Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part A 148 (2007) 591–598
CBP
www.elsevier.com/locate/cbpa

Muscle-specific calpain is localized in regions near motor endplates in differentiating lobster claw muscles

Scott Medler [a,*], Ernest S. Chang [b], Donald L. Mykles [a]

[a] Department of Biology, Colorado State University, Fort Collins, Colorado 80523, USA
[b] Bodega Marine Laboratory, University of California-Davis, Bodega Bay, California 94923, USA

Received 24 June 2007; received in revised form 31 July 2007; accepted 3 August 2007
Available online 15 August 2007

Abstract

Calpains are Ca^{2+}-dependent proteinases that mediate protein turnover in crustacean skeletal muscles. We used an antibody directed against lobster muscle-specific calpain (Ha-CalpM) to examine its distribution in differentiating juvenile lobster claw muscles. These muscles are comprised of both fast and slow fibers early in development, but become specialized into predominantly fast or exclusively slow muscles in adults. The transition into adult muscle types requires that myofibrillar proteins specific for fast or slow muscles to be selectively removed and replaced by the appropriate proteins. Using immunohistochemistry, we observed a distinct staining pattern where staining was preferentially localized in the fiber periphery along one side of the fiber. Immunolabeling with an antibody directed against synaptotagmin revealed that the calpain staining was greatest in the cytoplasm adjacent to synaptic terminals. In complementary analyses, we used sequence-specific primers with real-time PCR to quantify the levels of Ha-CalpM in whole juvenile claw muscles. These expression levels were not significantly different between cutter and crusher claws, but were positively correlated with the expression of fast myosin heavy chain. The anatomical localization of Ha-CalpM near motor endplates, coupled with the correlation with fast myofibrillar gene expression, suggests a role for this intracellular proteinase in fiber type switching.

Keywords: Calpain; Gene expression; Synaptotagmin; Molting; Crustacea; Skeletal muscle; Development; Muscle plasticity

1. Introduction

Skeletal muscles are highly plastic tissues, capable of completely remodeling themselves from fast to slow muscles, and *vice versa*. Several crustacean models exhibit dramatic examples of skeletal muscle plasticity, including the dimorphic lobster claw closer muscles (Govind, 1984; Govind et al., 1987). During juvenile development, both lobster claws begin with symmetrical muscles, composed of a central core of fast fibers surrounded by slow fibers. Over several molt cycles, the slow fibers of one claw, called the cutter, are replaced by fast fibers. In the contralateral crusher claw, all of the muscle fibers become slow fibers by the end of the juvenile stage of development. This type of complete transition from one fiber type to another requires two coordinated processes. First, the genes encoding different myofibrillar protein isoforms must be alternately turned off or on, depending on the specific transition taking place. Second, the unneeded myofibrillar proteins must be removed, as the new proteins take their place. This second process likely relies on the selective proteolysis of myofibrillar proteins by intracellular proteinases (Mykles, 1997; Pette and Staron, 2001), although little is known about the role of these proteinases during fiber switching. Fiber transformation in crustaceans also requires an extensive remodeling of the contractile apparatus, as fast and slow fibers differ in sarcomere length, thin to thick myofilament ratio, and Z-line thickness (Atwood, 1976; Mellon, 1992).

Much of what we know about muscle proteolysis comes from various models of disease-induced muscle atrophy (Mitch and Goldberg, 1996; Lecker et al., 1999; Jackman and Kandarian, 2004). Collectively, these studies have identified the ubiquitin/proteasome system as a major pathway responsible for the atrophy caused by conditions such as cancer

* Corresponding author. Department of Biological Sciences, 109 Cooke Hall, University at Buffalo, Buffalo, New York 14260, USA. Tel.: +1 716 645 2363 ext. 201.
E-mail address: smedler@buffalo.edu (S. Medler).

1095-6433/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2007.08.008

cachexia, sepsis, starvation, or cortisol administration (Mitch and Goldberg, 1996; Lecker et al., 1999). However, even studies identifying the ubiquitin/proteasome system as central in muscle atrophy concluded that other major proteolytic systems probably play a coordinate role in protein degradation (Tallandier et al., 1996; Huang and Forsberg, 1998; Eble et al., 1999). Calpains represent a large superfamily of calcium-activated proteinases that play a role in diverse cellular functions (Sorimachi et al., 1997; Mykles, 1998; Goll et al., 2003). In mammalian skeletal muscles, the well characterized m-calpain and μ-calpain are involved in the initial disassembly of myofibrillar proteins, which are subsequently degraded further by the ubiquitin–proteasome system (Lebart and Benyamin, 2006; Jackman and Kandarian, 2004).

In addition to the ubiquitous calpains that clearly play a role in muscle proteolysis, there are a number of tissue-specific calpains that have diverse functions (Goll et al., 2003). One of these, calpain 3 (Capn 3, also known as p94), in mammalian skeletal muscles is a muscle-specific form that plays a key role in the maintenance of normal muscle phenotype. Mutations of the Capn 3 gene in humans cause several types of limb girdle muscular dystrophy type 2A (Sorimachi et al., 1997; Kinbara et al., 1998; Kramerova et al., 2007). The precise role of Capn 3 in skeletal muscle function remains enigmatic, but a number of potential functions have been suggested, including a role in sarcomere development and remodeling (Kramerova et al., 2007). Capn 3 is localized with the titin molecule (also known as connectin) at the level of the Z-line in the sarcomere and appears to associate with molecular signaling complexes (Ojima et al., 2005; Kramerova et al., 2007). Capn 3 levels are fiber type specific, being about three times higher in mammalian fast muscles than in slow muscles and low frequency electrical stimulation leads to a rapid loss of Capn 3 in rabbit fast muscle (Jones et al., 1999; Sultan et al., 2001). These data suggest that Capn 3 is involved in mediating intracellular signaling processes. By analogy with Capn 3, we hypothesize that the muscle-specific calpain in lobsters may play a role in intracellular signaling pathways.

Crustacean skeletal muscles employ several intracellular proteinases to break down myofibrillar proteins during molt-induced atrophy, which results in the loss of 30–60% of the mass in the large claw muscles (Skinner, 1966; Mykles, 1992, 1998). Broadly, these include the Ca^{2+}-dependent proteinases (CDPs or calpains) and the ubiquitin–proteasome system (Mykles, 1992, 1998). The ubiquitin–proteasome system is clearly active in the breakdown of muscle proteins during molting in crabs and lobsters (Shean and Mykles, 1995; Koenders et al., 2002;), but almost certainly operates in conjunction with calpains during these processes (Mykles, 1992, 1998). In lobster skeletal muscles, four different calpain activities (designated CDP I, IIa, IIb, and III), each with their own specific proteolytic properties, are involved in the breakdown of myofibrillar proteins during muscle atrophy (Mattson and Mykles, 1993; Mykles, 1990; Mykles and Skinner, 1982, 1983, 1986). The calpains completely degrade all the myofibrillar proteins *in vitro* and *in situ* (Mattson and Mykles, 1993; Mykles, 1990; Mykles and Skinner, 1982, 1983) and their activities are elevated in atrophic claw muscles (Mykles and Skinner, 1982).

cDNAs encoding three crustacean calpains have been characterized. Calpain B (CalpB) has a domain organization similar to mammalian m-and μ-calpains and is expressed in all tissues; it appears to encode the CDP IIb activity (Kim et al., 2005). Calpain M (CalpM) and Calpain T (CalpT) encode atypical calpains and show more restricted tissue distributions than CalpB (Kim et al., 2005; Yu and Mykles, 2003). CalpM is a truncated protein that lacks the calmodulin-like Ca^{2+}-binding domain at the C-terminus, while CalpT has a novel T domain in place of the Ca^{2+}-binding domain (Kim et al., 2005; Yu and Mykles, 2003). CalpM is preferentially expressed in lobster and land crab skeletal muscles (Ha-CalpM and Gl-CalpM, respectively) (Yu and Mykles, 2003; Kim et al., 2005). The proteins in both species have an estimated mass of about 66 kDa and seem to correspond to the previously identified lobster CDP III (Yu and Mykles, 2003).

The ability of Ha-CalpM/CDP III to break down myofibrillar proteins and its high expression in skeletal muscles suggest that Ha-CalpM may play a role in restructuring the myofilament apparatus during fiber switching. In the current study, an antibody raised against a unique, N-terminal region of the Ha-CalpM protein (Yu and Mykles, 2003) was used to identify the intracellular location of the calpain in sections of 7th stage juvenile lobster claw muscles. In adults, Ha-CalpM has a uniform cytoplasmic distribution in cutter and crusher muscle fibers (Yu and Mykles, 2003). Differentiating cutter and crusher claws from different stages of the molt cycle (1 day post molt through 37 days postmolt) were examined. In addition, serial sections from some of these samples were labeled with an antibody raised against *Drosophila* synaptotagmin to identify motor synapses within the muscles. Together, these studies demonstrate that Ha-CalpM in differentiating lobster claw muscles is concentrated near motor endplates. In complementary analyses, we quantified Ha-CalpM mRNA levels in 9th and 10th stage juvenile claw muscles with real-time PCR and compared expression levels between developing cutter and crusher claws. These measurements demonstrate that Ha-CalpM expression is correlated with the expression of fast myosin heavy chain (MHC) in both fast and slow muscles.

2. Materials and methods

2.1. Animals and tissue preparation

Juvenile lobsters, *Homarus americanus*, were raised in the culture facility at the Bodega Marine Laboratory from larvae (Chang and Conklin, 1993). The left claw of 4th stage larvae was autotomized to induce differentiation of the right claw into the crusher type; the regenerated left claw differentiates into the cutter type (Govind and Pearce, 1989). These claws subsequently regenerate and were collected at later stages. 7th stage juvenile lobsters ($n=39$) were induced to autotomize both claws by gently squeezing the merus with forceps. The most proximal and distal regions of the claw propodus were removed to facilitate penetration of fixative and buffer. Claws were fixed in

3.7% formaldehyde in a buffered saline solution (0.5 M NaCl, 15 mM KCl, 10 mM EDTA and 25 mM HEPES–NaOH, pH 7.5) at 4 °C overnight. Claws were then rinsed several times in the saline without formaldehyde, dehydrated through a graded ethanol series, and embedded in paraffin. Sections of the claw closer muscles (10 μm) were placed on microscope slides and processed for immunohistochemistry. In complementary analyses, paired claws from 25 9th and 10th stage animals were collected and frozen for real-time PCR to quantify Ha-CalpM and fast MHC mRNA levels.

2.2. Immunohistochemistry

Claw muscle sections were heated to 60 °C for 5 min and then rehydrated through a graded ethanol series. Sections were then covered with a blocking solution containing 2% bovine serum albumin in PBS for 1 h at room temperature. The blocking solution was then replaced by the same solution containing anti-Ha-CalpM (Yu and Mykles, 2003) or anti-*Drosophila melanogaster* synaptotagmin (syt) (Mackler et al., 2002) serum (1:500) for 1 h. The anti-syt antibody was raised against an intra-vesicular (IV) domain of the protein and its specificity has been reported previously (Mackler et al., 2002). Sections were washed three times (5 min each) in Tris-buffered saline (TBS) with 0.05% Tween (TTBS) and incubated with blocking buffer containing biotinylated anti-rabbit immunoglobulin G (IgG) antibody (Vector Labs, Burlingame, CA, USA; 1:500) for 1 h at room temperature. Sections were washed three times in TTBS and incubated with an avidin/biotinylated alkaline phosphatase complex (ABC reagent, Vector Labs) for 30 min. Finally, sections were washed 3 times in TTBS and developed using NBT/BCIP reagent (Roche Molecular Biochemicals) as a substrate for the alkaline phosphatase enzyme. Sections were then washed several times in water, dehydrated through a graded ethanol series, twice washed in xylenes, and then mounted with Permount and a coverslip.

2.3. Western blot analysis of synaptotagmin

Adult lobster ventral nerve cord and juvenile cutter and crusher claw muscles were homogenized directly in SDS sample buffer (31.25 mM Tris pH 6.8, 12.5% (v/v) glycerol, 5% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.25% β-mercaptoethanol) on ice. Whole *Drosophila* heads were also homogenized in SDS sample buffer and used as a positive control. Proteins were separated electrophoretically on a discontinuous gel system (Bio-Rad Mini-Protean II) at 200 V for 45 min. The proteins were then transferred to PVDF membranes. Membranes were blocked in 2% non-fat milk in TTBS for 1 h before adding the anti-syt IV antibody (1:500 final concentration). The membrane was incubated with the antibody for 1 h and washed 3 times (5 min each with agitation) in TTBS. The membrane was then incubated with biotinylated anti-rabbit IgG (1:2000) in 2% non-fat milk in TTBS for 1 h and washed 3 times in TTBS. Finally, the membrane was incubated in ABC reagent (Vectastain, Vector Labs) for 30 min and again washed 3 times in TTBS. For development, the membrane was preincubated in the development buffer (0.1 M Tris, pH 9.5) and then placed in fresh NBT/BCIP (Roche Molecular Biochemicals) and kept in the dark until the blot developed.

2.4. Real-time PCR of Ha-CalpM

The methods for real-time PCR followed those reported by Yu and Mykles (2003) for amplification of Ha-CalpM. Briefly, sequence-specific primers designed to amplify Ha-CalpM (GenBank accession # AY124009) were used with cDNA obtained through reverse transcription of total RNA. A plasmid containing known amounts of Ha-CalpM cDNA insert was serially diluted and added to a master PCR mixture containing SYBR green (Light Cycler DNA Master SYBR Green I, Roche Molecular Biochemicals) and run on a Cepheid SmartCycler instrument. PCR conditions were the same as reported by Yu and Mykles (2003). Whole cutter and crusher claws from 9th and 10th stage juvenile lobsters were homogenized and used for RNA isolation (Medler et al., 2007). Log-transformed values were used to compare expression levels in cutter and crusher claws using a paired *T*-test. Log-transformed values were also used to examine the correlation between fast MHC and Ha-CalpM copy numbers.

Real-time PCR data were also obtained from adult lobsters from a previous study (Medler et al., 2005), although data for Ha-CalpM expression levels were not reported. In that study, lobsters were eyestalk ablated to experimentally elevate ecdysteroid levels, but this treatment had no discernable effect on fast MHC or Ha-CalpM expression levels. In the current report, we include these data because they offer a comparison of adult lobster muscles with the expression data from differentiating juvenile claw muscles. For comparison among muscle types, log-transformed values for copy number were compared by ANOVA, followed by a Bonferroni post-hoc test. The correlations between Ha-CalpM and fast MHC expression

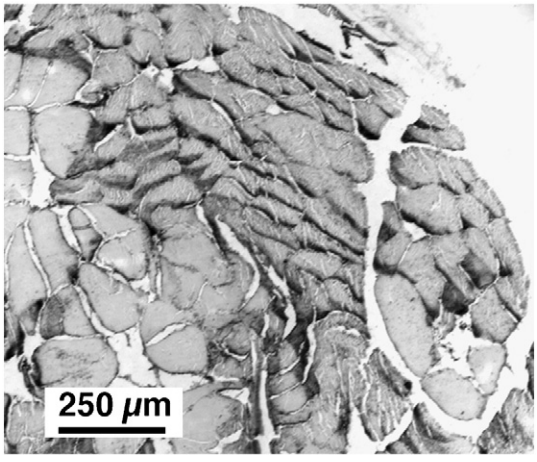

Fig. 1. Ha-CalpM localization in juvenile claw muscles. Ha-CalpM protein was localized within the muscles with immunohistochemistry using the anti-Ha-CalpM antibody (1:500 dilution). Staining was not evident in all fibers, but when present, was always found at the periphery of the fibers. Within a single region, staining tended to be on the same side of a group of fibers. This staining pattern was not correlated with claw type (cutter or crusher claw), nor with the stage of the molt cycle (number of days postmolt).

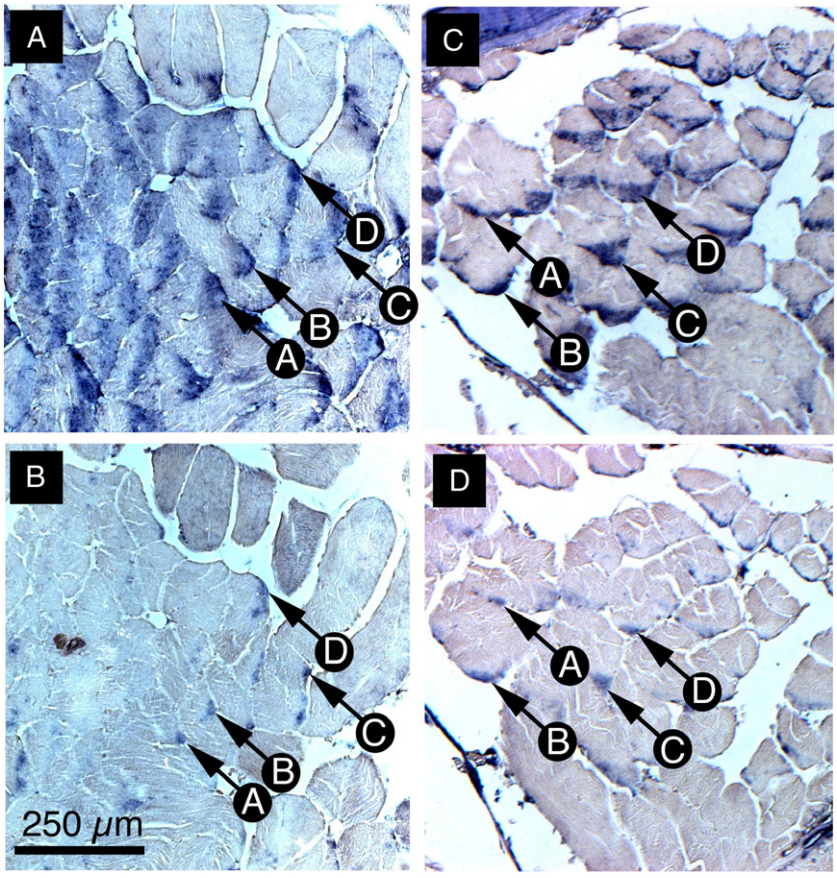

Fig. 2. Co-localization of Ha-CalpM and synaptotagmin in juvenile claw muscles. Serial sections of presumptive crusher claw muscle were labeled with either anti-Ha-CalpM antibodies (A, C), or anti-syt antibodies (B, D). The Ha-CalpM staining pattern was asymmetrical, with the Ha-CalpM concentrated toward one side of the muscle fibers. The syt staining was restricted to single points at the fiber periphery. Lettered arrows indicate the correspondence between Ha-CalpM and syt localization in the serial sections. Two different muscle regions are shown (A–B and C–D). In each case, both antibodies stain the same general region of the muscles, although the Ha-CalpM is within the muscle fiber itself, while the syt is at the fiber periphery.

levels in different muscle types were examined through simple linear regression of log-transformed values.

3. Results

3.1. Immunohistochemistry

Labeling with the anti-Ha-CalpM antibody led to distinct asymmetrical staining of muscle fibers in the majority (~85%) of the claw muscles sampled (Fig. 1). Staining regions were generally more restricted than that shown in Fig. 1, with only ~15% of all sections exhibiting such extensive staining throughout the whole section. Staining was always restricted to one of the peripheral edges of the fibers, and in regions of the claw muscle where staining was present, all of the fibers within that region had staining on the same side of the fibers. We did not find any correlation between muscle staining and muscle fiber type, nor was staining different between cutter and crusher claws. Furthermore, we did not detect any pattern related to the timing, or stage, within the molt cycle.

The anti-Syt IV antibody labeled discrete, small areas throughout the whole muscle. Some sections had little or no staining, while others had staining in several areas. In serial sections alternately labeled with either anti-Ha-CalpM or anti-Syt IV, the regions of staining always corresponded. In sections where several points of Syt-IV labeling were present, these points were directly adjacent to high Ha-CalpM staining (Fig. 2).

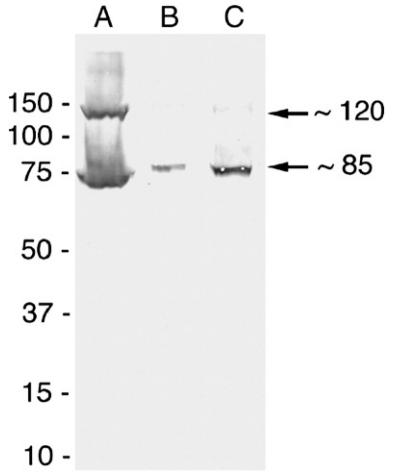

Fig. 3. Western blot demonstrating specificity of the anti-Syt IV antibody in lobster muscle. The antibody recognized similar-sized proteins (approximately 85 kDa) in (A) lobster nerve cord, (B) juvenile crusher claw muscle, and (C) juvenile cutter claw muscle. In addition, a larger (approximately 120 kDa) unknown protein was identified in the lobster ventral nerve cord sample.

3.2. Western blot of synaptotagmin

The anti-Syt IV antibody recognized similar-sized proteins (approximately 85 kDa) in lobster ventral nerve cord and in claw muscles from 10th stage juvenile lobster claws (Fig. 3). In addition, a larger (approximately 120 kDa) protein was observed in the ventral nerve cord. We found similar reactivity to a number of larger proteins in *Drosophila* whole head homogenates, in addition to the synaptotagmin labeling (data not shown). We did not observe cross-reactivity with any other proteins in the juvenile claw muscles.

3.3. Real-time PCR

Quantification of Ha-CalpM mRNA levels by real-time PCR indicated that expression levels did not differ between cutter and crusher claws (Fig. 4A). Further analyses indicated that expression levels did not differ as a function of timing in the molt cycle (data not shown). We also found that Ha-CalpM mRNA levels were significantly correlated with the expression of fast MHC in both cutter and crusher claw muscles: log Ha-CalpM = 0.699 + 0.409 * log fast MHC; $r^2 = 0.454$; $p < 0.0003$ (Fig. 4B). In addition, we found that Ha-CalpM expression was also correlated with mRNA levels of the fast muscle protein, P75 (data not shown). This is not surprising, as P75 and fast MHC expression is positively correlated (Medler and Mykles, 2003; Medler et al., 2007). However, expression levels of Ha-CalpM were not significantly correlated with other myofibrillar protein transcripts such as actin or slow MHC. In the muscles of young adult lobsters, Ha-CalpM mRNA levels were significantly higher in the predominantly fast cutter claw muscles than

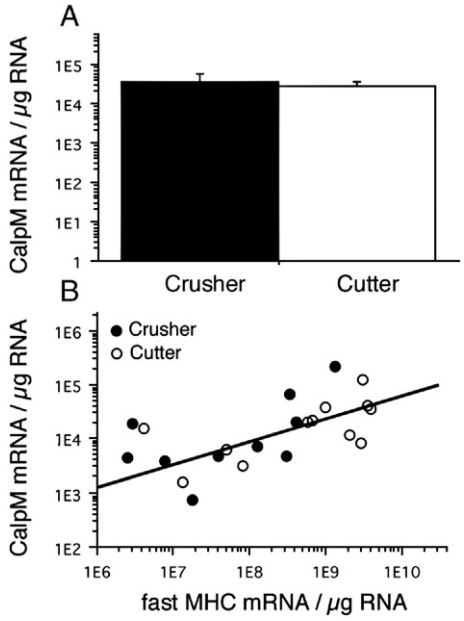

Fig. 4. Quantification of Ha-CalpM mRNA by real-time PCR in 9th and 10th stage juvenile claw muscles. (A) Ha-CalpM expression levels were not significantly different between cutter and crusher claw muscles. (B) In both claw types, the levels of Ha-CalpM mRNA were significantly correlated with the levels of fast MHC mRNA ($p < 0.009$). See Results section for regression equation.

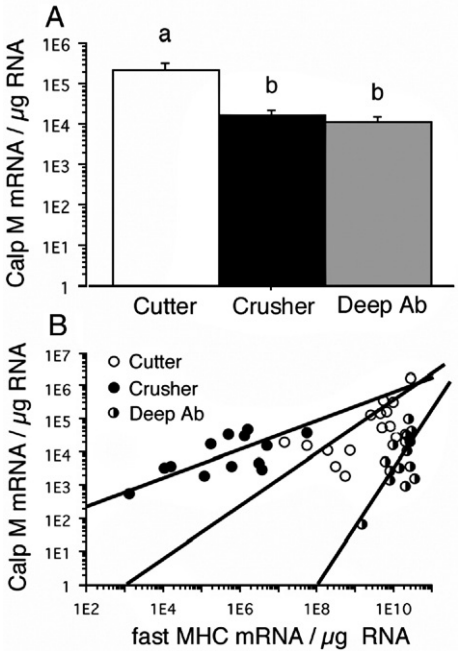

Fig. 5. Quantification of Ha-CalpM mRNA by real-time PCR in adult lobster muscles. (A) Ha-CalpM expression was significantly higher in the fast cutter claw muscles than in either crusher claw or deep abdominal muscles. (B) Significant correlations between Ha-CalpM and fast MHC were observed for each muscle type, with different specific relationships observed for each type. See Results section for specific regression equations. Means with different letters in (A) indicate significant differences as determined by a Bonferroni pairwise comparison.

in either the predominantly slow crusher claw or the exclusively fast deep abdominal muscles (Fig. 5A). There were also significant correlations between fast MHC and Ha-CalpM expression, with different specific relationships in different muscle types (Fig. 5B). In crusher claw muscles, log Ha-CalpM = 1.764 + 0.309 * log fast MHC; $r^2 = 0.426$; $p < 0.0155$. For cutter claw muscles: log Ha-CalpM = −0.731 + 0.561 * log fast MHC; $r^2 = 0.431$; $p < 0.0079$. For deep abdominal muscles: log Ha-CalpM = −9.66 + 1.324 * log fast MHC; $r^2 = 0.448$; $p < 0.0046$).

4. Discussion

We found that the lobster muscle-specific calpain (Ha-CalpM) is asymmetrically concentrated toward one side of the fibers within differentiating juvenile claw muscles (Fig. 1). This contrasts from our previous report of Ha-CalpM staining in adult lobster muscles, where uniformly low levels of cytoplasmic and nuclear staining were observed throughout both cutter and crusher muscles (Yu and Mykles, 2003). The highest concentration of the protein is localized in the region near the motor endplate, as indicated by the presence of synaptotagmin adjacent to the Ha-CalpM staining regions (Fig. 2). Synaptotagmin is a highly conserved synaptic protein central in the Ca^{2+}-sensitive release of neurotransmitter from the nerve terminal into the synaptic cleft (Mackler et al., 2002). We found that both the ventral nerve cord in adult lobsters and the claw muscles in juvenile lobsters expressed an ~85 kDa form of synaptotagmin,

while the adult nerve cord also expressed a ~120 kDa form (Fig. 3). By comparison, crayfish muscles express a 107 kDa isoform, while their nervous tissues possess an 87 kDa isoform (Cooper et al., 1995). In juvenile muscle sections, we identified synaptotagmin at discrete points at the periphery of muscle fibers, consistent with the position of motor synapses (Fig. 2B and D). This is similar to synaptotagmin labeling in crayfish motor neurons, where the protein is distributed in nerve terminals of excitatory and inhibitory motor neurons (Cooper et al., 1995; Msghina and Atwood, 1997). The Ha-CalpM staining pattern was not correlated with claw type (cutter *vs.* crusher) or with stage in the molt cycle (premolt, postmolt, or intermolt). The majority of all sections (~85%) exhibited similar staining patterns as those seen in Fig. 1, but in the majority of these the staining was restricted to only a small part of the section. This seemingly stochastic expression of Ha-CalpM in the juvenile claw muscles is consistent with temporal fluctuations in expression that might be related to changes in motor neuron activity.

We previously reported that Ha-CalpM is expressed at higher levels in fast cutter claw muscles and deep abdominal muscles, than in the slow crusher claw muscle (Yu and Mykles, 2003). This is similar to expression of Capn 3 in mammalian skeletal muscles, which is found at levels about three times higher in fast than in slow muscles, and where low frequency electrical stimulation leads to a rapid loss of the protein from fast muscles (Jones et al., 1999; Sultan et al., 2001). Here, we find that there is not a significant difference between the differentiating cutter and crusher claw muscles, but there is a significant correlation between Ha-CalpM and fast MHC mRNA levels in differentiating juvenile muscles (Fig. 4). Similar correlations are observed in fully differentiated muscles from adult lobsters, but the relationship between Ha-CalpM and fast MHC expression follows a different slope based on the muscle type (Fig. 5B). In previous studies, we observed that certain sets of genes are correlated in their expression, which is not surprising since specific fibers types are defined by the expression of a whole assemblage of proteins (Medler and Mykles, 2003; Medler et al., 2004, 2005, 2007; Mykles, 1985a,b, 1988). However, it is somewhat difficult to interpret the correlation between Ha-CalpM and fast MHC gene expression, since there is clearly not a restriction of the Ha-CalpM protein to fast fiber populations. Many fully differentiated lobster muscles co-express multiple MHC isoforms, including adult crusher claw muscles, which express significant levels of the fast MHC isoform (Medler and Mykles, 2003; Medler et al., 2005, 2007). It is possible that Ha-CalpM expression is causally linked to the regulation of fast MHC expression, so that elevation of Ha-CalpM stimulates an increase in fast MHC expression. However, it is also possible that both genes are simply controlled by common gene regulatory elements. In mammalian muscles, several slow muscle genes have upstream regulatory regions that share NFAT binding elements that all respond to the same calcineurin-NFAT signaling pathway (Chin et al., 1998).

The position of Ha-CalpM near the region of the motor endplate in differentiating lobster claw muscles raises many interesting questions about its function. One possibility is that Ha-CalpM is directly involved in mediating the hydrolysis of myofibrillar proteins during fiber switching. The role of different calpains in skeletal muscle remodeling is not well understood, but the emerging picture is that one function of the calpains is in the removal of obsolete myofibrillar proteins (Tallandier et al., 1996; Huang and Forsberg, 1998; Fareed et al., 2006). The activity of ubiquitous calpains (m-and μ-calpains) increases following electrical stimulation in transforming mammalian muscle fibers, and this increased activity is apparently related to a translocation of calpains to the cell membrane and myofibrillar components of the muscles (Sultan et al., 2000, 2001). In addition, the loss of muscle mass and characteristic shifts in myosin gene expression occurring during muscle atrophy are significantly reduced or blocked by administration of calpain inhibitors (Tidball and Spencer, 2002; Fareed et al., 2006). In crustacean claw muscles, calpains are responsible for the selective hydrolysis of thin filament proteins during molt-induced atrophy (Ismail and Mykles, 1992; Mykles and Skinner, 1981). In these muscles, four different calpain activities hydrolyze major myofibrillar proteins including myosin heavy chain, actin, troponins, and tropomyosin (Mattson and Mykles, 1993; Mykles and Skinner, 1982, 1983, 1986). Based on its size (62 kDa isoform in claw muscles and 68 kDa isoform in deep abdominal muscle), Yu and Mykles (2003) concluded that Ha-CalpM probably corresponds to CDP III, which degrades MHC to a greater extent than the thin filament proteins (Mattson and Mykles, 1993). As fibers undergo extensive remodeling in the developing lobster claws, it is necessary to remove the obsolete myofibrillar proteins, in addition to turning on new myofibrillar genes. Ha-CalpM may be involved in the selective removal of myofibrillar proteins as fast claw muscles become slow and *vice versa*. Activation of calpains in claw muscles cultured *in vitro* causes a rapid and preferential degradation of the Z-line (Mykles, 1990), which would facilitate removal and subsequent degradation of myofilaments.

Based on comparison with the mammalian muscle-specific calpain (Capn 3), Ha-CalpM may also be involved in complex signaling pathways involved in directing muscle development. In contrast to the role of the ubiquitous calpains (m- and μ-calpains), which are directly involved in myofibrillar degradation, Capn 3 appears to play a central role in the maintenance of normal muscle phenotype (Duguez et al., 2006). In fact, a number of specific mutations of the Capn 3 gene leading to a dysfunctional protein result in limb girdle muscular dystrophy type 2A (Sorimachi et al., 1997; Kinbara et al., 1998; Kramerova et al., 2007). Capn 3 seems to necessary for guiding muscle development and maintaining normal muscle phenotype (Kinbara et al., 1998; Duguez et al., 2006; Kramerova et al., 2007). Transgenic mice that over-express certain alternatively spliced forms of Capn 3 exhibit a number of features characteristic of developing muscles, and Capn 3 appears to be required for normal muscle remodeling in response to conditions such as reduced gravity (Spencer et al., 2002; Kramerova et al., 2007). The fact that Capn 3 binds to specific regions of the titin molecule within the sarcomere has also led to the hypothesis that this calpain participates in signaling complexes, which transduce mechanical stresses during muscle contraction into intracellular signals (Ojima et al., 2005). It is tempting to speculate that Ha-

CalpM may be involved in similar processes in lobster muscles; both Ha-CalpM and Capn 3 are preferentially expressed in skeletal muscles, although they differ in domain organization.

The concentration of the protein near muscle synapses and the seemingly stochastic expression of the protein suggest that it may be expressed, or translocated, in response to the activity of motor neurons. Govind and Pearce (1986) demonstrated that motor activity during a critical developmental window determines claw muscle asymmetry in juvenile lobsters. If motor activity is quiescent during this period, or if both claws are equally active, then both claws remain symmetrical for the lifetime of the lobster (Govind, 1992). This asymmetry is thought to develop initially in the central nervous system, with subsequent differences in motor activity driving specific fiber type changes in the differentiating claw muscles (Govind, 1992). The concentration of Ha-CalpM near the motor endplate of differentiating claw muscles raises the possibility that it is involved in directing muscle differentiation. We have shown that expression of slow-twitch (S_1) and slow-tonic (S_2) myofibrillar protein isoforms is correlated with synaptic properties, suggesting that increased motor neuron activity can drive the expression of the S_2 phenotype (Mykles et al., 2002).

In conclusion, we have found that a muscle-specific calpain is concentrated near the motor endplate of differentiating juvenile lobster claw muscles. This is distinct from the more generalized staining pattern previously reported in fully differentiated adult muscles (Yu and Mykles, 2003). Although the precise role of Ha-CalpM in lobster muscles is currently unknown, the results presented here are consistent with a role in linking muscle differentiation with the level of motor activity. This possibility should be readily testable in future studies, using experimental stimulation of the different motor neurons and then monitoring changes in Ha-CalpM expression.

Acknowledgements

We would like to thank Jennifer Torvaldson for assistance with the Ha-CalpM immunohistochemistry. We would also like to thank Dr. Noreen Reist for the generous donation of the Syt-IV antibody. The work was supported by a grant from the National Science Foundation (IBN-0077422) to DLM and an NRSA postdoctoral fellowship from NIH (AR 08597-01A1) to SM.

References

Atwood, H.L., 1976. Organization and synaptic physiology of crustacean neuromuscular systems. Prog. Neurobiol. 7, 291–391.

Chang, E.S., Conklin, D.E., 1993. Larval culture of the American lobster (*Homarus americanus*). In: McVey, J.P. (Ed.), CRC Handbook of Mariculture. CRC Press, Boca Raton, pp. 489–495.

Chin, E.R., Olson, E.N., Richardson, J.A., Yang, Q., Humphries, C., Shelton, J.M., Wu, H., Zhu, W., Bassel-Duby, R., Williams, R.S., 1998. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev. 12, 2499–2509.

Cooper, R.L., Hampson, D.R., Atwood, H.L., 1995. Synaptotagmin-like expression in the motor nerve terminals of crayfish. Brain Res. 703, 214–216.

Duguez, S., Bartoli, M., Richard, I., 2006. Calpain 3: a key regulator of the sarcomere? FEBS J 273, 3427–3436.

Eble, D.M., Spragia, M.L., Ferguson, A.G., Samarel, A.M., 1999. Sarcomeric myosin heavy chain is degraded by the proteasome. Cell Tissue Res. 296, 541–548.

Fareed, M.U., Evenson, A.R., Wei, W., Menconi, M., Poylin, V., Petkova, V., Pignol, B., Hasselgren, P.O., 2006. Treatment of rats with calpain inhibitors prevents sepsis-induced muscle proteolysis independent of atrogin-1/MAFbx and MuRF1 expression. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290, R1589–R1597.

Goll, D.E., Thompson, V.F., Li, H., Wei, W., Cong, J., 2003. The calpain system. Physiol. Rev. 83, 731–801.

Govind, C.K., 1984. Development of asymmetry in the neuromuscular system of lobster claws. Biol. Bull. 167, 94–119.

Govind, C.K., 1992. Claw asymmetry in lobster: case study in developmental neuroethology. J. Neurobiol. 23, 1423–1445.

Govind, C.K., Pearce, J., 1986. Differential reflex activity determines claw and closer muscle asymmetry in developing lobsters. Science 233, 354–356.

Govind, C.K., Pearce, J., 1989. Critical period for determining claw asymmetry in developing lobsters. J. Exp. Zool. 249, 31–35.

Govind, C.K., Mellon, DeF., Quigley, M.M., 1987. Muscle and muscle fiber type transformations in clawed crustaceans. Amer. Zool. 27, 1079–1098.

Huang, J., Forsberg, N.E., 1998. Role of calpain in skeletal-muscle protein degradation. Proc. Natl. Acad. Sci. U. S. A. 95, 12100–12105.

Ismail, S.Z.M., Mykles, D.L., 1992. Differential molt-induced atrophy in the dimorphic claws of male fiddler crabs, *Uca pugnax*. J. Exp. Zool. 263, 18–31.

Jackman, R.W., Kandarian, S.C., 2004. The molecular basis of skeletal muscle atrophy. Am. J. Physiol. Cell Physiol. 287, C834–C843.

Jones, S.W., Parr, T., Sensky, P.L., Scothern, G.P., Bardsley, R.G., Buttery, P.J., 1999. Fibre type-specific expression of p94, a skeletal muscle-specific calpain. J. Muscle Res. 20, 417–424.

Kim, H.W., Chang, E.S., Mykles, D.L., 2005. Three calpains and ecdysone receptor in the land crab *Gecarcinus lateralis*: sequences, expression and effects of elevated ecdysteroid induced by eystalk ablation. J. Exp. Biol. 208, 3177–3197.

Kinbara, K., Sorimachi, H., Ishiura, S., Suzuki, K., 1998. Skeletal muscle-specific calpain, p94. Biochem. Pharmacol. 56, 415–420.

Koenders, A., Yu, X., Chang, E.S., Mykles, D.L., 2002. Ubiquitin and actin expression in claw muscles of land crab, *Gecarcinus lateralis*, and American lobster, *Homarus americanus*: differential expression of ubiquitin in two slow muscle fiber types during molt-induced atrophy. J. Exp. Zool. 292, 618–632.

Kramerova, I., Beckmann, J.S., Spencer, M.J., 2007. Molecular and cellular basis of calpainopathy (limb girdle muscular dystrophy type 2A). Biochim. Biophys. Acta. 1772, 128–144.

Lebart, M.C., Benyamin, Y., 2006. Calpain involvement in the remodeling of cytoskeletal anchorage complexes. FEBS J. 273, 3415–3426.

Lecker, S.H., Solomon, V., Mitch, W.E., Goldberg, A.L., 1999. Muscle protein breakdown and the critical role of the ubiquitin–proteasome pathway in normal and disease states. J. Nutr. 129, 227S–237S.

Mackler, J.M., Drummond, J.A., Loewen, C.A., Robinson, I.M., Reist, N.E., 2002. The C2B Ca2+-binding motif of synaptotagmin is required for synaptic transmission in vivo. Nature 418, 340–344.

Mattson, J.M., Mykles, D.L., 1993. Differential degradation of myofibrillar proteins by four calcium-dependent proteinase activities from lobster muscle. J. Exp. Zool. 265, 97–106.

Medler, S., Mykles, D.L., 2003. Analysis of myofibrillar proteins and transcripts in adult skeletal muscles of the American lobster *Homarus americanus*: variable expression of myosins, actin and troponins in fast, slow-twitch and slow-tonic fibres. J. Exp. Biol. 206, 3557–3567.

Medler, S., Lilley, T., Mykles, D.L., 2004. Fiber polymorphism in skeletal muscles of the American lobster, *Homarus americanus*: continuum between slow-twitch (S1) and slow-tonic (S2) fibers. J. Exp. Biol. 207, 2755–2767.

Medler, S., Brown, K.J., Chang, E.S., Mykles, D.L., 2005. Eyestalk ablation has little effect on actin and myosin heavy chain gene expression in adult lobster skeletal muscles. Biol. Bull. 208, 127–137.

Medler, S., Lilley, T.R., Riehl, J.H., Mulder, E.P., Chang, E.S., Mykles, D.L., 2007. Myofibrillar gene expression in differentiating lobster claw muscles. J. Exp. Zool. 307A, 281–295.

Mellon, DeF., 1992. Connective tissue and supporting structures. In: Harrison, F.W., Humes, A.F. (Eds.), Decapod Crustacea. Microscopic Anatomy of Invertebrates, vol. 10. Wiley-Liss, New York, pp. 77–116.

Mitch, W.E., Goldberg, A.F., 1996. Mechanisms of muscle wasting. The role of the ubiquitin–proteasome pathway. N. Engl. J. Med. 335, 1897–1905.

Msghina, M., Atwood, H.L., 1997. Distribution and morphology of inhibitory innervation in crayfish (*Procamarus clarkii*) limb and abdominal muscles. Cell Tissue Res. 290, 111–118.

Mykles, D.L., 1985a. Heterogeneity of myofibrillar proteins in lobster fast and slow muscles: variants of troponin, paramyosin, and myosin light chains comprise four distinct protein assemblages. J. Exp. Zool. 234, 23–32.

Mykles, D.L., 1985b. Multiple variants of myofibrillar proteins in single fibers of lobster claw muscles: evidence for two types of slow fibers in the cutter closer muscle. Biol. Bull. 169, 476–483.

Mykles, D.L., 1988. Histochemical and biochemical characterization of two slow fiber types in decapod crustacean muscles. J. Exp. Zool. 245, 232–243.

Mykles, D.L., 1990. Calcium-dependent proteolysis in crustacean claw closer muscle maintained *in vitro*. J. Exp. Zool. 256, 16–30.

Mykles, D.L., 1992. Getting out of a tight squeeze — enzymatic regulation of claw muscle atrophy in molting. Am. Zool. 32, 485–494.

Mykles, D.L., 1997. Crustacean muscle plasticity: molecular mechanisms determining mass and contractile properties. Comp. Biochem. Physiol. 117B, 367–378.

Mykles, D.L., 1998. Intracellular proteinases of invertebrates: calcium-dependent and proteasome/ubiquitin-dependent systems. Int. Rev. Cyt. 184, 157–289.

Mykles, D.L., Skinner, D.M., 1981. Preferential loss of thin filaments during molt-induced atrophy in crab claw muscle. J. Ultrastructure Res. 75, 314–325.

Mykles, D.L., Skinner, D.M., 1982. Molt-cycle associated changes in calcium-dependent proteinase activity that degrades actin and myosin in crustacean muscle. Dev. Biol. 92, 386–397.

Mykles, D.L., Skinner, D.M., 1983. Ca2+-dependent proteolytic activity in crab claw muscle. Effects of inhibitors and specificity for myofibrillar proteins. J. Biol. Chem. 258, 10474–10480.

Mykles, D.L., Skinner, D.M., 1986. Four Ca2+-dependent proteinase activities isolated from crustacean muscle differ in size, net charge, and sensitivity to Ca2+ and inhibitors. J. Biol. Chem. 261, 9865–9871.

Mykles, D.L., Medler, S., Koenders, A., Cooper, R.L., 2002. Myofibrillar protein isoform expression is correlated with synaptic efficacy in slow fibres of the claw and leg opener muscles of crayfish and lobster. J. Exp. Biol. 205, 513–522.

Ojima, K., Ono, Y., Hata, S., Kowama, S., Doi, N., Sorimachi, H., 2005. Possible functions of p94 in connectin-mediated signaling pathways in skeletal muscle cells. J. Muscle Res. 26, 409–417.

Pette, D., Staron, R.S., 2001. Transitions of muscle fiber phenotypic profiles. Histochem Cell Biol 115, 359–372.

Shean, B.S., Mykles, D.L., 1995. Polyubiquitin in crustacean striated muscles: increased expression and conjugation during molt-induced atrophy. Biochim. Biophys. Acta. — Gene Struct. Expr. 1264, 312–322.

Skinner, D.M., 1966. Breakdown and reformation of somatic muscle during the molt cycle of the land crab, *Gecarcinus lateralis*. J. Exp. Zool. 163, 115–124.

Sorimachi, H., Ishiura, S., Suzuki, K., 1997. Structure and physiological function of calpains. Biochem. J. 328, 721–732.

Spencer, M.J., Guyon, J.R., Sorimachi, H., Potts, A., Richard, I., Herasse, M., Chamberlain, J., Dalkilic, I., Kunkel, L.M., Beckman, J.S., 2002. Stable expression of calpain 3 from a muscle transgene in vivo: immature muscle in transgenic mice suffests a role for calpain 3 in muscle maturation. Proc. Nat. Acad. Sci. U. S. A. 99, 8874–8879.

Sultan, K.R., Dittrich, B.T., Pette, D., 2000. Calpain activity in fast, slow, transforming, and regenerating skeletal muscles of rat. Am. J. Physiol. Cell. Physiol. 279, C639–C647.

Sultan, K.R., Dittrich, B.T., Leisner, E., Paul, N., Pette, D., 2001. Fiber type-specific expression of major proteolytic systems in fast-to slow-transforming rabbit muscle. Am. J. Physiol. Cell Physiol. 280, C239–C247.

Tallandier, D., Aurousseau, E., Meynial-Denis, D., Bechet, D., Ferrara, M., Cottin, P., Ducastaing, A., Bigard, X., Guezennec, C.Y., Schmid, H.P., Attaix, D., 1996. Coordinate activation of lysosomal, Ca2+-activated, and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle. Biochem. J. 316, 65–72.

Tidball, J.G., Spencer, M.J., 2002. Expression of a capastatin transgene slows muscle wasting and obviates changes in myosin isoform expression during murine muscle disuse. J. Physiol. 545, 819–828.

Yu, X., Mykles, D.L., 2003. Cloning of a muscle-specific calpain from American lobster *Homarus americanus*: expression associated with muscle atrophy and restoration during moulting. J. Exp. Biol. 206, 561–575.