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

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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.
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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

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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

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