The Journal of Physiology

Two novel/ancient myosins in mammalian skeletal muscles: MYH14/7b and MYH15 are expressed in extraocular muscles and muscle spindles

Alberto C. Rossi[1,2], Cristina Mammucari[1,2], Carla Argentini[1,2], Carlo Reggiani[3,4] and Stefano Schiaffino[1,2,4]

[1] *Department of Biomedical Sciences, University of Padova, Padova, Italy*
[2] *Venetian Institute of Molecular Medicine (VIMM), Padova, Italy*
[3] *Department of Anatomy and Physiology, University of Padova, Padova, Italy*
[4] *Institute of Neuroscience of the Consiglio Nazionale delle Ricerche, Padova, Italy*

The mammalian genome contains three ancient sarcomeric myosin heavy chain (MYH) genes, *MYH14/7b*, *MYH15* and *MYH16*, in addition to the two well characterized clusters of skeletal and cardiac MYHs. *MYH16* is expressed in jaw muscles of carnivores; however the expression pattern of *MYH14* and *MYH15* is not known. *MYH14* and *MYH15* orthologues are present in frogs and birds, coding for chicken slow myosin 2 and ventricular MYH, respectively, whereas only *MYH14* orthologues have been detected in fish. In all species the *MYH14* gene contains a microRNA, miR-499. Here we report that in rat and mouse, MYH14 and miR-499 transcripts are detected in heart, slow muscles and extraocular (EO) muscles, whereas MYH15 transcripts are detected exclusively in EO muscles. However, MYH14 protein is detected only in a minor fibre population in EO muscles, corresponding to slow-tonic fibres, and in bag fibres of muscle spindles. MYH15 protein is present in most fibres of the orbital layer of EO muscles and in the extracapsular region of bag fibres. During development, MYH14 is expressed at low levels in skeletal muscles, heart and all EO muscle fibres but disappears from most fibres, except the slow-tonic fibres, after birth. In contrast, MYH15 is absent in embryonic and fetal muscles and is first detected after birth in the orbital layer of EO muscles. The identification of the expression pattern of MYH14 and MYH15 brings to completion the inventory of the MYH isoforms involved in sarcomeric architecture of skeletal muscles and provides an unambiguous molecular basis to study the contractile properties of slow-tonic fibres in mammals.

(Received 3 September 2009; accepted after revision 25 November 2009; first published online 30 November 2009)
Corresponding author S. Schiaffino: Venetian Institute of Molecular Medicine, via G. Orus 2, 35129 Padova, Italy. Email: stefano.schiaffino@unipd.it

Abbreviations ALD, chicken anterior latissumus dorsi; EDL, extensor digitorum longus; Emb, embryonic; EO, extraocular; miR-499, micro-RNA 499; MYH, myosin heavy chain; Neo, neonatal; SOL, soleus; TA, tibialis anterior; VL, vastus lateralis.

Introduction

Until 10 years ago only eight sarcomeric myosin heavy chain (MYH) genes, associated in two highly conserved gene clusters, were known to be present in mammals (see Weiss *et al.* 1999*a*,*b*). Two tandemly arrayed genes, located in human chromosome 14 code for the cardiac myosins, α- and β-MYH, the latter being also expressed in slow skeletal muscle. Another gene cluster, located in human chromosome 17, codes for the six skeletal myosins, including the adult fast 2A-, 2X- and 2B-MYH, the developmental embryonic and neonatal/perinatal isoforms, and another isoform expressed specifically in extraocular (EO) muscles. More recently, the completion of the human genome project led to the identification of three additional sarcomeric *MYH* genes: *MYH14* (also called *MYH7b*, see below Nomenclature) located on human chromosome 20, *MYH15* on chromosome 3 and *MYH16* on chromosome 7 (Berg *et al.* 2001; Desjardins *et al.* 2002). The three new sarcomeric *MYH* genes differ significantly in sequence, size and exon–intron organization from the other sarcomeric *MYH*s and appear to correspond to ancestral *MYH*s (Desjardins *et al.* 2002; McGuigan *et al.* 2004; Ikeda *et al.* 2007). Orthologues of *MYH14* are found in fish, *Xenopus* and chicken genome, the chicken orthologue coding for a slow-type *MYH*,

© 2010 The Authors. Journal compilation © 2010 The Physiological Society DOI: 10.1113/jphysiol.2009.181008

called *slow myosin 2* (*SM2*), whereas orthologues of *MYH16* are not found in *Xenopus* or chicken but are present in fish and, surprisingly, in the ascidian, *Ciona intestinalis* (McGuigan *et al.* 2004; Garriock *et al.* 2005; Ikeda *et al.* 2007; Nasipak & Kelley, 2008). *MYH15* is the orthologue of *Xenopus* and chicken genes coding for ventricular MYH but no *MYH15* orthologue was detected in fish (McGuigan *et al.* 2004; Garriock *et al.* 2005; Ikeda *et al.* 2007).

The expression pattern of these three *MYH* isoform genes has not been characterized in mammals except for *MYH16*. *MYH16* codes for a myosin expressed in jaw muscles of carnivores, primates and marsupials, but is a pseudogene in humans (Schachat & Briggs, 1999; Berg *et al.* 2001; Qin *et al.* 2002; Stedman *et al.* 2004). MYH16 is often referred to as 'superfast', but a recent study shows that single cat and dog fibres containing exclusively this myosin isoform display a maximum shortening velocity similar to that of fast 2A fibres, namely intermediate between that of slow type 1 and fast 2B fibres, but exert more force than any other fibre type examined (Toniolo *et al.* 2008). In contrast, no information is available about expression and function of mammalian MYH14 and MYH15. Several EST matches for *MYH14* and *MYH15* were detected in humans, but these were not from cardiac or skeletal muscles (Desjardins *et al.* 2002). Garriock *et al.* (2005) were unable to detect MYH15 mRNA using reverse transcriptase–polymerase chain reaction (RT-PCR) on RNA from adult mouse heart and suggested that *MYH15* is probably a pseudogene in mammals. The issue is further confused by an early report (Nagase *et al.* 2000) on a MYH cDNA clone (designated KIAA1512) isolated from a human fetal brain library, located on chromosome 20 and apparently corresponding to the sarcomeric *MYH14* subsequently identified by Desjardins *et al.* (2002): transcripts of this gene were found to be expressed at high levels not only in human heart and skeletal muscle but also in brain and testis, and at lower but significant levels in ovary and kidney. The distribution of mammalian MYH14 transcripts is of special interest, in view of the finding that a specific micro-RNA, miR-499, is nestled within intron 19 of this gene in the mouse (van Rooij *et al.* 2008).

Here, we describe the expression pattern of *MYH14* and *MYH15* in mammalian muscles. This study was originally motivated by the search for the gene coding for slow-tonic myosin that we previously identified in mammalian muscles (Bormioli *et al.* 1979; Bormioli *et al.* 1980) on the basis of the reactivity with an antibody specific for chicken slow anterior latissimus dorsi (ALD). Slow-tonic muscle fibres present in amphibian muscles respond to stimulation with a long lasting contracture rather than a twitch and are characterized by multiple '*en grappe*' innervation rather than the single '*en plaque*' motor endplate typical of twitch muscle fibres (Morgan & Proske, 1984). Slow-tonic fibres with similar properties are also present in the chicken slow ALD muscle and in mammalian EO muscles and muscle spindles (Morgan & Proske, 1984). The presence of a distinct MYH in mammalian slow-tonic fibres was suggested by the finding that a polyclonal antibody raised against chicken ALD muscle myosin stained specifically multiply innervated fibres in human EO muscles and bag fibres in muscle spindles (Bormioli *et al.* 1979; Bormioli *et al.* 1980). Subsequent studies used monoclonal antibodies against chicken myosins to label as 'slow-tonic' a number of fibres in EO muscles and spindles (see Pedrosa & Thornell, 1990; Kucera *et al.* 1992), but the existence of a distinct gene coding for mammalian slow-tonic myosin was never established.

Methods

Nomenclature

The *MYH* gene located in human chromosome 20 was named *MYH14* in several studies of sarcomeric MYHs (Desjardins *et al.* 2002; McGuigan *et al.* 2004; Ikeda *et al.* 2007). However, the same denomination *MYH14* was subsequently given to another gene located in chromosome 19 (Leal *et al.* 2003) and coding for non-muscle myosin IIC (Golomb *et al.* 2004). To avoid confusion, the HUGO Nomenclature Committee termed the gene located in human chromosome 20 *MYH7b*. However, this denomination appears wholly inappropriate, since it suggests a non-existent relation of this gene with the *MYH7* gene coding for the MYH-β/slow expressed in mammalian slow-twitch muscle fibres. Given the wide use of the *MYH14* nomenclature for the sarcomeric myosin gene, it was recently suggested to use the name *MYH17* for the gene coding for non-muscle myosin IIC (Ikeda *et al.* 2007). We support this proposal, and therefore we will use hereafter the term *MYH14* for the chromosome 20 sarcomeric *MYH* gene. Genes names are written in italics and protein names in roman characters throughout the text, according to a widely accepted use.

Bioinformatics

Nucleotide sequences were identified and amino acid sequences were deduced using public databases and tools (see online Supplemental Material, Table S1). Sequences were aligned using the BLAST program available at NCBI. Physical maps were created using NCBI databases to obtain the syntenies reported in Fig. 1. However, the Ensembl genome browser (http://www.ensembl.org/) and JGI database (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html) were used to determine the gene order of *Xenopus tropicalis MYH* genes. Some *MYH* genes, for example rat *Myh15*, are not identified as such in public databases. In this case, known genes located upstream

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

and downstream of *MYH15* in the human genome were identified in the rat genome and genes in between were aligned to known putative orthologues using NCBI BLAST. Rat *Myh15* was found to correspond to a gene called RGD1565858 (see Table S1). A similar approach was also used for other *Myh14* and *Myh15* genes.

Muscle samples

Sprague–Dawley rats weighing approximately 300 g and CD-1 mice of approximately 30 g were bred in-house in a conventional colony, housed in controlled conditions of 25°C, 50% relative humidity, and a 12 h light (06.00–18.00 h) and 12 h dark cycle, with water and food available *ad libitum*, at the Animal Facilities of the Venetian Institute of Molecular Medicine. Three-month-old rats ($n = 12$), embryonic day (E)16 rats ($n = 6$), E20 rats ($n = 6$), three-month-old mice ($n = 12$) and E12.5 mice ($n = 6$) were used. Rats were killed by carbon dioxide (CO_2) gas asphyxation, whereas mice were killed by cervical dislocation by appropriately trained staff. Muscles were quickly removed and frozen in liquid nitrogen-cooled isopentane and stored at −80°C. All experimental protocols were reviewed and supervised by the Veterinary Service and Animal Care Committee of the University of Padova, in accordance with the D.Lgs. 116/92. Human vastus lateralis muscle biopsies were obtained from Neuromuscular Tissue Bank (NMTB) at the Regional Neuromuscular Center of the University of Padova approved by the Ethical Committee of the University of Padova in compliance with the *Declaration of Helsinki*. The vastus lateralis samples used in this study were evaluated as normal by the pathologist based on morphological and histochemical analyses of muscle sections. Human EO muscle samples were obtained from the Veneto Eye Bank Foundation (FBOV; Mestre-Venezia, Italy) according to the ethical recommendations of the Italian Transplantation Law. EO muscles were removed during the 6 h *post mortem*, rapidly frozen in isopentane chilled with liquid nitrogen and stored at −80°C until use. Human myocardial samples, obtained from cardiac surgery, were kindly donated by Professor C. Poggesi, University of Firenze. The samples were immediately frozen in liquid nitrogen and stored at −80°C.

Quantitative RT-PCR

RNA was extracted from frozen muscle samples using the Promega SV Total RNA Isolation System kit according to manufacturer's instructions (Promega Corp., Madison, WI, USA). Complementary DNA generated with Invitrogen SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was analysed by quantitative real-time RT-PCR using Qiagen QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) on an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA). Primers used in these experiments are reported in Table S2. Data were normalized to the expression of housekeeping genes. Quantitative real-time PCR on miR-499 was performed using the TaqMan miRNA assay kit (Applied Biosystems) according to the manufacturer's protocol using RNA isolated with Trizol (Invitrogen).

Antibodies, immunoblotting and immunofluorescence

Two peptides corresponding to the amino terminal sequences of rat MYH14 (residues 4–77) and MYH15 (residues 4–104) (Supplemental Fig. S1) were used to raise custom polyclonal antibodies (PRIMM, Milano, Italy), which were purified from rabbit serum by affinity chromatography on insolubilized immunogens. For immunoblotting, protein samples were resolved by SDS-PAGE, using either a standard protocol with 10% polyacrylamide gel or a specific protocol with polyacrylamide gel with a high glycerol concentration which allows the separation of MYH isoforms (Mizunoya *et al.* 2008). Immunofluorescence were performed on cryosections as described (Murgia *et al.* 2000). For EO muscles the rectus superior was used in most analyses, but similar results were obtained with other EO muscles. Reactivity of the Ab was shown to be specific by the use of peptide-saturated primary antibody controls, which demonstrated an absence of signal due to non-specific binding of primary antibodies in tissue immunohistochemistry. The polyclonal antibody raised against chicken ALD myosin, specifically reactive with multiply innervated slow-tonic fibres in mammalian EO muscles, was previously described (Bormioli *et al.* 1979; Bormioli *et al.* 1980). Monoclonal antibody S46, reactive with chicken slow myosin 1 and 2 (Miller *et al.* 1985; Page *et al.* 1992), was obtained from the Developmental Studies Hybridoma Bank (DSHB). A recent study showed that S46 is more specific for slow-tonic fibres in mammalian skeletal muscles than other anti-chicken myosin antibodies, which show partial cross-reaction with the MYH-β/slow present in slow-twitch muscle fibres (Sokoloff *et al.* 2007). Other antibodies used in the present study include mAbs BF-G6, specific for embryonic MYH (Schiaffino *et al.* 1986), BA-D5, specific for MYH-β/slow (Schiaffino *et al.* 1989) and BF-13, which is specific for developing and adult fast MYHs, but in immunoblotting is unreactive with 2B-MYH (our unpublished observations).

Results

Comparative genomics of *MYH14* and *MYH15*

Partial synteny analyses of sarcomeric *MYH* genes in vertebrates were previously reported (McGuigan

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

et al. 2004; Garriock *et al.* 2005; Ikeda *et al.* 2007). Using available data for different vertebrate classes, we re-examined the syntenic organization of *MYH14/7b* and *MYH15* genes and found that orthologues of mammalian *MYH14* and *MYH15* genes are present in birds and frogs (Fig. 1*A* and Table S1). In contrast, as reported in previous studies (McGuigan *et al.* 2004; Ikeda *et al.* 2007), orthologues of *MYH14,* but not of *MYH15*, genes were detected in the fish genome. Based on exon–intron analysis, it was suggested that a common precursor gave origin to the fish *MYH14* and to the *MYH14* and *MYH15* genes in other vertebrate classes (Ikeda *et al.* 2007). We re-examined the structure of *MYH14* and *MYH15* genes in different species and found additional evidence that supports this view but also points to a unique evolution of the mammalian *MYH15* gene. First, we noted that a conserved microRNA, miR-499, previously identified in mouse *MYH14* gene (van Rooij *et al.* 2008), is contained within the *MYH14* gene in all species (Fig. 1*A* and Table S1). Next, we focused on the structure of myosin loop 1, a surface loop which connects the 25 and 50 kDa domains in the myosin head and is located near the ATPase catalytic site. The sequence of loop 1 is known to vary between paralogous myosin isoforms, to be conserved between myosin orthologues and to affect some myosin properties, such as the rate of ADP release (Sweeney *et al.* 1998). The loop 1 sequence of zebrafish MYH14 is similar to that of MYH14 in other species but also to that of MYH15 in *Xenopus* and chick; in particular a specific ATKTGG signature is present at the 3′ end of loop 1 in all these myosins (Fig. 1*B*). In contrast, a completely different sequence is present in mammalian MYH15, which is much shorter than all other sarcomeric MYHs. These findings are consistent with the view that vertebrate *MYH14* and *MYH15*

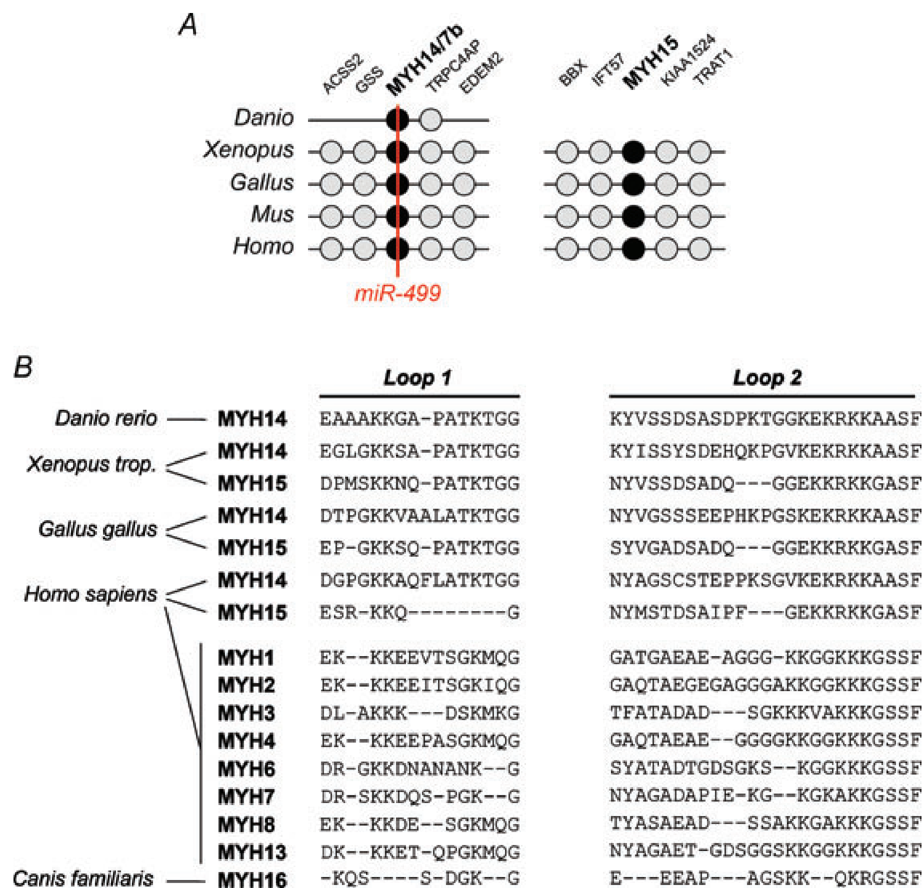

Figure 1. Syntenic organization of *MYH14/7b* and *MYH15* genes and amino acid sequence of MYH14 and MYH15 loops 1 and 2

A, physical maps showing syntenic organization of *MYH14/7b* and *MYH15* genes in fish, frog, chicken and mammals. The microRNA, miR-499, is contained within the *MYH14* gene in all species. Spacing of markers does not reflect actual scale. Gene organization information was obtained by NCBI and Ensembl Genome Browser databases (see Table S1 for gene ID). *B*, amino acid sequence of MYH14 and MYH15 loop 1 and loop 2 in different vertebrate species. The structure of the two surface loops in other human sarcomeric MYHs is also shown for comparison. The dog sequence is shown for MYH16, because human *MYH16* is a pseudogene.

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

genes derive from a common precursor similar to fish *MYH14*, but that the mammalian *MYH15* gene underwent a drastic structural remodelling possibly related to a divergent pattern of expression and function (see below). The divergent evolution of mammalian MYH15 is also apparent from sequence comparisons of the whole molecule: chicken MYH14 and MYH15 proteins have a much higher degree of similarity between them (83% identity) compared to the corresponding mammalian MYHs (human MYH14 *vs.* MYH15: 60% identity). The sequence of loop 2, another region of variability among MYH isoforms, shows a specific signature, KEKRKKAASF, at the 3′ end, which is identical among the various species, and is slightly different from the corresponding MYH15 sequence, EKKRKKGASF, which is also identical among the various species (Fig. 1*B*).

Expression of MYH14 and MYH15 in mammalian muscles at transcript level

Quantitative RT-PCR analysis was used to examine the expression pattern of MYH14 and MYH15 in mammalian muscles. MYH14 and miR-499 transcripts are present in rat heart, slow skeletal muscles and EO muscles but only at low levels in fast skeletal muscles (Fig. 2). In contrast, MYH15 transcripts are present at high levels in EO muscles but absent in fast and slow leg muscles and heart. The pattern of distribution of these MYH transcripts is similar in mouse muscles (Fig. S2A) and is completely different from that of typical slow and fast MYH, such as the *MYH7* gene, coding for MYH-β/slow, expressed in slow-twitch fibres, and *MYH4* gene, expressed in MYH-2B fibres. In human skeletal muscle MYH14 transcripts are present in EO muscles but also in vastus lateralis and in both atrial and ventricular myocardium, whereas MYH15 transcripts are detectable exclusively in EO muscles with minimal traces in myocardial tissues (Fig. S2B).

Expression of MYH14 and MYH15 in mammalian muscles at protein level

Polyclonal antibodies were raised against two peptides corresponding to the amino terminal sequences of rat MYH14 and MYH15, which are markedly different from the same regions of cardiac and skeletal MYHs (Fig. S1). By immunoblotting after standard polyacrylamide gel electrophoresis the antibody to MYH15 reacted selectively with MYH from rat EO muscles, whereas other striated muscles were completely unreactive (Fig. 3*A*). Western blots of MYHs separated in polyacrylamide gel containing high glycerol concentration show that the MYH band recognized by anti-MYH15 comigrates with the high mobility β/slow MYH band present in slow muscles (Fig. 3*B*). Analysis of serial transverse sections shows that MYH15 is present in almost all fibres of the orbital layer and in rare fibres of the global layer of EO muscles (Fig. 3*C*). This pattern of distribution is very similar to that of embryonic MYH, although a number of EO muscle fibres were reactive for MYH15 but not embryonic MYH and vice versa (Fig. S3). In contrast, MYH15 distribution is completely different from that of slow-tonic MYH, detected by anti-chicken ALD myosin, which is present in a minor population of fibres present in both the orbital and global layer, with the tendency for a weaker intensity of staining in positive global fibres (Fig. 3*C*). MYH15 is a sarcomeric myosin, as shown by immunofluorescence staining of A bands in longitudinal sections of EO muscles (Fig. S5). MYH15 is not detected in other cranial muscles, such as tongue and masseter (not shown). In fast and slow hindlimb muscles, anti-MYH15 stains exclusively rare small fibres, corresponding to the extracapsular polar regions of the nuclear bag fibres of muscle spindles, while both bag and chain intrafusal fibres are unreactive in the intracapsular region (Fig. 3*D*). This staining pattern contrasts with that of embryonic MYH, present mostly in the chain fibres, and of slow-tonic MYH, which is also expressed in the equatorial region of bag fibres but not in chain fibres (Bormioli *et al.* 1980; see also Walro & Kucera, 1999).

Next, we examined the expression of MYH14 protein. Western blots show no reactivity with different rat or

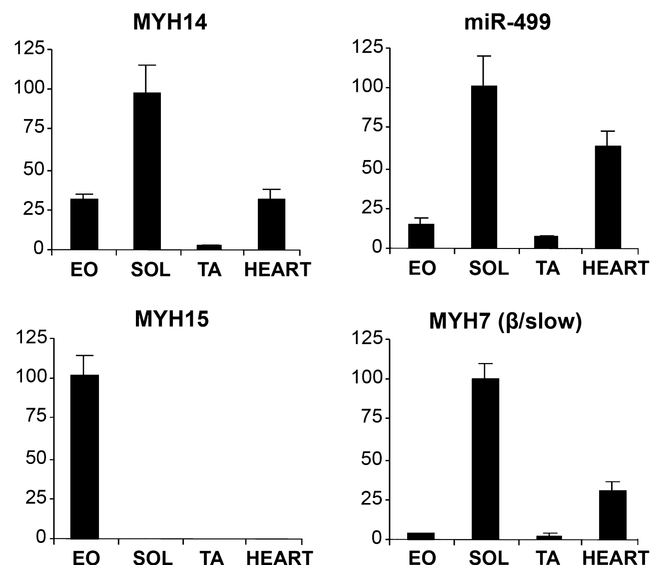

Figure 2. Quantitative RT-PCR analysis of different rat striated muscles with probes specific for *MYH14* and *MYH15* gene transcripts

The expression pattern of *MYH7*, coding for β/slow MYH, is shown for comparison. Transcripts levels are normalized to housekeeping genes and expressed as the percentage of the tissue with the highest expression level. Note that the distribution of miR-499 is similar to that of *MYH14*. EO: extraocular muscles; SOL: slow-twitch soleus muscle; TA: fast-twitch tibialis anterior muscle. Data are means ± S.E.M.; $n = 6$ (*MYH14* and *MYH15*), $n = 3$ (*MYH7* and mir-499).

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

mouse muscles, but give a strong reaction with chicken ALD MYH (Fig. 4*A*). In contrast, a specific reaction with MYH from human EO but not vastus lateralis muscles was easily detected (Fig. 4*B*). Glycerol gel analysis shows that the MYH band recognized by anti-MYH14 antibody migrates slightly slower than human β/slow MYH (Fig. 4*C*). By immunofluorescence, MYH14 is detected in a minor fibre population present in the orbital layer and occasional global layer fibres of rat EO muscles: these fibres correspond to slow-tonic fibres labelled by anti-ALD myosin and S46 antibodies and co-express MYH15 (Fig. 4*D*). In rat hindlimb muscles anti-MYH14 antibody reacts with different intensities with the two bag fibres of muscle spindles, strongly with bag 2 and weakly with bag 1 fibres, a pattern similar to that obtained with anti-ALD (Fig. 4*E*). In human EO muscles, MYH14 positive fibres are more numerous than in rat, which might explain the different results in Western blotting, and widespread in both orbital and global layers (Fig. 4*F*). MYH14 positive fibres correspond to slow-tonic fibres stained by anti-ALD (Fig. S4). Anti-MYH14 antibody stains specifically the sarcomere A-band in longitudinal sections of EO muscles (Fig. S5).

Developmental expression of MYH14 and MYH15

During development, the pattern of expression of MYH15 is completely different from that of MYH14. MYH15 is not detected by PCR in skeletal muscle or heart from E12

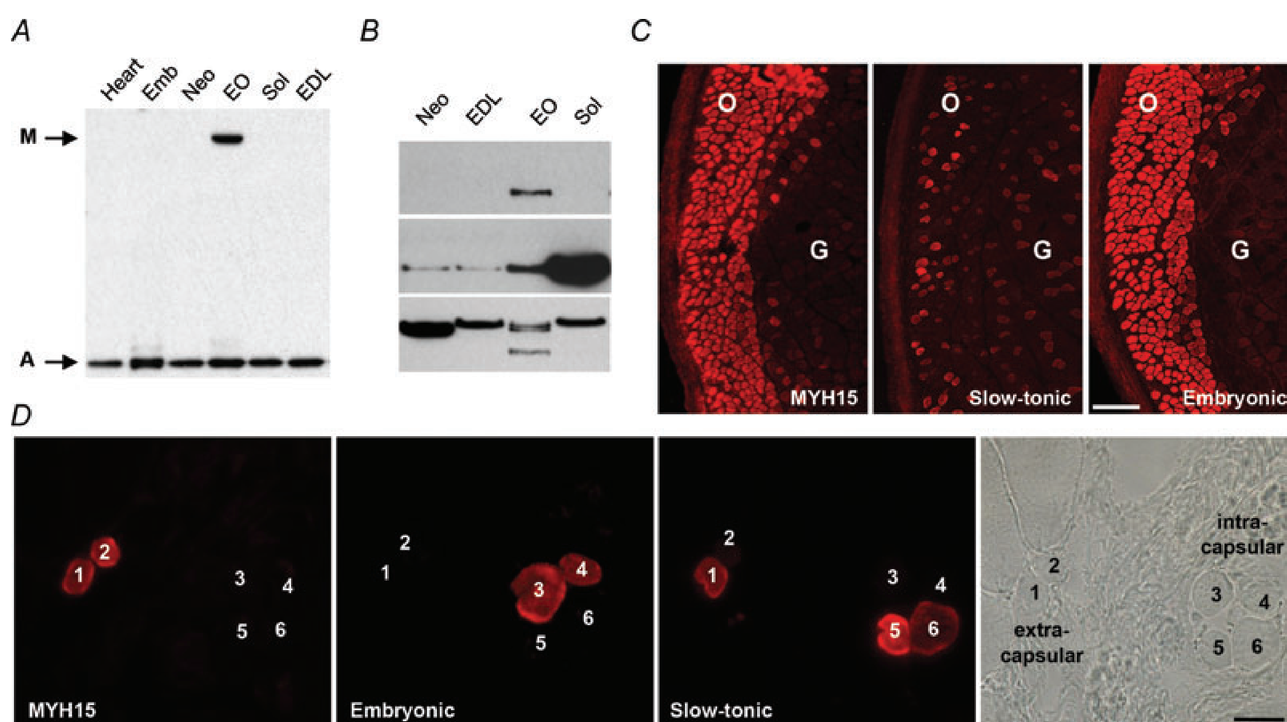

Figure 3. Distribution of MYH15 in adult muscles
A, Western blot analysis of homogenates from different rat striated muscles with anti-MYH15 antibody. Note specific reaction of this antibody with MYH (M) from EO muscles. The same blot was reacted with an antibody against α-actin (*A*) to demonstrate equal loading of all lanes. Emb: embryonic day 20 (E20) hindlimb skeletal muscles; Neo: neonatal hindlimb muscles; SOL: adult slow soleus muscle; EDL: adult fast extensor ditorum longus muscle. *B*, myosins from different rat muscles were separated by SDS-PAGE using glycerol gels, a procedure which allows separation of different MYH isoforms, blotted and reacted with antibodies against MYH15 (upper panel), MYH15 followed by anti-β/slow MYH (middle panel), or MYH15 followed by an antibody reactive with fast 2A- and 2X-MYH and developing MYHs (lower panel). Note that MYH15 co-migrates with MYH-β/slow. *C*, serial transverse sections of rat EO muscle reacted with antibodies specific for MYH15, chicken ALD MYH (slow-tonic) or embryonic MYH. Note that MYH15 protein, like embryonic MYH, is expressed in most fibres of the orbital layer (O) and rare fibres of the global layer (G), whereas slow-tonic MYH is present in a very minor fibre population mostly localized in the orbital layer. Scale bar, 100 μm. *D*, serial transverse sections of rat soleus muscle showing two muscle spindles, one cut through the intracapsular region and one through the polar extracapsular region, stained with antibodies specific for MYH15, chicken ALD MYH (slow-tonic) and embryonic MYH or examined by phase contrast microscopy (right panel). Note selective expression of MYH15 in the polar region of the two bag intrafusal fibres (fibres 1 and 2), whereas embryonic MYH is expressed in the chain fibres (fibres 3 and 4) and slow-tonic MYH is present in the intracapsular region of two bag fibres (fibres 5 and 6) and in the polar region of one bag fibre (fibre 1). Extrafusal fibres are unstained. Scale bar, 20 μm.

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

mouse embryos (Fig. S6A), nor by immunofluorescence in fetal and neonatal hindlimb muscles (not shown). MYH15 is also not found in fetal and neonatal EO muscles, being first detected at postnatal day 7 (P7) and at higher levels at P14 (Fig. 5). Since its first appearance at P7, MYH15 is exclusively present in the peripheral region of EO muscles, corresponding to the developing orbital layer (Fig. 5). In contrast, MYH14 transcripts are detected by PCR at low levels in skeletal muscle and heart from E12 mouse embryos (Fig. S6A). In fetal and neonatal rat muscles, MYH14 protein is detected by immunofluorescence in the rare fibres, also stained by anti-ALD and S46 antibodies, destined to become the bag 2 fibres of muscle spindles (Fig. S6B). MYH14 protein is expressed at high levels in all rat EO muscle fibres since E16 (Fig. S6C) but disappears from most fibres, except the slow-tonic fibres, during the first two weeks after birth (Fig. 5).

Discussion

The expression analysis of the two sarcomeric MYHs, MYH14 and MYH15, reported here completes the picture of sarcomeric *MYH* gene expression in mammalian striated muscles (Fig. 6). We find that *MYH14* gene, the orthologue of chicken *slow myosin 2*, is detected in both heart and slow skeletal muscles at the transcript level, but only in the slow-tonic fibres of EO muscles and in bag fibres of muscle spindles at the protein level. *MYH14* is, thus, the gene coding for the mammalian slow-tonic myosin, whose existence was previously

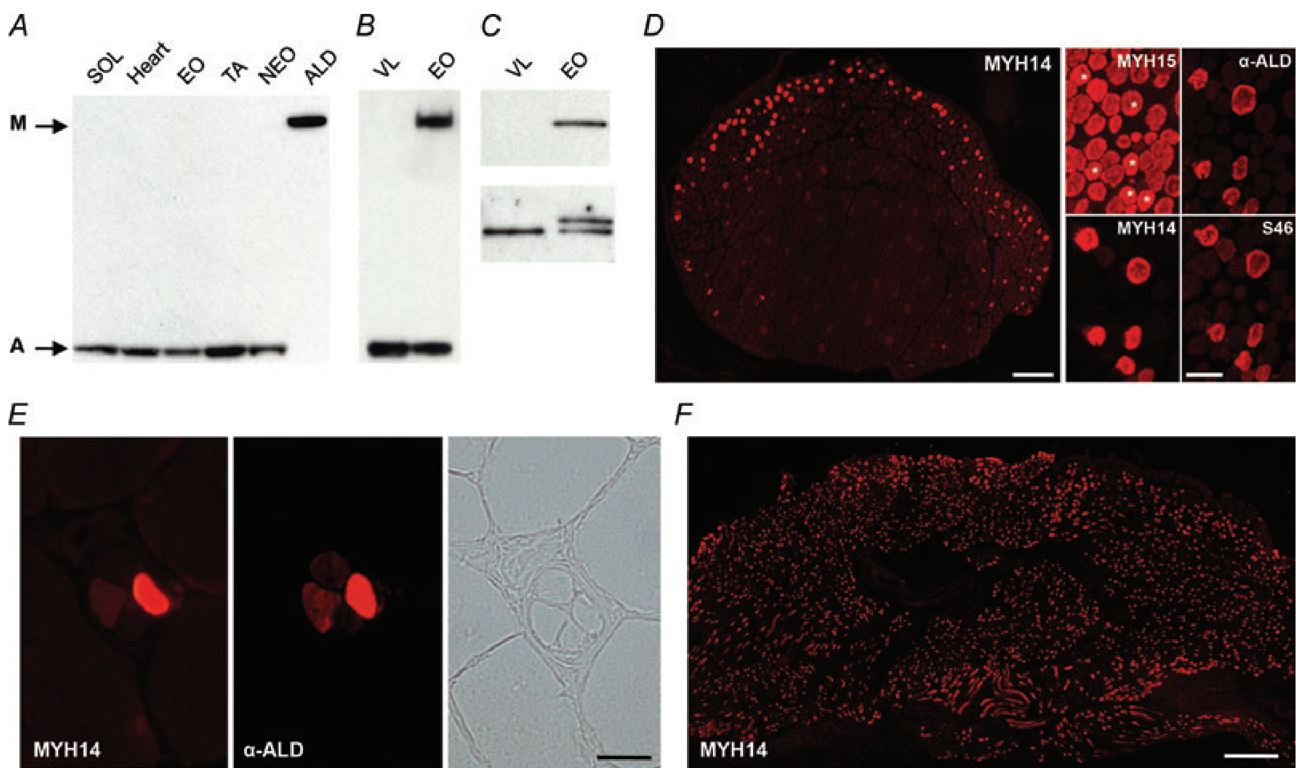

Figure 4. Distribution of MYH14 in adult muscles
A, Western blot analysis of homogenates from different rat striated muscles and chicken ALD muscle with anti-MYH14 antibody. The same blot was reacted with an antibody against α-actin to demonstrate equal loading of all lanes with the exception of ALD, that was about 50 times less loaded. *B*, Western blot of homogenates from human EO and vastus lateralis (VL) muscles with anti-MYH14 antibody. *C*, Western blot with human muscle myosins separated in glycerol gels, blotted and reacted with antibodies against MYH14 (upper panel), or MYH14 followed by anti-MYH-β/slow (lower panel). MYH14 corresponds to a band with lower electrophoretic mobility compared to MYH-β/slow. *D*, transverse sections of rat EO muscle reacted with antibodies specific for MYH14, showing few reactive fibres mostly localized in the orbital layer (left panel). These fibres correspond to slow-tonic fibres labelled by anti-ALD and S46 antibodies, as shown at high power in the right panels. Note that these fibres also co-express MYH15. Scale bar left panel, 100 μm; scale bar right panels, 25 μm. *E*, serial transverse sections of rat soleus muscle showing a muscle spindle cut through the intracapsular region, stained with anti-MYH14 or anti-ALD or examined by phase contrast microscopy (right panel). Note similar staining pattern of the two antibodies with stronger reactivity in one of the two bag fibres, corresponding to bag 2 fibre. Also note that surrounding extrafusal fibres are unstained. Scale bar, 20 μm. *F*, transverse sections of human EO muscle reacted with antibodies specific for MYH14, showing numerous reactive fibres. Scale bar, 500 μm.

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

Table 1. Expression pattern of mammalian *MYH14* and *MYH15* genes and their orthologues in different vertebrate classes[a]

		Cardiac muscle		Skeletal muscle		
		Embryonic	Adult	Embryonic	Adult	Refs
MYH14	Torafugu	+ mRNA	+ mRNA	+ mRNA	+ mRNA	b
	Xenopus	n.d.	n.d.	n.d.	+ slow-tonic m., EO m.	c
	Chicken (*SM2*)	+ conduction tissue	+ conduction tissue	+ slow m. (minor component)	+ slow m. (major component), EO m.	d
	Mammals	+ mRNA, low levels	+ mRNA − protein	+ mRNA, low levels + protein, EO m.	+ mRNA, slow m. + protein, EO m.	e
MYH15	*Xenopus* (*vMYH*)	+ conduction tissue	+ (mRNA)	−	−	f
	Chicken (*vMYH*)	+ ventricle	+ ventricle	+	+ EO m.	g
	Mammals	−	−	−	+ EO m.	h

[a]Abbreviations: m., muscle fibres; n.d., not determined; *vMYH*, ventricular *MYH*; *SM2*, *slow myosin 2*; EO: extraocular. The signs + (present) and − (absent) refer to analyses at the protein level unless indicated otherwise. [b]Ikeda *et al.* 2007. [c]Bormioli *et al.* 1980; Dieringer & Rowlerson, 1984. [d]Sartore *et al.* 1978; Matsuda *et al.* 1982; Gonzalez-Sanchez & Bader, 1985; Machida *et al.* 2002; our unpublished studies. [e]Bormioli *et al.* 1979; Bormioli *et al.* 1980; present study. [f]Garriock *et al.* 2005. [g]Masaki & Yoshizaki, 1974; Gorza *et al.* 1983; Sweeney *et al.* 1984; Bisaha & Bader, 1991; Stewart *et al.* 1991; our unpublished studies. [h]Present study.

postulated based on the reactivity of anti-chicken myosin antibodies but was never demonstrated. In contrast, *MYH15* gene, the orthologue of frog and chicken *ventricular MYH*, is expressed exclusively in a restricted region of EO muscles and in the extracapsular region of bag fibres of muscle spindles. The results of this study are of interest with respect both to *MYH* gene evolution in vertebrates and to the possible functional significance of these genes.

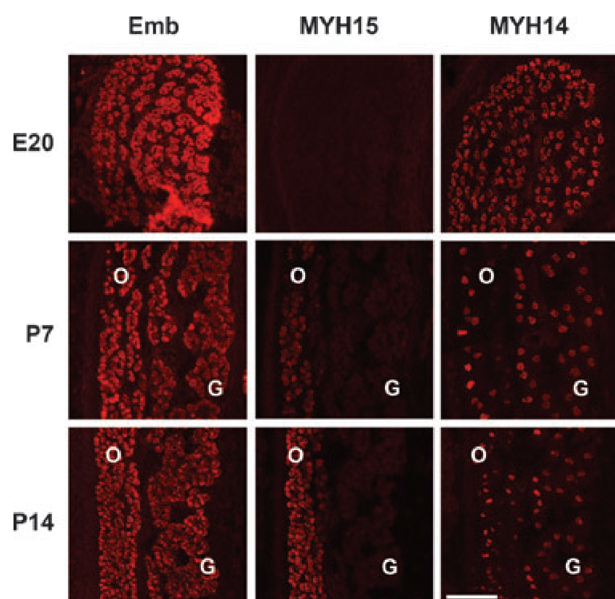

Figure 5. Serial sections of developing rat EO muscles stained for embryonic MYH (left panels), MYH15 (middle panels) or MYH14 (right panels)

Note that MYH15 is undetectable in fetal muscles (E20), is barely visible in the orbital layer at postnatal day 7 (P7) and is clearly expressed at P14. In contrast, MYH14 is expressed in all fibres in fetal EO muscles and disappears in most fibres except the slow-tonic fibres during early postnatal stages. Scale bar, 100 μm.

Evolutionary tinkering with sarcomeric myosin genes

It is of interest to compare the pattern of expression of mammalian *MYH15* gene with that of the orthologue *ventricular MYH* in frog and chicken (Table 1). The *Xenopus* ventricular MYH is expressed in both embryonic and adult heart and is never detected in skeletal muscle, with the exception of a weak transient expression in jaw muscles of stage 49 larvae (Garriock *et al.* 2005; Nasipak & Kelley, 2008). Immunocytochemical studies showed that chicken ventricular MYH is expressed in embryonic and adult heart, as well as embryonic and regenerating but not adult skeletal muscle (Masaki & Yoshizaki, 1974; Cantini *et al.* 1980; Gorza *et al.* 1983; Sweeney *et al.* 1984) and these findings were confirmed by analyses at the transcript level (Bisaha & Bader, 1991; Stewart *et al.* 1991). However, we found that this myosin is also expressed in peripheral fibres in adult chicken EO muscles not shown. The pattern of expression of MYH15 is still different in mammals, as this myosin is exclusively detected in adult EO muscles and in the extracapsular region of bag intrafusal fibres. This appears to be a striking case of evolutionary tinkering (Jacob, 1977), whereby a myosin gene used to control cardiac pumping function in amphibians is used to control eyeball movement in mammals. The change in functional specialization appears to correlate with major structural remodelling of the myosin molecule, including loop 1 structure: the sequence of *Xenopus* ventricular MYH is only 65% identical to mouse *MYH15*, whereas *Xenopus* atrial MYH and mouse α-MYH, which are both expressed in cardiac muscle and presumably have similar function, are 88% identical (Garriock *et al.* 2005).

The evolution of the *MYH14* gene is also remarkable and seems to parallel the evolution of the slow-tonic fibres, which represent a major slow fibre population in amphibian and avian skeletal muscles, whereas they are confined to the EO muscles in mammals concomitantly

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

with the emergence of slow-twitch fibres and β/slow MYH as the major slow component. These findings validate the notion that two distinct myosins are present in slow-twitch and slow-tonic muscle fibres (Bormioli *et al.* 1980). A striking aspect of the *MYH14* gene in mammals is the dissociation between the expression of the transcript, which is abundant in heart and slow-twitch muscles, at least in rat and mouse, and the corresponding protein, which is not detected in these tissues. To our knowledge this finding is unprecedented in the field of sarcomeric MYHs and points to a post-transcriptional level of MYH gene regulation that allows a wider expression of MYH14 transcript to both cardiac and skeletal muscles but restricts the expression of MYH14 protein to a specific minor population of muscle fibres in EO muscles and muscle spindles. The significance of this mismatch remains to be established. It is tempting to speculate that the unique pattern of *MYH14* gene regulation may be related to the presence within this gene of a microRNA, miR-499, which is also expressed in fish cardiac and skeletal muscle (Kloosterman *et al.* 2006) and might thus have maintained an evolutionary conserved function in all vertebrates. miR-499 has numerous potential targets, among which the transcription factor Sox6. Interestingly, Sox6 has been shown to repress slow gene expression in both mouse (Hagiwara *et al.* 2005, 2007) and zebrafish (von Hofsten *et al.* 2008). Thus the expression of MYH14 and miR-499 in slow but not fast muscles might play a role in the induction/maintenance of the slow phenotype by abrogating the Sox6-mediated repression of slow muscle-specific genes.

Functional significance of MYH14 and MYH15

It was previously suggested that MYH14 and MYH15 represent slow-type myosins based on sequence comparison of their motor domain which is more similar to that of slow than fast myosins (Desjardins *et al.* 2002). This conclusion is consistent with the fact that mammalian *MYH14* is orthologous to a chicken slow myosin (*SM2*), a major MYH component in the chicken slow ALD muscle, both with respect to sequence similarity (McGuigan *et al.* 2004) and syntheny (this paper), and is supported by the finding that MYH14 transcripts are expressed in cardiac and slow but not fast skeletal muscle. Finally, and more importantly, MYH14 is selectively expressed in multiply innervated EO fibres labelled by anti-ALD myosin antibody. The existence of a specific slow-tonic myosin in these fibres was first suggested based on the reactivity of a polyclonal antibody against chicken ALD muscle myosin (Bormioli *et al.* 1979; Bormioli *et al.* 1980) and later with different monoclonal anti-chicken myosin antibodies (see Pedrosa & Thornell, 1990; Kucera *et al.* 1992), many of which show partial cross-reactivity with the myosin β/slow present in slow-twitch fibres (Sokoloff *et al.* 2007). Therefore it was never possible to rule out the possibility that this immunoreactivity could be due to post-translational modifications of another myosin, e.g. MYH-β/slow, rather than to a distinct novel isoform. Our results provide the definitive evidence that slow-tonic MYH indeed exists as a specific MYH isoform coded by a distinct gene.

Circumstantial evidence supports the notion that also MYH15 is a slow-type myosin. First, the *MYH15* is orthologous to chicken *ventricular MYH* with respect to both sequence similarity (McGuigan *et al.* 2004) and synteny (Garriock *et al.* 2005), and it has been shown that ventricular *MYH* has a relatively low Ca^{2+}-ATPase activity, similar to that of the slow ALD muscle (Dalla Libera *et al.* 1979). However, direct enzymatic and *in vitro* motility assays with purified myosins will be necessary to provide definitive evidence about their functional properties. Interpretation of the physiology of the muscle fibres containing MYH14 or MYH15 is complicated by the fact that these myosins are always co-expressed with other myosins within the same muscle fibres, e.g. MYH15 is co-expressed with embryonic MYH in most orbital layer fibres and slow-tonic fibres co-express MYH14 and MYH15.

The selective expression of MYH15 in the orbital layer of EO muscles is consistent with the existence of major

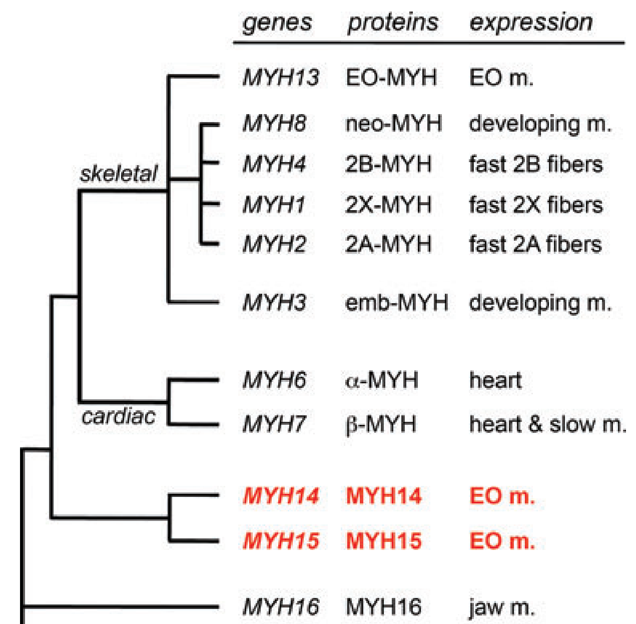

Figure 6. Scheme illustrating the evolutionary relationships among sarcomeric *MYH* genes in mammals, with the corresponding protein products and their expression pattern
The phylogenetic tree on the left is modified from that of the human MYH head domain (see McGuigan *et al.* 2004; Ikeda *et al.* 2007). Spacing and length of the branches do not reflect actual scale in this simplified scheme.

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

anatomical and physiological differences between orbital and global layers. It is now recognized that while global layer fibres insert directly on the globe, orbital layer fibres insert into pulleys, rings of dense collagen containing smooth muscle cells and elastin encircling EO muscles (Demer *et al.* 1995; Lim *et al.* 2007). According to the active pulley hypothesis, the orbital layer is predominantly involved in adjusting the pulley position, whereas the global layer is responsible for eye rotation. Analysis of gene expression profiles revealed that a large number of genes are differentially expressed in orbital *versus* global layer fibres (Budak *et al.* 2004; Khanna *et al.* 2004). However, *MYH15*, which was not identified in those studies, appears to be the only gene uniquely expressed in the orbital layer of EO muscles. On the other hand, MYH15 is not an embryonic muscle marker, since it is not expressed in embryonic and fetal muscles and is first detected after birth at P7 in the orbital layer of EO muscles and at higher levels at P14. This developmental pattern is the opposite of that of MYH14, which is widely expressed in EO muscles since early developmental stages but is down-regulated in most fibres, except the rare slow-tonic fibres, during the first two weeks after birth. These early postnatal stages correspond to the critical period when visual experience is required for the correct maturation of both sensory visual and oculomotor system. It will be of interest to determine whether visual deprivation during this critical developmental period affects the postnatal changes in the pattern of expression of MYH14 and MYH15 in EO muscles, as previously shown for the EO-specific myosin, MYH13 (Brueckner & Porter, 1998).

Conclusions

The findings reported in the present study complete the inventory of the myosin heavy chain isoforms which act as molecular motors of the contraction in mammalian striated muscle fibres. In particular, the present results provide an unambiguous and definitive identification of the myosin isoform expressed in slow-tonic fibres and identify a novel and unexpected myosin isoform present in the orbital layer of extraocular muscles. Such identification represents the basis for a thorough functional characterization.

References

Berg JS, Powell BC & Cheney RE (2001). A millennial myosin census. *Mol Biol Cell* **12**, 780–794.

Bisaha JG & Bader D (1991). Identification and characterization of a ventricular-specific avian myosin heavy chain, VMHC1: expression in differentiating cardiac and skeletal muscle. *Dev Biol* **148**, 355–364.

Bormioli SP, Sartore S, Vitadello M & Schiaffino S (1980). 'Slow' myosins in vertebrate skeletal muscle. An immunofluorescence study. *J Cell Biol* **85**, 672–681.

Bormioli SP, Torresan P, Sartore S, Moschini GB & Schiaffino S (1979). Immunohistochemical identification of slow-tonic fibres in human extrinsic eye muscles. *Invest Ophthalmol Vis Sci* **18**, 303–306.

Brueckner JK & Porter JD (1998). Visual system maldevelopment disrupts extraocular muscle-specific myosin expression. *J Appl Physiol* **85**, 584–592.

Budak MT, Bogdanovich S, Wiesen MH, Lozynska O, Khurana TS & Rubinstein NA (2004). Layer-specific differences of gene expression in extraocular muscles identified by laser-capture microscopy. *Physiol Genomics* **20**, 55–65.

Cantini M, Sartore S & Schiaffino S (1980). Myosin types in cultured muscle cells. *J Cell Biol* **85**, 903–909.

Dalla Libera L, Sartore S & Schiaffino S (1979). Comparative analysis of chicken atrial and ventricular myosins. *Biochim Biophys Acta* **581**, 283–294.

Demer JL, Miller JM, Poukens V, Vinters HV & Glasgow BJ (1995). Evidence for fibromuscular pulleys of the recti extraocular muscles. *Invest Ophthalmol Vis Sci* **36**, 1125–1136.

Desjardins PR, Burkman JM, Shrager JB, Allmond LA & Stedman HH (2002). Evolutionary implications of three novel members of the human sarcomeric myosin heavy chain gene family. *Mol Biol Evol* **19**, 375–393.

Dieringer N & Rowlerson A (1984). Fiber types in the extra-ocular muscles of the frog. *J Physiol* **353**, 50.

Garriock RJ, Meadows SM & Krieg PA (2005). Developmental expression and comparative genomic analysis of *Xenopus* cardiac myosin heavy chain genes. *Dev Dyn* **233**, 1287–1293.

Golomb E, Ma X, Jana SS, Preston YA, Kawamoto S, Shoham NG, Goldin E, Conti MA, Sellers JR & Adelstein RS (2004). Identification and characterization of nonmuscle myosin II-C, a new member of the myosin II family. *J Biol Chem* **279**, 2800–2808.

Gonzalez-Sanchez A & Bader D (1985). Characterization of a myosin heavy chain in the conductive system of the adult and developing chicken heart. *J Cell Biol* **100**, 270–275.

Gorza L, Sartore S, Triban C & Schiaffino S (1983). Embryonic-like myosin heavy chains in regenerating chicken muscle. *Exp Cell Res* **143**, 395–403.

Hagiwara N, Ma B & Ly A (2005). Slow and fast fibre isoform gene expression is systematically altered in skeletal muscle of the Sox6 mutant, p100H. *Dev Dyn* **234**, 301–311.

Hagiwara N, Yeh M & Liu A (2007). Sox6 is required for normal fibre type differentiation of fetal skeletal muscle in mice. *Dev Dyn* **236**, 2062–2076.

Ikeda D, Ono Y, Snell P, Edwards YJ, Elgar G & Watabe S (2007). Divergent evolution of the myosin heavy chain gene family in fish and tetrapods: evidence from comparative genomic analysis. *Physiol Genomics* **32**, 1–15.

Jacob F (1977). Evolution and tinkering. *Science* **196**, 1161–1166.

Khanna S, Cheng G, Gong B, Mustari MJ & Porter JD (2004). Genome-wide transcriptional profiles are consistent with functional specialization of the extraocular muscle layers. *Invest Ophthalmol Vis Sci* **45**, 3055–3066.

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

Kloosterman WP, Steiner FA, Berezikov E, de Bruijn E, van de Belt J, Verheul M, Cuppen E & Plasterk RH (2006). Cloning and expression of new microRNAs from zebrafish. *Nucleic Acids Res* **34**, 2558–2569.

Kucera J, Walro JM & Gorza L (1992). Expression of type-specific MHC isoforms in rat intrafusal muscle fibres. *J Histochem Cytochem* **40**, 293–307.

Leal A, Endele S, Stengel C, Huehne K, Loetterle J, Barrantes R, Winterpacht A & Rautenstrauss B (2003). A novel myosin heavy chain gene in human chromosome 19q13.3. *Gene* **312**, 165–171.

Lim KH, Poukens V & Demer JL (2007). Fascicular specialization in human and monkey rectus muscles: evidence for anatomic independence of global and orbital layers. *Invest Ophthalmol Vis Sci* **48**, 3089–3097.

Machida S, Noda S, Takao A, Nakazawa M & Matsuoka R (2002). Expression of slow skeletal myosin heavy chain 2 gene in Purkinje fiber cells in chick heart. *Biol Cell* **94**, 389–399.

Masaki T & Yoshizaki C (1974). Differentiation of myosin in chick embryos. *J Biochem* **76**, 123–131.

Matsuda R, Bandman E & Strohman RC (1982). The two myosin isoenzymes of chicken anterior latissimus dorsi muscle contain different myosin heavy chains encoded by separate mRNAs. *Differentiation* **23**, 36–42.

McGuigan K, Phillips PC & Postlethwait JH (2004). Evolution of sarcomeric myosin heavy chain genes: evidence from fish. *Mol Biol Evol* **21**, 1042–1056.

Miller JB, Crow MT & Stockdale FE (1985). Slow and fast myosin heavy chain content defines three types of myotubes in early muscle cell cultures. *J Cell Biol* **101**, 1643–1650.

Mizunoya W, Wakamatsu J, Tatsumi R & Ikeuchi Y (2008). Protocol for high-resolution separation of rodent myosin heavy chain isoforms in a mini-gel electrophoresis system. *Anal Biochem* **377**, 111–113.

Morgan DL & Proske U (1984). Vertebrate slow muscle: its structure, pattern of innervation, and mechanical properties. *Physiol Rev* **64**, 103–169.

Murgia M, Serrano AL, Calabria E, Pallafacchina G, Lomo T & Schiaffino S (2000). Ras is involved in nerve-activity-dependent regulation of muscle genes. *Nat Cell Biol* **2**, 142–147.

Nagase T, Kikuno R, Ishikawa K, Hirosawa M & Ohara O (2000). Prediction of the coding sequences of unidentified human genes. XVII. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. *DNA Res* **7**, 143–150.

Nasipak BT & Kelley DB (2008). The genome of the diploid anuran *Xenopus tropicalis* contains a novel array of sarcoplasmic myosin heavy chain genes expressed in larval muscle and larynx. *Dev Genes Evol* **218**, 389–397.

Page S, Miller JB, DiMario JX, Hager EJ, Moser A & Stockdale FE (1992). Developmentally regulated expression of three slow isoforms of myosin heavy chain: diversity among the first fibres to form in avian muscle. *Dev Biol* **154**, 118–128.

Pedrosa F & Thornell LE (1990). Expression of myosin heavy chain isoforms in developing rat muscle spindles. *Histochemistry* **94**, 231–244.

Qin H, Hsu MK, Morris BJ & Hoh JF (2002). A distinct subclass of mammalian striated myosins: structure and molecular evolution of 'superfast' or masticatory myosin heavy chain. *J Mol Evol* **55**, 544–552.

Sartore S, Pierobon-Bormioli S & Schiaffino S (1978). Immunohistochemical evidence for myosin polymorphism in the chicken heart. *Nature* **274**, 82–83.

Schachat F & Briggs MM (1999). Identification of two patterns of exon organization in the human striated muscle myosin heavy chain genes. *Mol Cell Biol* **10s**, 34a.

Schiaffino S, Gorza L, Sartore S, Saggin L, Ausoni S, Vianello M, Gundersen K & Lomo T (1989). Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J Muscle Res Cell Motil* **10**, 197–205.

Schiaffino S, Gorza L, Sartore S, Saggin L & Carli M (1986). Embryonic myosin heavy chain as a differentiation marker of developing human skeletal muscle and rhabdomyosarcoma. A monoclonal antibody study. *Exp Cell Res* **163**, 211–220.

Sokoloff AJ, Li H & Burkholder TJ (2007). Limited expression of slow tonic myosin heavy chain in human cranial muscles. *Muscle Nerve* **36**, 183–189.

Stedman HH, Kozyak BW, Nelson A, Thesier DM, Su LT, Low DW, Bridges CR, Shrager JB, Minugh-Purvis N & Mitchell MA (2004). Myosin gene mutation correlates with anatomical changes in the human lineage. *Nature* **428**, 415–418.

Stewart AF, Camoretti-Mercado B, Perlman D, Gupta M, Jakovcic S & Zak R (1991). Structural and phylogenetic analysis of the chicken ventricular myosin heavy chain rod. *J Mol Evol* **33**, 357–366.

Sweeney HL, Rosenfeld SS, Brown F, Faust L, Smith J, Xing J, Stein LA & Sellers JR (1998). Kinetic tuning of myosin via a flexible loop adjacent to the nucleotide binding pocket. *J Biol Chem* **273**, 6262–6270.

Sweeney LJ, Clark WA Jr, Umeda PK, Zak R & Manasek FJ (1984). Immunofluorescence analysis of the primordial myosin detectable in embryonic striated muscle. *Proc Natl Acad Sci U S A* **81**, 797–800.

Toniolo L, Cancellara P, Maccatrozzo L, Patruno M, Mascarello F & Reggiani C (2008). Masticatory myosin unveiled: first determination of contractile parameters of muscle fibres from carnivore jaw muscles. *Am J Physiol Cell Physiol* **295**, C1535–1542.

van Rooij E, Liu N & Olson EN (2008). MicroRNAs flex their muscles. *Trends Genet* **24**, 159–166.

von Hofsten J, Elworthy S, Gilchrist MJ, Smith JC, Wardle FC & Ingham PW (2008). Prdm1- and Sox6-mediated transcriptional repression specifies muscle fibre type in the zebrafish embryo. *EMBO Rep* **9**, 683–689.

Walro JM & Kucera J (1999). Why adult mammalian intrafusal and extrafusal fibres contain different myosin heavy-chain isoforms. *Trends Neurosci* **22**, 180–184.

Weiss A, McDonough D, Wertman B, Acakpo-Satchivi L, Montgomery K, Kucherlapati R, Leinwand L & Krauter K (1999*a*). Organization of human and mouse skeletal myosin heavy chain gene clusters is highly conserved. *Proc Natl Acad Sci U S A* **96**, 2958–2963.

© 2010 The Authors. Journal compilation © 2010 The Physiological Society

Weiss A, Schiaffino S & Leinwand LA (1999*b*). Comparative sequence analysis of the complete human sarcomeric myosin heavy chain family: implications for functional diversity. *J Mol Biol* **290**, 61–75.

Author contributions

S.S. and C.R. designed the research; A.C.R, C.M. and C.A. performed the research; S.S. wrote the paper with critical input from C.R. All authors read and approved the manuscript for publication.

Acknowledgements

This work was supported by grants from the European Union (MYORES and EXGENESIS to S.S.), the Italian Ministry of Education, University and Research (PRIN 2004 to C.R. and S.S.) and the Italian Space Agency (ASI, project OSMA to C.R. and S.S.). We thank Professor C. Poggesi (University of Florence), Drs. D. Camposampiero, M. Fanin and E. Pegoraro for samples of human muscles.

© 2010 The Authors. Journal compilation © 2010 The Physiological Society