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MYOSIN ISOENZYMES AS MOLECULAR MARKERS FOR MUSCLE PHYSIOLOGY

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SUMMARY

Myosin is a major component of skeletal muscle and it plays a central role in determining the physiological performance of adult tissue. Developing muscles contain myosin molecules which are different from the adult forms, and these isoenzymes have been found to be characteristic markers of the diverse physiological and pathological states of muscle tissue. The differences between these isoenzymes may be demonstrated by protein chemical, immunochemical and genetic evidence. The study of the sequential transitions between isoenzymes represents a means for characterizing the dynamic nature of muscle development, and neuronal and hormonal influences have been identified which contribute to the regulation of these transitions. The perspectives for future work include genetic studies, elucidation of the pathways relating extracellular signals to changes in gene expression, and the possibility that studies of myosin isoenzymes might further understanding of muscle pathology.

INTRODUCTION

From a reductionist point of view, the physiology of any system is determined by the collection of proteins present in the tissue. Some of the proteins will be required in many situations just to produce a viable cellular structure. Others will play a more tissue-specific role, accounting for the unique properties of a system either by conferring a special function or by fine-tuning the performance of the system. When considered in these terms, striated muscle tissue can be approached from a molecular viewpoint more readily than most other systems. This is because the contractile proteins are well studied molecules which clearly account for the mechanical function provided by muscle tissue. Actin and myosin play a central architectural and enzymatic role in contraction, while other well-characterized proteins, such as tropomyosin and troponin, serve a regulatory function to control and modulate contractility using Ca^{2+} as a signal.

Myosin has been the object of much study since the recognition of its central role in contraction in the 1940s. It has fascinated structuralists, biochemists and

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physiologists because of its ability to self-assemble into a filamentous system capable – in association with actin – of generating force, because of its enzymatic function that converts chemical energy into mechanical energy, and because of the clear relationship of this enzymatic activity to the contractile speed of muscle (Barany, 1967). The manner in which muscle tissue develops and acquires its adult physiological properties has also been studied from the point of view of myosin, although until recently the situation was less than clear. Nonetheless, over the last 10 years the concept has been developed that isoenzymes exist for myosin (and indeed other contractile proteins), each one having slightly different structures and – possibly – functions. This idea is now supported by several lines of evidence, including the most incontrovertible of all, the isolation of individual members of the myosin gene family. In this article, I will present this evidence concerning the myosin isoenzymes found in developing skeletal muscle, review current ideas on the formation of the adult phenotype and the mechanisms controlling myosin isoenzyme transitions, and finally attempt to indicate what directions future work might take.

A BRIEF HISTORICAL NOTE

Because the literature concerning the subject of foetal myosin makes for confusing reading, a brief historical indication of the ideas that were developed may be useful.

Buller, Eccles & Eccles (1960) established that newborn muscles (and by implication foetal muscles as well) contracted slowly, and thus when the high correlation between contractile speed and ATPase activity was established by Barany (1967), it seemed likely that foetal myosin either was slow muscle myosin or was very similar to it. Not without good justification, this idea became well entrenched, and the reasons for believing so were clearly set out by Close in his 1972 review.

In retrospect, the subsequent controversy must have had its origins in the observation that myosin from embryonic chickens or foetal mammalian muscle carries small subunits (called light chains) which are qualitatively indistinguishable from those of fast muscle myosin (Sreter, Holtzer, Gergely & Holtzer, 1972; Sreter, Balint & Gergely, 1975; Pelloni-Mueller, Erminy & Jenny, 1976; Rubinstein & Kelly, 1978). The implication was that since fast-type light chains associate with fast-type heavy chains in adult myosin (if only by definition), their existence in developing muscles must indicate the presence of fast myosin heavy chains.

Much of the literature in the 1970s dealt with the problem of developing muscle myosin in terms of whether the myosin (or more precisely the myosin heavy chain) was fast, slow or a mixture of both. The possibility that a distinct foetal myosin existed was certainly evoked (Trayer & Perry, 1966; Huszar, 1972; Sreter *et al.* 1975), but it was difficult to show convincingly that myosin in foetal muscles was not adult slow-type myosin (reviewed in Whalen, 1981).

The lack of clarity concerning the nature of the myosin heavy chain therefore reflected a lack of methodology to examine the proteins in question by any definitive biochemical technique. When Cleveland, Fischer, Kirschner & Laemmli (1977)

presented a method for obtaining partial polypeptide maps of even large proteins, several groups began to study myosin using this approach. Although a final offensive was launched to support the claim that embryonic myosin was none other than adult fast myosin (Rubinstein & Holtzer, 1979), the techniques used were inappropriate to the problem at hand. At the same time, data began to emerge independently from three laboratories that myosin in embryonic/foetal muscles was neither adult fast nor adult slow type myosin but indeed was a distinct form (Hoh & Yeoh, 1979; Rushbrook & Stracher, 1979; Whalen *et al.* 1979).

One further step was needed to produce the current picture of myosin isoenzymes in developing muscle. While attempting to pinpoint at what time in development the embryonic myosin disappeared and adult myosin appeared, we discovered that another myosin isoenzyme was found in newborn rat muscle which was distinct from the embryonic form and the known adult forms (Whalen *et al.* 1981). The existence of this 'neonatal' myosin pointed to the fact that several (at least two) myosin isoenzymes were appearing and disappearing sequentially in developing muscle. This situation has been found in human muscle (Fitzsimons & Hoh, 1981*a*) and chicken muscle (Bandman, Matsuda & Strohman, 1982), but more species and muscles types must be examined to assess the generality of this situation.

The scenario that has emerged concerning the major myosin isoenzymes in skeletal muscle is the following. Soon after their formation, muscle fibres contain an embryonic (or foetal) type of myosin, which is subsequently replaced by a neonatal isoenzyme. Eventually (e.g. 3–4 weeks after birth in rats and humans), adult myosins become established as the predominant species and they persist into adulthood.

MYOSIN ISOENZYMES ARE DIFFERENT PROTEINS ENCODED BY DIFFERENT GENES

The myosin isoenzymes can be defined by the type of large subunit they contain. This subunit, called the myosin heavy chain (MHC), accounts for about 85 % of the myosin molecule and carries the two important functional activities of myosin: the filament forming region is located in the C-terminal half of the molecule while the enzymatic ATPase activity resides in the N-terminal half (see Fig. 1). It is this heavy chain which has been found to be both highly conserved in general structural terms (Kavinsky *et al.* 1983) but also highly polymorphic, i.e. present as numerous isoenzymes in striated skeletal and cardiac muscle.

The two developmental isoenzymes (embryonic and neonatal) and the two principal adult forms (fast and slow) all have different MHC subunits. The most definitive data came initially from protein chemical and immunochemical approaches. The polypeptide mapping technique involves partially cleaving the large (2000 amino acids long) MHC into smaller fragments using proteolytic enzymes (Rushbrook & Stracher, 1979; Whalen *et al.* 1979, 1981; Bandman *et al.* 1982; Bugaisky, Butler-Browne, Sell & Whalen, 1984). Since these proteases have a strict specificity with respect to the amino acids at which they cut, fragments of different sizes from different myosins must indicate that the myosins being compared have a different

amino acid sequence. Antibodies to myosin heavy chain were also used in quantitative analysis to discriminate between different isoenzymes (Whalen *et al.* 1979, 1981). Since the antibodies recognize antigenic sites composed of amino acids, quantitatively different reactivity of two myosins with a given antibody also was strong evidence of differences in the polypeptide chains.

Emphasis was placed on determining if two heavy chain proteins have a different amino acid sequence because of the implication that they are the products of distinct genes. This was considered important in order to establish that the developmental programme controlling muscle differentiation involved sequential and differential expression of different myosin genes, rather than simply modulation of the quantity of myosin present, for example.

The most recent evidence concerns the physical isolation, using molecular cloning techniques, of parts of several MHC genes (Kavinsky *et al.* 1983; Umeda *et al.* 1983; Weydert *et al.* 1983; Wydro, Nguyen, Gubits & Nadal-Ginard, 1983; Mahdavi, Chambers & Nadal-Ginard, 1984; Periasamy, Wieczorek & Nadal-Ginard, 1984). In general, this genetic data has confirmed the scenario outlined by work on the heavy chain proteins, since distinct genes have been isolated for all four major isoenzymes of the rat (Wydro *et al.* 1983; Mahdavi *et al.* 1984; Periasamy *et al.* 1984). Recent studies using these cloned genes have also suggested the presence of several minor forms of MHC that would have been difficult to demonstrate by protein or immunochemical techniques (Umeda *et al.* 1983; Periasamy *et al.* 1984). These

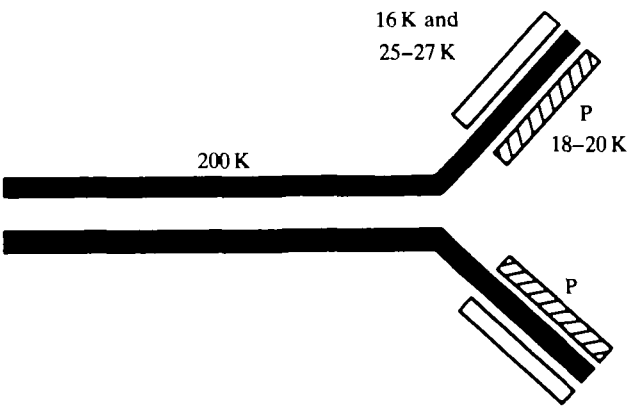

Fig. 1. Schematic representation of the myosin molecule and its subunits. The myosin heavy chain is represented by the solid black bar with a molecular weight of 200000 (200 K); each myosin molecule contains two heavy chains. The small subunits of myosin, called light chains, are present in four copies per myosin molecule, two of which can be phosphorylated (P; hatched bars) while the other two are not (open bars). These light chains are different in fast and slow muscles and have molecular weights as indicated in the figure. The C-terminal halves of the two myosin heavy chains (drawn as parallel bars in the figure) are highly α-helical, and they interact in a coiled-coil structure in the native molecule. This region of the molecule overlaps with the same region in other myosin molecules to form myosin filaments (see text and Kavinsky *et al.* 1983). The N-terminal halves of the heavy chains (shown in association with the light chains in the figure) are in the form of globular polypeptides, and they carry the enzymatic sites for ATP hydrolysis (see text).

approaches have also provided the best evidence to date that individual myosin genes can be independently regulated in different striated muscles. For example, the same gene encodes the heavy chain expressed in slow-contracting skeletal muscle and in ventricular cardiac muscle in rats (Lompré, Nadal-Ginard & Mahdavi, 1984). However, the gene is expressed in cardiac tissue at the foetal stage (and in certain pathological situations) while in skeletal muscle it is a major adult form (Lompré *et al.* 1984). Convincing evidence for this situation would have been very difficult to obtain by any other means for a protein as large as the myosin heavy chain.

Table 1 summarizes the current state of knowledge with regard to the numbers and types of myosin heavy chains found in vertebrates.

THE STUDY OF MYOSIN ISOENZYME TRANSITIONS

The myosin isoenzymes are molecular markers not only for different muscle and non-muscle tissue types, but also for the physiological state of muscle tissue. The adult forms are responsible for determining, at least in part, the performance of muscle tissue, while other forms are characteristic of developing muscle and possibly provide some special – as yet undiscovered – function for these muscles. These developmental forms are also re-expressed in regenerating muscle (Sartore, Gorza & Schiaffino, 1982; Maréchal, Schwartz, Beckers-Bleukx & Ghins, 1984) and regeneration is a feature of several muscle diseases (Fitzsimons & Hoh, 1981*a*). Finally, there is evidence that aberrant expression of the developmental forms occurs in some pathological situations independent of regeneration (Fitzsimons & Hoh, 1981*b*; Whalen, Toutant, Butler-Browne & Watkins, 1985).

Because of the association of certain myosin isoenzymes with different physiological or pathological states of muscle, the transitions between different isoenzymes and the control of these transitions are worthy of careful study. Since most physiological and

Table 1. *Myosin heavy chain isoenzymes*

Non-muscle	Smooth muscle	Striated muscle		
Brain 1	Gizzard 1	Skeletal:	embryonic	1
Platelet 1			neonatal	1
			adult fast	2
			adult super fast	1
			adult slow (β)	—
		Cardiac:	ventricular (α,β)	2
			atrial (α)	—

Minimum: 10 myosin heavy chains

This table summarizes the different myosin heavy chain isoenzymes that have been identified by protein chemical and molecular cloning approaches. References, in addition to those given in the text, are found in Whalen *et al.* (1983). Although the skeletal adult slow and the cardiac atrial isoenzymes have been listed, current molecular cloning data indicate that these two proteins are encoded by the same two genes that encode the cardiac ventricular α and β forms (Lompré, Nadal-Ginard & Mahdavi, 1984; Mahdavi, Chambers & Nadal-Ginard, 1984).

developmental studies concern the hindlimb muscles, current efforts normally take into account the four major forms found in these muscles: embryonic, neonatal, slow and fast.

It is essential for these studies to use a combination of biochemical and immunocytochemical approaches. Biochemical analysis is required to ascertain with some certainty which types of myosin are present in a given situation, while the immunocytochemical approach permits an evaluation of the type(s) of myosin contained in a single fibre. This cytochemical approach is indispensable for several reasons. First of all, muscle development is an asynchronous process. Fibres form over a period of several days in foetal mice and rats (Ontell, 1982), and the final phase of establishment of the adult configuration of the neuromuscular junction takes place over a week during the postnatal period (Thompson, Sutton & Riley, 1984). Therefore, developing muscles are composed of fibres at different stages of maturity. Secondly, muscles are only rarely homogeneous in phenotype either during development or at adult stages. As a result, when two or more myosin types can be found in a particular muscle by biochemical analysis, it is essential to know whether they are segregated into different fibres or whether they co-exist in the same fibres. Also, in some pathological situations only some of the muscle's fibres are involved, and their myosin content must therefore be assessed in the presence of a large number of unaffected fibres. Finally, in the study of myosin isoenzyme transitions one is essentially evaluating the co-existence of two myosin types in a given fibre population over a certain period of time. The immunocytochemical approach is currently the best one for such studies.

We have studied myosin isoenzyme transitions in rat and mouse muscle, initially with a view to understanding those transitions which take place in normal development. By then interfering with muscle development in different ways, we have been able to infer what mechanisms might control these isoenzyme transitions.

From the biochemical results obtained previously, it was suggested that in developing fast muscles, a transition from embryonic to neonatal myosin took place, followed by a neonatal–adult fast transition (Whalen *et al.* 1981). In principle, these transitions could either take place in an individual fibre, or alternatively neonatal myosin-containing fibres could degenerate and be replaced by fibres containing adult fast myosin. However, immunocytochemical investigation of the question showed that when neonatal and adult myosin were both present, they were often found in the same fibres (Butler-Browne *et al.* 1982). For example, at 3 weeks after birth in rats, about 50 % of the fibres are co-stained with antibodies to these two myosins, while the remainder of the fibres are stained by either one or the other antibody, in roughly equal proportions. These results suggest that we have observed the 3-week muscle at a time when it is undergoing an asynchronous neonatal–adult fast transition. The reasons why this transition might be occurring at different rates in different fibres are not yet understood, but may be related to some of the asynchronous aspects of fibre development referred to above.

We then asked whether the nerve was important for this neonatal–adult fast transition by simply cutting the nerves leading to the muscle at a time when neonatal

but not adult myosin was present (Butler-Browne *et al.* 1982). At 3 weeks after birth, adult fast myosin had appeared, neonatal myosin could still be detected, and both fast and neonatal myosin co-existed in the majority of the fibres. Thus the cytochemical analysis showed that the fibres were more homogeneous in phenotype than when the nerve was left intact. Although the important conclusion from these experiments is that continued innervation of the muscle is not required for the appearance of adult-type fast myosin, the immunocytochemical results have raised other questions concerning the role of the nerve in stimulating or retarding the disappearance of neonatal myosin in individual fibres.

We have also investigated the way in which slow-contracting muscle fibres develop, using the four major myosin isoenzymes as markers for the phenotype (Butler-Browne & Whalen, 1984). In muscles which are predominantly slow-contracting in adult animals, a phenotypic heterogeneity among the fibres can be observed in the foetal and newborn period; adult slow myosin is apparently present at this time (Lyons, Haselgrove, Kelly & Rubinstein, 1983; Butler-Browne & Whalen, 1984; Thornell *et al.* 1984; Whalen *et al.* 1984). Immunocytochemical analysis of the development of rat slow muscles (Butler-Browne & Whalen, 1984) suggested that fibres could be induced to accumulate slow myosin at any time in development. If the signal(s) for this induction did not arrive however, fibres would then follow the pathway of myosin transitions typical of fast fibres. These ideas are shown schematically in Fig. 2.

This scheme provides a unified point of view for the development of both fast and slow fibres. The denervation experiment described above suggests that the embryonic–neonatal–adult fast pathway is nerve-independent, while considerable evidence from other experimental models (reviewed by Jolesz & Sreter, 1981) strongly suggests that slow myosin accumulation is a nerve-driven process, at least in adult animals. It is definitely open to question whether the induction of slow myosin at foetal or newborn stages is nerve-dependent (see Whalen *et al.* 1985), and therefore a second mechanism might be required to account for the embryonic–slow transition illustrated in Fig. 2.

While the results of denervation experiments have suggested that the neonatal–adult fast transition is nerve-independent, recent investigations have revealed that it might be critically regulated by thyroid hormone levels. If rats are

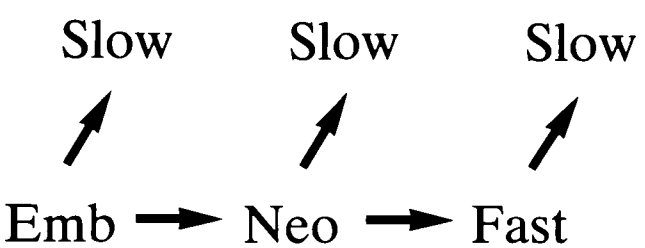

Fig. 2. Unified model for myosin isoenzyme transitions in developing fast and slow muscles. This model proposes that the induction of slow myosin can take place at any time in a developing muscle fibre provided that the signal for the induction of slow myosin is present. Otherwise the fibre will undergo the transition: embryonic (Emb)–neonatal (Neo)–adult fast. From Butler-Browne & Whalen (1984).

treated with anti-thyroid drugs during gestation and into the first month after birth, the hypothyroid offspring will accumulate neonatal myosin but not adult fast myosin (Gambke *et al.* 1983; Butler-Browne, Herlicoviez & Whalen, 1984). Normally of course, muscles of 1-month-old control rats would contain fast myosin as the predominant isoenzyme. This inhibitory influence of thyroid hormone levels seems to be restricted to only some myosin transitions: in developing slow muscles of hypothyroid rats, slow myosin is present in about half the fibres, as in control muscles, whereas neonatal myosin accumulates in the remaining fibres (Butler-Browne *et al.* 1984).

For further study of the hormonal control of myosin isoenzyme transitions, we have begun to investigate the myosin types in a genetic model of pituitary hypothyroidism, the dwarf mouse. In these mice, those anterior pituitary cells responsible for the production of growth hormone, thyroid-stimulating hormone, and prolactin are lacking, presumably due to a defect of development (Bartke, 1979). One particularly important aspect of this mutant is the absence of growth hormone production even in response to exogenous thyroid hormone (Slabaugh, Lieberman, Rutledge & Gorski, 1981). These two hormones often act together in producing a biological response; indeed the normal postnatal increases in growth hormone require correct levels of thyroid hormone (Seo, Wunderlich, Vassart & Refetoff, 1981). The dwarf mutant allows one to separate the effects of growth hormone from those of thyroid hormone since this mutant lacks growth hormone-producing cells.

Examination of myosin isoenzymes in dwarf mice of different ages shows that the neonatal–adult fast transition is considerably retarded. However, injections of thyroxine over a 2-week period will stimulate the accumulation of fast myosin, and the injected mice become indistinguishable from controls with respect to myosin isoenzyme content (Whalen *et al.* 1985). These observations lead us to conclude that the lack of thyroid hormone in these mutants is directly responsible for the effects on the neonatal–fast transition. Whether thyroid hormone acts directly on muscle tissue however is another question; since hypothyroidism has well-known effects on development of the nervous system, it seems at least possible that effects on muscle could be a secondary consequence. The dwarf mouse model should however be useful for further study of the phenomena.

PERSPECTIVES

The last 5 years have seen a substantial change in the way we view the topic of myosin isoenzymes. The developmental forms of myosin have been established as the predominant ones present in foetal and newborn muscle, and more generally the myosin isoenzymes are now seen as members of a large, highly conserved, multi-gene family. The structure, organization and regulation of the myosin genes will be the subject of considerable effort in the immediate future, and we will learn for example whether there is any genetic linkage of the genes which are expressed sequentially during muscle development. Although a few more myosin heavy chain isoenzymes will probably be discovered, mostly through the DNA cloning approach, the general outlines of the context into which they will fit is probably already established.

The earliest stages of muscle development remain to be investigated in detail, with respect to myosin content and its relationship to the morphological and temporal heterogeneity of muscle fibre formation. Two general categories of regulatory influences have been identified that seem to accompany the establishment of the adult muscle phenotype. However, it is inadequate simply to classify the myosin transitions as nerve- or hormone-dependent, since these ideas are not incisive enough to provide any real understanding of the control mechanisms. Some pathway must be found which connects the arrival of a signal at the muscles surface (i.e. a nerve impulse, or a hormone) with a change in its genetic expression in the nucleus.

In the field of muscle pathology, much more remains to be done just at the descriptive level, to determine under what circumstances the individual myosin isoenzymes are present. It is no longer anticipated that pathological muscle will contain some mutant form of myosin. However, the association of certain myosin forms with developing muscle, and the determination of muscle function in the adult by other myosin forms, lends support to the idea that further understanding of neuromuscular disease will come from investigation of these proteins.

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