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Contractile protein isozymes in muscle development: Identification of an embryonic form of myosin heavy chain

(denatured myosin/polypeptide mapping/antiserum to heavy meromysin/complement fixation/muscle cell cultures)

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ABSTRACT **The nature of the myosin heavy chain in embryonic muscle tissue, cultured muscle cells, and several adult muscles was investigated. After denaturation with sodium dodecyl sulfate, purified rat myosins were subjected to partial proteolytic cleavage or immunological analysis using micro-complement fixation. Three types of myosin heavy chains could be demonstrated by both approaches. Whereas adult muscles contain fast- or slow-type myosin heavy chains, embryonic tissue and cultured muscle cells harbor a distinct embryonic form. The existence of this distinct form further characterizes the isozymic transitions of contractile proteins during muscle development.**

During the differentiation of muscle tissue a number of isozymic transitions take place among the contractile proteins. It is now well established that nonmuscle cells, including the myoblast, contain contractile proteins (1, 2). The synthesis of the muscle-specific forms takes place around the time when myoblasts fuse to yield multinucleate myotubes (3–7). In the adult animal, muscle fibers are of two basic types, characterized by fast or slow contraction (8). These two fiber types also differ in the various contractile and regulatory proteins that they contain (9, 10). The existence of different isozymes in the adult poses the problem of ascertaining which forms characterize embryonic muscles. This problem has often been analyzed in terms of determining the adult forms present in developing muscle. For example, a mixture of the fast and slow types of the myosin small subunits, called light chains, has been found in future slow muscles, whereas the fast type is present in future fast muscles (7, 11–14). Recently we have shown that embryonic muscle tissue and cultured muscle cells contain a muscle-specific contractile protein distinct from the various adult forms. This protein was a myosin light chain of the LC1-type and was called $LC1_{emb}$ (7). The synthesis of this protein relative to that of the adult form decreases during the several days after cell fusion (15). This distinct form of light chain thus defines biochemically an embryonic stage of myotube development (15).

The nature of the large subunit, or heavy chain, of the myosin molecule during muscle development is less clear. A number of immunological studies have found that myosin in embryonic muscle reacts with antibodies to adult fast (13, 16), slow (16, 17), and cardiac (16) myosin. Amino acid sequence analysis of a myosin peptide isolated from 21-day-old rabbits demonstrated the presence of a sequence distinct from the adult skeletal and cardiac ones (18, 19). This form was attributed to fetal myosin although the corresponding peptide from slow skeletal muscle has not examined. Partial tryptic degradation of native myosin from embryonic muscle has suggested that the heavy chain is related to, although different from, both the fast and slow types (20, 21). However, such proteolytic cleavage could be influenced by the subunit composition of the myosin. None of these studies therefore was able to conclusively determine whether the myosin in embryonic tissue is composed of one or more types of adult myosin heavy chain or whether a distinct embryonic form that shares immunological and other properties with the adult forms is present.

We now report experiments that examine directly the nature of the myosin heavy chain in embryonic and several adult rat muscle tissues as well as in fused muscle cell cultures. After denaturation of the myosins with sodium dodecyl sulfate, they were subjected to partial proteolytic digestion (22) and to immunological analysis (23, 24). The proteolytic digestion products were analyzed by one- and two-dimensional gel electrophoresis. The resulting polypeptide maps show that cultured cells and embryonic muscle tissue harbor a form of myosin heavy chain that is distinguishable from the adult forms examined. Quantitative micro-complement fixation using antibodies to denatured adult skeletal and cardiac heavy meromyosin (HMM) reveals that the myosin from cultured muscle cells is different from the adult forms. By allowing native myosins to react with antibodies to native HMM, it is shown that cultured cell myosin is immunologically related to the other striated muscle forms. These and previous results demonstrate that in embryonic muscle development both a myosin heavy chain and one of the light chains ($LC1_{emb}$) differ from the adult forms.

MATERIALS AND METHODS

The methods used for cell culture, radioactive labeling, and gel electrophoresis have been described (7). Myosins were prepared from rat muscle tissue or cultured rat muscle cells (7). All preparations were chromatographed on Sepharose 2B in the presence of 1 mM ATP as a final step (7). The purest fractions were pooled, sodium dodecyl sulfate ($NaDodSO_4$) was added to a final concentration of 0.5% (wt/vol), and the fractions were stored at −80°C until needed. This method yielded myosin of acceptable purity as shown by the $NaDodSO_4$ gel profiles (Fig. 1).

Partial proteolytic cleavage of myosin was carried out as follows. When the cleavage reactions were to be analyzed by one-dimensional gel electrophoresis, the myosin solutions containing 0.5% $NaDodSO_4$ were heated for 2 min at 100°C. A protein concentration of 0.4 mg/ml was achieved by dilution with 0.5 M NaCl/0.02 M sodium phosphate, pH 6.5/0.1 mM $MgCl_2$/0.5% $NaDodSO_4$. Glycerol was added to 10% (vol/vol). Chymotrypsin or *Staphylococcus aureus* protease was added to a final concentration of 108 μg/ml or 15 μg/ml, respectively, and the reaction mixtures were incubated for 30 min at 37°C. Control incubations in the absence of protease showed that no significant proteolysis occurred. At the end of the reaction, $NaDodSO_4$ and 2-mercaptoethanol were added to final con-

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Abbreviations: HMM, heavy meromyosin; $NaDodSO_4$, sodium dodecyl sulfate; MHC, myosin heavy chain.

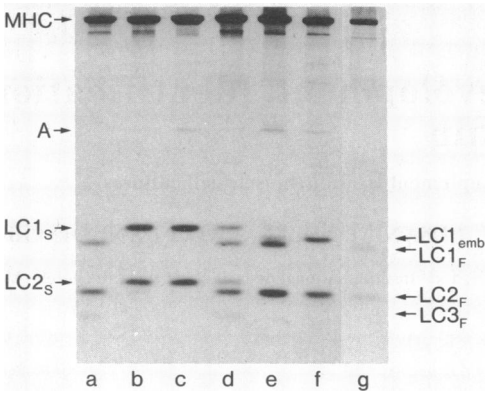

FIG. 1. $NaDodSO_4$ gel analysis of purified myosins. About 16 μg of each nonradioactive myosin was loaded and subsequently detected by Coomassie blue staining. The radioactive primary culture myosin (30,000 cpm) was detected by autoradiography. The myosins were from bulk tissue (a), soleus (b), cardiac tissue (c), diaphragm (d), 20-day embryonic bulk (e), L6 cultures (f), and primary cultures (g). The positions of the heavy chain (MHC), actin (A), and the various light chains (LC; see ref. 7) are indicated.

centrations of 1.0% and 5% (vol/vol), respectively, and the samples were heated for 2 min at 100°C. Approximately 16 μg of each myosin was analyzed on the one-dimensional gels. When analysis was to be carried out on two-dimensional gels, the cleavage reactions contained myosin at 1.2 mg/ml, no glycerol was added, and the samples were heated for only 1 min at 100°C. The chymotrypsin concentration was increased to 300 μg/ml. After incubation, the samples were frozen in liquid nitrogen, lyophilized, and then redissolved in isoelectric focusing buffer (7). Two-dimensional gels were run as previously described (7), using second dimension slab gels of 15% acrylamide and 0.087% bisacrylamide. Approximately 60 μg was loaded onto such gels.

The methods used for the production of antisera, the specificity of the antibodies, the preparation of tissue or cellular extracts, and the protocol for micro-complement fixation reactions have all been described (23–25). Myosins freshly prepared either according to Offer *et al.* (26) or after chromatography on Sepharose 2B were denatured and treated as before (24). Antigen and antibody controls were included in all experiments and never showed any pro- or anticomplement activity.

RESULTS

Partial proteolytic cleavage of denatured myosins

In order for the technique of partial proteolysis to be a valid probe of heavy chain structure, it is necessary that for a given myosin the light chains do not influence the pattern of enzymatic attack on the heavy chain. We have therefore compared the cleavage products of $NaDodSO_4$-treated bulk myosin with those of myosin heavy chain purified by $NaDodSO_4$ gel electrophoresis. The two samples gave identical patterns of proteolysis for the heavy chain (results not shown). Furthermore, once the myosin was denatured, the presence of the light chains during digestion did not influence the pattern of degradation, as shown by the fact that radioactive myosin was cleaved in the same manner regardless of the type of nonradioactive myosin present (see legend to Fig. 2).

The patterns of cleavage produced by chymotrypsin and *S. aureus* protease were compared for adult rat bulk (i.e., total), soleus, diaphragm, and cardiac tissue myosins. The myosins from 20-day embryonic bulk tissue, fused primary cultures, and fused cultures of the rat myogenic cell line L6 were also investigated. Analysis on one-dimensional gels showed that the two enzymes produced different patterns of digestion (Fig. 2). Three basic types of cleavage patterns can be observed by using either enzyme; the type obtained from adult bulk (predominantly fast) myosin, those given by soleus and cardiac myosin (slow-type myosins), and those found by using either embryonic bulk or cultured cell myosins. The myosin from diaphragm muscle, which is a natural mixture of fast and slow myosin as determined by immunology (9) or light chain content (Fig. 1, lane d), has a degradation profile that seems to be a mixture of the fast and slow patterns.

The cleavage products were also analyzed by two-dimensional gels. Fig. 3 shows the analyses of the chymotryptic deg-

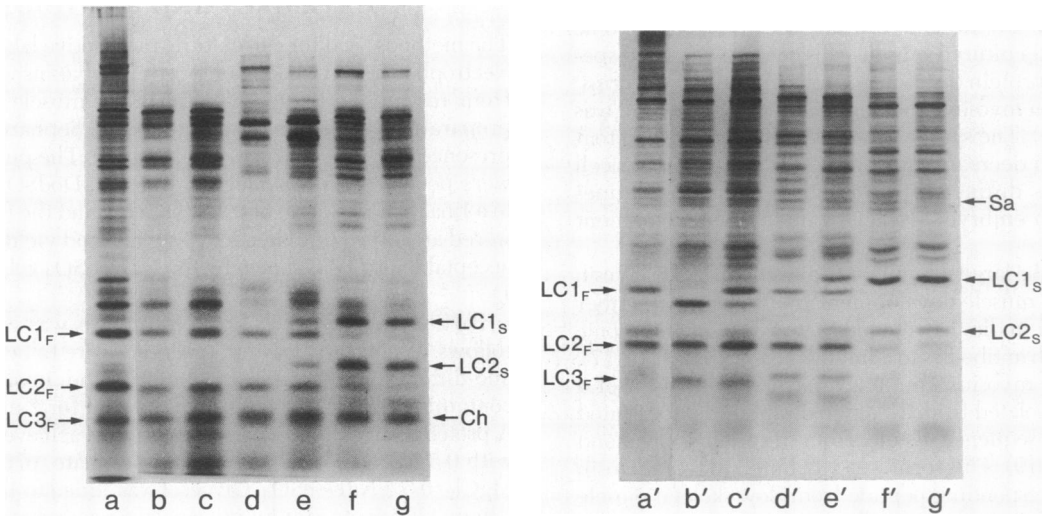

FIG. 2. One-dimensional analysis of myosin proteolytic cleavage products. Proteolysis was carried out with either chymotrypsin (a–g) or *S. aureus* protease (a'–g'). The myosins were from primary cultures (a, a'), L6 cultures (b, b'), embryonic bulk tissue (c, c'), bulk tissue (d, d'), diaphragm (e, e'), soleus (f, f'), and cardiac tissue (g, g'). The positions of the adult light chains, the high molecular weight subunit of chymotrypsin (Ch) and *S. aureus* protease (Sa) are indicated. In the chymotrypsin experiment, radioactive primary culture myosin was present along with each nonradioactive sample. The radioactive cleavage products shown in a are from the reaction mixture also containing diaphragm myosin (e). In the *S. aureus* protease experiment, radioactive myosin was included with L6, bulk, and soleus myosins; the radioactive cleavage products shown in a' are from the bulk myosin reaction (d'). Approximately 30,000 cpm were loaded in a and a'. The light chains were relatively resistant to both chymotrypsin and *S. aureus* protease except for $LC1_{emb}$, which appears to be converted to a polypeptide of slightly smaller molecular weight (see b').

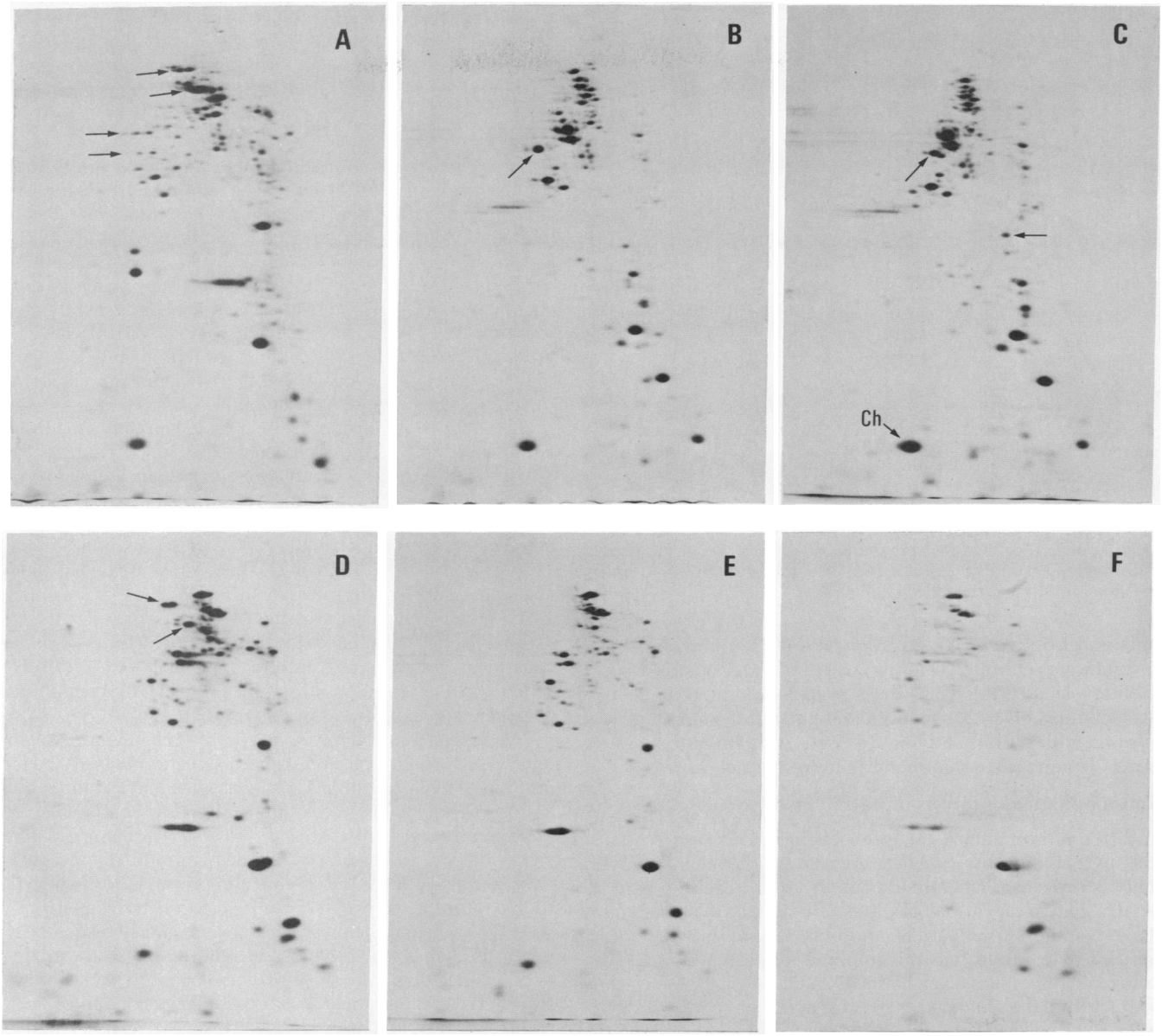

FIG. 3. Two-dimensional gel analysis of chymotryptic myosin cleavage products. The myosins used were from bulk tissue (*A*), soleus (*B*), cardiac tissue (*C*), 20-day embryonic bulk (*D*), L6 cultures (*E*), and primary cultures (*F*). The primary culture myosin was degraded in the presence of nonradioactive L6 myosin. Approximately 15,000 cpm was loaded in *F*. Gels are presented with the basic pH range to the left, and decreasing molecular weight from top to bottom. Excess chymotrypsin was added to the gel in *C* and its position is indicated. The corresponding spot can be seen in all the stained gels (*A–E*).

radation of a number of myosins. The three basic types of myosin cleavage patterns are clearly seen. In addition, the analysis of the bulk myosin degradation products revealed several polypeptides that were heterogeneous with respect to their isoelectric point (see arrows in Fig. 3*A*). The analyses of the soleus and cardiac myosin degradation patterns by the two-dimensional technique confirmed that the two are closely related but can definitely be distinguished by some of the polypeptides present (see arrows in Fig. 3 *B* and *C*). Myosins from embryonic tissue, primary cultures, and L6 cells have very similar cleavage patterns when analyzed by two-dimensional gel electrophoresis, but the patterns are different from those of the adult myosins. Small amounts of fast myosin cleavage products can also be seen in the high-resolution analysis of embryonic bulk myosin (see arrows in Fig. 3*D*).

Immunological analysis of denatured myosins

The existence of three myosin types was confirmed by an immunochemical approach using $NaDodSO_4$-treated myosins and antisera to $NaDodSO_4$-treated adult cardiac HMM (24). Such antibodies are specific to the denatured structure of cardiac myosin heavy chain because (*i*) the reactions given by $NaDodSO_4$- or guanidine hydrochloride-treated HMM are identical (24); (*ii*) the antibodies barely react with native HMM; and (*iii*) the antibodies strongly crossreact with peptides ranging from 76,000 to 29,000 daltons isolated from $NaDodSO_4$-denatured HMM but do not recognize lower molecular weight peptides (unpublished data). As shown in Fig. 4*A*, both denatured cardiac myosin and denatured soleus myosin reacted at the same antiserum dilution (1:4500) but with a difference in the amount of complement fixed (69% and 39%, respectively). In contrast, the antiserum had to be concentrated by a factor of 15 for denatured bulk myosin and of 18.7 for denatured L6 myosin in order to observe complement fixation of about 30%. Identical observations were made with two immune sera obtained from two different animals. By this immunological criterion, adult soleus and cardiac myosins seem therefore distinct but strongly related and both are very different from

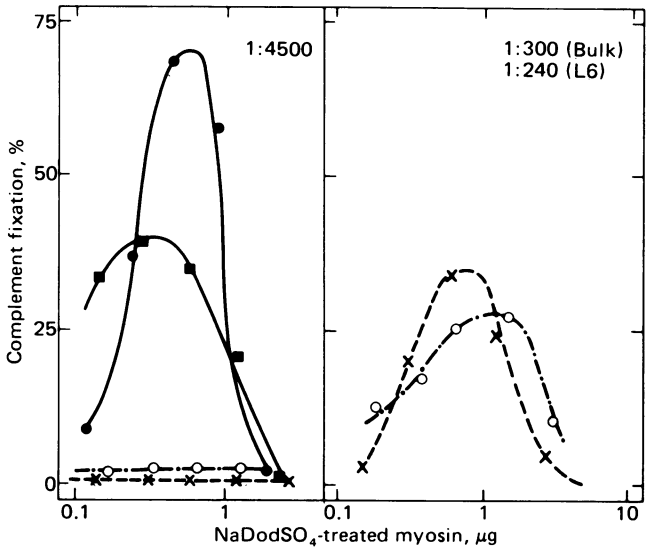

FIG. 4. Micro-complement fixation reactions with antiserum to $NaDodSO_4$-denatured rat cardiac HMM. The various $NaDodSO_4$-denatured myosins were from cardiac tissue (●), soleus (■), bulk tissue (×), and L6 cultures (○). The antiserum dilutions are indicated in this and the following figure.

bulk and L6 myosin. The differences between bulk and L6 myosin were confirmed by using an antibody to denatured bulk rabbit skeletal HMM. This heterologous antiserum, when used at a dilution of 1:120, gave greater than 75% complement fixation with rat bulk myosin. In contrast, L6 myosin barely fixed 20% under the same conditions (results not shown).

Immunological analysis of native myosins

A different immunological approach was used to verify that the myosin made by L6 cells was related to the other striated muscle myosins. The antibody employed had been prepared against native rat cardiac HMM, and its ability to detect epitopes common to rat cardiac and bulk myosins has been described (34). Because in this immune system an equivalent antigenic response is given by the isolated myosin or the crude extract from the same tissue (23), reactions were performed on extracts of bulk, soleus, and cardiac muscles as well as fused L6 cultures. Three different antisera were used with similar results. As shown in Fig. 5, all four extracts show extensive crossreaction with this antibody. The epitopes common to cardiac and bulk myosins are therefore also present on slow soleus myosin. The myosin in fused L6 cultures seems sufficiently homologous in its native state to share antigenic determinants with the other myosin types.

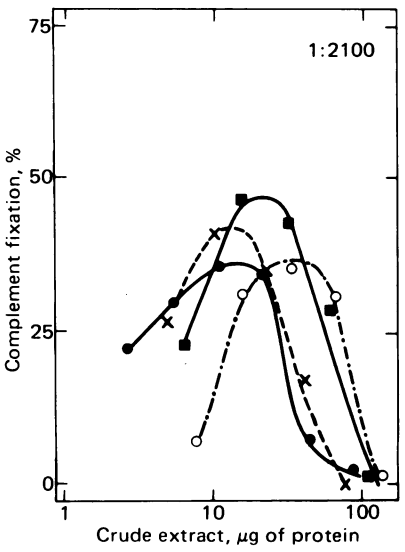

FIG. 5. Micro-complement fixation reactions with antiserum to native rat cardiac HMM. The extracts were from cardiac tissue (●), soleus (■), bulk tissue (×), or L6 cultures (○).

DISCUSSION

The myosin heavy chain is difficult to study biochemically as an intact protein because of its large size and particular properties of aggregation. Both chemical and enzymatic degradation have thus been used to fragment the heavy chain (20, 21, 27–31). The enzymatic cleavage of native myosin might be influenced by the subunit composition (see below), while chemical cleavage may introduce charge heterogeneity in the resulting peptides, rendering precise comparison difficult. The approach presented in this paper, partial proteolytic cleavage in the presence of $NaDodSO_4$, is a convenient means of studying different myosin types. Another approach is described, quantitative micro-complement fixation using antibodies to HMM. Complement fixation has been useful in detecting small structural differences for many proteins (32), including myosin (23, 33). The antigenic sites involved in the native HMM–anti-HMM immune system, though specific to the animal species (23), are common to the various muscle myosin isozymes (Fig. 5) for the same animal (34). In contrast, it appears likely that denaturation of the molecule by $NaDodSO_4$ exposes muscle type-specific regions that are less favorably exposed in the native molecule. Differences in the antigenic activity of native and denatured proteins have already been described (35–37). These differences allowed a double approach to the immunological analysis of the various myosins.

We have observed three basic types of striated muscle myosin heavy chain. One type corresponds to what could be called fast myosin heavy chain (MHC_F). In fact, we have studied bulk tissue myosin. This preparation, although containing predominantly fast light chains (see Fig. 1) is a mixture of fiber types (9) and therefore possibly a mixture of myosin heavy chains. Indeed, this is the only myosin preparation in which the polypeptide maps reveal obvious charge heterogeneity (Fig. 3*A*). This suggests the presence of several closely related heavy chain isozymes and is consistent with amino acid sequence studies that demonstrated two homologous peptides in bulk rabbit skeletal myosin (38). A second heavy chain type is that represented by slow and cardiac myosin ($MHC_{S(C)}$). Although the polypeptide maps of these two proteins are definitely related, the two-dimensional analysis shows that several peptides do differ (Fig. 3 *B* and *C*). Our immunological results on denatured soleus and cardiac myosins confirm these observations (Fig. 4*A*). A third myosin type differs from both MHC_F and $MHC_{S(C)}$. Polypeptide mapping shows that this heavy chain isozyme is found in embryonic muscle, either in tissue or in culture, and can be referred to as an embryonic heavy chain or MHC_{emb}. The slight crossreactions of bulk and L6 myosins with the antibody to denatured cardiac HMM do not necessarily indicate homologies between these two proteins. It shows only that they are immunologically very distant from the adult $MHC_{S(C)}$ group. The use of an antibody to denatured rabbit HMM has confirmed that MHC_F and HMC_{emb} are indeed different.

Because only obvious patterns of heterogeneity can be recognized with the two-dimensional technique, the number of isozymes discussed above is therefore a minimum. In addition to the four heavy chains observed by polypeptide mapping, and the known sequence heterogeneity of MHC_F (38), structural differences have also been found among the heavy chains of two cardiac myosin isozymes (31) as well as myosins from smooth muscle, brain, and platelets (27). Thus at least nine different myosin heavy chains can be identified in vertebrate

muscle and nonmuscle tissues by chemical or enzymatic cleavage and amino acid sequence data. Other immunological studies (39), as well as electrophoresis of native myosins (40), have suggested even more myosin isozymes.

Previous studies of the tryptic fragmentation pattern of native myosin from embryonic tissue (20, 21) had suggested the presence of a distinct form. However, this approach does not necessarily allow determination of the exact nature of the heavy chain. It is known that myosin from embryonic muscle contains two of the three adult fast light chains (see Fig. 1 and refs. 12, 13, and 20) as well as the embryonic light chain (7). The fragmentation of the heavy chain species in the native molecule could therefore be significantly influenced by the presence of the light chains. This type of phenomenon has been demonstrated by Weeds and Pope (41) for the chymotryptic cleavage of native myosin.

Antibodies to a number of adult myosins have been found to crossreact with the myosin in embryonic muscle (13, 16, 17). This approach has led to the suggestion that several adult types might be present in developing muscle fibers. Although our results with antibodies to denatured myosins show considerable differences between the L6 MHC_{emb} and the adult types, antibodies to the native molecules show that all forms have a number of antigenic sites in common. Thus this immunological relatedness may explain the results obtained by others.

The embryonic heavy chain that we have observed has been found in myogenic L6 cells, primary muscle cell cultures, and embryonic muscle tissue. A small amount of MHC_F cleavage products can be detected in the degradation of embryonic tissue myosin (Fig. 3*D*). This might suggest that a transition of heavy chain isozymes was occurring, as previously described for the embryonic and adult light chain proteins (15). Primary cultures and L6 cells do not seem to contain significant amounts of any adult MHC isozyme. Likewise, L6 cells do not synthesize the adult $LC1_F$ light chain, whereas the $LC1_{emb}$ protein is present (7, 15). Thus fused cultures of L6 cells synthesize the embryonic forms of both the myosin heavy chain and the LC1-type light chain but none of the adult forms of these proteins. This myogenic cell line does not seem to progress beyond the embryonic stage of muscle development, whereas primary cultures of rat muscle cells undergo a transition at least from the embryonic to adult forms of the LC1 light chains (7, 15).

It is also interesting to note that 20-day embryonic muscle is composed of largely the MHC_{emb} isozyme (Figs. 2 c and c′ and 3*D*). In myosin from this tissue the ratio of $LC1_F$ to $LC1_{emb}$ is at least 2:1 (result not shown). Thus in developing muscle tissue the $MHC_{emb} \rightarrow MHC_F$ transition occurs less rapidly than the $LC1_{emb} \rightarrow LC1_F$ one. This could be due simply to different rates of synthesis and breakdown for the heavy and light subunits or possibly to the differential effects of other factors, for example, innervation. The possible simultaneous presence of adult and embryonic heavy chain forms plus the adult and embryonic light chains during muscle development might allow the formation of a number of different myosin isozymes if all heavy and light chains could independently associate. The presence of embryonic heavy chain in primary cultures, L6, and embryonic bulk myosin shows that at least the adult light chains $LC1_F$ and $LC2_F$ can interact with a myosin heavy chain other than that with which they are normally associated in the adult. It is possible that, during development or in other situations, protein subunits not normally found together might be present simultaneously and thus assemble to create novel molecules with novel functions.

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