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RESEARCH ARTICLE

Patterns of tropomyosin and troponin-T isoform expression in jaw-closing muscles of mammals and reptiles that express masticatory myosin

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SUMMARY

We recently reported that masticatory ('superfast') myosin is expressed in jaw-closing muscles of some rodent species. Most mammalian limb muscle fibers express tropomyosin-β (Tm-β), along with fast-type or slow-type tropomyosin-α (Tm-α), but jaw-closing muscle fibers in members of Carnivora express a unique isoform of Tm [Tm-masticatory (Tm-M)] and little or no Tm-β. The goal of this study was to determine patterns of Tm and troponin-T (TnT) isoform expression in the jaw-closing muscles of rodents and other vertebrate species that express masticatory myosin, and compare the results to those from members of Carnivora. Comparisons of electrophoretic mobility, immunoblotting and mass spectrometry were used to probe the Tm and fast-type TnT isoform composition of jaw-closing and limb muscles of six species of Carnivora, eight species of Rodentia, five species of Marsupialia, big brown bat, long-tailed macaque and six species of Reptilia. Extensive heterogeneity exists in Tm and TnT isoform expression in jaw-closing muscles between phylogenetic groups, but there are fairly consistent patterns within each group. We propose that the differences in Tm and TnT isoform expression patterns between phylogenetic groups, which share the expression of masticatory myosin, may impart fundamental differences in thin-filament-mediated muscle activation to accommodate markedly different feeding styles that may require high force generation in some species (e.g. many members of Carnivora) and high speed in others (e.g. Rodentia).

Key words: masseter, temporalis, thin filament protein, troponin, tropomyosin.

INTRODUCTION

Skeletal muscle myosin consists of three pairs of subunits, two heavy chains (MHC) and two pairs of light chains (MLC). There is one pair of essential light chains (MLC1 and/or MLC3) and one pair of regulatory light chains (MLC2) in each myosin molecule in skeletal muscle. Fast and slow as well as additional isoforms of MLC1 and MLC2 exist. MLC3, expressed as a single isoform, is restricted to fast fibers. The composite hexameric myosin molecule is extremely diverse because of variations in the combinations of MHCs and of essential and regulatory MLCs. This diversity is, in large part, the basis for the broad spectrum of contractile properties among muscle fibers, as myosin is a major determinant of contractile properties of all types of muscle.

Masticatory myosin is an evolutionarily ancient protein that is expressed in the jaw-closing muscles of at least three vertebrate classes: mammals, reptiles and fish (reviewed in Hoh, 2002). Masticatory MHC (MHC-M) and MLC2 (MLC2-M) are unique isoforms that are restricted primarily to jaw-closing muscles in those species in which they are expressed (Rowlerson et al., 1981; Rowlerson et al., 1983a; Rowlerson et al., 1983b; Qin et al., 1994; Hoh, 2002; Hoh et al., 2006; Reiser et al., 2009; Reiser et al., 2010). The MLC1 isoform of masticatory myosin is an isoform that is also widely expressed in embryonic skeletal muscle and in adult atrial tissue (embryonic/atrial MLC1) (Reiser et al., 2009; Reiser et al., 2010).

Until recently, it was thought that masticatory myosin was absent in the order Rodentia, but expressed in jaw-closing muscles in species that have a carnivorous feeding style or in species with a diet consisting of tough vegetable matter (e.g. opossums) and/or a strong bite for defense (e.g. non-human primates) (reviewed in Hoh, 2002). An apparent paradox arose with the recent discovery of masticatory myosin in several rodent species (Reiser et al., 2009). Whereas rodents typically feed primarily by rapid nibbling of grasses, grains and nuts, many other species that express masticatory myosin, such as most members of Carnivora, typically strangulate prey (requiring sustained force generation), crush bones during feeding and frequently engulf large food items without extensive chewing. Muscle fibers that express masticatory myosin have unusually high force-generating ability (Kato et al., 1985; Saeki et al., 1987; Reiser and Bicer, 2007; Toniolo et al., 2008). It is not clear how masticatory myosin drives mandibular movements associated with food gathering and mastication in species with highly disparate feeding and chewing styles, such as carnivores and rodents. It is possible that other sarcomeric protein isoforms are expressed differently among the broad range of species that express masticatory myosin and these differences might underlie variations in masticatory kinematics.

The troponin (Tn) complex in muscle consists of three subunits: TnC, which binds Ca^{2+} ions released from the sarcoplasmic reticulum during activation; TnI, which has a role in inhibiting force generation in resting muscle; and TnT, which interacts with tropomyosin (Tm), another component of sarcomeric thin filaments. Extensive and well-characterized interactions between these proteins and actin, upon Ca^{2+} binding to TnC, expose myosin-binding sites on actin and allow crossbridge formation with subsequent force

generation. TnT and Tm are expressed as diverse families of isoforms through alternative RNA splicing (reviewed in Perry, 1998; Perry, 2001; Wolska and Wieczoreck, 2003; Gunning et al., 2005; Jagatheesan et al., 2010). Different isoforms of TnT and Tm are associated with variations in the Ca^{2+}-sensitivity or cooperativity of force generation in muscle, as determined by shifts in the force–[Ca^{2+}] relationship in fibers in which the surface membrane is removed or rendered hyperpermeable (e.g. Schachat et al., 1987; Greaser et al., 1988; Palmiter et al., 1996; Ogut et al., 1999; McCall et al., 2006; Biesiadecki et al., 2007; Jagatheesan et al., 2009). Furthermore, Tm isoforms can differentially modulate contractile properties through the TnT-binding domain, independent of differences in Ca^{2+}-sensitivity (Jagatheesan et al., 2004). We postulated that differences in Tm and TnT isoform expression patterns might exist in jaw-closing muscles between species with markedly different feeding styles and that these differences could facilitate either high force generation during sustained neural drive, possibly through enhanced Ca^{2+}-sensitivity, or high speed, through reduced Ca^{2+}-sensitivity, which could accommodate rapid contraction/relaxation cycles during nibbling.

The objective of this study was to determine whether there are differences in Tm and TnT isoforms in jaw-closing muscles of species that share the expression of masticatory myosin in jaw-closing muscles, yet differ with respect to feeding behavior. Differences in Tm and TnT isoform expression could reconcile the apparent paradox that masticatory myosin is expressed in species that use different styles of chewing. Multiple species, all of which express masticatory myosin (Reiser et al., 2009; Reiser et al., 2010), from several mammalian and reptilian orders were studied to determine whether there are consistent differences in the patterns of Tm and TnT isoform expression in jaw-closing muscles between groups of animals that differ with respect to feeding styles. Our results support the view that differences in Tm and TnT isoform expression patterns may impart fundamental differences in thin-filament-mediated muscle activation to accommodate markedly different feeding styles.

MATERIALS AND METHODS

Species selection

This study was conducted in accordance with a protocol that was approved by the Institutional Animal Care and Use Committee of Ohio State University. We studied multiple individuals from 27 species (Table 1). All of the animals from which samples were obtained were known (procured laboratory animals) or deemed (wild-caught animals) to be adults, based upon body size. Consistent results were obtained when multiple individuals of a given species were studied. The predominant or exclusive myosin isoforms in jaw-closing muscles of all of these species, except mouse and rat, were described in two recent reports (Reiser et al., 2009; Reiser et al., 2010). Mice and rats express primarily myosin isoforms in jaw-closing muscles that are characteristic of adult limb skeletal muscles (d'Albis et al., 1986; Eason et al., 2000; Widmer et al., 2002). All of the other species express masticatory myosin in jaw-closing muscles, except red squirrels and southern flying squirrels (Reiser et al., 2009). Masticatory myosin expression in jaw-closing muscles of some of these species has also been reported by others (Rowlerson et al., 1981; Rowlerson et al., 1983a; Rowlerson et al., 1983b; Sciote et al., 1995; Sciote and Rowlerson, 1998; Hoh, 2002).

Sample preparation

The procurement and preparation of samples were as described in previous reports (Reiser et al., 2009; Reiser et al., 2010). Samples of jaw-closing muscles (temporalis or masseter in mammalian species, and the pars profunda of the external adductor and the

Table 1. Species studied

Class	Order	Family	Common name, species	N^a	n^b
Mammalia	Rodentia	Muridae	FVB mouse, *Mus musculus* Linnaeus 1758	2	1
			Sprague Dawley rat, *Rattus norvegicus* (Berkenhout 1769)	2	1
		Sciuridae	Southern flying squirrel, *Glaucomys volans* (Linnaeus 1758)	3	1
			Red squirrel, *Tamiasciurus hudsonicus* (Erxleben 1777)	3	1
			Eastern fox squirrel, *Sciurus niger* Linnaeus 1758	2	1
			Eastern gray squirrel, *Sciurus carolinensis* Gmelin 1788	3	1
			Woodchuck, *Marmota monax* (Linnaeus 1758)	2	1
			Eastern chipmunk, *Tamias striatus* (Linnaeus 1758)	3	2
	Carnivora	Felidae	Domestic cat, *Felis catus* Linnaeus 1758	2	1
			Lion, *Panthera leo* (Linnaeus 1758)	1	1
		Canidae	Domestic dog, *Canis lupus familiaris* Linnaeus 1758	3	2
			Coyote, *Canis latrans* Say 1823	1	1
		Mephitidae	Striped skunk, *Mephitis mephitis* (Schreber 1776)	2	2
		Procyonidae	Northern raccoon, *Procyon lotor* (Linnaeus 1758)	3	2
	Primates	Cercopithecidae	Long-tailed macaque, *Macaca fascicularis* (Raffles 1821)	3	2
	Chiroptera	Vespertilionidae	Big brown bat, *Eptesicus fuscus* (Palisot de Beauvois 1796)	2	1
	Didelphimorphia	Didelphidae	Gray short-tailed opossum, *Monodelphis domestica* (Wagner 1842)	2	1
			Virginia opossum, *Didelphis virginiana* (Kerr 1792)	3	1
	Dasyuromorphia	Dasyuridae	Tiger quoll, *Dasyurus maculatus* Kerr 1792	1	1
	Diprotodontia	Acrobatidae	Feathertail glider, *Acrobates pygmaeus* (Shaw 1793)	1	1
		Petauridae	Sugar glider, *Petaurus breviceps* Waterhouse 1839	1	1
Reptilia	Crocodilia	Crocodylidae	American alligator, *Alligator mississippiensis* (Daudin 1802)	1	1
	Testudines	Chelydridae	Common snapping turtle, *Chelydra serpentina* (Linnaeus 1758)	2	2
		Trionychidae	Spiny softshell turtle, *Apalone spinifera* (Le Sueur 1827)	1	1
		Emydidae	Painted turtle, *Chrysemys picta* (Schneider 1783)	2	2
			Common map turtle, *Graptemys geographica* (Le Sueur 1817)	1	1
			Red-eared slider, *Trachemys scripta elegans* (Wied-Neuwied 1839)	1	1

[a]Total number of animals examined and from which consistent gel electrophoresis results were obtained.
[b]Total number of animals used for immunoblots.

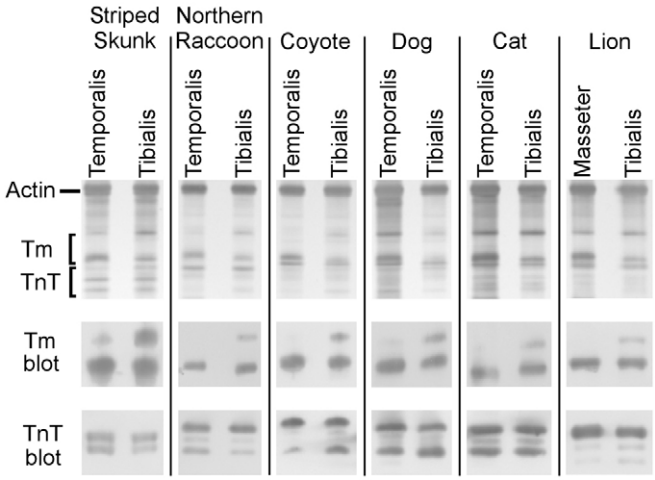

Fig. 1. Tropomyosin (Tm) and troponin-T (TnT) isoforms in the jaw-closing temporalis and fast limb tibialis of six species of Carnivora. Top: SDS gels loaded with temporalis and tibialis homogenates from striped skunk, northern raccoon, coyote, domestic dog, domestic cat and lion. Middle and bottom: western blots probed with anti-Tm and fast-type TnT antibodies, respectively.

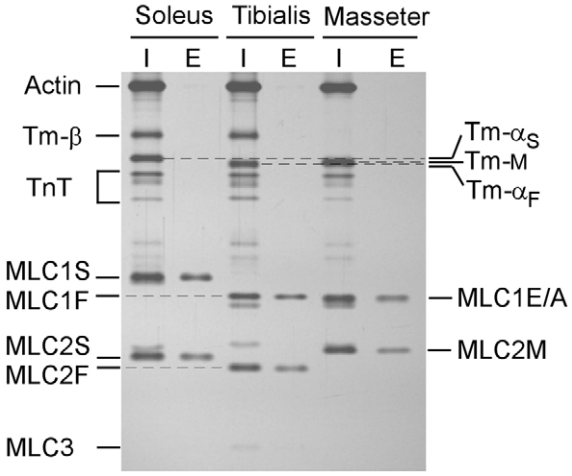

Fig. 2. Tm, TnT and myosin light chain (MLC) isoforms in single muscle fibers from northern raccoon slow soleus, fast tibialis and masseter muscles. Each fiber was cut in half lengthwise and myosin was extracted from one half. The proteins in the non-extracted, intact half and the extracted myosin from the other half were loaded in alternating lanes (I and E, respectively). The masseter fiber expressed embryonic/atrial MLC1 (MLC1E/A) and masticatory MLC2 (MLC2M), as recently reported (Reiser et al., 2010). The Tm in the masseter fiber (presumably Tm-M) had an electrophoretic mobility that was intermediate to that of fast and slow Tm-α (Tm-α_F and Tm-α_S) in the fast and slow limb fibers, respectively. The limb fibers were identified on the basis of MLC isoforms, with slow-type MLC1 and MLC2 (MLC1S and MLC2S) in the soleus fiber and fast-type MLC1 and MLC2 (MLC1F and MLC2F), as well as MLC3, in the tibialis fiber.

adductor mandibulae externus superficialis of reptilian species) and limb fast muscles of each species were used for this study, the latter for intraspecific comparisons. Pectoralis was used as a source of fast muscle in bat. Aliquots of the same samples used in the previous studies were frozen until analysis in the present study.

Gel electrophoresis and immunoblotting

Samples were run on 12% polyacrylamide gels that were either silver stained or used for immunoblotting (Reiser et al., 2009). Primary antibodies were a monoclonal anti-TnT, clone JLT-12 (T-6277, Sigma-Aldrich Co., St Louis, MO, USA), which recognizes fast-type TnT isoforms (Briggs et al., 1987; Galler et al., 1997), and a monoclonal anti-Tm, clone TM311 (T-2780, Sigma-Aldrich), which recognizes Tm-α, Tm-β and Tm-γ isoforms (Nicholson-Flynn et al., 1996). Gel staining and immunoblotting protocols were as described previously (Reiser et al., 2009). Scanning densitometry (Hoefer model GS 300 densitometer and GS365W software, version 3.01, Hoefer Inc., Holliston, MA, USA) was used to determine the relative amounts of Tm and TnT isoforms on immunoblots. Reported values were from individual samples. Myosin was extracted from several raccoon muscle fibers, as described previously (Bicer and Reiser, 2004), to assist in the identification of MLCs (in the extracted fraction) and Tm (in the non-extracted fraction) on gels.

Mass spectrometry

Homogenates of gray squirrel temporalis and tibialis were run on two-dimensional gels and excised proteins were analyzed by mass spectrometry, as described earlier (Bergrin et al., 2006; Reiser et al., 2010).

RESULTS

Carnivora

Two Tm isoforms were detected in the fast-twitch tibialis of all members of Carnivora that we examined (Fig. 1). The faster-migrating isoform predominated (54–81% of total Tm on the blots shown in Fig. 1) in the tibialis and was assumed to be Tm-α, and the slower-migrating isoform was assumed to be Tm-β. Others have reported that a unique Tm isoform (Tm-M), which has an electrophoretic mobility that is similar to Tm-α but is distinct from Tm isoforms in limb muscles, is predominantly expressed in the jaw-closing muscle of the domestic cat (Rowlerson et al., 1983a; Hoh et al., 1989; Kang et al., 2010). One Tm isoform, presumably Tm-M, was exclusively or predominantly (82–100% of total Tm) expressed in the temporalis muscle of the Carnivora species in this study. A slower-migrating Tm isoform was present, at a much lower level, in the temporalis of some of these species, as in the tibialis, and was assumed to be Tm-β. The Tm isoform in raccoon temporalis also appeared to be distinct (presumably Tm-M), based on electrophoretic mobility (Fig. 2).

The complement of fast-type TnT isoforms was virtually identical in the temporalis and tibialis within each species of Carnivora examined, although differences existed between species. Each species of Carnivora expressed two or three fast-type TnT isoforms in the temporalis and tibialis. The amount of the slowest migrating TnT isoform predominated in northern raccoon (75 and 82% of total TnT in the temporalis and tibialis, respectively), coyote (81 and 61% of total TnT in the temporalis and tibialis, respectively) and especially lion (90 and 88% of total TnT in the temporalis and tibialis, respectively) over other isoforms in the same species whereas no single TnT isoform predominated over others in domestic cat, domestic dog or striped skunk. Hence, Tm isoforms differed and TnT isoforms were similar in the jaw-closing and fast limb muscles of Carnivora. Furthermore, species differences exist within Carnivora with respect to the total number and predominance (relative amounts) of TnT isoforms in jaw-closing and fast limb muscle.

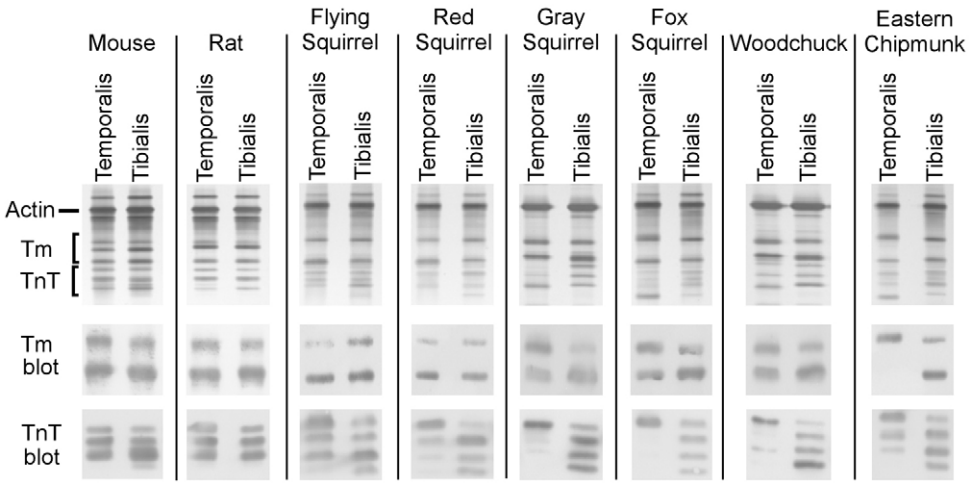

Fig. 3. Tm and TnT isoforms in the jaw-closing temporalis and fast limb tibialis of eight species of Rodentia. Top: SDS gels loaded with temporalis and tibialis homogenates from each of the eight species. Middle and bottom: western blots probed with anti-Tm and fast-type TnT antibodies, respectively. There are additional bands in the Tm region of the silver-stained gels that were not recognized by the anti-Tm antibody and are assumed, therefore, not to be Tm isoforms.

Rodentia

Two Tm isoforms were detected in the jaw-closing temporalis and fast limb muscles of all species of Rodentia examined, except eastern chipmunk (Fig. 3). The amount of the faster migrating Tm isoform, presumably Tm-α, predominated (61–77% of total Tm on the blots shown in Fig. 3) in the tibialis muscle of every rodent species studied and apparently the same isoform predominated (53–80% of total Tm) in the temporalis of those rodent species that do not express masticatory myosin (mouse, rat, flying squirrel and red squirrel). In contrast, the amount of the slower migrating Tm isoform, presumably Tm-β, predominated in the temporalis of those rodent species that express masticatory myosin: 54, 55, 64 and 100% of total Tm in fox squirrel, woodchuck, gray squirrel and eastern chipmunk, respectively. The same pattern (i.e. one Tm isoform) was observed in the temporalis of two eastern chipmunks.

The temporalis and tibialis were very similar in mouse and rat, with respect to the expression of three prominent TnTs. All of the members of the squirrel family that were examined had four TnT isoforms in the tibialis and three or four TnT isoforms in the temporalis. A single, slowly migrating TnT isoform predominated in the temporalis of eastern chipmunk (60% of total TnT), along with two less abundant isoforms; this isoform was almost exclusively expressed in the temporalis of woodchuck, gray squirrel and fox squirrel (81, 93 and 97% of total TnT, respectively), all of which express masticatory myosin. This same TnT isoform predominated in the temporalis of flying squirrel and red squirrel (53 and 66% of total TnT, respectively), but additional TnT isoforms were readily detected in the temporalis in these two species.

Mass spectrometry was used to strengthen the identification of the predominant Tm isoform in the temporalis of the gray squirrel as a representative of a rodent jaw-closing muscle that also expresses masticatory myosin (Reiser et al., 2009). Homogenates of gray squirrel temporalis and tibialis were separated on two-dimensional gels and the putative TM-β bands from both gels were excised. The gray squirrel temporalis protein migrated similarly, if not identically, to TM-β in tibialis, relative to actin and TM-α in the same samples (data not shown). The mass spectrometry result with the highest probability of a correct match was TM-β for both muscles, with highly significant (P<0.05) MOWSE scores of 792 (14 matching peptide) and 2803 (27 matching peptides), respectively. A MOWSE score of 56 corresponds to a 95% probability of the match being correct. The greater MOWSE score and greater number of peptides matched for the tibialis protein spot were due to the greater protein amount in this sample compared with the temporalis spot. All of the temporalis peptides fully matched peptide sequences in GenBank for cow, mouse, rat, rabbit and human TM-β. The sequence of TM-M is not known for any species; therefore, the mass spectrometry results do not exclude the possibility that this spot is TM-M. However, all of the peptides generated by tryptic digestion in gray squirrel temporalis fully matched peptides generated from the tibialis. It seems unlikely, if this band was not TM-β, that all of the generated peptides would completely match the peptides generated from tibialis TM-β and the known TM-β peptide sequences in cow, mouse, rat, rabbit and human. These results support the identification of the predominant TM band in gray squirrel temporalis as being TM-β. However, the possibility that this protein band is, or at least contains some, TM-M cannot be fully excluded.

Therefore, Tm isoforms were similar in the jaw-closing and fast limb muscle of all members of Rodentia that were studied, and TnT isoforms differed between jaw-closing and fast limb muscles, primarily in those rodent species that express masticatory myosin.

Big brown bat

A single Tm isoform was observed in the pectoralis of big brown bat (Fig. 4) and is presumably Tm-α, based on the fact that its migration is similar to Tm-α in other species. A single Tm isoform with the same mobility was observed in the temporalis. Whether the Tm isoform in the temporalis is Tm-α, Tm-M or another Tm isoform was not determined. Two apparently identical fast-type TnT isoforms were detected in big brown bat temporalis and pectoralis, and the amount of the same slower migrating isoform predominated in both muscles (93 and 91% of total TnT, respectively).

Long-tailed macaque

Two Tm isoforms were detected in macaque fast-twitch tibialis, with the amount of the faster-migrating isoform, presumably Tm-α (and the slower migrating isoform presumably being Tm-β), predominating (66% of total Tm). A single Tm isoform, with the same mobility as Tm-α in the tibialis, was detected in the temporalis (Fig. 5), but the identity of this isoform is not known.

The greatest number (five) of fast-type TnT isoforms and the most complex pattern of expression observed in any species in this study were found in long-tailed macaque. Four TnT isoforms were detected in the temporalis and five isoforms were found in the tibialis. The predominant (42% of total TnT) TnT isoform and an additional isoform in the tibialis were not detected in the temporalis.

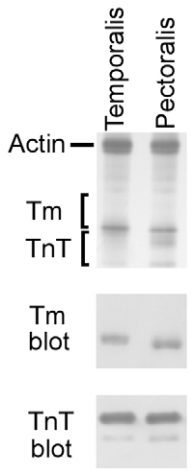

Fig. 4. Tm and TnT isoforms in the jaw-closing temporalis and fast, pectoralis of big brown bat. Top: SDS gels loaded with temporalis and pectoralis homogenates. Middle and bottom: western blots probed with anti-Tm and fast-type TnT antibodies, respectively.

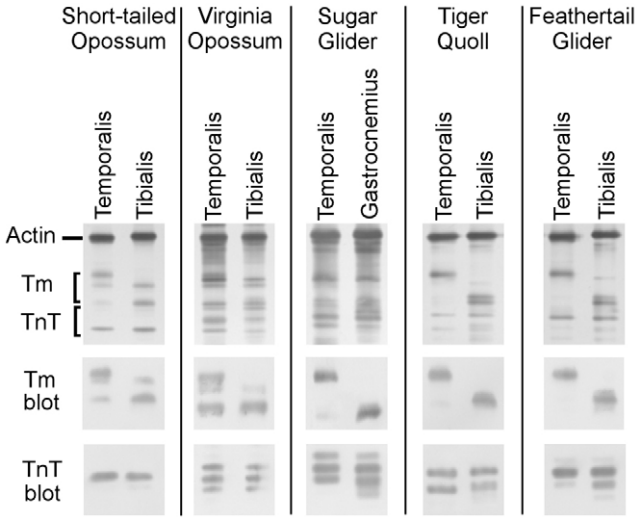

Fig. 6. Tm and TnT isoforms in the jaw-closing temporalis and fast limb tibialis of five species of Marsupialia. Top: SDS gels loaded with temporalis and tibialis homogenates from short-tailed opossum, Virginia opossum, sugar glider, tiger quoll and feathertail glider. Middle and bottom: western blots probed with anti-Tm and fast-type TnT antibodies, respectively.

The predominant (50% of total TnT) TnT isoform in the temporalis was also a prominent isoform in the tibialis, but another temporalis TnT isoform was not detected in the tibialis. Therefore, only three of the six fast-type TnT isoforms were expressed in both muscles. This is in marked contrast to all other eutherian mammals examined, in which the same TnT isoforms were present in jaw-closing and fast limb muscles.

Marsupialia

The Tm isoform expression pattern differed markedly between the temporalis and tibialis within each marsupial species (Fig. 6). The temporalis of short-tailed and Virginia opossums contained two TM proteins that co-migrated with TM-α and TM-β in the tibialis, plus a slower-migrating TM band that was not identified. This protein was not observed in the tibialis of either opossum species, but predominated (82% of total Tm) in short-tailed opossum temporalis and was a major band (46% of total Tm) in Virginia opossum temporalis. The temporalis of tiger quoll, sugar glider and feathertail glider expressed a very minor TM isoform that migrated similarly to TM-α in fast limb muscle (tibialis or gastrocnemius) of each species. The slowest migrating Tm isoform (not identified) predominated in the temporalis of tiger quoll, sugar glider and feathertail glider (97, 90 and 89% of total Tm, respectively) and was not observed in the fast limb muscle in these species.

The fast-type TnT isoform expression patterns were very similar in the temporalis and tibialis within each of the five marsupial species. The number of observed fast-type TnT isoforms varied, however, from one to four between marsupial species.

Therefore, TnT isoforms were similar and Tm isoforms differed between jaw-closing and fast limb muscles in Marsupialia, but the difference in Tm isoforms was very different from that in Carnivora.

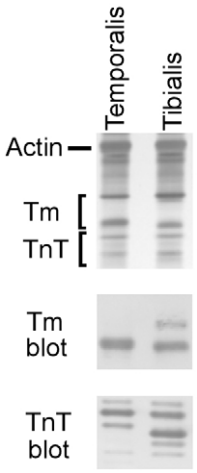

Fig. 5. Tm and TnT isoforms in the jaw-closing temporalis and fast limb tibialis of long-tailed macaque. Top: SDS gels loaded with temporalis and tibialis homogenates. Middle and bottom: western blots probed with anti-Tm and fast-type TnT antibodies, respectively. There are additional bands in the Tm region of the silver-stained gels that were not recognized by the anti-Tm antibody and are assumed, therefore, not to be Tm isoforms.

Reptilia

There was a distinct difference in Tm isoform expression between jaw-closing and tibialis muscles in American alligator and four of the turtle species examined. A faster-migrating Tm isoform constituted a major fraction (51–90%) of the total Tm in the jaw-closing muscles, but was not detected in the tibialis of alligator, painted turtle, snapping turtle, map turtle or red-eared slider (Fig. 7). A Tm isoform with lower electrophoretic mobility was detected in the tibialis of these species and this isoform co-migrated with the minor Tm isoform in the temporalis. Except for red-eared slider, an additional, even slower migrating Tm isoform was detected in the tibialis of each species examined. An apparent single Tm isoform predominated (97% of total Tm) in the jaw-closing and tibialis muscles of softshell turtle. The identity of this Tm isoform is not clear, as its electrophoretic mobility is intermediate between Tm-α and Tm-β of other reptilian species. The JLT-12 antibody did not recognize TnT isoforms in reptilian jaw-closing or tibialis muscles. However, examination of the SDS gel region in which TnT isoforms

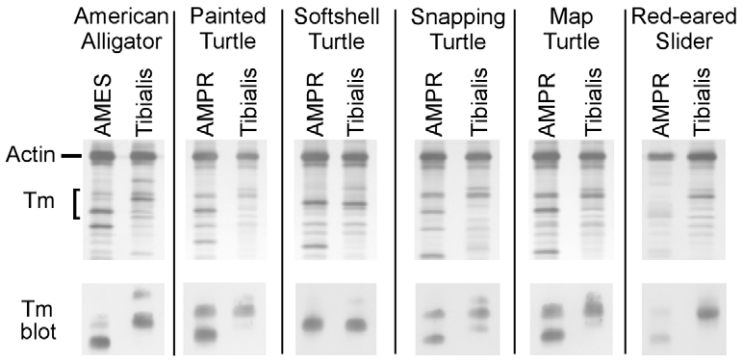

Fig. 7. Tm and TnT isoforms in the jaw-closing temporalis and fast limb tibialis of six species of Reptilia. Top: SDS gels loaded with homogenates of the fast tibialis and the jaw-closing pars profunda of the external adductor (AMPR) or the adductor mandibulae externus superficialis (AMES) of American alligator, painted turtle, sofshell turtle, snapping turtle, map turtle and red-eared slider. Bottom: western blots probed with anti-Tm antibody.

migrate in other vertebrate species revealed several major protein band differences between the jaw-closing and tibialis muscles.

DISCUSSION

The results of this study reveal clear differences in the isoform expression patterns of Tm and/or fast-type TnT between jaw-closing and fast limb muscles among species in several animal groups with markedly different feeding styles (Carnivora, Rodentia, Marsupialia and Reptilia). Except for four of the rodent species studied (mouse, rat, red squirrel and flying squirrel), all of the species in this study express masticatory myosin in their jaw-closing muscles. Generally, species within the class Reptilia, or within different orders of the class Mammalia, share similar patterns of Tm and TnT expression and exhibit consistent differences in the isoform expression patterns of these two proteins between jaw-closing and fast limb muscles. Whereas expression patterns of some thin filament proteins appear to be relatively constant in jaw-closing muscles, e.g. fast-type TnI and fast-type TnC predominate (S.B. and P.J.R., unpublished observations), and the skeletal muscle α-actin isoform is presumed to be expressed in both muscle groups of all of the species studied, the patterns of Tm and TnT isoform expression are highly variable. Interactions between Tm and TnT are essential during skeletal muscle activation. Therefore, variation in the isoform expression of these two proteins among muscle fibers could result in significant differences in contractile properties.

At least four genes encode the Tm isoforms that are expressed in skeletal and cardiac muscles (reviewed in Perry, 2001; Gunning et al., 2005; Jagatheesan et al., 2010) and one gene codes for multiple fast-type TnT isoforms in vertebrate skeletal muscle (Medford et al., 1984; Wilkinson et al., 1984; Breitbart et al., 1985; Hastings et al., 1985). The expression of these proteins is regulated by extensive alternative RNA splicing, which has the potential to yield complex patterns between muscles of a given organism or even between individual fibers within a specific muscle (e.g. Schachat et al., 1985). Differences in Ca^{2+}-sensitivity or cooperativity of force generation of striated muscle are associated with different isoforms of Tm and TnT (e.g. Schachat et al., 1987; Greaser et al., 1988; Palmiter et al., 1996; Gallon et al., 2006; McCall et al., 2006; Jagatheesan et al., 2009). It is possible, therefore, that significant differences in the Ca^{2+} regulation of force generation between jaw-closing and limb muscles within individual species and in jaw-closing muscles between species are associated with the differences in the Tm and TnT isoform expression patterns observed in this study. Modulation of muscle activation, independent of differences in Ca^{2+}-sensitivity of force generation, may also reside in differences between Tm isoforms (Jagatheesan et al., 2004). The predominance of apparent Tm-β in the temporalis of rodent species that express masticatory myosin is in sharp contrast to the apparent almost exclusive expression of Tm-M in the jaw-closing muscles of Carnivora. This observation suggests that there might be a fundamental difference in the regulation of activation in muscles that express masticatory myosin between Carnivora and Rodentia. Unequivocal determination of whether and how, specifically, the distinct patterns of Tm and/or TnT regulate or accommodate the kinetics and magnitude of force generation during masticatory motor functions awaits further study.

It is possible that masticatory myosin in different species functions differently due, for example, to possible non-identical sequence homology of the MHC and/or MLC subunits or species-related post-translational modification (e.g. glycosylation of cat MHC-M) (Kirkeby, 1996). The electrophoretic mobility of MHC-M varies between some species [e.g. fig. 2 in Reiser et al. (Reiser et al., 2009)], but the basis for this variation is not known. Therefore, it is possible that differences in the composite hexameric masticatory myosin molecule drive contractions differently between species, with high force generation in some species and high speed in others. With or without inherent differences of functional significance in masticatory myosin between species, it is possible that the thin-filament protein differences described in this report accommodate high force generation in some species and high speed in others during typical masticatory functions. The following model could functionally relate the expression of different patterns of thin filament protein isoforms to differences in feeding behaviors and mandibular kinematics. For those species in which high force during mastication appears to be important (e.g. many members of Carnivora), the complement of thin-filament proteins might impart high Ca^{2+}-sensitivity, which could accommodate contractions with high force generation and slow relaxation. A report of skinned fibers from cat temporalis having a higher Ca^{2+}-sensitivity than fibers in the jaw-opening digastric muscle (the latter do not express masticatory myosin) is consistent with this idea (Kato et al., 1985). However, members of Rodentia, with a different complement of thin-filament proteins, might have reduced Ca^{2+}-sensitivity in jaw-closing muscles, which could accommodate more rapid twitch-like contractions and relaxations for rapid nibbling.

Additional factors may contribute to differences in the Ca^{2+}-sensitivity of force generation in striated muscle, either in concert with or independent of variations in thin-filament protein isoforms. For example, phosphorylation of myosin binding protein-C (MyBP-C) in cardiac muscle apparently can result in the radial displacement of myosin heads toward actin and thereby potentially promote force generation (Colson et al., 2008). Evidence that myosin heads in resting dog jaw-closing muscle protrude closer to actin than in fast limb muscle and potentially contribute to high force generation during activation was recently reported (Yamaguchi et al., 2010). Phosphorylation of MLC2 results in an augmentation of Ca^{2+}-

activated force generation in skinned skeletal muscle fibers (Persechini et al., 1985) and skinned cardiac trabeculae (Morano and Ruegg, 1986), possibly through a mechanism that also allows myosin heads to protrude closer to actin prior to activation. A unique isoform of MLC2-M (see Introduction) and a unique isoform of MyBP-C (Wu et al., 2007; Kang et al., 2010) are expressed in jaw-closing muscles of at least some, if not all, species that express masticatory myosin. The electrophoretic mobility of MyBP-C in jaw-closing muscles of species that express masticatory myosin in this study appears to consistently differ from that of MyBP-C in limb muscles (S.B. and P.J.R., unpublished observation). It is possible that masticatory isoforms of MyBP-C and MLC2 also have a role in regulating the force or speed of contractions in jaw-closing muscles and contribute to potential differences in masticatory dynamics between phylogenetic groups through a thick filament-mediated mechanism (Harris et al., 2004; Stelzer et al., 2006; Luther et al., 2008; Colson et al., 2010) and/or through a mechanism involving interactions between MyBP-C and actin (Whitten et al., 2008; Shaffer et al., 2009). It is also possible that jaw-closing muscle fibers in all species that express masticatory myosin and a unique isoform of MyBP-C generate high force, and that rapid relaxation is facilitated in the squirrel family by a difference in Ca^{2+}-sensitivity, mediated by different thin-filament protein isoforms.

Marsupial jaw-closing muscles are noteworthy because their complement of Tm and TnT isoforms is intermediate between those in Carnivora and Rodentia. The ratio of Tm-β to Tm-α isoforms (relative amounts) is higher in the jaw-closing muscles of rodents that express masticatory myosin than in members of Carnivora, all of which [except red 'lesser' panda (Hoh, 2002)] express masticatory myosin. The Tm-β/Tm-α ratio is also high in marsupial jaw-closing muscles. However, whereas rodents that express masticatory myosin have strikingly different TnT complements in the temporalis and tibialis, Marsupialia and Carnivora share similar TnT patterns in the jaw-closing and tibialis muscles within each species. This adds to the diversity of thin-filament protein isoform expression patterns in jaw-closing muscles among mammalian species. The variation in Tm isoform expression in mammalian jaw-closing muscles that also express masticatory myosin is in marked contrast to the consistent predominance of Tm-α in mammalian fast-twitch limb muscles (Cummins and Perry, 1973). The greater variation in Tm isoform expression in jaw-closing muscles compared with fast limb muscle accommodates ostensibly greater variation in feeding dynamics than variation in the manner in which fast limb muscle drives terrestrial locomotion. The patterns of Tm and fast-type TnT isoform expression in jaw-closing muscles add to the growing list of characteristics that distinguish craniofacial muscles from limb muscles (e.g. Rowlerson et al., 1983b; Yamaguchi et al., 2010) (reviewed in Hoh, 2002; Sciote et al., 2003).

Feeding styles differ considerably among the marsupial species studied, e.g. the tiger quoll has a diet that largely consists of medium-sized (0.5–5.0 kg) vertebrates (Belcher, 1995) whereas the sugar glider consumes large amounts of exudates from plants – such as sap (accessed by gnawing on branches of trees, e.g. eucalyptus), gum and nectar – as well as insects (moths and beetles) (Smith, 1982). However, the expression pattern of Tm and TnT isoforms is essentially the same among the five species. Ostensibly, a complement of Tm and TnT isoforms that accommodates a carnivorous feeding style also imparts an advantage to nectarivorous and insectivorous marsupial species for occasional gnawing on plant material or breaking chitinous exoskeletons. Feeding styles also vary considerably among members of Carnivora (e.g. lions are apex predators whereas raccoons are omnivores), but similar Tm and TnT expression patterns exist in jaw-closing muscles. Determination of contractile properties of jaw-closing muscle fibers will be very valuable for understanding the significance of the diversity of thin-filament protein isoform expression in the masseter and temporalis muscles of different species.

Normal adult guinea pig masseter expresses predominantly the Tm-α isoform and there is a small, but significant, increase in Tm-β following 1 week of an open bite (imposed with a biplate cemented to the mandibular incisors), along with a significant increase in the MLC1F/MLC3 ratio (Ohnuki et al., 1999). Lower Tm-α and MLC3 levels are associated with slower contraction rates among fast-type muscle fibers (Schiaffino and Reggiani, 1996). Therefore, the shift towards greater relative levels of Tm-β in the jaw-closing muscles of those rodent species that express masticatory myosin might reflect an adaptation that is associated with slower contractions in fast fibers and possibly favor higher force generation instead.

Wieczorek and co-workers manipulated Tm isoform expression in mouse myocardium in a series of studies (reviewed in Jagatheesan et al., 2010) to examine the role of fast-type Tm-α, slow-type Tm-α (also referred to as Tm-γ or TPM 3) and Tm-β in the regulation of contractile properties of striated muscle. They reported that Tm-β is associated with a slowing of relaxation and an increase in the Ca^{2+}-sensitivity of force generation compared with control myocardium, which expresses predominantly fast-type Tm-α. Slow-type Tm-α increases isometric contraction and relaxation rates and decreases Ca^{2+}-sensitivity in transgenic mouse hearts (Jagatheesan et al., 2010). Our results show that the jaw-closing muscles of members of Carnivora express little or no Tm-β, which suggests that contractions could be faster and that the Ca^{2+}-sensitivity of force generation would be lower. However, in the jaw-closing muscles of at least some members of Carnivora (raccoon, domestic dog and domestic cat), a unique Tm isoform is expressed (Rowlerson et al., 1983a; Hoh et al., 1989; Kang et al., 2010), to the virtual exclusion of other Tm isoforms. Others have shown that the Ca^{2+}-sensitivity of force generation is higher in skinned fibers from cat jaw-closing muscle than in fast-type limb muscle (Kato et al., 1985; Saeki et al., 1987) and that isometric contractions in cat jaw-closing muscle are twice as fast as in limb muscle (Taylor et al., 1973; Hoh and Hughes, 1988). Therefore, it is not clear how the different Tm and TnT isoforms in jaw-closing muscles in different phylogenetic groups that express masticatory myosin regulate contractile properties, either independently or through interactions between these two proteins that have integral roles during activation. Unambiguous identification of specific Tm and TnT isoforms in jaw-closing muscles will be essential to gaining a more complete understanding of the Ca^{2+}-activation characteristics in these muscles.

Species-related differences in TnT and Tm isoform expression were identified on the basis of electrophoretic mobility, immunoblot detection and mass spectrometry. A limitation of this study is that the specific isoforms of Tm that are expressed in the jaw-closing and limb muscles of species that we studied have not been fully characterized. Given that multiple isoforms of Tm and TnT can be generated through alternative RNA splicing, identification of specific isoforms of either protein, based on one-dimensional gel electrophoretic mobility and/or immunoblotting alone, will not unambiguously identify all isoforms. Additional isoforms of either Tm or TnT may be revealed with two-dimensional gel electrophoresis (e.g. Heeley et al., 1995). Undetected species-related differences in post-translational modifications (e.g. phosphorylation) may also modulate activation. However, two Tm

characteristics have been consistently reported (e.g. Pieples and Wieczorek, 2000) since the original observations of Cummins and Perry (Cummins and Perry, 1973; Cummins and Perry, 1974): the amount of the electrophoretically faster migrating Tm-α is greater than the amount of Tm-β in mammalian skeletal muscle, and the Tm-α/Tm-β ratio is greater in mammalian fast compared with slow muscle. Therefore, based on electrophoretic mobility, it appears that Tm-β is exclusively or predominantly expressed in some of the muscles examined (e.g. squirrel family, sugar glider, tiger quoll and feathertail glider). Tm is present along the entire length of sarcomeric thin filaments as a dimer. If Tm-β is the exclusive or predominant isoform in some jaw-closing muscles, then it presumably is present, in large part, as ββ dimers in the same muscles. This would be unusual but not unprecedented, as others (Holtzer et al., 1992) have reported that Tm-ββ dimers are present in mammalian skeletal muscle, but at relatively low levels (≤10% of total Tm), as well as in avian smooth muscle (Strasburg and Greaser, 1976). The apparent exclusive expression of Tm-β in some muscles, reported here, is a novel finding. The adult mouse heart normally expresses almost exclusively Tm-α. Very high overexpression of Tm-β in the hearts of transgenic mice has marked deleterious functional and structural consequences, and results in death at a very early (neonatal) age (Muthuchamy et al., 1998). The rates of force generation and relaxation are profoundly reduced in ventricular strips from these mice. Therefore, if jaw-closing muscles of some species that express masticatory myosin also express high levels of Tm-β, then it will be very important to determine how this subserves efficient contraction and relaxation in these muscles. Despite the lack of identification of each isoform, the present results reveal marked differences in Tm and TnT isoform expression between jaw-closing and fast limb muscles and consistent patterns within, but different between, phylogenetic groups. These differences have the potential to affect the magnitude and kinetics of force generation and, thereby, contribute to specific contractile properties of jaw-closing muscles among species with disparate feeding behaviors and diets.

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