Characterization of a Myosin Heavy Chain in the Conductive System of the Adult and Developing Chicken Heart

ARLENE GONZÁLEZ-SÁNCHEZ and DAVID BADER
Department of Cell Biology and Anatomy, Cornell University Medical College, New York 10021

ABSTRACT A monoclonal antibody (anterior latissimus dorsi 58 [ALD58]; antimyosin heavy chain, MHC) directed against myosin from slow tonic muscle was found to react specifically with the striated muscle cells of the conductive system in the adult chicken heart. This monoclonal antibody was used to study the expression of myosin in the conductive system of the adult and developing heart. Using immunofluorescence microscopy with ALD58, muscle cells of the conductive system were demonstrated in both the atria and ventricles of the adult heart as previously shown by Sartore et al. (Sartore, S., S. Pierobon-Bormioli, and S. Schiafino, 1978, Nature (Lond.), 274: 82–83). Radioactive myosin from adult atria and ventricles was precipitated with ALD58 and subjected to limited proteolysis and subsequent peptide mapping. Peptide maps of ALD58 reactive myosin from atria and ventricles were very similar, if not identical, but differed from peptide maps of ordinary atrial and ventricular myosin. The same antibody was used to study cardiac myogenesis in the chick embryo. When ALD58 was reacted with myosin isolated from atria and ventricles at selected stages of development in radioimmunoassays, reactivity was not observed until the last week of embryonic life (>15 d of egg incubation). Thereafter concomitant and progressively increased reactivity was observed in atrial and ventricular preparations. Also, no ALD58 positive cells were observed in immunofluorescence studies of embryonic hearts until 17 d of egg incubation. Primary cell cultures of embryonic hearts also proved to be negative for this antibody. This study demonstrates that an epitope recognized by ALD58 associated with an antimyosin heavy chain of striated muscle cells of the adult heart conductive system is absent or present in only small amounts in the early embryonic heart.

Studies of myosin heavy chain (MHC) polymorphism have determined that multiple isoforms of this protein are expressed within the adult heart (4–6, 14, 18, 20, 21, 27, 28, 30, 34–37, 39, 45). Sartore et al. (35) and Dalla Libra et al. (6) have demonstrated that the myosins of atrial, ventricular, and Purkinje fiber myocytes are immunologically distinct within the myocardium of the adult chicken. Biochemical and immunochemical studies of mammalian ventricular myosin demonstrated the existence of two species of MHC which are differentially expressed during development and selectively accumulated in response to the hormonal state of the animal (4, 14, 20). In addition, molecular cloning of ventricular MHC has shown the presence of two mRNAs encoding the heavy chain which are selectively responsive to hormone levels (30, 39). Electrophoresis of atrial myosin on nondenaturing gels also has resolved two different myosins in mammalian hearts (5, 20).

The expression of MHCs in the cardiac conductive system has been much more difficult to study than atrial or ventricular myosins. Sartore et al. (35) used immunochemical techniques to demonstrate myosin polymorphism in Purkinje fibers of the atria and ventricles of the adult chicken heart.

1 Abbreviations used in this paper: ALD, anterior latissimus dorsi; McAb, monoclonal antibody; MHC, myosin heavy chain.
While myosins isolated from bovine Purkinje fibers and ventricular myocardium have been shown to have similar ATPase activities and heavy chain peptide maps, an additional myosin light chain was noted for Purkinje fibers which was not present in ordinary ventricular muscle (34, 45). Morphological studies have demonstrated that Purkinje fibers of adult hearts have fewer and less well organized myofilaments and a more extensive network of intermediate filaments than observed in ordinary ventricular myocardium (12, 32, 41).

While it is apparent that the myosin of Purkinje fibers in the adult heart is distinct from that of atrial and ventricular myocardium, only a limited number of studies have addressed the developmental history of the cardiac conductive system as a whole (7, 15, 22, 23) and using myosin as a specific marker for its constituent cells (45). This is due in part to the limited amount of striated muscle in the developing conductive system and the distribution of these cells within the early embryonic heart. Some of these problems can be circumvented by the use of monoclonal antibody technology. In the present study, a monoclonal antibody (McAb) generated against the MHC of slow tonic muscle (anterior latissimus dorsi; ALD), and specifically cross-reactive with Purkinje fibers in the adult chicken was used to examine the expression of MHCs in adult and developing chicken hearts.

MATERIALS AND METHODS

Preparation of Antibodies Used: The McAbs used in this study were the result of three separate cell fusions. Monoclonal antibodies ALD19 and ALD58 were produced by the fusion of splenocytes from mice immunized with adult myosin from the anterior latissimus dorsi (slow tonic muscle), and nonsecreting myeloma cells (P3NP, the gift of Dr. M. Scharf, Albert Einstein Medical College) (38). The production of antibodies MF20 and BI have been previously reported (1, 13, 16).

Protein Preparation: Myosin was prepared from adult and embryonic white leghorn chickens (Spafas, Norwich, CT) as previously described (1). McAbs used in radioimmunoassays and affinity chromatographic experiments were affinity purified on myosin or goat antimouse IgG-conjugated Sepharose 4B columns prepared according to the manufacturer's instructions (Pharmacia). These analyses were carried out as previously described (1) (16).

Primary Cardiac Cultures: Hearts from chick embryos at 7 d of egg incubation were dissociated and cultured for the procedure for trypsin digestion used by De Haan (9). Cultures were grown on gelatinized polystyrene coverslips and maintained in Eagles' 199 medium and 15% fetal bovine serum (Gibco Laboratories, Grand Island, NY). Media was changed after the initial 24 h and every other day thereafter.

Radioimmunoassay, Immunoautoradiography, and Immunofluorescence Microscopy: These analyses were carried out as previously described (1, 16).

Radioiodination of Myosin and Affinity Chromatography: 100 µg of atrial or ventricular myosin in 25 µl of 3 M NaCl, 10 mM NaH2PO4, 10 mM Na2HPO4, pH 7.4 (HSB) was reacted with 1 µCi Na125I (New England Nuclear, Boston, MA) for 1 h using the Enzymobead reagent and the manufacturer's specifications (Bio-Rad, Rockville Center, NY). The reaction mixture was centrifuged for 3 min in a Beckman microfuge (Beckman Instruments, Palo Alto, CA) and the supernatant applied to a Sephadex G-25 column (0.5 x 20 cm) equilibrated with HSB. The radiolabeled myosin was recovered from the void volumes of these columns and spun for 10 min in a Beckman airfuge (30 psi). Protein concentrations and specific activities were determined for the high speed supernatants. Thereafter the solution was made 0.1% BSA from a 1% stock of BSA in 0.3 M NaCl and stored at 4°C. Preparations were used within 7 d of radioiodination.

Affinity chromatography of myosin was performed in the following manner: McAbs were exhaustively dialyzed against HSB containing 0.1 M phenylmethylsulfonyl fluoride, 0.1 mM diithiothreitol, and 1 mM EDTA (Rx B) at 4°C. These solutions were passed through columns of goat anti-mouse (GAM) immunoglobulins conjugated to Sepharose 4-B columns until unbound anti-

bodies could be detected in the effluent using a radioimmunoassay as previously described (1). At this point, the columns were washed with 10 column volumes of Rx B and stored at 4°C (<2 d) prior to reaction with radioiodinated myosin.

Radioiodinated myosin was diluted to 107 cpm/ml and applied to 25 to 75 µl of McAb-GAM-Sepharose 4B beads packed in 200 µl disposable Gilson micro-pipette tips. Myosin was applied until no further counts could be bound as determined by counting the pipette tip in a gamma counter. The column was then washed until the effluent radioactivity reached background levels after which the bound counts were eluted dropwise with 1% SDS, 100 mM Tris, pH 6.8, 1 mM EDTA. Radioactivity was adjusted to 0.25 to 1 x 106 cpm/µl and myosin added to a final concentration of 0.1 mg/ml. These samples were used for peptide mapping with Staphylococcus aureus V8 protease (Miles Laboratories, Elkhart, IN). Digestion was carried out in the presence of SDS (2) and terminated by boiling after making the solution 5% β- mercaptoethanol, and the resulting peptides were analyzed by gel electrophoresis.

Miscellaneous Techniques: Protein concentration was determined by the method of Lowry et al. (29). Polyacrylamide gels were run employing the buffer system of Laemmli (26). Chick embryos were staged by the system of Hamilton and Hamburger (19).

RESULTS

As part of a study of myosin heavy chain heterogeneity in the adult and embryonic chicken heart, McAbs from several cell fusions were screened against myosin isolated from the atra and ventricles of the adult chicken heart. Several of the McAbs used here have been reported in previous studies of MHC in skeletal and cardiac muscle (1, 13, 16, 38). One McAb studied, ALD58, was shown to react with the MHC of ALD muscle in immunoblotting experiments while exhibiting extremely low reactivity with the MHCs of adult ventricles and atria even after long film exposures (Fig. 1). Nevertheless, this McAb exhibited reactivity with DEAE column purified myosin in radioimmunoassay analysis (Fig. 4). For the other McAbs used in this study: (a) MF 20 reacts with all striated muscle cells of the heart (16); (b) BI reacts with only atrial muscle; (c) A19 reacts with only ventricular muscle (Zhang, Y., S. Shafiq, and D. Bader, manuscript in preparation).

Immunofluorescence microscopy was used to determine the cellular distribution of MHCs recognized by several McAbs. MF20 which has been shown to bind to the light meromyosin subfragment of myosin (1) was found to be reactive with all striated muscle cells of the adult chicken atrial and ventricular myocardium (Fig. 2). In contrast to MF20, ALD58 bound only to a small population of striated muscle cells of both the atria and ventricles (Fig. 2). The intensity of staining with ALD58 varied among reactive muscle cells (Fig. 2). These same cells often exhibited a greater intensity of staining with MF20. Previous studies using polyclonal antibodies reactive with Purkinje fiber and ventricular myosins, not unlike MF20, have also noted this increased

![Coomassie Brilliant Blue-stained 7.5% gel and accompanying immunoblot of adult heart myosin with ALD58. MHCs of adult (A), atria (At), and ventricles (V) are indicated.](image-url)
immunofluorescent intensity of Purkinje fibers (35). At present, it has not been determined whether this is due to the plane of sectioning or variations in MHC content of different myocytes. Background fluorescence with ALD58 was somewhat higher in the ventricle than the atrium. ALD58 positive cells which were located primarily in the subendocardial connective tissue, around blood vessels, and singly in the substance of the myocardium, have a large cell diameter and peripherally located myofibrils when viewed in cross-section. Truex et al. (43) and Sartore et al. (35) have previously described these striated muscle cells as members of the Purkinje fiber system in the adult chicken heart.

Radiolabeled myosins prepared from adult atrial and ventricular tissues were reacted with McAbs as described above. B1, an antibody specific for the atrial MHC, selectively bound atrial myosin, its counterpart A-19, an McAb specific for ventricular myosin (Zhang, Y., S. Shafiq and D. Bader, manuscript submitted for publication), reacted with the ventricular preparation. Myosin from both atrial and ventricular preparations reacted with ALD58 (Fig. 3), although the ratio of counts per minute bound to counts per minute applied to affinity columns of ALD58 was usually less than ten times than that for either B1 or A19 with their reactive myosin isoform. Peptide maps of MHCs reactive with these different McAbs varied markedly (Fig. 3). It is interesting to note that the peptide maps of myosin bound by ALD58 from atria and ventricles were very similar if not the same and that these maps were significantly different from A-19 and B-1 reactive myosin. Varying enzyme concentration or time of digestion did not produce any obvious differences between myosin bound by ALD58 from the atria and ventricles.

Appearance of the MHC Recognized by ALD58 during Cardiac Myogenesis

To determine whether or not the MHC epitope recognized by ALD58 in adult Purkinje fibers was present in the embryonic heart, myosin was prepared from atria and ventricles of hearts at selected stages of development and were reacted with ALD58 and MF20 in solid phase radioimmunoassay. As seen in Fig. 4, ALD58 bound both adult atrial and ventricular preparations but exhibited a greater reactivity with the latter samples (discussed below). No binding of this antibody was observed with myosin from embryonic atria or ventricles before 12d of egg incubation (Fig. 4). Thereafter, antibody reactivity gradually increased concurrently in both chambers.
FIGURE 3 Peptide maps of myosin digests from adult ventricles and atria bound by A-19 (A) and B-1 (B), respectively, and ALD58 with the ventricular (C) and atrial (D) preparations. 10 µg of carrier myosin and 10^5 cpm of affinity-bound radioactive myosin were digested with S. aureus Vp protease for 30 min at room temperature.

FIGURE 4 Radioimmunoassay analysis of adult and embryonic atrial and ventricular myosin with ALD58 and MF20. Serial dilutions (3 x; from 0.4 mg/ml) of myosin isolated from adult (○), 2-d posthatching (△), 17-d embryonic (□), and 12-d embryonic atria (●), and ventricles were reacted with McAbs, MF20, and ALD58, at 100 µg/ml.

Monoclonal Antibody Reactivity with Cultured Embryonic Cardiac Myocytes

These experiments were undertaken: (a) to determine whether embryonic cardiac muscle cells grown in cell cultures express the Purkinje MHC recognized by ALD58; and (b) to ensure that a small population of cells reactive with ALD58 had not been missed in our previous in vivo analyses. (This point was of great importance because immunofluorescence positive cells are more easily identified in monolayer cultures.) Cardiac muscle cells shown in Fig. 5 were indicative of the results obtained with embryonic cardiac cultures at 2, 3, and 6 d after plating. While all cardiac myocytes, often varying greatly in morphology, were positive for MF20, no cells were stained positively with the anti-Purkinje MHC antibody.

DISCUSSION

The present study shows that the MHC of adult slow tonic muscle shares a common epitope with the MHC of Purkinje muscle fibers of the adult chicken heart. This epitope on the MHC could not be detected in the embryonic heart (<12 d of egg incubation) using any of our assay systems. At present it can not be determined whether the MHC recognized by
ALD58 is expressed in very low levels or is completely absent in the early stages of cardiac myogenesis. Lastly, the present data suggest that the accumulation of the MHC identified by this McAb occurs simultaneously in the developing atria and ventricles during the later stages of embryonic life.

A variety of biochemical, structural, and immunoochemical studies have shown that muscle cells of the Purkinje fiber system contain cytoskeletal and contractile proteins distinct from those of atrial and ventricular myocytes (12, 32, 34, 35, 41, 45). Sartore et al. (35) and Saito et al. (34) have demonstrated in chickens and cattle that myosin of Purkinje fibers differs from other heart myosins in its immunoochemical and light chain subunit properties. Conversely, Whalen et al. (45) have used peptide mapping to show the similarity between MHCs of adult bovine Purkinje and ventricular myocytes, while demonstrating variation in their light chain composition. The present study indicates that at least one MHC of adult chicken Purkinje fibers is immunologically distinct from those of atrial and ventricular muscle (Figs. 2 and 3). Whether or not multiple MHC isoforms are present in Purkinje fibers as previously suggested (35), cannot be determined from the present data. In addition, we have not yet determined whether the MHC recognized by ALD58 in the heart is identical to one of the MHC expressed in the ALD muscle (31). It is interesting that the myosins from atrial and ventricular preparations bound by ALD58, presumably derived from the Purkinje fibers of these two heart chambers, are very similar when analyzed by limited proteolysis and SDS PAGE. These experiments provide evidence for the relatedness of ALD58 positive cells in the atrium and the ventricle.

Sartore et al. (35) have used polyclonal antisera to demonstrate the presence of Purkinje fibers in the atria and ventricles of the adult chicken. The present data agree with their study. While a cellular conductive system has been postulated for the atria as well as ventricles (11, 24), the precise function of ALD58 positive cells in the atria remains to be established.

The epitope recognized by ALD58 could not be detected in the early embryonic heart prior to 12d of incubation using a variety of immunoochemical techniques. This suggests that an MHC expressed in the Purkinje fibers of the adult chicken heart is either not present or is present in very small amounts in the embryonic heart both in vivo and in vitro (Figs. 4 and 5). Several points must be considered with this interpretation. First, the present data do not determine whether the immunoochemical differences between adult and embryonic preparations are due to the expression of two different MHC gene products or to a developmentally regulated post-translational modification of the MHC. Both situations have been previously reported for developing skeletal muscle (2, 22, 44). We do not believe that conformational variations between adult and embryonic myosin can explain these differences because ALD58 reacts with high activity for denatured atrial and ventricular myosin in radioimmunoassay (data not shown). Several laboratories have demonstrated that embryonic and adult ventricular myocardial cells preferentially express different myosin isoforms in several species (4, 5, 27, 30, 39). Conceivably, the same situation exists in the Purkinje fibers of the chicken heart where one MHC isoform predominates in embryonic fibers while another MHC is expressed in adult cells. Alternatively, these modified muscle fibers which do not have an abundance of myofibrils in adult hearts (21, 31, 41), may not produce enough myosin in embryonic hearts to be detected using the present techniques. This latter hypothesis seems less plausible in that previous work (7) has shown that Purkinje fibers of 7-d embryonic hearts have myofibrils and that these specialized myocytes may even be precocious with respect to myofibrillogenesis (15). We have been unable to demonstrate positive immunoochemical staining of cardiac myocytes with our adult Purkinje specific McAb in tissue sections, cell cultures, or cell isolates (Zadeh, unpublished results) from 7-d embryonic chick hearts. Thus, while a protracted differentiation of contractile proteins in the heart conductive system in general may explain the immunoochemical heterogeneity of MHCs of developing Purkinje fibers, it seems unlikely. Further study with McAbs specific for the MHC of Purkinje fibers will be necessary to resolve these points.

Earlier studies of Rawles (33), Cavanaugh (3), and De Haan (8, 10) have shown that distinct populations of myocytes differentiate early in cardiac myogenesis, but definitive identification of Purkinje fibers in the earliest stages of heart development is lacking. Using McAbs specific for ventricular and atrial MHCs, Sweeney et al. (40) and Gonzalez-Sanchez and Bader (16) have demonstrated the presence of ventricular and atrial specific MHCs in myocytes during the initial stages of cardiac myogenesis. Our study demonstrates that an MHC present in adult Purkinje fibers is not present in early embryonic hearts, whether the cellular precursors of the adult Purkinje fiber system are present in the early stages of cardiogenesis has not been determined. Evidence that Purkinje fibers of the adult chicken atria and ventricles express the same or very similar MHCs is not proof that these two populations of muscle cells are identical. However, expression of the same MHC would suggest that these two groups of myocytes may be related by cell lineage and/or physiological control. While it has been proposed that a direct cellular linkage exists between the sinoatrial and atrioventricular nodes and the Purkinje system of the ventricles of the mammalian heart (11, 24), it is uncertain if the muscle cells of this system are derived from a common cell lineage or are simply recruited from myoblasts of the surrounding myocardium during cardiogenesis.

While a comparatively large number of studies have documented the influence of cholinergic innervation on the expression of MHCs in skeletal muscle, relatively little is known about the possible effects of autonomic innervation on MHC expression in cardiac muscle. Recently, Toyota and Shimada (42) have reported a shift in the expression of troponin after addition of sympathetic neurons or extracts to cultures of cardiac myocytes. Innervation of Purkinje fibers in the developing heart may trigger the expression of a specific MHC in these myocytes. Indeed, it is intriguing that the appearance of sympathetic innervation in the developing chick atria corresponds to or slightly predates the accumulation of the MHC in Purkinje fibers as detected by ALD58 (25). Further investigation may elucidate the possible influence of innervation on MHC expression in muscle cells of the developing heart.

We thank Dr. S. A. Shafiq and Dr. D. A. Fischman for allowing us to screen their libraries of McAbs and Barry Zadeh, Maryann Matheson, Gudrin Mayard, and Cynthia Burton-Brown for their assistance during this study.

This work was supported in part by grants from the National Institute of Health (AM31305) and the New York Heart Association.

Dr. Bader is an Andrew W. Mellon Teacher-Scientist.

Received for publication 20 June 1984, and in revised form 13 September 1984.
