodium pentobarbital and the skin over the PM was incised. A metal rod cooled in liquid nitrogen was pressed to the muscle surface.

Figure 1. Specificity of HV11 antibody. (a) ELISA using myosins from adult chicken ventricle ( ), 12 day fetal PM, 12 day neonatal PM, adult PM, adult ALD, adult biceps brachii, and adult gastrocnemius. Results are the average of triplicate determinations. Only myosins from the adult ventricle reacted with HV11 antibody. (b) Immunoblot using NA4 mAb (upper panel) and HV11 mAb (lower panel). Myosins from 14 day fetal PM (1), 12 day neonatal PM (2), adult PM (3), 12 day neonatal ALD (4), adult ALD (5), adult biceps brachii (6), adult ventricle (7), and adult gastrocnemius (8) were separated on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose, and reacted with mAbs as described in methods. NA4 mAb reacted with all of these myosins while HV11 mAb reacted only with ventricular myosin. (c) Immunocytochemical staining of adult ventricle cryosections with HV11 antibody and (d) immunocytochemical staining of adult atrial cryosections with HV11 antibody. All myocytes in the ventricle reacted with HV11 mAb while only an occasional myocyte in the atria react with the antibody (indicated by arrow).
for 10 seconds. Four to 15 spots were injured on each side of the PM and 2-3 areas on the ALD. The skin was sutured and after three days, muscle was removed for immunocytochemistry as described below.

**Myosin preparation**

Myosin was prepared as described in Bandman et al., [6]. Muscle taken from adult chicken heart ventricle, PM and ALD was homogenized and washed in low salt buffer, 20mM potassium chloride, 2mM potassium phosphate buffer, 2mM ethylene glycol bis (β-amino-ethyl ether), N,N’-tetra acetic acid (EGTA), pH 6.8. Myosin was then extracted with a high salt buffer composed of 40mM sodium pyrophosphate, 2mM EGTA, 1mM magnesium chloride, pH 9.5, for 1 h on ice. Crude myosin was obtained by precipitation in low salt buffer and stored in high salt buffer containing 50% glycerol at -200 C.

**Immunoblots of myosin and myosin peptide maps**

Immunoblots of myosin were performed as previously described [3]. Immunoblots of myosin heavy chain peptide maps were prepared as previously described with a few modifications [4]. Myosin extracts were digested with Staphylococcus aureus V8 protease (10 ng/ml) and chymotrypsin (2100 μg/ml) for 30 minutes at 37°C and the peptides were separated on 7.5% SDS-polyacrylamide gels. The peptides were transferred to nitrocellulose and incubated with ascorbic fluids diluted 1:5000 in 5% nonfat dry milk in PBS [21]. Bound antibody was detected with diaminobenzidine following incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulin (1:100) (Dako Laboratories).

**Immunocytochemistry**

Tissue preparation and cryosectioning was performed as previously described [13]. Tissue sections of adult regenerating muscle, whole embryos and embryonic limb bud were air dried. Embryonic sections were fixed with 100% methanol for 5 minutes. The sections were blocked with 2% horse serum, 5mM EDTA, 0.1% sodium azide in PBS for 15-30 minutes and incubated with primary antibodies in 1% horse serum PBS as described [13]. The sections were then incubated with biotinylated rabbit anti-mouse IgG followed by biotin-avidin-peroxidase complex diluted in 1% horse serum PBS according to a Vectastain ABC kit (Vector Laboratories). Staining was visualized with diaminobenzidine (DAB) in 100 mM Tris buffer and 0.01% H2O2, pH 7.2. Sections were mounted in Permount (Fisher) and viewed on a Nikon Diaphot microscope.

For double immunofluorescence, sections were incubated first with EB165 mAb at 1:2000. Samples were subsequently incubated with rabbit anti-mouse IgG conjugated to rhodamine (Vector Laboratories). Sections were then incubated with mouse IgG (10 mg/ml) prior to incubating with HV11 mAb directly conjugated to fluorescein (1:1000). Sections were then mounted in 90% glycerol, n-propyl gallate (2 mg/ml) and viewed with a Leitz fluorescent microscope.

Staining of stage 8-10 chicken embryos was performed on intact embryos as described by Dent et al. [15] with some modifications. Embryos were removed from the egg and fixed with a 20% dimethylsulfoxide (DMSO)/80% methanol solution for 2 h. Embryos were then bleached overnight at room temperature in 20% DMSO/10% H2O2/70% methanol solution. The embryos were washed extensively with PBS and incubated with 2% horse serum PBS for 30 min as a blocking agent. Embryos were incubated with primary antibodies overnight at room temperature, and washed for at least 1 h in PBS. Binding of antibodies with the Vectastain ABC kit (Vector Laboratories) was as previously described except washing times were increased to facilitate removal of unbound antibody and detecting agents.

**Results**

**Antibody specificity**

The specificity of mAb HV11 is illustrated in Figure 1. In ELISA, the antibody reacted with myosin from the adult chicken ventricle but not with myosins from skeletal muscles of late fetal or older chickens (Figure 1a). On immunoblots HV11 antibody reacted with MHC from the adult chicken ventricle, but not MHCs from skeletal muscles (Figure 1b). Identical results were obtained with quail myosins (results not shown). On thin sections of the adult chicken heart, HV11 antibody reacted with ventricular myocytes (Figure 1c), but only an occasional atrial myocyte (Figure 1d). Thus this antibody appears to react with a MHC epitope primarily present in the adult ventricle.

Monoclonal antibody NA4 reacts with all sarcomeric MHCs in both avian (Figure 1b) and mammalian myosins (data not shown). Monoclonal antibody EB165 reacts with all embryonic fast MHCs [4,5,13].

**HV11 antibody reacts with differentiating muscle cells in culture**

Myoblasts from 11 day embryonic PM were grown in culture as described in methods. At 24 h, only an occasional myoblast reacted with HV11 antibody (Figure 2a). By 48 h most myoblasts which were beginning to fuse into myotubes were positive for the HV11 epitope (Figure 2b), as were the myotubes present in 72 and 96 h cultures (Figure 2c and 2d). As previously described [12], myotubes in similar cultures were also stained by mAb EB165.

Myogenic cultures were also prepared from 5 day (stage 26) chicken embryo limb buds. Many differentiated muscle cells in these cultures have a unique morphology as compared to differentiated muscle cells from 11 day fetal myoblasts [8]. In cultures of hindlimb bud cells grown for 6 days, HV11 antibody reacted with differentiated muscle cells that exhibited both the early and late morphology [8] (Figure 3). These cells also reacted with other monoclonal antibodies. EB165 appeared to react with essentially all of the differentiated cells, while mAbs NA1 and NA2 which are specific to slow MHCs [5] reacted with only a few myocytes and myotubes (data not shown). This latter observation is consistent with previous studies which have reported that 2 types of muscle cells can be distinguished in these cultures based on their fast and slow MHC composition [27,26].

In order to demonstrate that the MHC that is detected by HV11 mAb in differentiating muscle cultures is the same as that found in the adult chicken ventricle, myosin was prepared from 3 day old cultures and digested with 2 different proteases. These myosin digests, along with similar digests of adult ventricular myosin, were then subjected to SDS-PAGE, transferred to nitrocellulose and reacted with HV11 antibody. As shown in Figure 4a and 4b, the peptide fragments containing the HV11 epitope from the myosin digests from the cultured
Figure 2. Differentiating muscle cells derived from 11 day fetal myoblasts react with HV11 mAb. Cell cultures were fixed and reacted with HV11 mAb at 24 h (a), 48 h (b), 72 h (c), and 96 h (d). At 24 h only an occasional differentiated muscle cell reacted with the antibody (indicated by arrow). With increasing culture time more mononucleated cells and newly fused myotubes produced myosin that reacted with HV11.

Figure 3. Differentiating muscle cells derived from stage 26 hindlimb bud myoblasts reacted with HV11 mAb. Cell cultures were fixed at 6 days and reacted with HV11 antibody as described in methods. Muscle cells exhibiting both an early (arrow) and late (double arrow) morphology reacted with the mAb.

Figure 4. Immunoblots of peptide maps incubated with HV11 mAb. Myosins from 3 day old cell cultures (1) and adult ventricle (2) were reacted with S. aureus V8 protease (a) and chymotrypsin (b) as described in methods. The similarity of the peptide map blots suggests that HV11 mAb is reacting with the same MHC in both cell cultures and adult ventricle.
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![Images of embryos stained with antibodies](images)

**Figure 5. Whole embryo mounts incubated with HV11 and EB165 mAbs. Stage 8 (a,b), 9 (c,d), and 10 (e,f) embryos were incubated with HV11 mAb (a,c,e) and Eb165 mAb (b,d,f) as described in methods. No cells in stage 8 embryos reacted with either antibody. At stage 9, heart primordia reacted with HV11 mAb (arrow in c) but not EB165 mAb (arrow in d). The developing heart in stage 10 embryos (e,f) stained with both antibodies.**

Cells were electrophoretically identical to the peptide fragments containing the HV11 epitope from the ventricular myosin digests. The similarity of the immunoblots of the MHC peptide maps using 2 different proteases suggests that HV11 is reacting with the same MHC in cell cultures and in adult chicken ventricles.

**HV11 antibody reacts with developing chicken cardiocytes and skeletal muscle fibers in vivo**

Monoclonal antibodies were incubated with stage 8 - 10 chicken embryos as described in methods. As shown in Figure 5 stage 8 embryos do not stain with either HV11 (Figure 5a) or EB165 (Figure 5b) mAbs. By stage 9, the heart primordia stained with HV11 mAb (Figure 5c), but not with EB165 mAb (Figure 5d). At stage 10 the developing heart stains with both mAbs (Figure 5 e, f). As shown in Figure 6 a, b the somitic myotomes of stage 17 embryos also stain with both of these antibodies. However, we consistently observed that in the more recently differentiated myotomes (those more posterior) more myotubes stain with mAb HV11 than stain with mAb EB165, while in the more anterior somites which differentiate first, the myotubes stain equally with both antibodies (data not shown). As shown in Figures 6 c, d, both HV11 and EB165 mAbs reacted with early myotubes in the stage 24 limb bud. Thus essentially all differentiated cardiac myocytes and skeletal myotubes in the embryo reacted with HV11 mAb. In the heart and in somitic myotomes staining with the anti-ventricular MHC mAb appeared to precede that of anti-embryonic fast MHC mAb.

**HV11 antibody reacts with differentiating myotubes in regenerating fast and slow muscle**

Regeneration was induced in adult fast PM and adult slow ALD muscles as described in methods and after 3 days the regenerating area was removed and cryosectioned. In the fast muscle HV11 antibody reacted with newly differentiated
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Figure 6. Double immunostaining of stage 17 somitic myotomes and stage 24 limb buds with HV11 and EB165 antibodies. Cryosections of stage 17 embryos (a,b) and of stage 24 limb buds (c,d) were double immunostained with HV11 and EB165 mAbs as described in methods. (a,c) HV11 fluorescence and (b,d) EB165 fluorescence. In the somitic myotome shown in a and b, HV11 antibody reacted with more myotubes than did EB165, while somitic myotomes more anterior to the one shown, reacted equally with both HV11 and EB165 mAbs. In the myotomes of the stage 24 limb bud, essentially all myotubes reacted with both EB165 and HV11 mAbs.

myotubes in the regenerating area (Figure 7a). EB165 reacted with these same fibers as well as the existing adult fibers that contain adult fast MHC which also contains the EB165 epitope (Figure 7b) [13]. An antibody to neonatal MHC did not react with any fibers at this stage of regeneration and an antibody specific to adult fast MHC did not react with HV11 positive fibers but reacted with the pre-existing myofibers (data not shown). In regenerating slow muscle, HV11 antibody (Figure 7c) and EB165 (Figure 7d) antibody reacted with the newly differentiated myofibers.

Discussion
In this report we demonstrate that an adult ventricular-specific MHC antibody reacts with differentiating muscle cells that we studied in vivo and in vitro. In cell cultures where it was feasible to obtain sufficient myosin for biochemical analyses, we show that the MHC that is expressed in differentiating cells has the same immunopeptide map as adult ventricular myosin. Our results thus support a previous conclusion by Sweeney et al. [38] that a "ventricular-like" MHC is expressed by newly formed muscle cells irrespective of their future myosin composition. Based on the similarity of the immunopeptide maps in our study, we propose that the same isoform expressed in adult ventricle is also expressed in newly differentiated skeletal muscle cells. Further analyses with isoform specific nucleic acid probes will be necessary to confirm this hypothesis. The recent sequencing of the cDNA encoding this isoform should expedite this work [35].

Expression of ventricular MHC precedes that of embryonic fast MHC in the developing heart and in the somitic myotomes as well. However in myotubes of the embryonic limb bud and those of adult regenerating muscle we found that most cells in the stages examined reacted with both HV11 and EB165 antibodies. It is unclear whether this indicates different programs of MHC expression by these myotubes. The inability to detect ventricular MHC prior to that of embryonic fast MHC in the limb bud and satellite cell derived myotubes in vivo and the occasional fiber that reacted with EB165 but not HV11 mAb, may be due to the fact that myotubes in these preparations are at various stages of development. In cell culture where differentiation is more synchronous it has recently been observed that myotubes derived from adult satellite cells react with HV11 prior to reacting with antibodies to embryonic myosins [19, 20]. Furthermore, QM cells, which are a maturation defective avian myogenic line, differentiated into muscle cells that accumulated MHCs that reacted with HV11 antibody but not EB165 antibody [1]. These observations provide further evidence that the expression of the ventricular MHC may precede that of embryonic fast MHC.

Based on the reactions of future fast and slow muscles with mAbs, Sweeney et al. [38] concluded that fibers that will ultimately contain different myosins arise from a "primordial fiber". This conclusion differs from previous studies by Stockdale and coworkers [37] which used different mAbs to show that myotubes of differing MHC content arise from myoblasts that are committed to distinct myogenic lineages. It seems likely that these disparate conclusions are the result of using different antibodies and methodologies. The evidence provided by Sweeney et al. [38] that all differentiating muscle cells have a common MHC "phenotype" is based on immunocytochemical evidence only. While such experiments can demonstrate the presence or absence of MHC epitopes, they do not
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Figure 7. Regenerating myotubes in fast and slow muscle react with HV11 and EB165 mAbs. Regeneration was induced in the adult PM and ALD muscles as described in methods and cryosections from the regenerating areas of the PM (a,b) and of the ALD (c,d) were reacted with HV11 (a,c) and EB165 (b,d) mAbs. In both regenerating fast and slow muscles, newly differentiated myotubes reacted with both mAbs.

demonstrate that specific MHC proteins are present. Furthermore, since it is also possible that MHCs are present that do not react with any of the antibodies employed for the study, cells with a common immunochemical MHC phenotype may actually differ in their myosin composition. Finally, it is also possible that myogenic stem cells have an initial program of MHC expression in common but are nevertheless committed to distinct subsequent programs of MHC expression.

We propose that the expression of ventricular MHC is a requisite part of the decision to differentiate into skeletal muscle of all myogenic lineages. The diversity of MHC content observed in different myotubes derived from cloned myoblasts grown under identical conditions [25] as well as the differing MHC content of chicken primary myofibers [14, 26] may thus be explained if differences in MHC expression occur subsequent to the expression of the ventricular MHC. The basis for these differences may include both intrinsic factors such as distinct lineages [37] as well as external signals, such as position within the embryo [10, 24], innervation [13], activity [9], and hormones [11, 16]. Thus cells exhibiting a common MHC phenotype early in differentiation may still be committed to distinct programs of MHC expression.

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