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Nucleotide and protein sequences for dog masticatory tropomyosin identify a novel *Tpm4* gene product

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Abstract

Jaw-closing muscles of several vertebrate species, including members of *Carnivora*, express a unique, “masticatory”, isoform of myosin heavy chain, along with isoforms of other myofibrillar proteins that are not expressed in most other muscles. It is generally believed that the complement of myofibrillar isoforms in these muscles serves high force generation for capturing live prey, breaking down tough plant material and defensive biting. A unique isoform of tropomyosin (Tpm) was reported to be expressed in cat jaw-closing muscle, based upon two-dimensional gel mobility, peptide mapping, and immunohistochemistry. The objective of this study was to obtain protein and gene sequence information for this unique Tpm isoform. Samples of masseter (also a jaw-closing muscle), tibialis (with predominantly fast-twitch fibers), and the deep lateral gastrocnemius (predominantly slow-twitch fibers) were obtained from adult dogs. Expressed Tpm isoforms were cloned and sequencing yielded cDNAs that were identical to genomic predicted striated muscle Tpm1.1_{St(a,b,b,a)} (historically referred to as α Tpm), Tpm2.2_{St(a,b,b,a)} (β Tpm) and Tpm3.12_{St(a,b,b,a)} (cTpm) isoforms (nomenclature reflects predominant tissue expression (“St”—striated muscle) and exon splicing pattern), as well as a novel 284 amino acid isoform observed in jaw-closing muscle that is identical to a genomic predicted product of the *Tpm4* gene (δ Tpm) family. The novel isoform is designated as Tpm4.3_{St(a,b,b,a)}. The myofibrillar Tpm isoform expressed in dog masseter exhibits a unique electrophoretic mobility on gels containing 6 M urea, compared to other skeletal Tpm isoforms. To validate that the cloned Tpm4.3 isoform is the Tpm expressed in dog masseter, *E. coli*-expressed Tpm4.3 was electrophoresed in the presence of urea. Results demonstrate that Tpm4.3 has identical electrophoretic mobility to the unique dog masseter Tpm isoform and is of different mobility from that of muscle Tpm1.1, Tpm2.2 and Tpm3.12 isoforms. We conclude that the unique Tpm isoform in dog masseter is a product of the *Tpm4* gene and that the 284 amino acid protein product of this gene represents a novel myofibrillar Tpm isoform never before observed to be expressed in striated muscle.

Keywords

Tropomyosin; Masseter; Dog; Jaw-adductor; Myosin; Masticatory

Introduction

Tropomyosin (Tpm) is a component of muscle thin filaments and has an important role in mediating calcium regulation of contraction (reviewed by Gunning et al. 2008). Tpm blocks the interaction of myosin and, therefore, contraction in the absence of calcium. Upon stimulation of striated muscle, the release of calcium from the sarcoplasmic reticulum enters the cytosol where it binds to and activates the troponin complex. This, in turn, results in movement of Tpm from its blocking state, exposing the myosin-binding sites on actin, allowing the formation of force-generating cross-bridges and contraction of muscle. Striated muscle activation is, therefore, a complex process involving multiple protein–protein interactions between the subunits of the troponin complex, Tpm, actin and myosin. Alterations of these proteins can significantly impact muscle activation.

Virtually all myofilament proteins, including Tpm, are expressed as families of isoforms and isoform expression patterns of these proteins are driven by isoform-specific genes or by alternative RNA splicing. Different muscle fiber types are commonly identified on the basis of this myofibrillar protein isoform complement, with myosin heavy chain (MHC) isoform typically being the main defining characteristic. Generally, fast and slow fibers, which differ on the basis of MHC isoform composition, also differ with respect to Tpm isoform composition. While cellular Tpm can be expressed as isoforms of either 248 or 284 amino acids, myofibrillar muscle Tpm isoforms are exclusively of the 284 amino acid class. Sarcomeric Tpm isoform expression is especially heterogeneous in vertebrate muscle (Perry 2001; Gunning et al. 2005). Fast muscle fibers express predominantly a Tpm1 gene product at varied amounts with a Tpm2 gene product (previously referred to as α Tpm and β Tpm, respectively (see Geeves et al. 2015 for recently proposed tropomyosin nomenclature). Slow muscle fibers predominantly express the same Tpm2 product and a product of the Tpm3 gene (previously referred to as γ Tpm).

The evolutionarily ancient masticatory myosin is expressed in jaw-closing muscles of virtually all members of *Carnivora*, all primates except humans, and multiple species of several other vertebrate orders, including rodents (gray and fox squirrels), reptiles (e.g., crocodiles and alligators) and fish (sharks) (Rowlerson et al. 1981, 1983a, b; Hoh 2002; Hoh et al. 2006; Reiser et al. 2009, 2010). This myosin, which is composed of a unique MHC (MHC-Mast), unique regulatory light chain and the embryonic/atrial isoform of essential light chain, is associated with very high force generation and intermediate shortening velocity (Kato et al. 1985; Reiser and Bicer 2007; Toniolo et al. 2008). These contractile properties of masticatory myosin serve well the feeding style of species that capture live prey and employ tearing and engulfing, with little or no chewing, for ingestion of food, or species that consume tough plant material, as well as for defensive biting.

MHC-Mast is regarded as being a unique isoform for several reasons. The homology of MHC-Mast with other MHC isoforms is much lower than the homologies among fast and

slow isoforms (Qin et al. 2002). Additionally, the gene that codes MHC-Mast is located in the genome remotely from the tandem arrays of genes that code fast and slow isoforms (Desjardins et al. 2002). In addition to this unique myosin isoform, a Tpm isoform of unique electrophoretic mobility from that of Tpm in fast or slow fibers was initially reported in cat jaw-closing masseter and temporalis muscles (Rowlerson et al. 1983a). Co-expression of this unique masticatory Tpm and MHC may both contribute to the unique contractile properties of this muscle. The present project was designed to address two major questions: (1) whether the Tpm associated with MHC-Mast is an isoform that was previously identified as being expressed in any other muscle or other tissue and (2) whether this Tpm isoform is coded by one of the *Tpm* genes that codes for the recognized sarcomeric Tpm isoforms (Tpm1, Tpm2, and Tpm3) or by a novel sarcomeric expressed *Tpm* gene.

Materials and methods

Samples

Tibialis (predominantly fast fiber type), deep portion of the lateral gastrocnemius (predominantly slow fiber type), left ventricle (a standard for the identification of Tpm1.1, previously referred to as “ α -Tpm”) and the jaw-closing masseter were obtained from adult domestic dogs (mongrel hounds, male, body mass 20–25 kg) immediately following euthanasia. The care and use of the dogs were approved by the Institutional Animal Care and Use Committee of The Ohio State University. All of the dogs were normal and healthy and had served as controls for other, unrelated studies. Based upon analysis of MHC isoform composition by gel electrophoresis (not shown), masticatory myosin predominates throughout the dog masseter (~70 % of total MHC), with slow myosin constituting most of the remaining MHC.

Gel electrophoresis and Western blot

A homogenate was prepared from a portion of each sample with urea sample buffer (8 M urea, 2 M thiourea, 75 mM dithiothreitol, 3 % SDS, 0.05 % bromophenol blue, 50 mM Tris-HCl, pH 6.8), as described in Blough et al. (1996). Aliquots of each sample were run on gels to separate Tpm isoforms in each sample. The inclusion of urea in separating gels, at a concentration of 6 M, was originally shown by Sender (1971) to modify the electrophoretic mobility of Tpm. It was recently reported that gels with 6 M urea yield separation of Tpm1.1, Tpm2.2 (previously β -Tpm) and Tpm3.12 (previously γ -Tpm) skeletal muscle isoforms in dog striated muscles (Bicer and Reiser 2013). Therefore, unless specified, separating gels in this study contained 6 M urea. Separating gels consisted of 12 % total acrylamide with a 200:1 acrylamide-to-*N,N'*-methylene-bis-acrylamide cross-linking ratio and pH 9.3. The stacking gels consisted of 4 % total acrylamide with a 50:1 cross-linking ratio and pH 6.8. The gels (16 cm $\times$ 18 cm) were run at 30 mA per gel in Hoefer SE600 units (Hoefer Scientific Instruments, San Francisco, CA) at 18 °C until the dye in the sample buffer migrated to the bottom of the plates (typically ~3 h). Some gels were either silver-stained (Blough et al. 1996) or stained with Coomassie Blue, and others were used for transfers to nitrocellulose for immunoblots (Bicer and Reiser 2013). The blots were probed with a monoclonal Tpm primary antibody, TM311 (T-2780, Sigma-Aldrich Co., St. Louis, MO; diluted 1:500) which is known to recognize Tpm1.1, Tpm2.2 and Tpm3.12 in multiple

species (Schevzov et al. 2011). We previously showed that this antibody recognizes dog Tpm1.1, Tpm2.2 and Tpm3.12, as well as the unique Tpm isoform in dog jaw-closing muscles (Bicer and Reiser 2013). Bundles of fibers were also isolated by dissection immediately after euthanasia from a portion of the tibialis, deep gastrocnemius and masseter and were stored at -20°C in relaxing solution with 50 % (v/v) glycerol to prepare skinned fibers (Bergrin et al. 2006). Single skinned fibers were subsequently isolated by dissection in relaxing solution and individually placed in microcentrifuge tubes to which gel sample buffer (Blough et al. 1996) was added (2 μl of buffer per ηl of fiber volume; fiber volume calculated from fiber length and estimated fiber width measurements).

Twenty skinned single fibers, isolated from the tibialis ($n = 2$), deep gastrocnemius ($n = 2$), superficial masseter ($n = 8$) and deep masseter ($n = 8$), were run on a gel (without urea) that was designed to separate myosin light chain isoforms. The results from this gel were used to identify limb fast and slow fibers, as well as fibers in the masseter that express masticatory myosin or slow myosin, based upon the distinct electrophoretic mobility of myosin light chains in each fiber type (Bicer and Reiser 2004; Reiser et al. 2010). Representative limb fast-type and slowtype fibers and fibers from the masseter that expressed either masticatory myosin or slow-type myosin were identified on this gel and aliquots of the selected fibers were run on a gel that included 6 M urea. Protein bands were transferred and probed with TM311 antibody to examine Tpm isoforms in these identified single fibers.

Cloning and sequencing

Cloning was conducted similar to that previously described with slight modification (Biesiadecki et al. 2002). Total RNA was isolated from dog tibialis cranialis and masseter muscles using ReliaPrep RNA Tissue Miniprep System (Promega, Madison, WI), according to the manufacturer's instructions. Muscle-specific cDNA libraries containing all mRNA expressed in the given muscle were generated employing the Reverse Transcription System (Promega, Madison, WI) by two-step RT-PCR with a cDNA Cloning Primer (5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTTV-3') according to the manufacturer's instructions. Tpm-specific cDNAs were then amplified from this library by PCR with degenerative dog Tpm primers (Dog Tpm Fwd, 5'-GCCATATGGASGCSATCAAGAARAAGATGC-3') and a 3' RACE reverse primer (5'-GGCCACGCGTCGACTAGTAC-3'). The PCR conditions were as follows: initial denaturation at 94°C for 5 min.; 30 cycles of 94°C for 20 s., $55\text{--}60^{\circ}\text{C}$ for 30 s., and 68°C for 90 s.; and final elongation at 72°C for 7 min. PCR products of approximately 1.1 kb were gel-purified with the QIAEX II Gel Extraction Kit (Qiagen) and cloned into the pCR-Blunt II-TOPO vector using Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Transformation of One Shot competent *Escherichia coli* cells (Invitrogen) and subsequent plasmid minipreps were performed using the Pure-Link Quick Plasmid Miniprep Kit (Invitrogen). Resultant clones were sequenced by The Ohio State University Plant-Microbe Genomics Facility.

Protein expression

The Tpm4.3 cDNA insert was subcloned into the pET17b vector at the NdeI and EcoRI restriction sites and the resulting plasmid sequence verified. Rosetta 2(DE3) competent *E.*

coli cells (Novagen) were transformed with the pET17b Tpm4.3 plasmid. Recombinant Tpm was expressed overnight and the cells were harvested by centrifugation. Resulting bacterial pellets were homogenized in urea sample buffer and subjected to fractionation by urea gel electrophoresis and immunoblotting, as described above.

Results

It was initially observed that the Tpm expressed in dog masseter co-migrates with Tpm1.1 on gels without urea (Bicer et al. 2011). We, therefore, sought a gel format that yielded clear separation of the masseter Tpm from the sarcomeric Tpm isoforms known to be expressed in skeletal muscle—Tpm1.1, Tpm2.2 and Tpm3.12. Sender (1971) reported that the inclusion of 6 M urea in separating gels dramatically altered the electrophoretic mobility of Tpm. Electrophoresis with gels containing 6 M urea yielded clear separation of all four Tpm isoforms in dog striated muscle (Fig. 1). The effect of urea on the electrophoretic mobility of dog Tpm isoforms is illustrated in Fig. 2. The Tpm bands on the gel were identified on the basis of the pattern observed on the immunoblots. Sarcomeric Tpm isoforms have a molecular mass ~30–35 kDa and it has been broadly demonstrated that these isoforms migrate ahead of actin (~43 kDa) on separating gels without urea, in accordance with their molecular mass. The migration of Tpm on gels with urea is markedly impeded and all of the Tpm isoforms in dog striated muscle migrate in what is estimated to be the 70–80 kDa molecular mass range, based upon migration relative to that of actin which has a molecular mass of ~43 kDa. These results demonstrate the predominance of a Tpm isoform with unique electrophoretic mobility that is distinct from that of the Tpm1.1, Tpm2.2 and Tpm3.12 in jaw-closing masseter (Fig. 1). Minor amounts of additional Tpm isoforms, identical in electrophoretic mobility to Tpm2.2 and Tpm3.12, were also observed in the masseter. The difference from other skeletal muscle Tpm isoforms in electrophoretic mobility indicates this masseter Tpm is a unique isoform consistent with earlier reports of a unique Tpm in jaw-closing muscles that express masticatory myosin (Introduction).

Multiple Tpm isoforms were detected in all of the limb muscle homogenates. A single Tpm isoform was detected in the left ventricle and it is well known that mammalian hearts express predominantly Tpm1.1 (reviewed by Wolska and Wieczorek 2003). We tested whether the predominant, uniquely-migrating Tpm isoform in dog masseter is expressed in fibers that also express masticatory myosin. Dog single masticatory muscle fibers, with the embryonic/atrial isoform of myosin light chain 1 (MLC1) and a unique regulatory myosin light chain (MLC2) isoform, which are associated with masticatory MHC (Reiser et al. 2010), were identified by gel electrophoresis on the basis of MLC isoform composition (Fig. 3). An aliquot of the same fiber was then run on a gel with 6 M urea, together with single fast and slow fibers from limb muscle and slow fibers from the deep portion of the dog masseter. The Tpm region was transferred and the membrane was probed with the anti-Tpm antibody. The results demonstrate that the unique Tpm isoform in dog masseter is, in fact, expressed in fibers that express masticatory myosin (Fig. 4). Fibers of each type—masticatory, limb fast and limb slow—had distinct Tpm isoform expression patterns.

To determine the composition of this unique masseter Tpm and its relation to other skeletal muscles, the Tpm isoforms expressed in the dog masseter, fast tibialis cranialis and the

predominantly slow deep portion of the lateral gastrocnemius were cloned. Amplification of total reverse-transcribed mRNA isolated from these muscles with a Tpm-specific primer yielded DNA of appropriate size to code Tpm. Sequencing of these clones identified four distinct Tpm transcripts. Comparison of these sequences against coding exons predicted from the deposited dog genomic sequence (Gene ID: 478332) identified these transcripts as four distinct dog *Tpm* gene products. Further alignment of the tibialis cranialis expressed sequences demonstrated that these were identical to the predicted Tpm1.1 (NCBI accession number XM_864907) and Tpm2.2 (NCBI accession number XM_847850) gene transcripts, while the masticatory expressed sequences were identical to the predicted Tpm3.12 (NCBI accession number XM_005622243) and Tpm4 (NCBI accession number XM_861034) transcripts from the genomic sequence of the domestic dog, *Canis lupus familiaris*. The 3' UTR of the cloned cDNA was also shown to be identical to that of the corresponding gene-specific genomic sequence following exon 9 of each gene product, further verifying that these four cloned Tpm cDNAs resulted from their corresponding *Tpm* genes (data not shown). These cloned dog cDNA sequences have been submitted to NCBI; Tpm1.1 accession number KR822486, Tpm2.1 accession number KR822487, Tpm3.12 accession number KR822488 and Tpm4.3 accession number KR822489. Translation of the cloned cDNAs predict Tpm1.1_{St(a,b,b,a)}, Tpm2.2_{St(a,b,b,a)}, Tpm3.12_{St(a,b,b,a)} and a novel Tpm4.3_{St(a,b,b,a)} isoform (Fig. 5; Table 1). Consistent with the cDNA sequencing, the deduced amino acid sequence of dog Tpm4.3 differs from that of dog Tpm1.1, Tpm2.2, Tpm3.12 and the predicted dog Tpm4.1, non-muscle, cytoplasmic isoform. The four cloned dog isoforms markedly differ from each other, especially in periods 5 and 6, which are important for calcium-regulation of muscle activation (see Discussion).

To date, there is no demonstration of a Tpm4.3 isoform expressed in mammalian skeletal muscle. To confirm this cloned Tpm4.3 isoform is the unique Tpm expressed in the masseter muscle, the cloned Tpm4.3 was bacterially expressed and its migration was determined on a gel with 6 M urea. Western blot of the expressed recombinant Tpm4 demonstrates that it has an electrophoretic mobility identical to that of the native dog masseter-specific Tpm (Fig. 6). Together these results demonstrate that the unique Tpm expressed in the dog masseter is a Tpm4 isoform and, therefore, represents the first demonstration of a sarcomeric Tpm4.3 isoform expressed in mammalian skeletal muscle.

Discussion

A unique Tpm isoform was reported, more than thirty years ago, to be expressed in the jaw-closing masseter and temporalis muscles of the domestic cat (Rowlerson et al. 1983a). It was shown, in the same report, that the unique Tpm isoform was expressed in muscle fibers that also express masticatory myosin, with these fibers now being referred to as “masticatory” fibers. Rowlerson and coworkers also demonstrated that, while cat limb muscle fibers exhibited two Tpm spots on 2-D gels, individual masticatory fibers contained only a single Tpm spot that migrated with the Tpm1.1 isoform from limb muscle fibers (Rowlerson et al. 1983a). Peptide mapping distinguished the masseter Tpm as a distinct Tpm isoform that varied from the traditional Tpm1, Tpm2 and Tpm3 skeletal muscle isoforms. Kang et al. (2010) reported that, in the domestic cat, this Tpm isoform is expressed in the masseter but not limb muscles, based upon immunohistochemical observations. The jaw-closing muscle

specific expression has led to the designation of this Tpm as a unique “masticatory” isoform. The amino acid composition of the unique masticatory Tpm was previously unknown. Our data demonstrate this unique masticatory Tpm is a novel isoform of the *Tpm4* gene.

It appears from multiple studies that the Tpm isoform that is expressed in muscles that also express masticatory myosin is at least primarily restricted, if not completely confined, to those muscles. For example, Kang et al. (2010) reported that cat masseter fibers that express masticatory myosin were strongly stained with an antibody that recognized a unique, masticatory Tpm isoform and that this isoform was not detected in slow fibers in the same muscle or in limb fast and slow fibers. Except for faint staining on the blot shown in Fig. 1 of the present study, which may be non-specific background stain, a band corresponding to Tpm4.3 was never observed in limb muscles on gels or immunoblots in which all Tpm isoforms were separated (e.g., Figs. 2, 4).

For most species that have been studied, it appears that isoforms of troponin-C (TnC) and troponin-T (TnT) that are expressed in muscles that express masticatory myosin are the same as those in limb fast muscle fibers (Rowlerson et al. 1983a; Bicer et al. 2011). However, the complement of TnT isoforms in jaw-closing muscles that express masticatory myosin is very different from that in limb muscles in some species (Bicer et al. 2011) and in one species, long-tailed macaque, one relatively abundant fast-type TnT isoform in the temporalis muscle was not detected in limb fast muscle. There do not appear to have been any reports describing troponin-I (TnI) isoforms in muscles that express masticatory myosin. However, the electrophoretic mobilities of TnC and of TnI in masticatory fibers are essentially identical to those in limb fast fibers in multiple species (e.g., Virginia opossum, raccoon, domestic dog, domestic cat and lion; P. Reiser, unpublished observations).

Masticatory Tpm is a novel skeletal Tpm4.3 isoform

The early proliferation of different Tpm protein isoform names and the initial lack of information from molecular biological techniques confounded the understanding of the gene origin of Tpm isoforms. Heeley and co-workers (1983, 1985) reported the presence of a Tpm isoform in adult rabbit and rat skeletal muscles that they designated as “ γ ” and “ δ ” isoforms in their reports. However, their designation of Tpm as “ δ ” was based upon the isoform’s different amino acid composition and not its transcription from the *Tpm4* gene (later also designated as Tpm- δ). To date, there has not been an identification of a *Tpm4* transcribed protein isoform in any mammalian skeletal muscle.

Of the four *Tpm* genes, only the *Tpm1*, *Tpm2* and *Tpm3* gene products have been reported to be expressed in skeletal muscle (Geeves et al. 2015; Perry 2001). While cellular Tpm can be expressed as isoforms of either 248 or 284 amino acids, regulatory myofibrillar muscle Tpm isoforms are exclusively of the 284 amino acid size. Vertebrate Tpm isoforms from these genes are determined by alternate exon splicing of exons 1, 2, 6 and 9. Exons 1a, 2b, 6b and 9a predominate in striated muscle expressed isoforms. To date, the *Tpm4* gene has been demonstrated to encode 248 and 284 amino acid isoforms, including exon 9d, that are primarily expressed in the cytoplasm. The expression of a 284 amino acid Tpm4 isoform containing exon 9a is, however, supported by the presence of this exon in humans and other vertebrates. The dog masticatory Tpm4.3 isoform is novel in that its amino acid sequence is

identical to the predicted cytosolic Tpm4.1 except that it contains the striated muscle exon 9a (Fig. 5). Since the dog Tpm4.1 isoform containing exon 9d is not a sarcomeric isoform, the inclusion of exon 9a in the Tpm4.3 isoform may be critical to Tpm4 isoform incorporation into striated muscle as a regulatory protein.

Regarding the verified Tpm4 gene products, only the Mexican Axolotl (*Ambystoma mexicanum*) (Spinner et al. 2002) and African clawed frog (*Xenopus laevis*) (Hardy et al. 1995) have been reported to express a muscle Tpm4 isoform containing exon 9a. The amino acid sequences of Tpm in these two species are 95 and 93 %, respectively, identical to the dog Tpm4.3 and are identical in the exon 9 encoded sequence. Unlike the dog Tpm4.3, *Ambystoma* Tpm4 is expressed only in the heart, while *Xenopus* Tpm4 is expressed primarily in the heart and at a very low level in skeletal muscle. While the function of the *Xenopus* Tpm4 is unknown, knockdown of the *Ambystoma* Tpm4 isoform lead to disrupted myofibril organization during embryonic development of the heart in the presence of normal Tpm1 and Tpm2 expression. These findings suggest that exon 9a is critical to myofilament structure and support a role for Tpm4.3 as a unique isoform that conveys specialized myofilament properties required in unique muscles. The current findings demonstrate that the predominant Tpm expressed in dog masseter is a unique 284 amino acid Tpm4.3 isoform containing exon 9a and establish Tpm4.3 as a novel Tpm isoform in vertebrate striated muscle.

Regions in Tpm involved with calcium-mediated activation

All known vertebrate sarcomeric Tpm isoforms consist of 284 amino acids, with seven repeating periods existing along the length of the molecule. Periods 5 and 6 are especially critical for calcium activation of muscle, including myosin-induced binding of Tpm to actin and cooperative binding of myosin to actin (Hitchcock-DeGregori et al. 2001, 2002; Singh and Hitchcock-DeGregori 2006). Periods 5 and 6 consist of 77 amino acids and are coded by most of exon 5, all of exons 6 and 7 and part of exon 8. Alignment of the four cloned dog Tpm isoforms demonstrates that over one-third of the amino acid differences between these isoforms occur within periods 5 and 6 (Fig. 5). Furthermore, within a 59 amino acid stretch, spanning adjacent regions of periods 5 and 6, there is a 58 % amino acid residue difference, compared to a 22 % residue difference throughout the other regions of the molecule. This 2.6-fold greater heterogeneity in periods 5 and 6 suggests the potential of this region to impart fibertype specific modulation of calcium activation, based upon Tpm isoform expression. Studies to determine the effect of these different Tpm isoforms on calcium regulation of the thin filament and muscle force development will be necessary to ultimately determine the effect of these varied dog Tpm isoforms on contractile regulation.

Conclusions

The present report is the first to definitively demonstrate the expression of a 284 amino acid Tpm4.3 isoform containing exon 9a in any mammalian skeletal muscle. This is consistent with the expression of isoforms of other sarcomeric proteins that are restricted primarily to jaw-closing muscles of some vertebrate species, including MHC-Mast (Rowlerson et al. 1981, 1983a, b; Hoh 2002; Hoh et al. 2006; Reiser et al. 2009, 2010), masticatory myosin

light chain 2 (Qin et al. 1994) and a unique myosin binding protein-C (Kang et al. 2010). The expression of Tpm4.3 in jaw-closing muscle fibers of domestic dogs, and likely other species that express MHC-Mast, has the potential to contribute to the unique contractile properties of these fibers which generate unusually high force per unit of cross-sectional area and are typically used for sustained contractions during the capture of live prey and feeding, for the breakdown of tough plant material and for defensive biting.

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Fig. 1.

Tropomyosin (Tpm) immunoblot of a gel containing 6 M urea. Homogenates of the deep portion of the lateral gastrocnemius (DG), tibialis (Tib), masseter (Mass) and left ventricle (LV) were run and the separated proteins were transferred and probed with the anti-Tpm antibody TM311. The order of migration of dog Tpm isoforms on gels with 6 M urea was determined in Bicer and Reiser (2013). The LV was used as a standard for Tpm1.1. The unique Tpm isoform in the masseter (Tpm4.3) migrates slightly faster than Tpm1.1

Fig. 2. Coomassie-stained SDS gel containing 6 M urea. Samples consisted of homogenates of the deep portion of the lateral gastrocnemius (DG), tibialis (Tib), masseter (Mass) and left ventricle (LV). *MHC* myosin heavy chain, *Tpm* tropomyosin

Fig. 3.

Silver-stained gel, without urea, loaded with single skeletal muscle fibers. *Lane 1*, limb fast fiber; *lane 2*, limb slow fiber; *lane 3*, masseter fiber that expressed masticatory myosin. Fiber type was based upon the myosin light chain (MLC) isoform patterns (Reiser et al. 2010). *MHC* myosin heavy chain, *Tpm* tropomyosin

Fig. 4.

Tropomyosin (Tpm) immunoblot of single skeletal muscle fibers. *Lanes 1 and 2*, limb fast fibers; *lanes 3 and 4*, masseter fibers that expressed masticatory myosin; *lanes 5 and 6*, masseter slow fibers; *lanes 7 and 8*, limb slow fibers. The gel from which the proteins were transferred contained 6 M urea and the resultant membrane was probed with the anti-Tpm antibody TM311

Fig. 5.

Deduced amino acid alignment of the cloned dog Tpm1.1, Tpm2.2, Tpm3.12 and Tpm4.3 isoforms and the predicted dog Tpm4.1_{sk(a.b.b.d)} isoform (GeneBank XM_852641.1). “-” denotes the amino acid is identical to that of the Tpm4.3 protein sequence. Periods 5 and 6, which are particularly important for actin-binding and for cooperative binding of myosin to actin and interactions with troponin (see “Discussion” section), are highlighted in blue and red, respectively. The region in periods 5 and 6 that exhibits high amino acid divergence is denoted with a dashed box (see “Discussion” section)

Fig. 6.

Tropomyosin (Tpm) immunoblot. *Lane 1*, bacterially-expressed cloned Tpm from masseter; *lane 2*, masseter homogenate; *lane 3*, tibialis homogenate; *lane 4*, deep gastrocnemius homogenate; *lane 5*, bacterially-expressed Tpm3.12. The gel from which the proteins were transferred contained 6 M urea and the resultant membrane was probed with the anti-Tpm antibody TM311

Table 1

Calculated properties of cloned dog Tpm isoforms

	Molecular weight (Da)	Isoelectric point
Tpm1.1 _{st(a,b,b,a)}	32,710	4.74
Tpm2.2 _{st(a,b,b,a)}	32,837	4.70
Tpm3.12 _{st(a,b,b,a)}	32,687	4.72
Tpm4.3 _{st(a,b,b,a)}	32,839	4.75