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The superfast extraocular myosin (MYH13) is localized to the innervation zone in both the global and orbital layers of rabbit extraocular muscle

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Summary

Extraocular muscles (EOMs) are the most molecularly heterogeneous and physiologically diverse mammalian striated muscles. They express the entire array of striated muscle myosins, including a specialized myosin heavy chain MYH13, which is restricted to extraocular and laryngeal muscles. EOMs also exhibit a breadth of contractile activity, from superfast saccades to slow tracking and convergence movements. These movements are accomplished by the action of six ultrastructurally defined fiber types that differ from the type IIa, IIb, IIx and I fibers found in other skeletal muscles. Attempts to associate different eye movements with either the expression of different myosins or the activity of particular EOM fiber types are complicated by the molecular heterogeneity of several of the fiber types, and by electromyography studies showing that the majority of extraocular motor units participate in both fast and slow eye movements. To better understand the role of MYH13 in ocular motility, we generated MYH13-sequence-specific antibodies and used SDS-PAGE to quantify the regional distribution of myosin in EOM and to characterize its heterogeneity in single fibers. These studies demonstrate that MYH13 is preferentially expressed in the majority of orbital and global fibers in the central innervation zone of rabbit EOM. Many individual fibers express MYH13 with the fast IIb myosin and varying amounts of IIx myosin. The differential localization of MYH13, coupled with specialization of the sarcoplasmic reticulum and thin filament systems, probably explains how activation of the endplate band region enables the majority of EOM fibers to contribute to superfast contractions.

Key words: myosin, extraocular, EOM, muscle, neuromuscular junction, innervation, rabbit.

Introduction

The extraocular muscles (EOMs) execute a complex set of motions, including hyperfast (<6.5 ms), low-tension saccades as well as slow, low-tension contractions that keep distant objects in the visual field and permit convergence of the eyes for focusing (Barmack, 1977; Close and Luff, 1974; Cooper and Eccles, 1930; Fuchs et al., 1985). To perform this variety of movements, EOMs have undergone a number of adaptations that distinguish them from the skeletal muscle fibers of limb and epaxial muscles. These adaptations include a decrease in fiber and myofibril dimensions, amplification of the sarcoplasmic reticulum and the Ca^{2+}-reuptake system, and specialization of six fiber types, unlike those in other skeletal muscles (Blank and Schachat, 1999; Chiarandini and Davidowitz, 1979; Spencer and Porter, 1988). EOM fibers also differ from limb and epaxial skeletal muscles in their high-frequency pattern of neuromuscular activation and the presence of both singly innervated twitch fibers and multiply innervated tonic fibers (Chiarandini and Davidowitz, 1979; Spencer and Porter, 1988). Gene expression profiling has revealed additional differences between EOM and limb muscles in the expression of many genes, including those involved with aerobic metabolism, development, growth, calcium homeostasis, intracellular signaling and neuromuscular transmission (Cheng and Porter, 2002; Fischer et al., 2002; Porter et al., 2001).

In trunk and limb skeletal muscles, distinct fiber types expressing different myosins are recruited for fast and slow muscular activity. A similar division of labor does not occur in extraocular muscles, because several extraocular fiber types can be members of a single motor unit (Gurahian and Goldberg, 1987; Shall and Goldberg, 1995), and motor units are recuited on the basis of the amount of work required rather than the type of movement they execute (Dean, 1996; Scott and Collins, 1973; Shall and Goldberg, 1992).

The specialized physiology of EOM fibers is at least partly determined by the array of myosin isoforms and the complex pattern of differential myosin expression in EOM (Brueckner et al., 1996; Jacoby et al., 1990; Rushbrook et al., 1994; Wieczorek et al., 1985). In addition to all the isoforms present in skeletal and cardiac muscles, EOM express a specialized myosin heavy chain, first identified by Wieczorek et al. (1985). The ‘extraocular myosin’ or MyHC-EO isoform is also expressed in the superfast laryngeal muscles, and was termed myosin IIL (Briggs and Schachat, 2000; DelGaudio et

al., 1995; Lucas et al., 1995; Merati et al., 1996). This gene was mapped to the cluster of six fast and developmental myosin heavy chain genes on chromosome 17 (Weiss et al., 1999; Winters et al., 1998) and given the genomic designation *MYH13*. We propose using the designation MYH13 to simplify the nomenclature and avoid the implication of muscle-specificity. In addition, this usage is more consistent with its phylogenetic relationship to the other striated myosin genes (Briggs and Schachat, 2000; Schachat and Briggs, 2002).

MYH13 cDNA sequences are highly conserved across species (Briggs and Schachat, 2000), but the sequence and gene structure of *MYH13* differs radically from other members of the fast/developmental cluster of *MYH* genes, suggesting a considerable period of genomic insulation from the other *MYH* genes (Schachat and Briggs, 2002). In addition, analysis of their phylogenetic relationships showed that *MYH13* was the first specialized myosin to arise in the fast/developmental cluster after the divergence of the ancestral skeletal and cardiac myosins (Schachat and Briggs, 2002). That observation, coupled with the limited tissue-specific expression of MYH13 in two extraordinarily fast contracting muscles, suggests that it has evolved to fulfill a highly specialized function.

MYH13 is thought to play a role in the high velocity saccadic contractions observed in whole EOM. The contractile properties of individual EOM fibers were compared by measuring the f_{min} (frequency at which the dynamic stiffness of a muscle fiber is at a minimum) values, which reflect the rate of cross-bridge cycling. Li et al. (2000) showed that some EOM fibers exhibit higher values of f_{min} than any observed in limb muscles, which express only the fast isoforms of MyHC, IIb, IIx and IIa. Although the myosin composition of fibers was not analyzed in that study, their observations suggest that MYH13 contributes to these faster contractile properties. However, MHY13 makes up only 20–30% of the total myosin in rabbit EOM (Briggs and Schachat, 2000), and it is difficult to understand how MYH13 could dominate certain aspects of contraction in EOM unless it is highly enriched in a limited region of the muscle that could be differentially activated.

Longitudinal variation in the expression of fast, embryonic and slow MyHCs has been reported in EOMs (Brueckner et al., 1996; Jacoby et al., 1990; Kranjc et al., 2000; McLoon et al., 1999; Rubinstein and Hoh, 2000; Wasicky et al., 2000), with fast-reactive isoforms more prominent in the central region and developmental isoforms more abundant towards the ends of the muscle. MYH13 has been localized to the orbital layer fibers in the rat (Brueckner et al., 1996), and more specifically to a relatively broad region spanning the band of motor endplates in the orbital layer (Rubinstein and Hoh, 2000). Other studies with MYH13-specific antibodies and *in situ* hybridization have produced a spectrum of results for its distribution in the orbital and/or the underlying global layer of muscle fibers (Brueckner et al., 1996; Lucas et al., 1995; Sartore et al., 1987). These differences in distribution may reflect the use of different probes, analysis of different regions of the muscle or species-specific differences. If it is the latter, it may provide important insights into the use or recruitment of fibers in different layers. Such differences may be particularly important in light of the active pulley hypothesis proposed by Demer and colleagues, which proposes that global and orbital fibers have distinctive roles in ocular motility (Demer, 2002; Demer et al., 1995; Khanna and Porter, 2001). Global fibers act directly on the globe to move the eye, whereas orbital layer fibers insert on a sheath of connective tissue that acts as a pulley to control the plane of rotation of the eye. Defining the distribution of MYH13 in these layers will have major implications in determining its potential roles in EOM function.

Here we have used a combination of techniques, including antibodies and SDS-PAGE, providing an independent approach to localizing myosin expression that does not depend on probe specificity, to provide the first quantitative data on the distribution of MYH13 and the other myosin isoforms in different regions of rabbit EOM. This information provides a new basis for assessing the role of MYH13 in the contractile properties of these unique muscles.

Materials and methods

Tissue preparation and biochemical analysis

Animal procedures were approved by the Institutional Animal Care and Use Committee at Duke University. New Zealand White rabbits were euthanized by barbiturate overdose. Adult muscles were dissected from 1.8–2.5 kg male rabbits and fetal samples were taken at 27 days gestation. For SDS-PAGE analysis of myosin heavy chains, myofibrils were prepared from pulverized frozen muscles (Briggs et al., 1987; Schachat et al., 1988). Single muscle fibers were dissected from muscle bundles stored at –20°C in rigor:glycerol (Schachat et al., 1985b). To compare the orbital and global regions by SDS-PAGE, sections (120 μm thick) were cut with a sliding microtome, then placed on a slide and allowed to dry. The regions were separated along connective tissue boundaries under a dissecting microscope. Samples were prepared for electrophoresis, and the myosin heavy chain isoforms were resolved on SDS-polyacrylamide gels, with 7.4% polyacrylamide and 35% glycerol as described previously (Briggs and Schachat, 2000; Talmadge and Roy, 1993). The gels were stained with Coomassie Brilliant Blue G250, except for those containing single fibers, which were stained with silver (Schachat et al., 1985a). This gel formula was optimized to provide good resolution of EO, IIb and embryonic myosin, which migrates slightly faster than IIx. This migration position was confirmed by comparison with fetal muscle standards that contain embryonic and perinatal isoforms (not shown). To detect the perinatal (also known as neonatal) isoform, EOM samples were also analyzed using a different formula gel (8% acrylamide, 30% glycerol), on which both perinatal and embryonic isoforms migrate in the more typical positions above IIa, as shown previously (Janmot and d'Albis, 1994; McKoy et al., 1998). On those gels, perinatal myosin was detectable in trace amounts (less than 1% of the total myosin)

in some but not all EOM samples. Western blotting using the Vector ABC kit with horseradish peroxidase and chloronapththol substrate was carried out as described (Briggs et al., 1990). Adsorbed EO-1 and EO-2 antisera were used at 1:900 dilutions.

Preparation of RNA and reverse transcription-polymerase chain reaction

RNA was prepared as described previously (Chomczynski and Sacchi, 1987) from three regions of the EOM: the central region containing the orbital layer's endplate band, and the distal and far distal regions spanning approximately 4 mm each. These boundaries do not correspond exactly to those used for protein analysis, but the data obtained is completely consistent. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with the Titan One-Tube kit (Roche Molecular Biochemicals), using only 28 cycles of PCR to ensure that the amplification remained in the linear range. Equal amounts of RNA (20 ng) were used. Primers were GGTCCTCCTCAGCCTGGTACGTCAT and AGAAGGCCAARAARGCCAT, which generated a 320 bp product specific for MYH13.

Preparation of antibodies

Antibodies specific for MYH13 were prepared in chickens (Cocalico, Inc.) using bacterially expressed peptides. Peptides corresponding to the distal C-terminal end of the myosin rod (EO-1, amino acids1827–1938) and the hinge region (EO-2, amino acids 1210–1382) were selected because they exhibit high antigenic indices and the greatest sequence divergence with other myosin isoforms. The cDNA sequences encoding these peptides were amplified from rabbit MYH13 clones (GenBank accession no. AF212147; Briggs and Schachat, 2000) with PCR primers that introduced the restriction sites *Nde*I (underlined) or *Bam*HI (lower case). Primers for EO-1 were GGAATTCCATATGGTACGTGAGCTGGAAAC and GCTCCTCTCTCCTGCAGGTGT and for EO-2, GAATTC-CATATGCTCGGGGAGCAGATTG and GggatccTATTT-GGTCCTCCACT. PCR was performed for 28 cycles of 94°C for 1 min; 58°C for 1 min; 72°C for 1 min. The PCR products were digested with *Nde*I and *Sac*I, which cleaved at an internal site of EO-1 or with *Nde*I and *Bam*HI (EO-2). They were then directionally cloned into the pET17 vector, and the accuracy of the nucleotide sequence was confirmed. The plasmids were transformed into BLR (DE3) bacteria, and protein expression was induced with 0.2 mmol l^{-1} isopropylthio-β-D-galactoside (IPTG). Bacterial lysis and removal of contaminating proteins by incubation at 100°C was performed essentially as described (McNally et al., 1991). EO-1 was purified by anion-exchange chromatography and preparative SDS-PAGE. EO-2 was purified by hydroxyapatite and anion-exchange chromatography. Antisera were tested by ELISA and western blotting to identify maximal production of specific antibodies. Immunoglobulins were then purified from egg yolks (Akita and Nakai, 1993), and adsorbed against immobilized fast (IIa, IIb, IIx) and slow myosins to enhance specificity.

Immunohistochemical analysis

Muscle samples were infiltrated with PBS (10 mmol l^{-1} sodium phosphate, 150 mmol l^{-1} NaCl, pH 7.4)/30% sucrose, embedded, and frozen in liquid nitrogen. Sections (5 μm thick) were cut and frozen until use. Staining was performed according to the manufacturer's instructions using the Vector ABC kit. Sections were blocked with 2% normal goat serum in PBS, and incubations were performed for 30–60 min, followed by 3× 10 min washes with PBS. The primary antibody, adsorbed EO-1, was diluted 1:100 (v:v). The secondary antibody was biotinylated anti-chicken IgG, and the antibody reaction was visualized with horseradish peroxidase and diamino benzidine (DAB) as substrate. For localization of neuromuscular junctions, slides were blocked as above and incubated with Alexa 488-alpha-bungarotoxin (10 μg ml^{-1}) for 60 min, followed by 3×20 min washes with PBS. Slides were examined using a Zeiss Axioplan 2 microscope with visible light and fluorescein filters.

Results

MYH13 and IIb are expressed primarily in the central region of rabbit EOM that spans the endplate band

Myofibrils prepared from rabbit EOMs express MYH13, the fast isoforms IIa, IIb, IIx, and smaller amounts of embryonic, perinatal and slow/cardiac alpha and beta MyHCs. Overall, MYH13 makes up approximately 30% of the total myosin in rabbit EOM (Briggs and Schachat, 2000). To examine the longitudinal distribution of myosin isoforms, superior rectus was cut into six transverse sections as shown (Fig. 1A). The MyHCs present in each section were resolved by SDS-PAGE and the relative amounts of each MyHC were quantified by densitometry (Fig. 1B,C). The relative expression levels of all the MyHC isoforms changes along the length of EOM. MYH13 (EO) and IIb myosin are enriched in the central region containing the endplate band, where they make up 50% and 35% of the total, respectively, but they are virtually absent from the proximal and distal ends of the muscle (Fig. 1Ci). In those regions, IIa and slow/cardiac isoforms predominate (Fig. 1Cii), which is consistent with previous immunohistochemical studies (Brueckner et al., 1996; Jacoby et al., 1990; McLoon et al., 1999). At its highest level in the proximal and distal ends of the muscle, the embryonic isoform is approximately 10% of the total myosin.

Additional isoforms, including alpha cardiac, slow tonic and perinatal have been reported in EOM. With this type of gel, alpha and beta cardiac isoforms comigrate, and the amount of slow/tonic is too low to be detected. The perinatal isoform is not well resolved with the conditions optimized to resolve IIb and MYH13, but it was analyzed using different conditions (see Materials and methods), in which both perinatal and embryonic isoforms migrate in the more typical positions above IIa (d'Albis et al., 1989; Janmot and d'Albis, 1994). Under those conditions perinatal myosin was not detected in the samples shown in Fig. 1 (not shown). Trace amounts were, however, detected in other EOMs, indicating variable

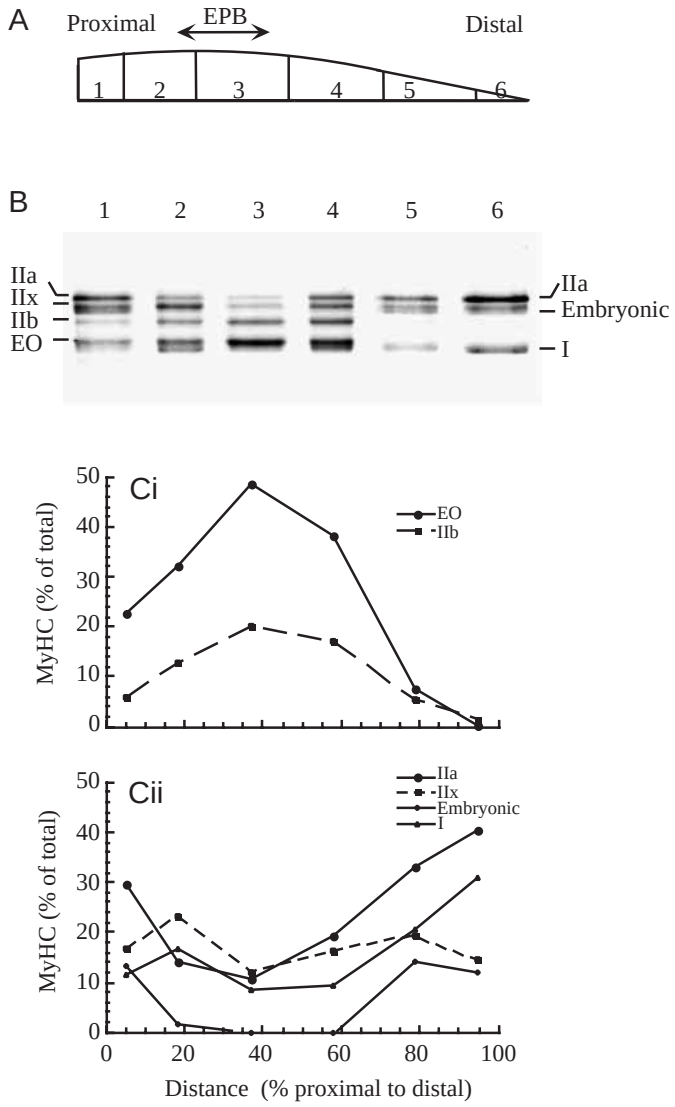


Fig. 1. Longitudinal variation in myosin heavy chain (MyHC) expression detected by high resolution SDS-PAGE. (A) Superior rectus muscle was cut into six parts as shown. The approximate location of the endplate band region (EPB) is marked. (B) Myofibrils prepared from each region identified in A were analyzed by SDS-PAGE and stained with Coomassie Blue G-250. The bands were identified by comparison with the migration positions in muscles of known myosin composition (Briggs and Schachat, 2000), and as described in Materials and methods. EO myosin is MYH13. (C) Relative amounts of each MyHC were determined by scanning with visible light and densitometry.

expression at levels less than 1% of the total myosin in rabbit EOM.

These observations demonstrate that EOM fibers express a complex and varying complement of the fast MyHCs. In addition to the longitudinal variation in MYH13 expression, MyHC-IIx levels are higher at the proximal range of MYH13 expression, but the ratio of MyHC-IIb to MYH13 is higher distal to the endplate region, indicating that they are regulated differently from MYH13 (Fig. 1Ci). Although the individual

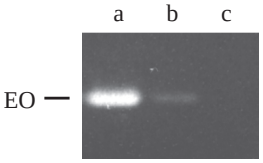


Fig. 2. MYH13 mRNA is also localized to the central region of the muscle. Primers specific for MYH13 mRNA were used in semiquantitative RT-PCR of RNA prepared from different sections of the muscle. The resulting 320 bp product is abundant in the central region (a), but greatly diminished in the distal (b) and far distal (c) regions. The muscle used for RNA preparation did not include as much of the proximal region as that used for the protein samples.

regulatory mechanisms of the isoforms remain to be defined, the amplification of MYH13 in the endplate region band supports the observations that innervation plays a critical role in regulating MYH13 expression (Brueckner and Porter, 1998; Kranjc et al., 2001).

MYH13 mRNA is also localized to the central region of EOM

For independent confirmation that MYH13 varies along the length of EOM, we examined the mRNA levels for MYH13 as a function of position along the muscle. Specific primers were developed from the distinctive 3′ region of the rabbit cDNA sequence (Briggs and Schachat, 2000) and used in semiquantitative RT-PCR of RNA prepared from different sections of the muscle (Fig. 2). Similar to the results obtained by protein analysis, the resulting 320 bp product obtained from MYH13 mRNA was abundant in the central region containing the endplate band, but greatly diminished in the distal and far distal regions of the muscle.

Production of two antibodies specific for MYH13

Antibodies against MYH13 were generated to determine the distribution of MYH13 among the different fiber types in EOM. Comparison of MyHC sequences identified two highly divergent regions of MYH13 (EO-1, amino acids 1827–1938, and EO-2, amino acids 1210–1382). Peptides containing those regions were bacterially expressed and used to produce antibodies in chickens. After preadsorption with skeletal myosins, these antibodies both specifically recognized MYH13 on western blots (Fig. 3). Their specificity provides the first direct evidence that the distinct myosin heavy chain resolved by SDS-PAGE in EOM is the product of the *MYH13* gene.

MYH13 is expressed in both the orbital and global layers of rabbit EOM

The specificity of EO-1 for MYH13 *in situ* was established in frozen muscle sections (Fig. 4A). It does not crossreact with the IIb isoform present in adductor magnus, β/I in semitendinosus, the IIa, IIx, β/I and ‘α-like’ isoforms in diaphragm (Hamalainen and Pette, 1997), or with the embryonic or perinatal isoforms present in fetal muscles (gestational day 27).

Transverse sections of EOM reveal two distinct layers, a superficial orbital layer, and the underlying global layer, which are distinguished by the smaller diameter fibers and the lower number fibers per fascicle present in the orbital layer. When

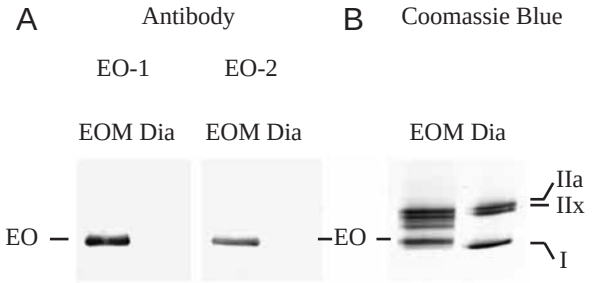


Fig. 3. Production of two antibodies specific for MYH13. Comparison of MYH13 with other MyHC sequences identified two highly divergent regions (EO-1, amino acids 1827–1938, and EO-2, amino acids 1210–1382). (A) Antibodies directed against those regions recognized only MYH13 on western blots containing extraocular muscle (EOM) and diaphragm muscle (Dia). (B) The migration positions of the myosin isoforms are shown on a duplicate gel stained for protein with Coomassie Blue G-250.

EO-1 is used to stain slides from the central region of EOM, nearly all fibers in the orbital surface layer are labeled (Fig. 4B). Many fast global fibers also express MYH13, but their staining intensity is heterogeneous and somewhat less intense than in the orbital layer. The negative fibers in the global region are labeled by antibodies against slow/cardiac or IIa myosin isoforms. Previous studies have shown that the slow-reactive fibers in the global layer also express the α-cardiac and slow/tonic isoforms (Pierobon-Bormioli et al., 1979; Rushbrook et al., 1994), and the absence of reaction with those fibers here confirms that EO-1 recognizes only MYH13. Thus, similar to findings in several other species (Sartore et al., 1987), MYH13 is expressed in both orbital and global layers of rabbit EOM, and may play a key role in the function of fibers in both layers.

Localization of the innervation zone

In the orbital layer, the motor endplates of the singly innervated fibers (SIFs) are arranged in a relatively narrow region in the proximal half of the muscle (Chiarandini and Davidowitz, 1979). Approximately 10% of the fibers in EOM are multiply innervated fibers (MIFs), and some of them have similar *en plaque* neuromuscular junctions in that region, as well as multiple sites of *en grappe* innervation elsewhere in the muscle (Jacoby et al., 1989). To confirm that the central region of the muscles analyzed by SDS-PAGE, RT-PCR and immunohistochemistry above (Figs 1, 2 and 4) contained the zone of innervation, we labeled semiserial sections with EO-1 and fluorescently labeled alpha-bungarotoxin (Fig. 5). As in Fig. 4, many of the fibers in this region contain MYH13 (Fig. 5A). In addition, numerous endplates containing the labeled acetylcholine receptors are present across the orbital layer (Fig. 5B), confirming the location of the endplate band. Many fibers in the global layer also contain endplates, but they are not as evenly distributed. That observation is consistent with the broader range of endplate distribution along the length of the

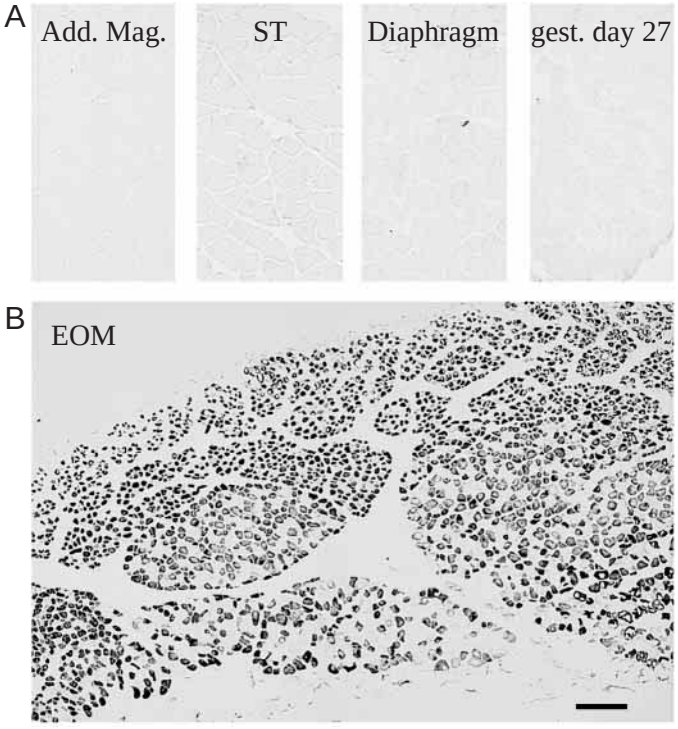


Fig. 4. Distribution of MYH13 in global and orbital layers of rabbit extraocular muscle (EOM). (A) The specificity of EO-1 was established by staining sections from control muscles that express the other skeletal and cardiac MyHC isoforms as described in text: adductor magnus (Add. Mag.), semitendinosus (ST), Diaphragm and fetal muscle (gest. day 27). (B) In the central region of EOM containing the endplate band, EO-1 stains most fibers in the orbital region near the top of the section, as well as many fibers in the global layer in the lower portion of the section. Scale bar, 200 μm.

muscle reported for global fibers (Davidowitz et al., 1996). These observations confirm that the highest levels of MYH13 expression span the endplate band.

In the distal portion of the muscle, expression of MYH13 terminates in orbital fibers but continues in some global fibers

To further define the distribution of MYH13 relative to the innervation zone, frozen muscle sections from the endplate band and more distal regions were stained with EO-1. Fig. 6A shows a section within the endplate band, where MYH13 is expressed in both orbital and global layers. In the distal region of the muscle, MYH13 is still abundant in global fibers, when many orbital fibers are negative (Fig. 6B). More distally, where orbital fibers are negative, MYH13 persists at low to moderate levels in some fibers of the global region (Fig. 6C).

SDS-PAGE confirms that MYH13 is expressed in the global layer

To isolate a sample containing only global-layer fibers, the inner global layer (light gray) was dissected along connective tissue boundaries from a 120 μm frozen section (Fig. 7A). The remaining portion (dark gray) contained the outer (or intermediate) global layer and the orbital surface layer.

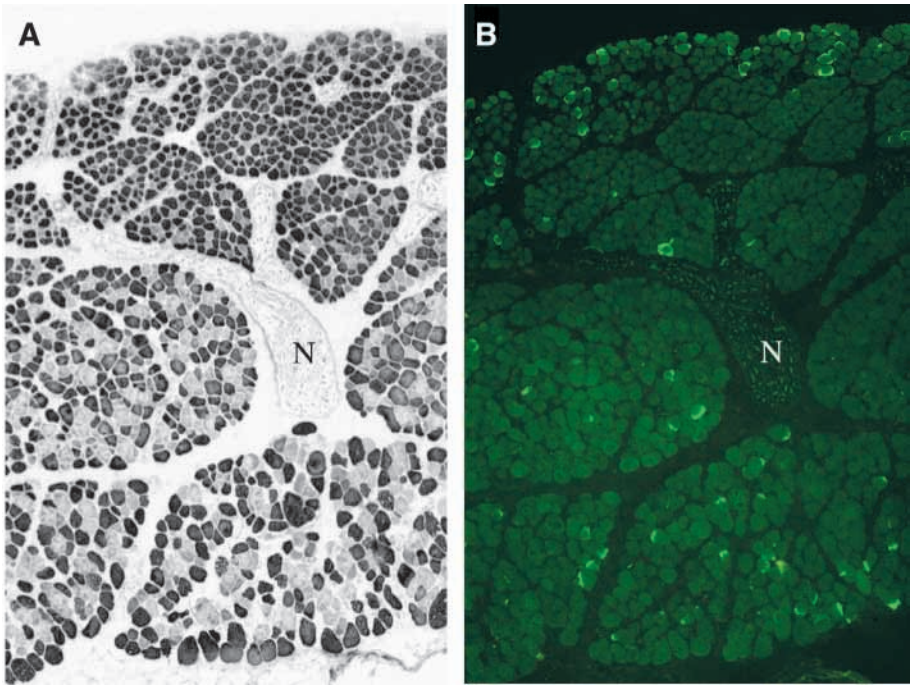


Fig. 5. Localization of the endplate band (innervation zone). (A) High levels of MYH13 in this region were detected by staining with EO-1. (B) In a semiserial section the neuromuscular junctions were detected by labeling acetylcholine receptors with fluorescently labeled alpha-bungarotoxin. N, nerve.

Analysis by SDS-PAGE (Fig. 7B) shows that MYH13 (EO) is abundant in both layers. Myosin heterogeneity in this narrow section is just as complex as in the 2–3 mm sections analyzed in Fig. 1, indicating that the overall myosin composition changes relatively gradually across the length of the muscle.

MYH13 is coexpressed with other myosin isoforms in individual global fibers

One potential explanation of the staining heterogeneity of global fibers (Fig. 4) is that other isoforms are also expressed in those fibers. This idea was tested by SDS-PAGE analysis of individual fibers dissected from the global region (Fig. 8). No 'pure' fibers containing only MYH13 were observed. Most of the fibers form what appears to be a continuum of MYH13 expression with other fast myosin isoforms, particularly IIb, the myosin isoform associated with the fastest-contracting fibers in skeletal muscles. MYH13 is less abundant in fibers expressing IIa and/or IIx. A few fibers express only IIa myosin and probably correspond to red singly innervated fibers (Spencer and Porter, 1988). It thus appears that multiple myosin isoforms are expressed in most fibers in the rabbit global layer.

These quantitative molecular studies provide a critical complement to immunolocalization because they provide a means of assessing the relative contributions that each myosin isoform can make to the complex function of EOM. The differential expression of MYH13 in the innervation region of EOMs may allow it to make a critical contribution to the contractile features of saccades.

Discussion

This study provides the first quantitative analysis of the regional distribution of myosin heavy chains in extraocular muscle. This information is particularly important as the relationship between myosin expression, fiber type and physiological function is more complex in EOM than in other skeletal muscles. EOMs express all the striated myosins, and their novel and diverse fiber types are recruited in both slow vergence and tracking movements and superfast saccades (Pierobon-Bormioli et al., 1979; Rushbrook et al., 1994; Wieczorek et al., 1985) (Scott and Collins, 1973; Shall and Goldberg, 1992). Our particular focus is on the distribution of the superfast myosin heavy chain MYH13, which is implicated in saccadic contractions. Despite its relatively low abundance in the whole muscle (approximately 30 %), we find that MYH13 is preferentially expressed in the central innervation zone of the majority of both global and orbital layer fibers and that it colocalizes with the IIb MYH in global fibers. The presence of MYH13 and IIb myosin, the fastest of the limb and trunk myosins, in most of the fibers spanning the central innervation zone suggests that they may have some functional overlap or synergy in driving the fastest ocular movements.

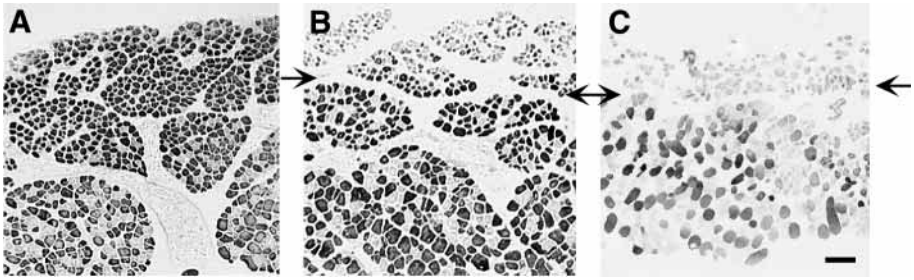
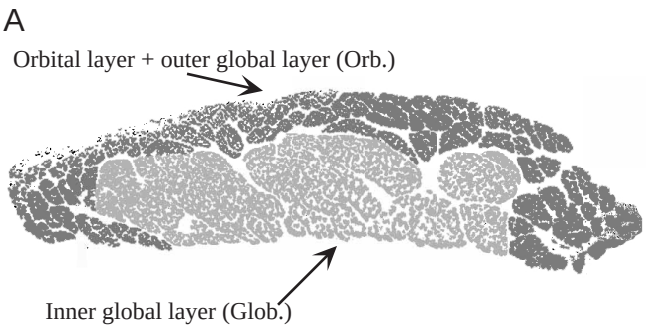


Fig. 6. Expression of MYH13 persists in distal portions of the global layer. Sections taken from the endplate and more distal regions were stained with EO-1. (A) A portion of the section shown in Fig. 5, within the endplate band, (B) approximately 3 mm distal, and (C) approximately 6 mm distal to (A), where the muscle is considerably thinner because many fibers do not extend the full length of the muscle. Scale bar, 100 μm. Orbital (upper) and global (lower) layers are marked by arrows.



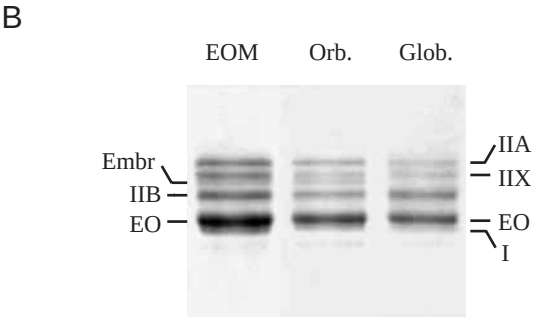


Fig. 7. Molecular confirmation that MYH13 is present in both orbital and global layers. (A) Schematic illustration of dissection of a 120 μm frozen section into two samples: the inner global layer (Glob.; light gray) and the outer (or intermediate) global layer plus the orbital surface layer (Orb.; dark gray). (B) The myosin isoforms present in EOM and in the two dissected samples (Orb. and Glob.) were analyzed by SDS-PAGE, which showed that MYH13 is present in the global as well as orbital layer.

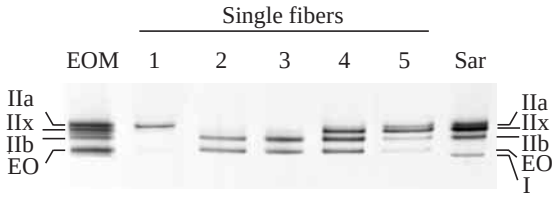


Fig. 8. SDS-PAGE analysis of single extraocular muscle (EOM) fibers. Single fibers were dissected from the global region in the central region of the muscle. The five representative fibers show that MYH13 (EO) is coexpressed with different levels of IIb and, at lower levels, with IIx and IIa. Sar, sartorius.

The presence of MYH13 and IIb myosin in a majority of the fibers spanning the innervation zone offers a potential explanation for a puzzling aspect of EOM physiology: how the many diverse fiber types of EOM can all participate in the fastest contractile movements of EOM (Scott and Collins, 1973; Shall and Goldberg, 1992). If the roles and recruitment order of motor units in EOM were like those of limb and epaxial muscles, then each extraocular motor unit would be composed of fibers expressing a single myosin, and it would be differentially recruited for specific subsets of fast or slow ocular movements. However, this simple relationship between myosin expression, motor units and function does not occur in EOM: virtually all EOM motor units – despite differences in morphology and innervation – can participate in saccades, tracking and vergence movements, and recruitment depends on the amount of work required rather than on the speed of the movement (Scott and Collins, 1973). Moreover, the first motor units recruited in saccades may comprise specialized ‘bilayer’ motor units that activate both orbital and global layer fibers (Shall and Goldberg, 1995). These observations could be reconciled with the heterogeneity of myosin isoforms in most EOM fibers if a majority of fibers express kinetically fast myosin over a narrow region, allowing that region to respond uniformly, despite expression of kinetically slower isoforms in other regions. The fast kinetics of MYH13, inferred from its expression in the extremely fast-contracting laryngeal muscles as well as the higher rate of cross-bridge cycling observed in EOM fibers (Li et al., 2000), suggests that the presence of MYH13 in the majority of both orbital and global fibers within the innervation zone may allow diverse EOM fibers to participate in the extremely fast saccades.

If the role of MYH13 in saccades depends on its rapid cycling time and the majority of fibers can participate in saccades, it is surprising that less than 35 % of the total myosin is composed of MYH13. In other skeletal muscles, a fast myosin must comprise at least 80 % of the total myosin to dominate contractile kinetics of individual fibers (Cuda et al., 1997; Reiser et al., 1985). In the central innervation region, MYH13 and IIb myosin together reach such high levels (Figs 1, 8), but it is also possible that in a more localized region, about the neuromuscular junction, MYH13 alone may reach these levels.

Several functional mechanisms based on this localized concentration of MYH13 can be envisaged. The absence of MYH13 in rat global fibers, which are activated in driving saccades, led Rubinstein and Hoh (2000) to propose an indirect role for MYH13 to enable saccades by increasing the rate of relaxation, thus reducing the tension in the orbital layer opposing the saccade. Thus, for a lateral saccade, the rapid cycling kinetics of MYH13 in the central region of the medial rectus orbital fibers would enable that region to relax rapidly, thereby reducing the tension opposing the contraction of the lateral rectus muscle when it initiates a saccade. By contrast, the presence of MYH13 in global as well as orbital fibers in rabbit, coupled with its coexpression with the fast IIb myosin, would allow MYH13 and global fibers to play a direct role in moving the eye during saccades. And MYH13 expression in the orbital layer would enable it to contribute to either or both of the rapid movements in saccades and the repositioning of the pulley. We propose that the localized expression of MYH13, in synergy with IIb myosin and increased levels of sarcoplasmic reticulum, result in a differentially responsive and very fast contracting region of extraocular muscle. Neither mechanism is exclusive in the rabbit, and each is consistent with the distribution of MYH13 in these species. To resolve these issues, further studies correlating the species-specific differences in MYH13 expression and the pattern of recruitment of EOM motor units need to be pursued.

With regard to potential species-specific differences in MYH13 expression, our finding that MYH13 is expressed in both the global and orbital layers is consistent with the immunolocalization described by Sartore et al. (1987) in several species, but it differs from other studies that describe different orbital or global localizations (Lucas et al., 1995; Brueckner et al., 1996). This variation may reflect subtle variation in probe specificity, antigen accessibility or true species-specific differences. These uncertainties emphasize the importance of confirming localization results by an independent technique such as SDS-PAGE. Our SDS-PAGE analysis of the myosin isoforms present in dissected orbital and global layers of the muscle and in single fibers from the global layer (Figs 7, 8) confirms the distribution of MYH13 in rabbit EOM.

Longitudinal changes in the expression of cardiac/slow, developmental and fast myosin have been detected previously by immunolocalization (Brueckner et al., 1996; Jacoby et al., 1990; McLoon et al., 1999; Rubinstein and Hoh, 2000). But those studies could not distinguish among all the fast subtypes because specific antibodies for IIb and IIx were unavailable. Here, quantitative differences in the distribution of all the fast myosins were detected by SDS-PAGE. Fig. 1 shows that IIb and MYH13 are expressed in the central region, whereas IIx is distributed relatively uniformly and IIa is expressed primarily in the proximal and distal regions of the muscle.

Segmental expression of myosins along the length of muscle could arise in several ways. In the orbital layer, many fibers extend to the end of the layer, so the decrease in MYH13 in the distal region of the muscle reported here, and by Rubinstein and Hoh (2000), clearly indicates that different myosin isoforms are expressed along the length of individual fibers. By contrast, most previous studies of fast myosin isoforms reported that only one fast isoform is expressed in each fiber in the global layer. Homogeneity of myosin isoform expression in single fast global fibers is not supported by the present results. Some fibers express IIa uniformly, but the majority of global fibers that express MYH13 also express IIb and varying amounts of IIx (Fig. 8). These observations, coupled with the immunolocalization of MYH13 (Fig. 5), suggest either that MYH13 and IIb are coexpressed in single fibers, or that there is a transition from high levels of MYH13 expression near the neuromuscular junction to IIb and then other fast isoforms in more distal regions of the fibers.

Interpreting the changes in myosin distribution along the length of the muscle is complicated by the fact that many fibers do not extend full length, and some are present only in the proximal or distal portions of the muscle (Davidowitz et al., 1977, 1996). For MYH13, the broader distribution in global fibers (Fig. 6) may result from several factors. The endplates in the global layer are spatially distributed over a two- to threefold wider range than in the orbital layer (Davidowitz et al., 1996), so if MYH13 expression is regulated by proximity to the endplate, then a broader region of high MYH13 expression would be expected in the global layer. In addition, two of the global singly innervated fiber types, the red and intermediate fibers, terminate early, and only 20% of the third type, the ‘pale’ fibers, continue to the distal region. Another group of global fibers originates in the distal half of the muscle, and they are innervated at a secondary zone approximately two-thirds of the way down the muscle (Davidowitz et al., 1996, 2000). Thus, continued MYH13 expression may result from expression in the relatively few fibers that extend that far, as well as the influence of a broader innervation zone and the presence of distally innervated fibers.

Muscle fibers distant from the endplate region may also be heterogeneous. Quantitative analysis showed that the embryonic and perinatal isoforms are relatively minor components of distal and proximal regions in rabbit EOM (Fig. 1). The perinatal isoform makes up at most 1% of the myosin in the distal section, and the proportion of embryonic myosin is approximately 5% overall (Briggs and Schachat, 2000) and only 12% in farthest distal section. Similarly, these isoforms were below the limits of detection by SDS-PAGE analysis of rat EOM (Kranjc et al., 2000). However, immunolocalization studies have reported that these developmental isoforms are expressed in 50–90% of the fibers in this region of the muscle (Brueckner et al., 1996; McLoon et al., 1999). If these isoforms are present in a large percentage of fibers, then they only represent a small fraction of the total myosin in each fiber – perhaps too small to have a significant effect on physiology. For this reason a combination of both quantitative data and immunolocalization will be required to fully define expression patterns and assess the relative functional roles of the different isoforms.

The complex patterns of myosin expression described in extraocular muscle have no parallels in other adult vertebrate muscles, but segmental heterogeneity has been reported in individual fibers during development. Rosser et al. (2000) found that in the innervation-dependent transition from neonatal to adult myosin isoforms, adult isoforms are first expressed at the neuromuscular junction and gradually spread down the length of the fiber. In contrast to that dynamic transition, the segmental heterogeneity in EOM appears to be a stable situation in which expression of the novel myosin MYH13 is induced by an innervation-dependent signaling pattern that is attenuated along the length of the fiber.

The localized expression of MYH13 and its mRNA in the central region of rabbit EOM, spanning the innervation zone, suggests that MYH13 transcription is regulated by the activity of the extraocular motor nerves (Brueckner and Porter, 1998; Kranjc et al., 2001). The particular dependence of MYH13 on appropriate neural activity was demonstrated by the loss of MYH13 expression after paralysis of rat EOM with botulinum toxin, coupled with the failure to recover its expression after the muscle was reinnervated and the expression of the other myosin isoforms had returned to near normal (Kranjc et al., 2001). The molecular mechanism of this regulation has not been established. Although MYH13 colocalizes with IIb in EOM, it is clear that they are independently regulated because the *MYH13* gene sequence lacks many of the upstream regulatory elements in the proximal promoter of IIb that

regulate its expression in fast skeletal muscles (Briggs and Schachat, 2000).

Segmental expression of MYH13 and the other myosin isoforms may be the molecular explanation for how different regions of EOM muscle fibers can have diverse contractile properties. Expression of EO MyHC in almost all fast fibers in the EPB region may permit those fibers to perform a common function such as saccades. However, the extensive coexpression of MYH13 with IIb and other fast myosins in individual fibers raises the question of whether kinetic differences of myosin isoforms are the primary determinant of rapid EOM contractions. The localized high levels of MYH13 and IIb myosin are probably the key to the initial velocity of shortening in saccades, but the short contraction times and low tension that characterize saccades more likely result from other special adaptations. The small fiber and myofibril size and high density of sarcoplasmic reticulum (Chiarandini and Davidowitz, 1979; Spencer and Porter, 1988), which at the molecular level result in a differential increase in the proteins associated with calcium reuptake (Blank and Schachat, 1999), would allow the central region of the muscle to have more rapid rates of tension generation and relaxation. These specializations probably account for the low tension generated in saccades, as they would reduce the duration and amplitude of the calcium transient and shorten the contraction time in exchange for a reduction in twitch tension. Such a trade-off of tension for more rapid contraction times would be further enhanced by the high levels of expression of TnT3f observed in EOM (Briggs et al., 1988), the fast TnT isoform associated with the most graded tension increase in response to increases in intracellular calcium (Schachat et al., 1987).

Investigations are continuing to define more precisely how the diverse patterns of myosin expression are regulated in EOM fibers and to correlate myosin expression with fiber physiology.

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