Detection of a Ventricular-specific Myosin Heavy Chain in Adult and Developing Chicken Heart

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Abstract. In the present study, a monoclonal antibody (McAb), ALD19, generated against myosin of slow tonic muscle, was shown to react with the heavy chain of ventricular myosin in the adult chicken heart. With this antibody, it was possible to detect a ventricular-specific myosin during myocardial differentiation and to show that the epitope recognized by ALD19 was present from the earliest stages of ventricular differentiation and maintained throughout development only in the ventricle. A second McAb, specific for atrial myosin heavy chain (MHC) (Gonzalez-Sanchez, A., and D. Bader, 1984, *Dev. Biol.*, 103:151– 158), was used as a control to detect an atrial-specific myosin in the caudal portion of the developing heart at Hamburger-Hamilton stage 15. It was found that the appearance of ventricular MHC predated the expression of atrial MHC by ~1 d in ovo and that specific MHCs were always differentially distributed. While a common primordial MHC may be present in the early heart, this study showed the tissue-specific expression of a ventricular MHC during the initial stages of heart development and its differential accumulation throughout development.

SEVERAL recent studies have shown the heterogeneity of myosin heavy chain (MHC)[1] expression in the hearts of adult avians and mammals (4, 5, 11, 13, 14, 16, 20, 21, 26–28, 30, 34). In addition, biochemical (4), immunochemical (4, 5), and molecular cloning (21, 30) techniques have detected two different MHCs in mammalian ventricles, which exist as either homo- or heterodimers of myosin (24), and whose expression is responsive to the hormonal state of the animal (4, 10, 16, 30). In mammals, one of the major cardiac MHCs (beta-form) is identical to the slow skeletal isoform (22), and the major atrial MHC is identical to the beta-form of the ventricles (18). The avian heart has been shown to have distinct atrial, ventricular, and conductive system MHCs (6, 11, 22, 27, 32). In contrast, the ventricular MHC of the avian heart is immunochemically distinct from the slow tonic isoform (32), and the atrial MHC is a distinct isoform in that it has no ventricular counterpart (6, 11, 12).

Cardiac myogenesis is also typified by the diversity of myosin components. Electrophoretic analysis of native myosin has shown that the expression of at least two different ventricular MHCs is developmentally regulated within the ventricles of several different species (5, 20), whereas other species, including some avians, exhibit a single myosin throughout development (23). The accumulation of specific ventricular MHC isoforms appears to be transcriptionally controlled (21, 30).

[1] *Abbreviations used in this paper:* McAb, monoclonal antibody; MHC, myosin heavy chain; RIA, radioimmune assay; GAM, goat anti–mouse immunoglobulin.

Although previous studies have documented the expression of myosin components during the later stages of embryonic life and in the adult organism, little is known about the expression of MHC during the initial stages of cardiac myogenesis. Ebert and co-workers (8, 9) used anti-sera generated against adult heart myosin to demonstrate myosin early in the formation of the heart, but could not differentiate between different isoforms of myosin that might be expressed due to the polyclonal nature of their antibody preparation. The study of myosin polymorphism in the early embryonic heart is complicated by the lack of specific reagents to study MHC expression and the small amount of tissue available for study.

We have used monoclonal antibody (McAb) technology to examine the expression of MHCs during the initial phases of avian cardiac myogenesis (11, 12). In one study (11), an atrial-specific MHC was detected at or near the onset of atrial myogenesis, and the expression of this MHC was confined to atrial-forming tissues. Sweeney et al. (32) have demonstrated the antigenic relatedness of MHCs in the early heart and hypothesized that the first MHC produced in all differentiating striated muscle may be related to the cardiac ventricular myosin. In this study, an McAb reactive with an MHC of the adult chicken ventricle was used to determine the distribution of MHCs within the developing heart. The present data demonstrate the tissue-specific distribution and accumulation of a ventricular-specific MHC within the ventricles of the differentiating heart from the initial stages of cardiac myogenesis.

Materials and Methods

Protein Preparation

Myosins were prepared from dissected atria and ventricles by homogenization in ice-cold 150 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4 (pH 7.2) including 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Triton X-100 (10:1; vol/wt) with an Omnimixer (Sorvall, two 10-s bursts at top speed). The homogenate was spun at 10,000 rpm in an SS34 rotor for 10 min and the resulting pellet was resuspended in the aforementioned buffer (10:1; vol/wt). The cycle of centrifugation and extraction was repeated until the resulting supernatant was clear (usually four or five cycles). The final pellet was resuspended in the homogenization buffer without Triton X-100, centrifuged, and the pellet extracted in cold 0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4 (3:1; vol/wt) for 10 min. The extraction was terminated by centrifugation (10,000 rpm, SS34 rotor, 10 min), and the supernatant was diluted 10× with cold distilled H_2O. The precipitant was collected by centrifugation (10,000 rpm, SS34 rotor, 10 min). The pellet was made 0.5 M NaCl, 5 mM $MgCl_2$, 5 mM ATP and spun at 45,000 rpm for 2 h in a Ti70.1 rotor (Beckman Instruments, Inc., Palo Alto, CA). Crude myosin was collected from the resulting supernatant by precipitation ($i < 0.05$) and subsequent centrifugation (10,000 rpm, SS34 rotor, 10 min). Myosin was purified to homogeneity by ion exchange (DEAE) chromatography using a linear KCl gradient (0.0–0.5 M) (25). Purified myosin was stored at 5–10 mg/ml in 0.5 M NaCl and 50% glycerol at −20°C. To assay for the appearance of myosins in the early heart (stages 9–21 [15]), specific regions of the heart were micro-dissected and extracted in 10 µl of 0.5 M NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4 (pH 7.6) with 0.1% Triton X-100 for 10–16 h. The tissue/extract in buffer was then spun in a microfuge for 10 min, and the supernatant was collected and dried for solid-phase, indirect radioimmunoassay (RIA).

Immunoglobulins from monoclonal cell lines were purified from conditioned cell culture media by affinity chromatography on columns of Sepharose 4B conjugated with specific myosins or goat anti-mouse immunoglobulins (GAMs) (Cappel Laboratories, Cochranville, PA). Antibodies were eluted with 0.2 M glycine (pH 2.2) into equal volumes of 0.2 M Tris (pH 8.1), and protein-containing fractions were immediately dialyzed against 150 mM NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4 (pH 7.2), concentrated to 5–10 mg/ml by ultra-filtration and stored at −20°C until use.

Immunochemical Analysis and McAbs

RIAs, immunoradiography, and immunofluorescence microscopy were done using published methods (1, 11, 19). The McAbs used here were produced from several different cell fusions. Myosin from anterior latissimus dorsi (McAb ALD19) or pectoralis (McAbs MF20 and B1) muscles were used as antigens. These cell lines have been recloned several times without any change in antibody specificity. Details of this production have been published (1, 11, 29). There specificities are discussed below (Results).

Radiolabeling of Myosin and Affinity Chromatography

Myosin from adult or 5-d embryonic ventricles recovered after ion exchange chromatography was radiolabeled with ^{125}I (New England Nuclear, Boston, MA) by the Enzymobead reagent (BioRad Laboratories, Richmond, CA). DEAE-purified myosin was precipitated in 10 vol of cold distilled H_2O after the pH was adjusted to 6.8. The precipitated myosin was collected by centrifugation and redissolved in 0.3 M NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.4 at a concentration of 4 mg/ml. 100 µg of this preparation was iodinated using the manufacturer's directions.

Radiolabeled myosin was separated from free iodine on a Sephadex G-25 column (0.5 × 20 cm) equilibrated in 0.3 M NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4 (pH 7.2). Fractions that contained myosin were collected and spun in an airfuge (Beckman Instruments, Inc.) for 10 min at 30 psi. Specific activities of supernatants were determined and made 0.1% in bovine serum albumin from a 10 mg/ml stock in 0.3 M NaCl. Samples were used with 7 d of iodination.

GAM (Cappel Laboratories) was covalently linked to Sepharose 4B using the manufacturer's instructions (Pharmacia Fine Chemicals, Piscataway, NJ). Hybridoma supernatants that contained McAbs were passed over GAM beads until the beads were saturated as determined from RIA for the presence of unbound McAb in the column effluent. Radiolabeled myosin was reacted with these antibody/bead complexes batch-style in 1.5-ml Eppendorf tubes for 2–24 h at 4°C. After reaction the beads were washed with the buffer that contained 0.3 M NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.4 until no cpm were detected in the wash buffer. Bound cpm were eluted in 1% sodium dodecyl

sulfate, 10 mM Tris, pH 6.8, 1 mM EDTA. Counts were adjusted to 10^4–10^5 cpm/µl and 1 mg/ml with the appropriate myosin.

Bound radioactive myosin was analyzed by peptide mapping using *Staphylococcus aureus* protease (Miles-Yeda, Rehovot, Israel) and alpha-chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) according to Bandman et al. (2). Protein/enzyme ratios were adjusted to permit total digestion of the MHC. Digests were applied to 10% polyacrylamide gels, and after electrophoresis, the gels were dried and used to expose x-ray film (Kodak X-OMAT AR-5).

Results

Specificity of McAb ALD19 for Ventricular MHC

McAbs generated from several cell fusions were screened for their reactivities with cardiac myosin. One McAb, ALD19, which was generated against ALD myosin, was studied in greater detail. Immunoblots with ALD19 showed strong reactivity with ventricular MHC, whereas very faint reactivity with atrial MHC was detected in some preparations with long film exposures (Fig. 1). To determine the cellular distribution of heavy chains recognized by ALD19, tissue sections were treated for immunofluorescence microscopy. As seen in Fig. 2, all ventricular myocytes were stained by ALD19, whereas atrial cells were negative for this antibody. Occasional ALD19, immunofluorescence positive cells were seen in the atria near their junction with the ventricles. McAb B1, specific for atrial MHC (11), was used as a control in the present study and recognized MHCs present in atrial myocardium, but was negative for all cells of the ventricle (Fig. 2). Endothelial cells, smooth muscle, and all elements of the connective tissue were completely negative for all anti–MHC antibodies. The reactivities of these antibodies have not been extensively tested with skeletal muscles other than the ALD and pectoralis muscles. These results demonstrate the specificity of ALD19 for ventricular myocytes in the heart.

Reaction of ALD19 with Ventricular Myosin Isolated from Embryonic Hearts

To examine possible antigenic similarities or differences between adult and embryonic ventricular MHC, ALD19 was reacted with preparations of myosin at selected stages of development. Binding of this McAb was confined primarily to the ventricles in early and late embryonic life, while slight reactivity of ALD19 with high concentrations of atrial myosin was detected in some preparations. This reactivity may be due to contamination of the atrial preparation with ventricular myosin or minor cross-reactivity of ALD19 with atrial myosin. B1, an antibody that recognizes only atrial MHC, showed no binding to ventricular myosin over all protein concentrations tested. When tissue sections of embryonic
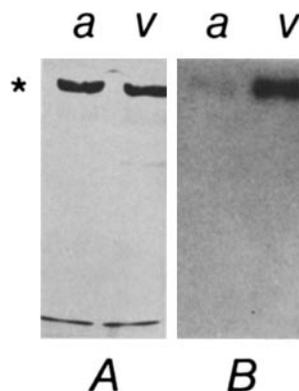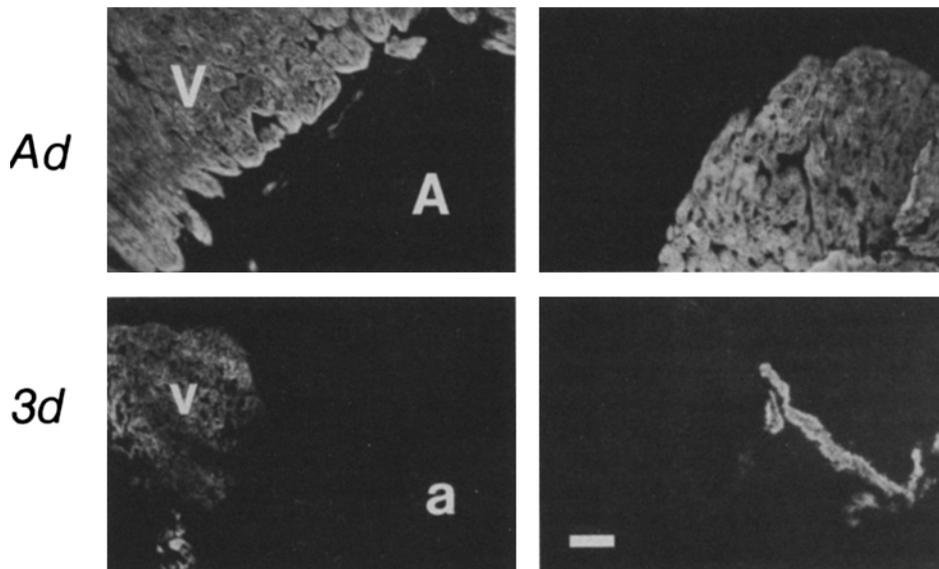

Figure 1. Coommassie Brilliant Blue–stained gel (*A*) shows myosin prepared from atria (*a*) and ventricles (*v*) of adult chickens. Asterisk denotes heavy chain. Accompanying blot (*B*) shows specificity of ALD19 for the ventricular MHC.

ALD-19   B-1

Figure 2. Immunofluorescence microscopy with ALD19 and B1. Serial sections of adult and 3-d embryonic hearts reacted with these antibodies show specificity of ALD19 and B1 for the ventricle and atrium, respectively. Atrium (*A*, *a*); ventricle (*V*, *v*). Bar, 100 μm.

hearts were reacted with anti–MHC antibodies, the pattern of immunoreactivity was the same as that detected in the adult (Fig. 2). Within the limits of the light microscope, all ventricular muscle cells were stained with ALD19 at all stages tested, while a limited number of atrial myocytes at atrioventricular junction were positive with this antibody.

Radiolabeled embryonic (5 d) and adult ventricular myosins were reacted with ALD19 and peptide mapped to examine the structure of these proteins. Under conditions in which the heavy chain was completely digested by V8 protease or chymotrypsin, the radioactive bands in embryonic and adult preparations appeared homologous in molecular weight (Fig. 3, *A* and *B*). Differences in the labeling intensity of several bands between the two preparations could be seen in the V8 protease digests (Fig. 3*A*), but these variations were most probably due to differences in the amount of radioactivity loaded as all bands could be identified in both embryonic and adult myosins. In addition, variant bands, which were always of low intensity, were readily visible upon longer film exposure and, invariably, matched the accompanying digest. Varying enzyme concentration or time of digestion did not produce any diversity in bands of the digestion patterns between the two samples.

Appearance of the Epitope Recognized by ALD19 during the Initial Stages of Heart Formation

RIA of isolated regions of the forming heart were run in parallel with analyses previously published (11) to establish the earliest stage at which ALD19 reacted with heart myosin. (The reaction of atrial-specific antibody B1 is provided here as a reference.) The first significant reactivity of ALD19 with the tubular heart was observed in the presumptive ventricle (region 3) at stage 10 (Fig. 4). Limited reactivity was also observed in atrial forming tissues, but this reactivity was not positively confirmed using immunofluorescence microscopy (data not shown). Reactivity of ALD19 increased rapidly and was confined to ventricular tissues (Fig. 4). Binding of B1 was observed ~1–1.5 d later in the caudal-most regions of the
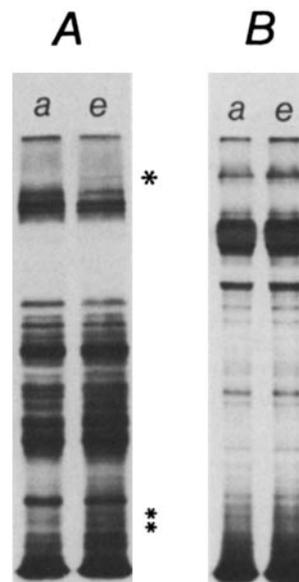
Figure 3. (*a*) Peptide maps of ALD19 affinity bound 5-d embryonic and adult ventricular myosin produced by digestion with *S. aureus* V8 protease. 10 μg of carrier myosin and 50,000 cpm of affinity bound myosin were digested with 0.1 mg/ml of V8 protease. Adult (*A*) and 5-d embryonic (*B*) preparations are noted. Asterisks denote bands that differ in intensity between the two preparations. (*b*) Peptide maps of ALD19 affinity bound 5-d embryonic and adult ventricular myosin digested with chymotrypsin. All conditions were similar except myosin was digested by chymotrypsin.

developing heart. Thereafter, B1 only bound to atrial tissues throughout development (Figs. 2 and 4).

Discussion

In the present study, a McAb (ALD19) generated against the MHC of a slow tonic muscle is shown to react with the MHC of ventricular myocardium. At no time does this McAb react with myosin from the embryonic or adult atria. In addition, it is possible to detect the tissue-specific distribution of the ventricular MHC recognized by ALD19 from the earliest stages of cardiac myogenesis. Thus, while the expression of a common "primordial" MHC throughout the myocardium of the developing heart has been noted (32), the present study demonstrates the tissue-specific appearance and accumulation of a ventricular MHC during the earliest stages of ventricular cardiac myogenesis.
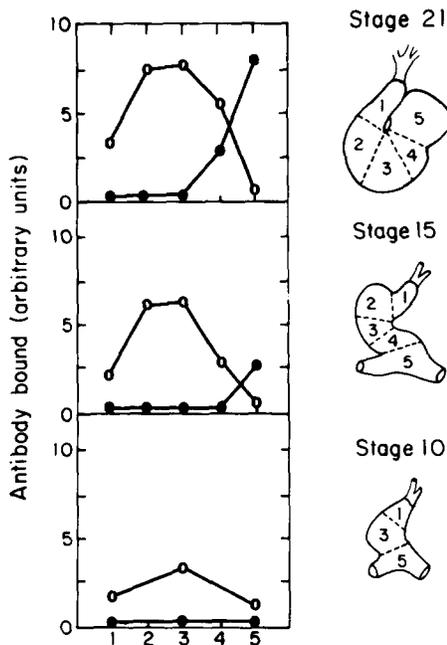
Figure 4. RIA analysis of early embryonic hearts with ALD19 (O) and B1 (●). Tissues were isolated and prepared for RIA as previously reported (11). Horizontal axis represents regions assayed, whereas the vertical axis represents the amount of radioactive GAM bound. Each point is the average of at least three individual hearts.

Previous studies have used a variety of techniques to demonstrate the diversity of MHCs present in the atria and ventricles of adult mammals and avians (4, 5, 10–14, 16, 20, 21, 26, 27, 30). In addition, differential expression of myosin (20) and MHCs (5, 21, 30) in developing hearts has been observed in many species, while in chickens it appears that a single myosin is expressed in both embryonic and adult ventricles (23). From peptide mapping experiments presented here (Fig. 3), it appears that the myosin reactive with ALD19 in the 5-d embryonic and adult ventricle are remarkably similar, if not identical, even in different digestive systems. The minor variations in peptide maps between embryonic and adult preparations were limited to slight differences in band intensity in the V8 protease digests and not to unique bands in either preparation. In addition, our RIA and immunofluorescence data demonstrate that the epitope recognized by ALD19 is present throughout development. For these reasons, we feel that the myosin recognized by ALD19 in the chambered embryonic and adult ventricle are the same protein. Still, the present data do not determine whether all of the ventricular myosin in the developing heart is reactive with this McAb. It is possible that sarcomeric myosins non-reactive with ALD19 are constantly or transiently present in the ventricular myocardium. This point must be considered in light of the studies of Sweeney et al. (32), which suggest that all striated muscle (both skeletal and cardiac) of the early avian embryo transiently produce an antigenically related MHC. Future studies will be necessary to determine the total number of MHC isoforms expressed in the developing ventricle of the chicken.

An MHC reactive with ALD19 is first detected at stage 10 in the developing heart. This reactivity is confined primarily to that portion of the heart tube destined to become the ventricle. Earlier morphological studies have demonstrated

that this region of the heart is the first to exhibit cross-striated myofibrils (19), sporadic contractions (17) and, in addition, is the first region where significant numbers of myocardial cells withdraw from the cell cycle (10). Thus, it is possible that the first sarcomeric, cardiac MHC is a "ventricular-like" MHC. Our immunofluorescence data reveal that this MHC, recognized by its reactivity with ALD19, is confined to the ventricle. We consider the limited reactivity of ALD19 with atrial-forming tissues (region 5, stage 10) in RIAs (Fig. 4) due to contamination of these preparations with ventricular cells in that ALD19 immunofluorescence-positive cells were never detected in the developing atria. Sweeney et al. (32, 33) describe two McAbs specific for adult ventricular myosin that react with the cardiac and myotomal myogenic cells in the stage 15 of the chick embryos. They propose that all myogenic cells of striated muscles at this stage contain a common "primordial" myosin. With ALD19, however, a ventricular myosin can be identified in stage 10 embryos which is clearly antigenically different from atrial myosin that is first detectable at stage 15 and is reactive with the B1 antibody. Thus, at stage 15 there are already two different tissue-specific myosin isoforms in the developing myocardium differentially distributed in the heart. While these data demonstrate the tissue-specific distribution of MHCs in the differentiating ventricles and atria, we cannot exclude the co-expression of a common "primordial" MHC as previously proposed by Sweeney et al. (32, 33). Indeed, coexistence of MHC isoforms in developing cardiac muscle has been documented for several species (13, 30). It is possible that the earliest stages of avian cardiac myogenesis include the synthesis of both nonspecific and tissue-specific, sarcomeric MHC.

Several studies have suggested the heterogeneous nature of cardiac myoblasts which give rise to the heart myocardium (3, 7, 24). These investigations noted that cardiac myoblasts derived from the cardiogenic plate beat at different rates and, from these results, it was postulated that cardiac myoblasts are heterogeneous in nature. Ebert and co-workers (8, 9) have demonstrated that these myocytes accumulate myosin early in heart development. From the present data and previous reports from this laboratory (11), it appears that tissue-specific expression of ventricular and atrial MHCs is a feature of early cardiogenesis. Still, the cellular and molecular factors that give rise to the apparent heterogeneity of MHC expression remain unresolved.

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