The Journal of Experimental Biology 208, 3177-3197
Published by The Company of Biologists 2005
doi:10.1242/jeb.01754

Three calpains and ecdysone receptor in the land crab *Gecarcinus lateralis*: sequences, expression and effects of elevated ecdysteroid induced by eyestalk ablation

H.-W. Kim[1], E. S. Chang[2] and D. L. Mykles[1,*]

[1]*Department of Biology, Colorado State University, Fort Collins, CO 80523, USA* and [2]*Bodega Marine Laboratory, University of California, Davis, Bodega Bay, CA 94923, USA*

*Author for correspondence (e-mail: don@lamar.colostate.edu)

Accepted 15 June 2005

Summary

Crustacean muscle has four calpain-like proteinase activities (CDP I, IIa, IIb and III) that are involved in molt-induced claw muscle atrophy, as they degrade myofibrillar proteins *in vitro* and *in situ*. Using PCR cloning techniques, three full-length calpain cDNAs (Gl-CalpB, Gl-CalpM and Gl-CalpT) were isolated from limb regenerates of the tropical land crab *Gecarcinus lateralis*. All three had highly conserved catalytic (dII) and C_2-like (dIII) domains. Gl-CalpB was classified as a typical, or EF-hand, calpain, as the deduced amino acid sequence had a calmodulin-like domain IV in the C-terminus and was most similar to *Drosophila* calpains A and B. Based on its estimated mass (~88.9 kDa) and cross-immunoreactivity with a polyclonal antibody raised against Dm-CalpA, Gl-CalpB may encode CDP IIb, which is a homodimer of a 95-kDa subunit. It was expressed in all tissues examined, including skeletal muscle, heart, integument, gill, digestive gland, hindgut, nerve ganglia, gonads and Y-organ (molting gland). Both Gl-CalpM and Gl-CalpT were classified as atypical, or non-EF-hand, calpains, as they lacked a domain IV sequence. Gl-CalpM was a homolog of Ha-CalpM from lobster, based on similarities in deduced amino acid sequence, estimated mass (~65.2 kDa) and structural organization (both were truncated at the C-terminal end of dIII). It was expressed at varying levels in most tissues, except Y-organ. Gl-CalpT (~74.6 kDa) was similar to TRA-3 in the nematode *Caenorhabditis elegans*; domain IV was replaced by a unique 'T domain' sequence. It was expressed in most tissues, except eyestalk ganglia and Y-organ. The effects of elevated ecdysteroid, induced by eyestalk ablation, on calpain and ecdysone receptor (Gl-EcR) mRNA levels in skeletal muscles were quantified by real-time PCR. At 1 day after eyestalk ablation, Gl-EcR and Gl-CalpT mRNA levels increased 15- and 19.3-fold, respectively, in claw muscle but not in thoracic muscle. At 3 days after eyestalk ablation, Gl-EcR and Gl-CalpT mRNA levels in claw muscle had decreased to 2.8-fold and 4.3-fold higher than those in intact controls, respectively, suggesting a feedback inhibition by ecdysteroid. There was no significant effect of eyestalk ablation on Gl-CalpB and Gl-CalpM mRNA levels. Gl-CalpT and Gl-EcR mRNA levels were significantly correlated in both claw and thoracic muscles from intact and eyestalk-ablated animals. The data suggest that Gl-CalpT is involved in initiation of claw muscle atrophy by ecdysteroids. Premolt reduction in claw muscle mass and concomitant remodeling of the sarcomere probably result from post-transcriptional regulation of calpains.

Key words: Crustacea, Arthropoda, calpain, tissue distribution, steroid hormone, molting, ecdysone, gene expression, mRNA, ecdysone receptor, DNA sequence, amino acid sequence, cloning, cDNA, muscle atrophy, skeletal muscle.

Introduction

Calpains are a diverse family of intracellular Ca^{2+}-dependent cysteine proteinases containing a papain-related catalytic domain (Goll et al., 2003; Mykles, 1998, 1999). In mammals, at least 14 calpain genes have been characterized (Goll et al., 2003; Suzuki et al., 2004). Calpains have also been identified in plants (Margis and Margis-Pinheiro, 2003), yeast (Futai et al., 1999), fungus (Denison et al., 1995), schistosomes (Andresen et al., 1991), *Hydra* (GenBank #DN636661), nematode (Barnes and Hodgkin, 1996), *Drosophila* (Farkas et al., 2004; Jékely and Friedrich, 1999; Spadoni et al., 2003), trout (Salem et al., 2004) and lobster (Yu and Mykles, 2003). Calpains have a highly conserved cysteine protease domain (dII), which contains a catalytic triad (Cys, His, Asn) and two highly conserved Ca^{2+}-binding regions required for Ca^{2+}-dependent activity (Moldoveanu et al., 2002, 2004). However, in some calpains, the catalytic residues are mutated (e.g. mammalian Capn6 and *Drosophila* CalpC), which suggests that these calpains lack protease activity (Friedrich et al., 2004;

Goll et al., 2003). The NH_2-terminal domain (dI) varies in length and sequence between different calpain genes. Autolysis of the N-terminus is associated with activation of some calpains (Goll et al., 2003). Most calpains have a C_2-like domain (dIII) that mediates Ca^{2+}-dependent phospholipid binding and activation (Alexa et al., 2004; Fernandez-Montalvan et al., 2004; Friedrich et al., 2004; Hosfield et al., 1999).

Calpain genes have been organized into two general categories, based on the presence ('typical' or 'EF-hand' calpains) or absence ('atypical' or 'non-EF-hand' calpains) of a calmodulin-like domain (dIV) in the C-terminus (Goll et al., 2003; Huang and Wang, 2001; Suzuki et al., 2004). Domain IV contains five EF-hand motifs, the first three of which (EF-1, EF-2 and EF-3) bind Ca^{2+} with varying affinities (Hosfield et al., 1999). The fifth EF-hand (EF-5) mediates dimerization with a 28-kDa regulatory subunit in vertebrate heterodimeric calpains (Maki et al., 2002). Atypical calpains are either truncated, e.g. lobster CalpM, nematode CPL-1, mammalian nCl-2′ and *Drosophila* Dm-CalpA′ (Sorimachi et al., 1993; Theopold et al., 1995; Yu and Mykles, 2003), or have the C-terminal region replaced by a different sequence, e.g. the 'T domain' in mammalian calpain 5 (Capn5) and nematode TRA-3 (Barnes and Hodgkin, 1996; Dear et al., 1997) or the 'SOL domain' in mammalian SOLH and *Drosophila* SOL or Calpain D (Delaney et al., 1991; Friedrich et al., 2004; Kamei et al., 1998). SOL and SOLH have six Zn-finger motifs in the N-terminal region, suggesting that they are DNA-binding proteins (Delaney et al., 1991; Kamei et al., 1998).

Calpains are involved in the selective degradation of myofibrillar proteins during a molt-induced atrophy in the large claws of decapod crustaceans (Mykles, 1992, 1998). Over several weeks, there is as much as a 78% reduction in muscle mass, which facilitates withdrawal of the appendages at molt (Mykles and Skinner, 1982a; Skinner, 1966). Crustacean calpains degrade myofibrillar proteins to acid-soluble products and show about 2-fold greater activity in atrophic muscle (Mykles, 1990; Mykles and Skinner, 1982b, 1983). Lobster muscle contains four calpain activities, which were initially termed Ca^{2+}-dependent proteinases (CDP I, CDP IIa, CDP IIb and CDP III; native masses 310, 125, 195 and 59 kDa, respectively; Mykles and Skinner, 1986). All require millimolar Ca^{2+} for full activity *in vitro* and are inhibited by cysteine protease inhibitors (Mykles and Skinner, 1983, 1986). CDP I has not been well characterized and it is the least efficient of the four calpains in degrading myofibrillar proteins (Mattson and Mykles, 1993). CDP IIb is a homodimer of a 95-kDa subunit and is related to Dm-CalpA, as determined by immunological analysis (Beyette et al., 1993; Beyette and Mykles, 1997). In western blots, CDP IIa reacts with an antibody directed to a peptide sequence in the active site of mammalian calpains but not with polyclonal antibodies raised against mammalian μ- and m-calpains or Dm-CalpA (Beyette et al., 1997). These results indicate that CDP IIa differs in structure from typical calpains. CDP IIa and CDP IIb are the most effective in degrading myofibrillar proteins, such as myosin heavy and light chains, actin, tropomyosin and troponin (Mattson and Mykles, 1993). A cDNA encoding CDP III was cloned and characterized in lobster (Ha-CalpM; Yu and Mykles, 2003). Ha-CalpM lacks the calmodulin-like domain in the C-terminus that is characteristic of typical calpains. It is highly expressed in skeletal muscle, but its mRNA and protein levels do not change significantly over the molting cycle. It may be involved in restructuring and/or maintaining contractile structures in crustacean skeletal muscle (Yu and Mykles, 2003).

Molt-induced muscle atrophy is coincident with increasing concentrations of ecdysteroids (ecdysone, 20-hydroxyecdysone and related compounds) in the hemolymph (Skinner, 1985). In the large claw muscles of fiddler crabs, ecdysone receptor (Up-EcR) mRNA level is increased significantly in the premolt stage (Chung et al., 1998b). (We use the term 'ecdysone receptor' as per common usage; however, we recognize that it is actually an ecdysteroid receptor.) This suggests that the molting hormone, 20-hydroxyecdysone, initiates and sustains the elevated myofibrillar protein degradation that results in the reduction of muscle mass. Ecdysteroids are steroid hormones that regulate growth, development, reproduction and molting in arthropods (Chang, 1993; Kozlova and Thummel, 2000). Ecdysteroids regulate gene transcription after binding to a nuclear ecdysone receptor (EcR). In insects, EcR heterodimerizes with ultraspiracle (USP), a vertebrate RXR ortholog, to form a functional hormone receptor (Yao et al., 1993); it binds to a specific response element to induce expression of early response genes, such as *E74* and *E75* (Thummel, 1996). The products of these early response genes are usually transcription factors that initiate an ecdysteroid cascade reaction (Huet et al., 1995).

Here, we report the cloning of cDNAs encoding three calpain genes (Gl-CalpM, Gl-CalpB and Gl-CalpT) and ecdysone receptor (Gl-EcR) isolated from the tropical land crab *Gecarcinus lateralis*. The deduced sequences of the three calpains were compared with those of calpains from other species. The expression of the three calpains in nine tissues was quantified by real-time RT-PCR. The effects of elevated ecdysteroid, induced by eyestalk ablation, on calpain and EcR mRNA levels in claw and thoracic muscles were determined. The results suggest that Gl-CalpT is involved with the initiation of myofibrillar proteolysis induced by elevated ecdysteroid.

Materials and methods

Animals

Adult blackback land crabs, *Gecarcinus lateralis* Fréminville, were collected from San Miguel Reserve near Fajardo, Puerto Rico. They were kept in covered plastic cages with aspen bedding moistened with tap water and were maintained at 27°C, 50% humidity and a 12 h:12 h L:D cycle. They were fed cat chow, carrots and lettuce twice a week. All eight walking legs were autotomized in some animals to induce limb regeneration and molting (Skinner, 1985). The regeneration index, which is the ratio of the limb bud length to carapace width, expressed as a percentage, was used to assess

limb bud growth (Skinner, 1985). Eyestalks were ablated to derepress the Y-organs to increase ecdysteroid levels in the hemolymph (Skinner, 1985). Wounds were cauterized with a heated spatula to minimize loss of hemolymph.

Cloning of calpains and ecdysone receptor (EcR) cDNAs

Partial calpain cDNAs were initially obtained by nested RT-PCR using degenerate primers directed to highly conserved sequences in the protease domain of a wide variety of calpains in the GenBank database (http://www.ncbi.nlm.nih.gov), including those from nematode (GenBank accession #NP502751), fruit fly (#NP477047 and #AAD04331), rat (#NP058813) and human (#AAH08751). Conserved sequences were identified by aligning proteins using the ClustalW program (http://www.ebi.ac.uk/clustalw/index.html). Two sets of degenerate primers were designed to anneal to DNA sequences encoding LG(N/D/E)CW(L/F), EKA(Y/F)AK or (G/R)HAY(T/S)(V/I) in the catalytic domain: CPN F1, (G/A)II (C/T)T(A/G/T/C) GG(A/G/T/C) (G/A)A(A/G/T/C) TG(C/T) TGG; CPN F2, GA(G/A) AA(G/A) GC(A/G/T/C) (C/T)A(C/T) GC(A/G/T/C) AA(G/A); CPN R1, IA(C/T) (A/G/T/C)(G/C)(A/T) (G/A)TA (A/G/T/C)GC (G/A)TG (IC); CPN R2, (C/T)TT (A/G/T/C)GC (G/A)T(G/A) (A/G/T/C)GC (C/T)TT (C/T)TC. Partial sequences encoding EcR were initially obtained by nested RT-PCR using degenerate primers directed to highly conserved sequences in the DNA-binding (domain C; MMRKCQ and CRL(K/R)KC) and ligand-binding (domain E; EYALL(T/A)A and DQI(A/T)LLK) domains of EcR cDNAs in the GenBank database, as described above: EcR F1, ATG MGN MGN AAR TGY CAR; EcR F2, TGY MGN YTN VSN AAR TG); EcR R1, CNG YNA RNA RNG CRT AYT C; EcR R2, TTN ARN ARN GYD ATY TGY TC. To obtain more of the 3′ sequence in the EcR ORF, a second round of nested PCR was done using two sequence-specific forward primers (cEcR F1 and cEcR F2; Table 1) and two degenerate primers to highly conserved sequences (AEIWDV and PFLAEI) in domains E and F (EcR R3, CNG YNA RNA RNG CRT AYT C; EcR R4, DAT YTC NGC NAR RAA NGG). All primers were synthesized and purified by Integrated DNA Technologies, Inc. (Des Moines, IA, USA).

Total RNA was isolated from intermolt claw muscle or primary limb regenerates (regeneration index 13–16; Skinner, 1985) using a Qiagen RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA). cDNA was synthesized according to the manufacturer's protocol using the SuperScript II RNase H-reverse transcriptase first strand synthesis system (Invitrogen, Inc., Carlsbad, CA, USA). Briefly, 12 μl of a mixture containing 1 μl oligo (dT)12–18 (500 μg ml^{-1}), 1 μg total RNA and 1 μl 10 mmol l^{-1} dNTPs was heated to 65°C for 5 min and chilled on ice for 1 min. 5× First-Strand Buffer (4 μl), 2 μl 0.1 mol l^{-1} DTT and 1 μl RNaseOUT recombinant ribonuclease inhibitor (40 units μl^{-1}; Invitrogen, Carlsbad, CA, USA) were added, and the mixture was incubated at 42°C for 2 min. The reaction was initiated by the addition of 1 μl (200 units) of SuperScript II and incubated at 42°C for 50 min. The reaction was inactivated by heating at 70°C for 15 min.

PCR was performed using an ABI 9600 thermal cycler (Perkin-Elmer, Inc., Wellesley, MA, USA). Claw muscle cDNA was used for the EcR PCR, and limb regenerate cDNA was used for the calpain PCR. The reactions contained 3 μl cDNA, 3 μl 10× Takara EX Taq buffer (Takara, Inc., Madison, WI, USA), 2 μl 250 μmol l^{-1} dNTPs, 1 μl forward primer (either CPN F1, EcR F1 or cEcR F1), 1 μl reverse primer (CPN R1, EcR R1 or EcR R3), 0.2 μl Takara EX Taq DNA polymerase (5 units μl^{-1}) and 18.8 μl PCR-grade deionized water. Initial denaturation (95°C for 5 min) was followed by 35 amplifying cycles (either 95°C for 30 s, 55°C for 30 s and 72°C for 30 s for calpain or 95°C for 30 s, 53°C

Table 1. *Primers used in 3′ and 5′ RACE for cloning land crab calpains B, M and T and ecdysone receptor*

Name	Sequence	T_m (°C)	Description	Location in cDNA (bp #)
cCalpM R1	5′-CCGTTGGACATGATGCTTTCTGG-3′	59.9	Gl-CalpM 5′ RACE first round RP	1081–1103
cCalpM R2	5′-TCCACCATACTCTCGTTGATGTTC-3′	58.0	Gl-CalpM 5′ RACE nested RP	924–947
cCalpM F1	5′-GGCGGGAACATCAACGAGAG-3′	59.0	Gl-CalpM 3′ RACE first round FP	918–937
cCalpM F2	5′-CTCGCTCATAGGTTGTGCCATCG-3′	61.2	Gl-CalpM 3′ RACE nested FP	1034–1056
cCalpB R1	5′-CAGTAAAGTCCTCCATTGCCTCACAG-3′	60.9	Gl-CalpB 5′ RACE first round RP	865–920
cCalpB R2	5′-TTGGTCACTAGGCACTATTTGATAGAAG-3′	58.2	Gl-CalpB 5′ RACE nested RP	655–682
cCalpB F1	5′-GCTATGATGGATGTGGATCGCTC-3′	58.8	Gl-CalpB 3′ RACE first round FP	2069–2091
cCalpB F2	5′-GATGACTTCATCATGTGCTCTGTG-3′	57.4	Gl-CalpB 3′ RACE nested FP	2303–2326
cCalpT R1	5′-GAAGGTGAGGATGGTGCGTCTCAAGA-3′	63.9	Gl-CalpT 5′ RACE first round RP	1343–1365
cCalpT R2	5′-TATGTTGATTTTGCGGCAAGCGGTGATTC-3′	64.0	Gl-CalpT 5′ RACE nested RP	1060–1088
cCalpT F1	5′-GACGATTGGAGGCTATGTGGATGAC-3′	60.7	Gl-CalpT 3′ RACE first round FP	894–918
cCalpT F2	5′-CTCTCTGATGTGCTGTGCCATAACTCC-3′	62.6	Gl-CalpT 3′ RACE nested FP	972–998
cEcR F1	5′-GTTTGAGCAGCCAACTGAAGCAGATG-3′	62.3	EcR 3′ RACE first round FP	380–405
cEcR F2	5′-ATTCAGGCACATAACCGAGATGACGATC-3′	62.2	EcR 3′ RACE nested FP	455–482
cEcR R1	5′-CATCTGCTTCAGTTGGCTGCTCAAAC-3′	62.3	EcR 5′ RACE first round RP	380–405
cEcR R2	5′-CACTGGACTAATAGGAGCAGCCTTGTC-3′	62.2	EcR 5′ RACE nested RP	258–284

Abbreviations: EcR, ecdysone receptor; FP, forward primer; RP, reverse primer; T_m, melting temperature.

for 30 s and 72°C for 1 min for EcR) and final extension at 72°C for 7 min. For the second PCR reaction, 0.2 µl of the first PCR reaction was used, in conjunction with one of four primer pairs (CPN F1/CPN R2, CPN F2/CPN R1, EcR F2/EcR R2 or cEcR F2/EcR R4). Other reaction components and PCR conditions were the same as those in the first reaction.

The PCR products were separated by 1.2% agarose gel electrophoresis and stained with ethidium bromide. The PCR products were purified from the gel slices using the Qiaquick Gel Extraction kit (Qiagen, Inc.), ligated into TA cloning vector with the TOPO TA Cloning kit (Invitrogen, Inc.) and transformed into One Shot TOP 10 *E. coli* strain (Invitrogen, Inc.). Transformants were first selected by blue–white colony selection on LB agar plates containing 50 µg ml^{-1} ampicillin (Sigma-Aldrich, Inc., St Louis, MO, USA) and subjected to PCR with T7 and M13-reverse vector primers to verify sizes of inserts. Plasmids were purified using the Qiagen Spin Mini prep kit and sequenced using T7 and M13-reverse vector primers (Davis Sequencing, Inc., Davis, CA, USA). If needed, gene-specific primers were used to obtain the complete sequences of both strands.

RACE (rapid amplification of cDNA ends) was used to obtain full-length mRNA sequences. Poly (A^+) RNA was isolated from claw muscle or limb regenerate total RNA using the Oligotex mRNA kit (Qiagen, Inc.). For the 3′ sequence, the Invitrogen 3′ RACE System was used. Briefly, first-strand cDNA synthesis reactions contained 200 ng claw muscle or limb regenerate poly (A^+) RNA and adaptor primer (5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT-TT-3′). First-round PCR on 20 ng cDNA included a universal amplification primer (5′-CUACUACUACUAGGCCACGCG-TCGACTAGTAC-3′) and gene-specific forward primers (Table 1) at the following conditions: denaturation at 96°C for 5 min, 35 amplification cycles (96°C for 30 s, 60°C for 30 s and 72°C for 2 min) and extension at 72°C for 10 min. Nested PCR on 30 µl of each reaction was conducted with gene-specific primers (Table 1) and abridged universal amplification primer (5′-GGCCACGCGTCGACTAGTAC-3′) using the same conditions as the first-round PCR. Gel-purified PCR products were cloned into the TA vector and sequenced as described above.

SMART^{TM} RACE cDNA Amplification kit (BD Biosciences, Inc., Palo Alto, CA, USA) was used to obtain the 5′ sequences of each gene. The first-strand cDNA synthesis reaction contained 3 µl poly(A+) RNA (50 ng for calpain primers and 100 ng for EcR primers), 1 µl 10 mmol l^{-1} 5′ CDS primer [5′-(T)25N-1N-3′] and 1 µl 10 mmol l^{-1} SMART II A oligo (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′) and was incubated at 68°C for 2 min. After chilling the reaction for 2 min on ice, 2 µl 5× First-Strand buffer (250 mmol l^{-1} Tris-HCl, pH 8.3; 375 mmol l^{-1} KCl; 30 mmol l^{-1} $MgCl_2$), 1 µl 20 mmol l^{-1} DTT, 1 µl 10 mmol l^{-1} dNTPs and 1 µl PowerScript reverse transcriptase (BD Biosciences Clontech, Mountain View, CA, USA) were added. The reaction was covered with 20 µl paraffin oil and incubated at 42°C for 1.5 h in an ABI 9600 thermal cycler. The reaction mixture was diluted 10-fold with autoclaved distilled water and used for first-round PCR with 10× Universal Primer A Mix (0.4 mmol l^{-1} 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCA-ACGCAGAGT-3′ and 2 mmol l^{-1} 5′-CTAATACGACTCA-CTATAGGGC-3′) and gene-specific reverse primers (Table 1) under the following conditions: denaturation at 96°C for 5·min, 35 amplification cycles (either 96°C for 30 s, 65°C for 15 s and 72°C for 3 min for calpains or 96°C for 30 s, 66°C for 15 s and 72°C for 3 min for EcR) and extension at 72°C for 10 min. Second-round PCR was conducted using nested gene-specific primers (Table 1) and nested universal primer A (10 mmol l^{-1}, 5′-AAGCAGTGGTATCAACGCAGAGT-3′). The PCR conditions were the same as those used for first-round PCR. Gel-purified PCR products were cloned into the TA vector and sequenced as described above. Continuous sequences of three calpains were obtained by PCR using primer pairs to the start and stop codons to verify the sequence of each ORF.

Phylogenetic relationships between calpain sequences were determined with Treeview, which is a program that displays ClustalW result files (http://taxonomy.zoology.gla.ac.uk/rod/treeview/treeview_manual.html).

Calpain and EcR expression by RT-PCR

Total RNA was isolated from tissues using the RNeasy mini kit according to the manufacturer's instructions (Qiagen, Inc.).

Table 2. *Forward and reverse primers used for qualitative RT-PCR analysis of calpain B, calpain M and calpain T mRNA levels in land crab tissues*

Name	Sequence	T_m (°C)	Location in cDNA (bp #)
rtCalpM F1	5′-CTCGCTCATAGGTTGTGCCATCG-3′	59.9	1034–1057
rtCalpM R1	5′-AATCCTGATAACTCATCCAGAACTCTC-3′	55.9	1318–1343
rtCalpB F1	5′-AACCTAACCCTCAACCGTCGCCTC-3′	62.8	631–655
rtCalpB R1	5′-CCAAGGATTTCGGATGCGGAC C-3′	60.5	1146–1168
rtCalpT F1	5′-CTACAAGGACCAAGAGTACGGAGAC-3′	58.1	617–642
rtCalpT R1	5′-CTTGAGACGCACCATCCTCACCTTC-3′	59.9	1127–1152

Abbreviations: T_m, melting temperature.
GenBank accession numbers: Gl-CalpM, AY639152; Gl-CalpB, AY639153; Gl-CalpT, AY639154.

Total RNA (1 μg) was DNase-treated (Invitrogen, Inc.) and reverse-transcribed using the SuperScript II RNase H-reverse transcriptase first strand synthesis system (Invitrogen, Inc.). End-point PCR reactions were performed in an ABI 9600 thermal cycler using TaKaRa Ex Taq HotStart polymerase (Takara, Inc.), gene-specific primers (Table 2) and either 2 μl (calpains) or 5 μl (EcR) of the first strand cDNA mixture as template. The PCR conditions for calpains were an initial denaturation at 95°C for 4 min, 35 polymerization cycles (denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s), and final extension at 72°C for 2 min. PCR reactions were analyzed by separating all of the 20 μl reaction volume on 2% agarose ethidium bromide-stained gels. Primers were designed using IDT BioTools program (http://biotools.idtdna.com/gateway).

For quantitative RT-PCR, transcript levels of the three calpains (Gl-CalpB, Gl-CalpM and Gl-CalpT) and ecdysone receptor (Gl-EcR) were determined by real-time PCR using a Cepheid Smart Cycler instrument (Cepheid, Sunnyvale, CA, USA) and sequence-specific primers (Medler and Mykles, 2003; Yu and Mykles, 2003). Transcript levels of the four genes were normalized to the mRNA level of elongation factor 2 (EF2), which was constitutively expressed. The PCR products were ligated into TOPO 2.1 vector using TOPO TA Cloning kit (Invitrogen, Inc.). Standard curves were generated using serial dilutions (10 fg to 10 ng) of plasmids containing either Gl-CalM, Gl-CalpB, Gl-CalpT or Gl-EF2 inserts (Medler and Mykles, 2003; Yu and Mykles, 2003). Reaction mixtures contained 2 μl LightCycler FastStart Reaction Mix (Roche, Indianapolis, IN, USA; 10× buffer, Fast Taq DNA polymerase and dNTPs), 2 μl 25 mmol l^{-1} $MgCl_2$, 2 μl cDNA template, 1 μl forward primer (Table 3; 10 pmol μl^{-1}), 1 μl reverse primer (Table 3; 10 pmol μl^{-1}) and 12 μl PCR-grade water. The PCR conditions were denaturation at 96°C for 5 min, 40 amplification cycles (96°C for 20 s, 65°C for 15 s and 72°C for 30 s), and melting curve detection (60°C+0.2 deg. s^{-1}). PCR products were evaluated by melting temperature analysis and separation on 2% agarose ethidium bromide-stained gels (Medler and Mykles, 2003; Yu and Mykles, 2003).

Statistical analyses

The Statview 5.0.1 Program (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. One-way analysis of variance (ANOVA) was used to compare the copy numbers determined by real-time PCR. Values were log-transformed due to high levels of variability between samples and to correct for the correlation between mean and variance (Medler and Mykles, 2003). Pair-wise post-ANOVA comparisons used a Bonferroni test with an experiment-wise error rate of 0.05.

Results

cDNAs encoding calpains B, M and T

Nested PCR with degenerate primers generated products of the expected size from cDNA synthesized from primary limb regenerate mRNA. Our rationale was that since the limb regenerate contained various tissues (e.g. muscle, epidermis, nerve, connective tissue, hemocytes, blood vessels), a greater number of calpain species would be obtained than by using a single tissue. After cloning into a plasmid vector, 10 different clones were selected for sequencing. The calpain-like sequences encoding a part of the protease domain fell into four categories: the first was similar to lobster CalpM (accession #AY124009); the second was similar to *Drosophila* CalpA (accession #NP477047) and CalpB (accession #NP524016); the third was similar to human Capn5 (accession #CAA71584) and *C. elegans* TRA-3 (accession #NP502751); and the fourth was similar to *C. elegans* clp-1 (accession #NP741237). 3′ and 5′ RACE using nested sequence-specific primers yielded full-length sequences of three of the four initial sequences, designated Gl-CalpB, Gl-CalpM and Gl-CalpT (*G. lateralis* calpains B, M and T). Expression of each calpain in limb regenerates was confirmed by RT-PCR. However, the clp-1-

Table 3. *Forward and reverse primers used for quantifying calpains, ecdysone receptor and elongation factor 2 mRNAs by real-time PCR*

Name	Sequence	T_m (°C)	Location in cDNA (bp #)
RcEF2 F1	5′-TTCTATGCCTTTGGCCGTGTCTTCTC-3′	62.6	11–36
RcEF2 R1	5′-TGATGGTGCCCGTCTTAACCAGATAC-3′	62.1	214–239
RcCalpM F1	5′-GGCGGCTGCAGGAATTACATTAACAC-3′	62.2	1498–1523
RcCalpM R1	5′-TGTACCTGAAGAAATCGACGTCCAGC-3′	62.2	1713–1738
RcCalpB F1	5′-GTTCAACTTTGAGGGCTTCAGCAAGG-3′	62.2	2023–2048
RcCalpB R1	5′-GATAACCAGCAGAGTTGAGGGCTTGA-3′	62.3	2209–2234
RcCalpT F1	5′-TCTCTGATGTGCTGTGCCATAACTCC-3′	62.3	973–998
RcCalpT R1	5′-TGATGCAGAGACCTGTGACCATTCTG-3′	62.2	1202–1227
RcEcR F1	5′-CACGAAGAATGCCGTGTACCAGTGTA-3′	62.3	35–60
RcEcR R1	5′-CATCTGCTTCAGTTGGCTGCTCAAAC-3′	62.4	380–405

Abbreviations: EcR, ecdysone receptor; EF2, elongation factor 2; T_m, melting temperature.

GenBank accession numbers: Gl-EF2, AY552550; Gl-CalpM, AY639152; Gl-CalpB, AY639153; Gl-CalpT, AY639154; Gl-EcR, AY642975.

Table 4. *Characteristics of cDNAs encoding land crab calpains*

cDNA	cDNA length (bp)	Translated ORF: Length (amino acids)	Translated ORF: Estimated mass (kDa)
Gl-CalpB	2740	754	88.89
Gl-CalpM	1677	558	65.23
Gl-CalpT	2639	639	74.56

like sequence was not amplified and therefore not characterized further; it may have resulted from contamination of the limb regenerate mRNA with mRNA from a parasitic nematode that occurs in some individuals. The full ORFs were confirmed by PCR using specific primer sets containing the start codon and stop codon sequences for each calpain.

The characteristics of the cDNAs encoding the three land crab calpains are summarized in Table 4. The domain organization of the land crab calpains is compared with that of calpains from lobster (Ha-CalpM), *Drosophila* (Dm-CalpA and Dm-CalpB) and nematode (TRA-3) in Fig. 1. The DNA and deduced amino acid sequences of Gl-CalpB, Gl-CalpM and Gl-CalpT are presented in Figs 2, 3 and 4, respectively. The deduced amino acid sequence of Gl-CalpB was 61% identical to Dm-CalpB, 51% identical to Dm-CalpA and 50% identical to human Capn3 (accession #NP-775110.1). The deduced amino acid sequence of Gl-CalpM was most similar to that of Ha-CalpM (66% identity) and Dm-CalpA (48% identity). The amino acid sequence of Gl-CalpT was 47% identical to human Capn5 (accession #O15484) and 41% identical to *C. elegans* TRA-3 (accession #AAB60256).

Gl-CalpB had the four-domain organization of typical calpains: N-terminal (dI), catalytic (dII), C_2-like (dIII) and calmodulin-like (dIV) (Figs 1, 2). Gl-CalpM had the conserved catalytic and C_2-like domains but lacked the C-terminal calmodulin-like domain (Figs 1, 3). A minor, alternatively spliced variant of Gl-CalpM (Gl-CalpM′) was obtained with 3′ RACE; the deduced polypeptide is truncated 958 bp from the start codon, which results in the absence of the catalytic asparagine in domain II (Fig. 3). Gl-CalpT resembled other calpains from domains I to III, but dIV was replaced with a T domain found in Capn5 and TRA-3 (Figs 1, 4).

Multiple amino acid sequence alignment showed high similarity in domains II and III in calpains from nematode (TRA-3), arthropods (Gl-CalpM, Gl-CalpB, Gl-CalpT, Dm-CalpA and Dm-CalpB) and mammals (calpains 1 and 3) (Fig. 5). All have the conserved catalytic triad (C, H, N) and two non-EF hand Ca^{2+}-binding sites in dII. A C_2-like sequence containing an acidic loop in dIII was common to all calpains. The major difference between the two CalpM sequences was that Ha-CalpM had two acidic amino acid insertions, one (DDSDD) near the end of dII and the other (DDDDDDDDDDRG) in the C_2 acidic loop region, that were absent in Gl-CalpM. Unlike a muscle-specific mammalian

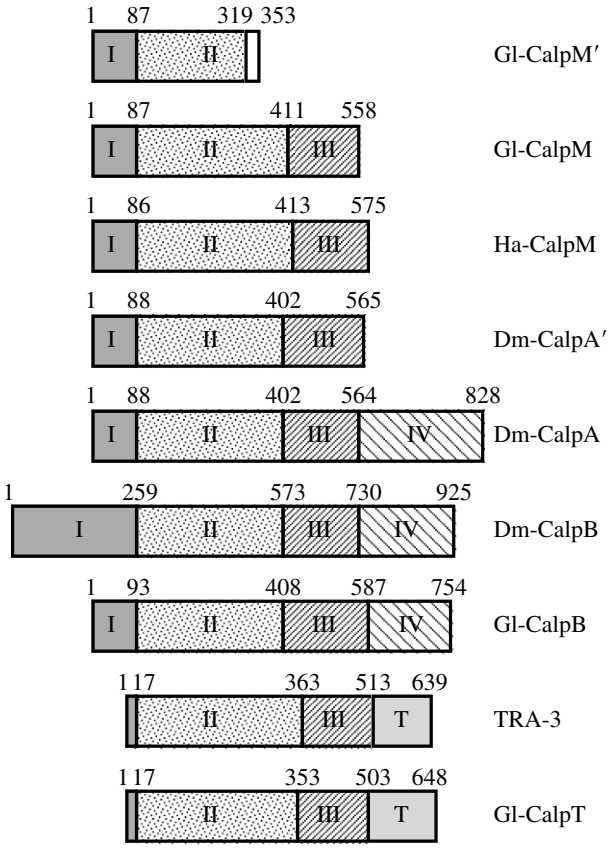

Fig. 1. Domain organization of calpains from arthropods and nematode. Calpains from land crab (Gl-CalpM, Gl-CalpB and Gl-CalpT), *Drosophila melanogaster* (Dm-CalpA and Dm-CalpB) and *Caenorhabditis elegans* (TRA-3) are depicted. All calpains share conserved proteolytic (II) and C_2-like (III) domains. The N-terminal domain I varies in sequence and length among different calpains. Calpains differ in the C-terminal region. 'Typical' calpains (e.g. Gl-CalpB, Dm-CalpA and Dm-CalpB) have a calmodulin-like domain (IV) containing five EF-hand motifs. 'Atypical' calpains either lack domain IV (Gl-CalpM and Ha-CalpM) or have domain IV replaced with a T domain (Gl-CalpT and TRA-3). Gl-CalpM′ and Dm-CalpA′ are truncated proteins resulting from alternative mRNA splicing of Gl-CalpM and Dm-CalpA, respectively. Amino acid residues, numbered from the N-terminus, indicate the boundaries between the domains.

Capn3, calpains from arthropods and nematode lacked an insertion sequence in dII. Domain IV, when present, was well conserved from arthropods to mammals, although Dm-CalpA had an insertion sequence between EF-1 and EF-2 not found in any other calpain (Fig. 6). The sequence of the T domain of Gl-CalpT was similar to that of other 'T domain' calpains (Fig. 7).

To study the sequence relationships of the three land crab calpains, a phylogenetic analysis was done based on the deduced amino acid sequences of dII and dIII of calpains from arthropods, nematode and mammals (Fig. 8). The sequences clustered in four groups. TRA-3, Capn5 and Gl-CalpT were clustered as a distinct group, even though the T domain

```
AGTTCTTCCTCAACTACTGCGCGTTCAAGTAATAAACCGCTGAAACCGAACACCAGAGACGCTTCGTTAATAACTGGAGTGTTTGCCCAAAGAAGACAACAACG
CCCCTGCACAAACGAACGCCAAGTAACCCCTTGAACACAGACGAGACAGCCAAACCCCGACG
   ATG GAC GAA GAG GAG GCG CAG TAC AAC GAA GAG GAG GAG CAG GAG GAG GAG AAC GAA GAG GGA GAG GAA GAG GAG GTC
  1 M   D   E   E   E   A   Q   Y   N   E   E   E   E   Q   E   E   E   N   E   E   G   E   E   E   E   V
   GAT GAG TCA TAC GAT CGT GTA GAC AAT CCC ATT GGA GAC GAT ACT ATA GAA AAG ACA CTC TAT GAC GAT GAG GGA AAT
 27 D   E   S   Y   D   R   V   D   N   P   I   G   D   D   T   I   E   K   T   L   Y   D   D   E   G   N
   GAG ATG TTC TTC GTT TTC GAT AAG TTC TAC ATG TTT GGG GAG CGT GGA TCA GGC CTT CGT CCC CGC GGC CAG GTG CAG
 53 E   M   F   F   V   F   D   K   F   Y   M   F   G   E   R   G   S   G   L   R   P   R   G   Q   V   Q
   GAT TTC TAT GAG CTA CGG CAA CAG TGT CTT GAT AAT GGC ACA CTC TTC GAG GAC CCT GAC TTC CCG GCT GAA GAC ACC
 79 D   F   Y   E   L   R   Q   Q   C   L   D   N   G   T   L   F   E   D   P   D   F   P   A   E   D   T
   TCC ATC TTC TTC TCT CGC AGC CCA CCT AAG CCT TTC GAG TGG AAG AGG CCT CAT GAA ATC ACA GAT GAG CCA CAA CTT
105 S   I   F   F   S   R   S   P   P   K   P   F   E   W   K   R   P   H   E   I   T   D   E   P   Q   L
   TTT ATT GAT GGA GCC ACT CGC TTT GAT GTC AAG CAA GGA GAG CTG GGT GAC TGT TGG CTG CTG GCT GCT GTG GCA AAC
131 F   I   D   G   A   T   R   F   D   V   K   Q   G   E   L   G   D   C   W   L   L   A   A   V   A   N
   CTA ACC CTC AAC CGT CGC CTC TTC TAT CAA ATA GTG CCT AGT GAC CAA GGC TTT GGA GAT AAC TAT GCT GGC ATC TTC
157 L   T   L   N   R   R   L   F   Y   Q   I   V   P   S   D   Q   G   F   G   D   N   Y   A   G   I   F
   CAC TTC AGG TTT TGG CAG TAT GGC CGC TGG GTA GAT GTT GTG GTG GAT GAC CGA CTT CCC ACC TTC TAT GGC CGA CTG
183 H   F   R   F   W   Q   Y   G   R   W   V   D   V   V   V   D   D   R   L   P   T   F   Y   G   R   L
   GTC TTC ATG CAC TCA GAG GAG AAG AAT GAG TTC TGG TCT GCC CTA GCA GAG AAG GCA TAT GCC AAG TTG CAT GGA TCA
209 V   F   M   H   S   E   E   K   N   E   F   W   S   A   L   A   E   K   A   Y   A   K   L   H   G   S
   TAT GAG GCA CTG AAG GGA GGC ACC ACC TGT GAG GCA ATG GAG GAC TTT ACT GGC GGT GTG TCT GAG ATC TAT GAC CTT
235 Y   E   A   L   K   G   G   T   T   C   E   A   M   E   D   F   T   G   G   V   S   E   I   Y   D   L
   ACT AAG GCT CCC CCG AAC CTG TTC AAC ATC ATG CTG AAG GCC TAC CAG AGA GGC TCC CTG ATG GGC TGC TCT ATT GAT
261 T   K   A   P   P   N   L   F   N   I   M   L   K   A   Y   Q   R   G   S   L   M   G   C   S   I   D
   CCA GAC CCC AAT GTG GTG GAG GCA CGC TGT GAC AAT GGT CTG ATT CGT GGT CAT GCA TAC TCC ATC ACC CGC ATC AAG
287 P   D   P   N   V   V   E   A   R   C   D   N   G   L   I   R   G   H   A   Y   S   I   T   R   I   K
   TAC TGT GAC ATT GAG ACA CCT AGG GTT TCT GGT AAG ATC CCC CTG GTC CGC ATC CGA AAT CCT TGG GGC AAT GAG GCT
313 Y   C   D   I   E   T   P   R   V   S   G   K   I   P   L   V   R   I   R   N   P   W   G   N   E   A
   GAG TGG GTT GGC TCT TGG AGT GAC AAA AGT CCT GAG TGG CAG TTC ATT CCT CCT GAG GAG AAA GAA GAG ATG GGC CTC
339 E   W   V   G   S   W   S   D   K   S   P   E   W   Q   F   I   P   P   E   E   K   E   E   M   G   L
   ACC TTT GAA CAT GAT GGA GAG TTC TGG ATG TCC TTC AAG GAC TTC CTT ACC AAT TTC ACC ATG CTG GAG ATG ACC AAC
365 T   F   E   H   D   G   E   F   W   M   S   F   K   D   F   L   T   N   F   T   M   L   E   M   T   N
   CTG AAT CCT GAC TCC CTG GAG GAT GAG GAC ATC ACT GGT TCT GTC CAG CAC AAG TGG GAG ATG AGT GTC TTT GAA GGG
391 L   N   P   D   S   L   E   D   E   D   I   T   G   S   V   Q   H   K   W   E   M   S   V   F   E   G
   GCC TGG ATC AGG GGC TCT ACT GCT GGT GGT TGC AGG AAT TTC CTT GAT TCA TTC TGG CAC AAC CCG CAG TAC AGG ATC
417 A   W   I   R   G   S   T   A   G   G   C   R   N   F   L   D   S   F   W   H   N   P   Q   Y   R   I
   ACC CTG AGT GAG GTG GAT GAT GAT GAT GAT GAC AAC AAA TGC ACA GTG ATT GTG GCA CTG ATG CAG AAG AAT CGC CGT
443 T   L   S   E   V   D   D   D   D   D   N   K   C   T   V   I   V   A   L   M   Q   K   N   R   R
   TCA CAA AGG AAG CTT GGC CTG GAG TGC CTT ACT ATT GGT TTT GCA ATT TAT CAT TTG CGA GAC CCA GAC AGT GTT CCT
469 S   Q   R   K   L   G   L   E   C   L   T   I   G   F   A   I   Y   H   L   R   D   P   D   S   V   P
   CGG CCA CTG GAC CTG AAC TTC TTC AAG TAC TCA GCA TCA GTT GCT CGC TCC CCA TCT TTC ATC AAC ATG AGG GAG GTT
495 R   P   L   D   L   N   F   F   K   Y   S   A   S   V   A   R   S   P   S   F   I   N   M   R   E   V
   TCA TGT CGT TTC AAG CTG CCC CCT GGC ACT TAC TGT ATT GTG CCT TCT ACC TTT GAG CCA AAT GAG GAC GGA GAA TTT
521 S   C   R   F   K   L   P   P   G   T   Y   C   I   V   P   S   T   F   E   P   N   E   D   G   E   F
   ATT TTG AGA GTC TTC TCT GAA AAA GCT AAT GAG ATG GAA GAA AAT GAT GAA GAT GTA GGC TTC GGA CAG GTG GAT GAC
547 I   L   R   V   F   S   E   K   A   N   E   M   E   E   N   D   E   D   V   G   F   G   Q   V   D   D
   AGG GTC CGA CCT GAG GAT GAT GCA CAG GAA GTG GAG GCT GAT GAG AGG ATC AAC GCC TTC TTC AGG AAA GTG GCT GGC
573 R   V   R   P   E   D   D   A   Q   E   V   E   A   D   E   R   I   N   A   F   F   R   K   V   A   G
   GAT GAC CTG GAG ATT GAC TGG AAG GAG CTG CAG GAT GTC CTC AAC TTT GCC CTG AAA AGA GAG TTC AAC TTT GAG GGC
599 D   D   L   E   I   D   W   K   E   L   Q   D   V   L   N   F   A   L   K   R   E   F   N   F   E   G
   TTC AGC AAG GAT GTA TGT CGC AGC ATG ATT GCT ATG ATG GAT GTG GAT CGC TCA GGA AAG CTG GGC CTC CAA GAA TTC
625 F   S   K   D   V   C   R   S   M   I   A   M   M   D   V   D   R   S   G   K   L   G   L   Q   E   F
   CTG CAG TTG TGG ATG GAT ATC AGA GTG TGG AAG AAT GCC TTC AAG CTA TAT GAC AAG GAC AGC TCT GGC CAA CTG TGT
651 L   Q   L   W   M   D   I   R   V   W   K   N   A   F   K   L   Y   D   K   D   S   S   G   Q   L   C
   TCA TTT GAA CTG CGT CAA GCC CTC AAC TCT GCT GGT TAT CGC CTC AAC AAC CAT ATT TGT GAC GCC CTC ATG CTC CGC
677 S   F   E   L   R   Q   A   L   N   S   A   G   Y   R   L   N   N   H   I   C   D   A   L   M   L   R
   TAT GGG GAT CGG GAT GGC AAA GTG TCA TTT GAT GAC TTC ATC ATG TGC TCT GTG AAG CTG AAA ACC ATG ATG GAA ATC
703 Y   G   D   R   D   G   K   V   S   F   D   D   F   I   M   C   S   V   K   L   K   T   M   M   E   I
   TTC CAG GAG AGG GAC CCC GAC AGG ACT ACG AAG GCC ACC TTC AGC CTT GAG GAG TGG GTA GAA AAC ACT ATG TAC TCA
729 F   Q   E   R   D   P   D   R   T   T   K   A   T   F   S   L   E   E   W   V   E   N   T   M   Y   S
   TAG
    *
AACTACATCAGCTTATTTACTGTTGAGCTAAATTTAATTAGCTTTACTATAATGAAAGAATCTTTATAATTATGTATGGTGATTTTTTGGGAGTTTTGCCTAATT
GCATATTCAGACTTGTGTTGTGAAATGATGATGTCTTTTAATGCCAAATTTAAGGGCCCATTCTGTTAGGCTTATCATTGAACTGTGCTCTTACATTTTAGCCAT
GGTCTAAGTAAAATAATTACTGGATCAGGCTAATGTTGAAAATGCAGTGTTTCACTATACAAACCCGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 2. The complete sequence of calpain B cDNA from land crab (Gl-CalpB). The sequence (2740 bp) contained a full open reading frame (167–2431 bp) encoding a protein of 754 amino acids with a predicted mass of 88.89 kDa (GenBank accession #AY639153). Locations of degenerate primer sites for initial nested RT-PCR are underlined. Three amino acid residues comprising the conserved catalytic triad (C, H, N) in domain II are in bold. The asterisk indicates the stop codon. The poly(A) signal in the 3′ UTR is in bold and underlined.

sequences were not included in the analysis. Mammalian Capn1 and Capn3 were grouped together and were more closely related to calpains with an EF-hand domain. Crustacean-specific Ha-CalpM and Gl-CalpM were grouped separately from arthropod A/B-type calpains (Gl-CalpB and Dm-CalpA and B).

cDNA encoding ecdysone receptor

The initial 700-bp product from nested PCR using degenerate EcR primers was 95% identical to the deduced amino acid sequence of the EcR from fiddler crab (accession #AAC33432). 3′ RACE using sequence-specific primers failed. If the 3′ UTR in the Gl-EcR is as long as it is in fiddler crab EcR (~4 kb), it

may be difficult to amplify with 3′ RACE. Additional 3′ sequence was obtained by PCR using gene-specific forward primers (cEcR F1 and cEcR F2; Table 1) and degenerate primer sets (EcR F3 and EcR F4; see Materials and methods). 5′ RACE yielded a 300-bp product, which extended the 5′ sequence another 100 bp. The consensus partial sequence of Gl-EcR was 1005 bp and encoded a deduced 335-amino acid sequence encompassing part of the C domain and all of the D and E domains (Fig. 9). The partial amino acid sequence of Gl-EcR was aligned with full-length sequences of EcR-encoding genes from fiddler crab (Up-EcR) and locust (Lm-EcR) (Fig. 10). The Gl-EcR sequence was 93% identical with homologous regions of Up-EcR and 66% identical with Lm-EcR.

Tissue expression of calpain mRNAs

End-point RT-PCR showed that most tissues expressed the three calpains (Fig. 11). All tissues expressed Gl-CalpB. The molting gland (Y-organ) did not express Gl-CalpM or Gl-CalpT. Eyestalk ganglia did not express Gl-CalpT. The mRNA levels in nine tissues were quantified by real-time PCR (Fig. 12). Three general expression patterns were observed. In skeletal muscle (Fig. 12A,B), Gl-CalpM and Gl-CalpB were expressed at significantly higher levels than Gl-CalpT; there was no significant difference between Gl-CalpM and Gl-CalpB. In heart, gill, thoracic ganglion, digestive gland and testis, Gl-CalpB was expressed at higher levels, often significantly, than Gl-CalpM and Gl-CalpT; there was no

```
                                                                                           5'-ACTCCGAGGCGTG
    ACAGACTCCAGCCCACTTCTCCCCCGATTTGAGTGCATATCTCCTGTGTTTACGTCCCTCTCTCCGCAGTGACCCCAGCGGTATAACAAGCAGGTGTAGGTGC
    CTCAGCCGACCACTGCCATCCAAAACCCTTAACAACCCGCCCAAGCAGACCAAGAAGAGCCGGGCAGCCGCTGACCCCGGCAGGAGAGGGAAGCAGTAGCAGA
    ATG TCG GCC CGC GAG ATT GTG GAA GAG CCG TGC GTG CTT CGG CAG CAG CAC CAA CAC GAT GAC CGC CAC TGC CGC TGT
  1 M   S   A   R   E   I   V   E   E   P   C   V   L   R   Q   Q   H   Q   H   D   D   R   H   C   R   C
    TAC AAG GGT GGC GCA GAC CGC ACC CTT CTG GAC AAC ATT GAG CCG GTG TCC CTA CAG AAG GAG ACC TAC TAC AGC CGC
 27 Y   K   G   G   A   D   R   T   L   L   D   N   I   E   P   V   S   L   Q   K   E   T   Y   Y   S   R
    GTC ACT AAC GAG TAC ACG AAG CAG AGA CAG GTG AGG GCT GCC AGT AGA CCC ATC AAA AAG GGC TTC CAG GTG TTA CGC
 53 V   T   N   E   Y   T   K   Q   R   Q   V   R   A   A   S   R   P   I   K   K   G   F   Q   V   L   R
    CAG GAG ACC CTC GCA AGT GGC AAG CTG TAT CGT GAC CCA GAC TTC CCG CCT AAC GAC TAC TCC ATT AAC TTC AGC GGC
 79 Q   E   T   L   A   S   G   K   L   Y   R   D   P   D   F   P   P   N   D   Y   S   I   N   F   S   G
    ATC ACA CGG AGG ACC TAC GAG TGG AAG CGA CCC CTT GAG ATG GTC CAG AAC CCA CAA TTC TTC ATC GAC GGA GCC ACG
105 I   T   R   R   T   Y   E   W   K   R   P   L   E   M   V   Q   N   P   Q   F   F   I   D   G   A   T
    AGA TTT GAT ATA CAG CAA GGA GAA CTG GGT GAC TGT TGG CTC CTG GCG GCC GTT TCC AAC TTG ACA CTC CAC CCA CAC
131 R   F   D   I   Q   Q   G   E   L   G   D   C   W   L   L   A   A   V   S   N   L   T   L   H   P   H
    CTG TTT CAC GTG GTG GTG CCC CGG GAT CAA GGC TTC ACA CAC CTG TAC GCC GGG ATC TTT CAT TTC AAG TTC TGG CAG
157 L   F   H   V   V   V   P   R   D   Q   G   F   T   H   L   Y   A   G   I   F   H   F   K   F   W   Q
    TAC GGG AGG TGG CAG GAG GTG GTG ATT GAC GAC TTG TTA CCC ACA CAC AAC GGC CGC CTC GTC TTT ATG CAC TCC CGC
183 Y   G   R   W   Q   E   V   V   I   D   D   L   L   P   T   H   N   G   R   L   V   F   M   H   S   R
    AAC TCC AGC GAG TTC TGG TGT GCG CTA CTG GAA AAA GCC TAC GCC AAG CTG TAC GGA GGC TAC GAG GCA CTA CGC GGC
209 N   S   S   E   F   W   C   A   L   L   E   K   A   Y   A   K   L   Y   G   G   Y   E   A   L   R   G
    GGG AAC ATC AAC GAG AGT ATG GTG GAC CTG ACG GGC GGC GTG GTG GAG CTC ATC GAC CTC CGC TCC CCG GCT TCT AAC
235 G   N   I   N   E   S   M   V   D   L   T   G   G   V   V   E   L   I   D   L   R   S   P   A   S   N
    CTC TTC CCC AGG CTC CTG AAG GCA TGC CGC AGG GGC TCG CTC ATA GGT TGT GCC ATC GAG TCT GAT CAC CTC GGC GTG
261 L   F   P   R   L   L   K   A   C   R   R   G   S   L   I   G   C   A   I   E   S   D   H   L   G   V
    CGC CCA GAA AGC ATC ATG TCC AAC GGC CTC ATC GTG CGC CAC GCC TAC TCC ATC ACC CGC GTC ACC GCC ATC TCC CTC
287 R   P   E   S   I   M   S   N   G   L   I   V   R   H   A   Y   S   I   T   R   V   T   A   I   S   L
    GCC ACG GCC CCT AAA CTC AAG GGC GAG GCG CAT CTG GTT CGC CTG CAT AAC CCC TGG GGG AAC GAG ACC GAG TGG AAG
313 A   T   A   P   K   L   K   G   E   A   H   L   V   R   L   H   N   P   W   G   N   E   T   E   W   K
    GGC TCC TGG AGT GAC AAG TCC CCC GAG TGG AAC GCC GTG CCA CCT GGG GAG AGA CAG CGC CTT GGC CTC ACC TTT AAT
339 G   S   W   S   D   K   S   P   E   W   N   A   V   P   P   G   E   R   Q   R   L   G   L   T   F   N
    GAC GAT GGA GAG TTC TGG ATG AGT TAT CAG GAT TTC CTC AAA AAC TTC ACC ACC GTT GAG ATC TGT GAC GTC AAC CCG
365 D   D   G   E   F   W   M   S   Y   Q   D   F   L   K   N   F   T   T   V   E   I   C   D   V   N   P
    GAC TTC TGC GAC GAG GAG AAC GGC AAC GGC GGC GGA GTG CCC GAG GAT GTG GAG CGC GGC TGG AAG GTG ACC ATG TAC
391 D   F   C   D   E   E   N   G   N   G   G   G   V   P   E   D   V   E   R   G   W   K   V   T   M   Y
    GAG GGG GCC TGG GTC GCT AAT CAC ACG GCG GGC GGC TGC AGG AAT TAC ATT AAC ACG TTC GCG AGA AAC CCG CAG TAC
417 E   G   A   W   V   A   N   H   T   A   G   G   C   R   N   Y   I   N   T   F   A   R   N   P   Q   Y
    ACC ATC GAG CTA ATG GAC CCC GAC GAG GAA GAT GAC GAC GAC TTC TGC ACC GTC ATC GTC TCG CTC ATG CAG AAG AAC
443 T   I   E   L   M   D   P   D   E   E   D   D   D   D   F   C   T   V   I   V   S   L   M   Q   K   N
    GTG CGG CAG CTG AAG CGT TAC GGC GTG GAC TAC GTG CCC ATC GGG TTC ACG CTG TAC AAG CTC CCG CCC GGC CGC CAA
469 V   R   Q   L   K   R   Y   G   V   D   Y   V   P   I   G   F   T   L   Y   K   L   P   P   G   R   Q
    CCT GGA ATG AAG CTG GAC GTC GAT TTC TTC AGG TAC AAC GCC AGC TGC GCC AAA GTC CCC TAC TTC CTC AAC ACA CGG
495 P   G   M   K   L   D   V   D   F   F   R   Y   N   A   S   C   A   K   V   P   Y   F   L   N   T   R
    GAG GTC ACG ACG AGG TTC CGA ATG CCA GCT GGC CAC TAC GTC ATC ATC CCG TCC ACC TTC GAG CCA GAG ATG ACG GGA
521 E   V   T   T   R   F   R   M   P   A   G   H   Y   V   I   I   P   S   T   F   E   P   E   M   T   G
    GAG TTT CTC TTG AGG GTC TTC ACT GAG ATC AGA CAG TAA
547 E   F   L   L   R   V   F   T   E   I   R   Q   *

TGGGATGCTGGGGTCATCTCGAGTAGACCTGAGGCTGAACACACACACACACGCACTGGGATGAGGCAAAAGTATTGGGTGCTTTGGTTTTCCCCCTTCCTTCG
CTCTTCCCCTCGTCCCTGTCTTCCACCCGCCCAATATGGCATGTGTAGACTGAGGCTGAACACACACACACACACACACACACACACACATACACACTATTG
TCTCAGCGTTGGACTGTGGGGAAAGGGTCAGATGGCGCCCTTCACCCCCCTCTCGTGCTGCCACAGTGAGCCTCGCCCCTTCATGGGTTGTTACTCGCTTCCTCTC
ATCAGTATTGTCAGGTGAAAGGTTACATTGTTTCTGGACTTTTTCCGCCGGGCATTATTTATTTCACCCTTCGCCGTGTGCCAAGTCTTCGCCAGCCCCTCGTTCA
CTGGGTCCCGCTGGTCACATCACCACTCATTCTCGCGTGTCATCACTCATCACCTTGGCGAGGAAGGCAGGACAACATTTCGAGTGATATTATAAGGTATTAAGAG
TATTGTGAATGGCTGCTGTGTTGAGTGAAATGCAGCCGCTGTGTGTTTTGATATTTTAATGAAATCAATAAATAATATTTGTTAAAAAAAAAAAAAAAAAAAAA
```

Fig. 3. The complete sequence of calpain M cDNA from land crab (Gl-CalpM). The sequence (2531 bp) contained a full open reading frame (220–1896 bp) encoding a protein of 558 amino acids with a predicted mass of 65.23 kDa (GenBank accession #AY639152). Locations of degenerate primer sites for initial nested RT-PCR are underlined. Three amino acid residues comprising the conserved catalytic triad (C, H, N) in domain II are in bold. The asterisk indicates the stop codon. The poly(A) signal in the 3′ UTR is in bold and underlined. The arrow indicates the position of a putative alternative splicing site that produces a truncated isoform (Gl-CalpM′; GenBank accession #AY644674).

significant difference between Gl-CalpM and Gl-CalpT (Fig. 12C–G). In ovary and integument, Gl-CalpM was expressed at higher levels than Gl-CalpB or Gl-CalpT, although the difference in the means was only significant in ovary; there was no significant difference between Gl-CalpB and Gl-CalpT in either tissue (Fig. 12H,I). Interestingly, the striated muscles showed different patterns. In skeletal muscle, Gl-CalpM and Gl-CalpB were expressed about 100-fold and 20-fold higher than Gl-CalpT in claw and thoracic muscles, respectively (Fig. 12, compare A and B with C). By contrast, Gl-CalpB was expressed in heart about 5-fold higher than Gl-CalpM and about 2.6-fold higher than Gl-CalpT. Gonadal tissues also differed in expression pattern. Gl-CalpM was expressed in ovary about 4.4-fold higher than Gl-CalpB and about 10.5-fold higher than Gl-CalpT, while Gl-CalpB was expressed in testis about 100-fold higher than Gl-CalpM and about 32-fold higher than Gl-CalpT (Fig. 12, compare G with H).

Effects of eyestalk ablation on calpain and EcR expression in skeletal muscles

As claw muscle atrophy coincides with elevated ecdysteroid levels during premolt, the effects of ecdysteroids, induced by eyestalk ablation, on the expression of the EcR and the three

```
5′-GGCCAGTCGTGCCTCAGACAATTGCAAGGGTGTGTTCACTAATTTCACGTGCTAGGAGTGAGTGGCCTCGACGCCTAAATATTCAAAGAGGATTAATTATT
   AGTAGTGTCCATCAAGCTGATAGGTGAAGACCTGATGCCTACGGTATCAACATCCTACAGAGCACAAGGGCTTGAATGAAGTGAAATAACAGTACGAAGAA
   GAGAGCAAGGCAACACCACACACCCTCGAAAGGACAGGCAACAAGCAGGTGGAAGTGTGGAGTTGTGAGGATAATGTACAGGTAGCTTAAACTTGTTCACA
  ATG GGT CTG TTC AGC TCC ACC AAG AAC TTC CGC GGC CAA GAC TAC GCC AAA CTG AAG AAG GAT TGT CTC CAT AGA GGA
1  M   G   L   F   S   S   T   K   N   F   R   G   Q   D   Y   A   K   L   K   K   D   C   L   H   R   G
  GAA AAG TTC AGT GAT CCC AAG TTC CCT CCA CGT GAC TCC TCA TTG TAC TTC TCA AAG CAG CCA CCT GGA GTT GTC ACC
27 E   K   F   S   D   P   K   F   P   P   R   D   S   S   L   Y   F   S   K   Q   P   P   G   V   V   T
  TGG AAG AGG CCT CAT GAA ATA TTA GAC AAG CCT CAG CTC TTC ATT GAG GGT GCA AGT GCC AAA GAT GTG ACT CAA GGT
53 W   K   R   P   H   E   I   L   D   K   P   Q   L   F   I   E   G   A   S   A   K   D   V   T   Q   G
  CAG CTA GGC AAC TGC TGG TTT GTT GCA GCA TGT GCC ACT CTT GCT GGT GTC AAG GAA CTC TGG CAC AAG GTC ATC CCA
79 Q   L   G   N   C   W   F   V   A   A   C   A   T   L   A   G   V   K   E   L   W   H   K   V   I   P
  GAC TAC AAG GAC CAA GAG TAC GGA GAC TTG CAT CCA GGC ATC TTC CAC TTC CGG TTC TGG AGG TTC GGG GAG TGG GTG
105 D   Y   K   D   Q   E   Y   G   D   L   H   P   G   I   F   H   F   R   F   W   R   F   G   E   W   V
  GAG GTG GTG GTG GAT GAC CTG CTT CCC ACC ATT GAG GGG CAG CTT ATC TTC ACT CAC TCT AAG GAG AGA GGA GAA TTC
131 E   V   V   V   D   D   L   L   P   T   I   E   G   Q   L   I   F   T   H   S   K   E   R   G   E   F
  TGG TGT GCC TTA CTG GAG AAA GCT TAT GCC AAA CTT TAT GGC TCA TAT GAA GCA CTT GAG GGA GGT AAT CTC AGT GAT
157 W   C   A   L   L   E   K   A   Y   A   K   L   Y   G   S   Y   E   A   L   E   G   G   N   L   S   D
  GCC CTG GTG GAC CTC ACA TCT GGA GTG TCA GCC CAT CTA GAC CTG ACG ATT GGA GGC TAT GTG GAT GAC TTT GAG AAG
183 A   L   V   D   L   T   S   G   V   S   A   H   L   D   L   T   I   G   G   Y   V   D   D   F   E   K
  CGG AAG CAA CTA TTC AAA ATG ATG TCC AAG GAA ATG AAT GAA CAC TCT CTG ATG TGC TGT GCC ATA ACT CCA CAC AGC
209 R   K   Q   L   F   K   M   M   S   K   E   M   N   E   H   S   L   M   C   C   A   I   T   P   H   S
  CAT GAG GAG GCA GAG ATG CGA ACA AAC GTA GGA CTG GTG AAA GGC CAT GCC TAT GGA ATC ACC GCT TGC CGC AAA ATC
235 H   E   E   A   E   M   R   T   N   V   G   L   V   K   G   H   A   Y   G   I   T   A   C   R   K   I
  AAC ATA GGA GAT ACA GGC CTC TTC TCC ATC TTC AAG GGC GCC CAG AAG GTG AGG ATG GTG CGT CTC AAG AAC CCT TGG
261 N   I   G   D   T   G   L   F   S   I   F   K   G   A   Q   K   V   R   M   V   R   L   K   N   P   W
  GGA GAG AAA GAG TGG AAT GGG GCC TTC AGT GAT GGA TCA CCA GAA TGG TCA CAG GTC TCT GCA TCA GAG CGA CAG AAG
287 G   E   K   E   W   N   G   A   F   S   D   G   S   P   E   W   S   Q   V   S   A   S   E   R   Q   K
  CTT GGC CTA ACC TTT GAG GAT GAT GGT GAA TTC TGG ATG ACT TTT GAA GAT TTT CTA GAG CAC TTC ACT GAC CTT TCC
313 L   G   L   T   F   E   D   D   G   E   F   W   M   T   F   E   D   F   L   E   H   F   T   D   L   S
  ATA TGC TTC CTT ATC AAC ACC AAG TTC TTG AGC TTC AGC AAG ACT TGG CAT GAG ACT GTC TTC TTT AAT GGT TGG ACT
339 I   C   F   L   I   N   T   K   F   L   S   F   S   K   T   W   H   E   T   V   F   F   N   G   W   T
  ATT GGT GTT CGA GGC CAT AAC TCG GAC AGG GCA GGA GGA TGC CCA AAC CAC AAG GAT ACT TTT TTG CGC AAC CCT CAG
365 I   G   V   R   G   H   N   S   D   R   A   G   G   C   P   N   H   K   D   T   F   L   R   N   P   Q
  TTT AGG TTT GAT ATT AAG GAG GAA ACA GAT GAC GTT GTG TTC CAG CTG ATG CAG AAG GAT GCC AGG GAA CGT AAA CAA
391 F   R   F   D   I   K   E   E   T   D   D   V   V   F   Q   L   M   Q   K   D   A   R   E   R   K   Q
  GAA GGA ATG CAA AAC CTT GTC ATA GGT TTT CAC ATA ATG AGG GTT GAA GAA AAC AGG AAA TAC AGA GTC CAC CGG ATC
417 E   G   M   Q   N   L   V   I   G   F   H   I   M   R   V   E   E   N   R   K   Y   R   V   H   R   I
  CAT GAT GCA GTG GCC ACA TCT GAC TAT ATC CGC ACT AGA GGA ATC TTC CTG AGG GAG CAG CTA AAG CAA GGC CGC TAT
443 H   D   A   V   A   T   S   D   Y   I   R   T   R   G   I   F   L   R   E   Q   L   K   Q   G   R   Y
  GTC ATA ATT CCC ACA ACT TTC AAG CCA GAT GAG ACA GGG GAG TTT CTT CTG AGA ATA TTC ACC TCA AAA GAT CCA GAT
469 V   I   I   P   T   T   F   K   P   D   E   T   G   E   F   L   L   R   I   F   T   S   K   D   P   D
  GCC AAA GAG CCG ATC AGA GAC CAA CCG AAG AGT CCC TGG TAC TCT TGC TTC AAG AAG GCT GTT ATG GTT ACC ACA ATC
495 A   K   E   P   I   R   D   Q   P   K   S   P   W   Y   S   C   F   K   K   A   V   M   V   T   T   I
  ACT GTG AAG TGT GCC AGT AAC TTG GAG AAA CAA TCT GCA TTT GGA GGT GAT GCT GAT GCA TAT TGC ATC ATA AAG TGC
521 T   V   K   C   A   S   N   L   E   K   Q   S   A   F   G   G   D   A   D   A   Y   C   I   I   K   C
  GGG GGA GAA ACT GTT CGG ACA CCA GTG AAA GGA CAG CAA TCC TAT ATG ACA CCA CAG CCA TTT TAC CGT GCT AAG CCT
547 G   G   E   T   V   R   T   P   V   K   G   Q   Q   S   Y   M   T   P   Q   P   F   Y   R   A   K   P
  GAA CAG CCC ATT GTG ATT GAA ATC TGG AAC AGC AAC ATG TTA GTG GAT GGC TTC ATT GGG CGA GCT GAG GTG ACT GCA
573 E   Q   P   I   V   I   E   I   W   N   S   N   M   L   V   D   G   F   I   G   R   A   E   V   T   A
  CCT ATC AAT CCA AAC TAT ACC CAG GTT CAA CTT TCA CTG TAT GGC AGA CGC AAG GAG AAA ACT GTA GAG AAA CAA GGG
599 P   I   N   P   N   Y   T   Q   V   Q   L   S   L   Y   G   R   R   K   E   K   T   V   E   K   Q   G
  CAC CTT CTG GTG CAA GTG TAC AGT GAT GAC CTG ACC AGC ATC TAG
625 H   L   L   V   Q   V   Y   S   D   D   D   L   T   S   I   *
  CTGCCACCTACAGAAAGCAGTTGATGTTGAGAAATATCCTACATTATCAAAAATGCTTTTCATTCAAACAAGTGTTTGTTATTTTGCATTAATATGAAATTTGTTA
  CAGTATTTTATATAACGAGGAGGGAAAAGTGGAAATATTCTCAAATGTTGACTGATCACCACAAAAATGAGTCATGTGTGTGTGTCAGAGCTGGGCACCACAAA
  CGGAAAATTACTTCTGCAAACGCAAACCCGCAAACTTGAACTCATGCCTTCCACAAACGCAAACTCGCAAACTTTATGATTTCTGTGACACCGCAAACACAAATCC
  GCAAGTGCAAACCATTTTCCGATTTTAACTATAAAAGGTATCCAAGACATTAAAAACAAGGTTAAATAAAGCAATACTGTAAAAAAAAAAAAAAAAAA-3′
```

Fig. 4. The complete sequence of calpain T cDNA from land crab (Gl-CalpT). The sequence (2639 bp) contained a full open reading frame (304–2223 bp) encoding a protein of 639 amino acids with a predicted mass of 74.56 kDa (GenBank accession #AY639154). Locations of degenerate primer sites for initial nested RT-PCR are underlined. Three amino acid residues comprising the conserved catalytic triad (C, H, N) in domain II are in bold. The asterisk indicates the stop codon. The poly(A) signal in the 3′ UTR is in bold and underlined.

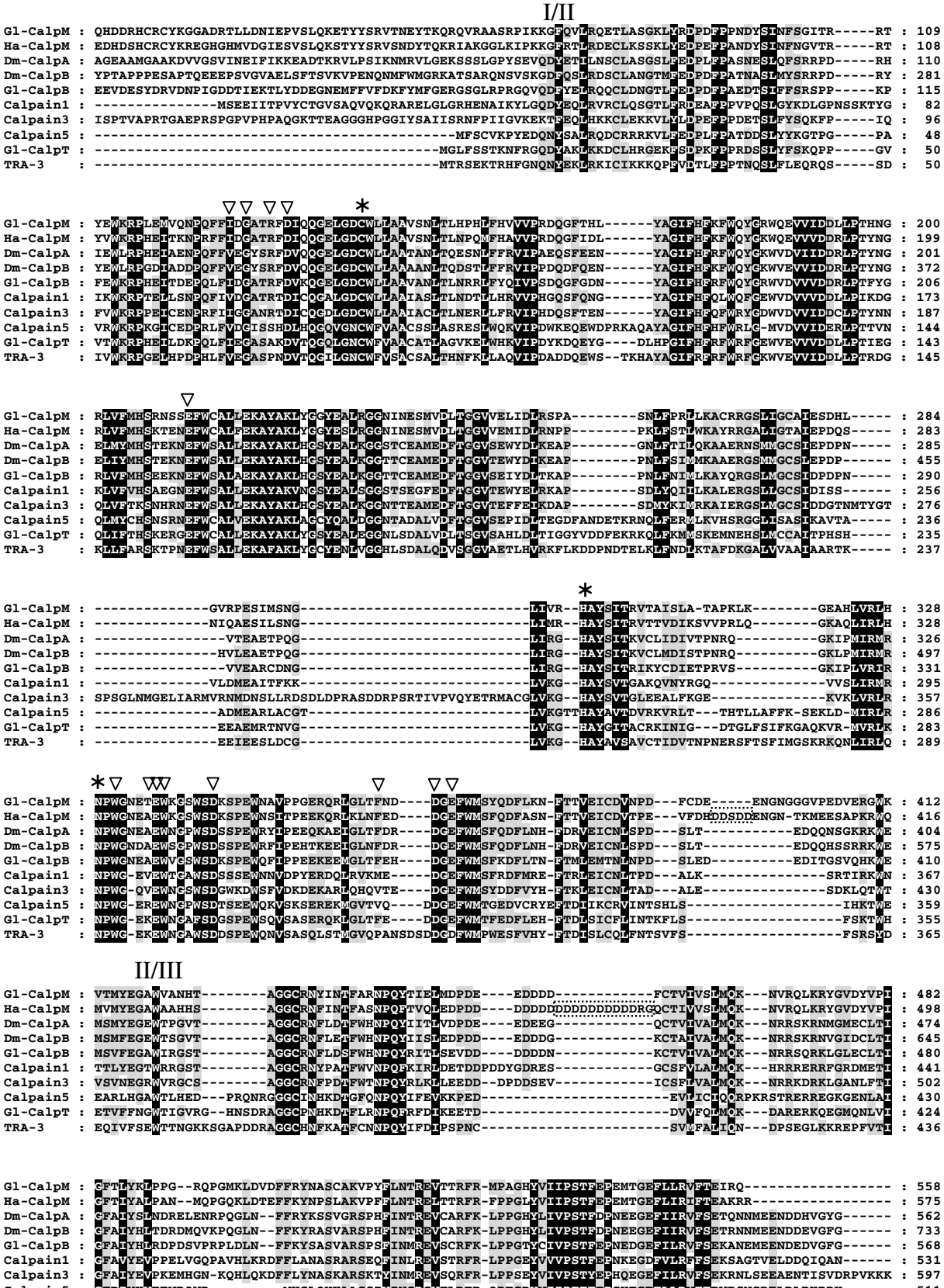

Fig. 5. See next page for legend.

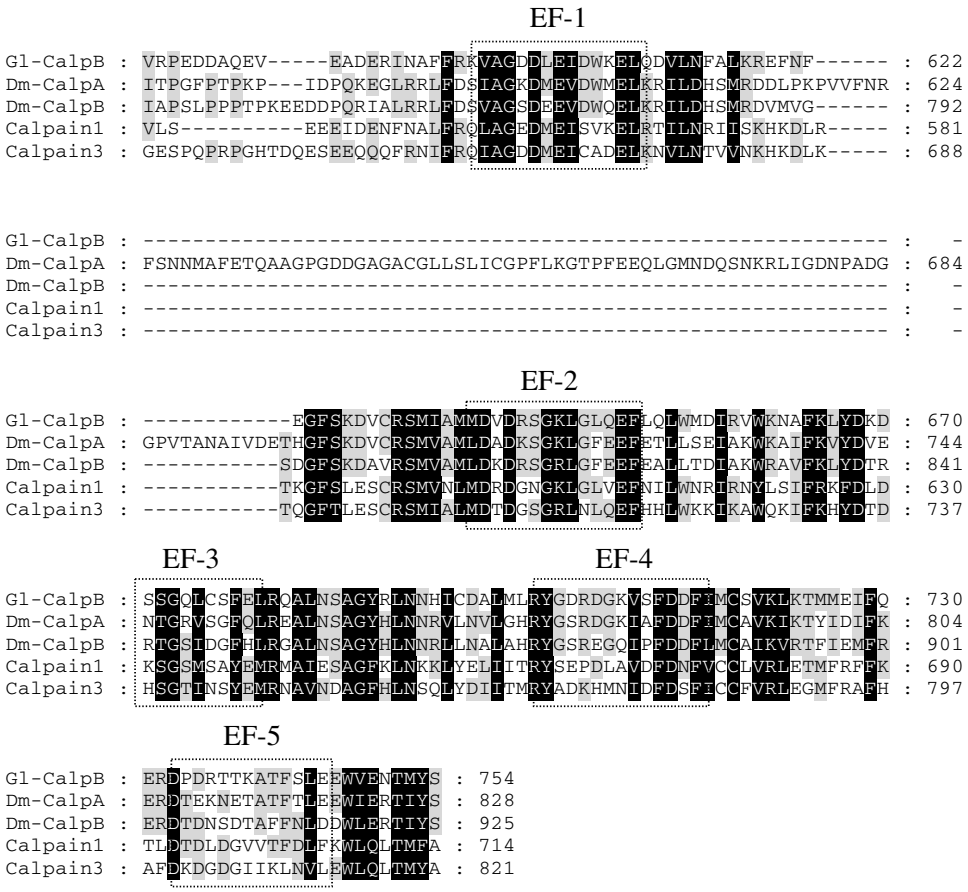

Fig. 6. Comparison of amino acid sequences in domain IV of 'typical' calpains from arthropods and human. Deduced amino acid sequences were aligned using ClustalW (see Materials and methods). Broken lines indicate gaps for optimizing alignment. Amino acid residues that are identical or similar in all the sequences are highlighted in black; gray background indicates identical or similar amino acids in most of the sequences. Numbers at the right indicate amino acid positions, numbered from the N-terminus of each protein. Domain IV is a calmodulin-like domain that contains five EF-hand motifs (boxes with broken line). Dm-CalpA contains a unique insertion sequence between EF-1 and EF-2. The putative EF-5 region was the least conserved between the calpain sequences. Accession numbers are the same as those given in the legend to Fig. 5.

calpains were determined in claw and thoracic muscles. The thoracic muscle served as a non-induced control, as it does not undergo premolt atrophy (Moffett, 1987). EcR mRNA was used as an indicator of tissue sensitivity to molt induction, as it is upregulated in response to elevated ecdysteroids in insects (Sun et al., 2003).

Fig. 5. Comparison of the deduced amino acid sequences in domains II and III of calpains from arthropods, nematode and human. Amino acid sequences were aligned using ClustalW (see Materials and methods). Broken lines indicate gaps for optimizing alignment. Amino acid residues that are identical or similar in all the sequences are highlighted in black; gray shading indicates identical or similar amino acids in most of the sequences. Asterisks indicate residues of the catalytic triad (C, H, N). Open inverted triangles indicate conserved residues in two non-EF-hand Ca^{2+}-binding regions in domain II. Roman numerals indicate boundaries between domains. Numbers at the right indicate amino acid positions, numbered from the N-terminus of each protein. Boxes with broken lines indicate locations of two acidic amino acid expansions found in Ha-CalpM, but not in Gl-CalpM; one (DDSDD) is positioned near the end of domain II and the other (DDDDDDDDDDRG) is located in the acidic loop region in domain III. Human calpain 3 contains a unique insertion sequence in domain II. Accession numbers: *C. elegans* TRA-3, NP502751; Dm-CalpA, NP477047; Dm-CalpB, NP524016; human calpain 1, AAH08751; human calpain 3, NP058813; human calpain 5, JC5772; Ha-CalpM, AAM88579.

The X-organ/sinus gland complex in the eyestalk is the primary source of molt-inhibiting hormone (MIH), a neuropeptide that inhibits ecdysteroid synthesis and secretion by the Y-organs. Eyestalk ablation, therefore, is used as an effective strategy to rapidly stimulate the Y-organs and chronically increase the level of ecdysteroids in the hemolymph (Skinner, 1985). Ecdysteroid levels were significantly higher one day after eyestalk ablation (80.8±22.3 ng ml^{-1}, mean ± 1 S.D., N=4) compared with intact controls (31.9±5.9 ng ml^{-1}, N=5; P=0.002). Ecdysteroid levels in 3-day eyestalk-ablated animals were not determined, although previous work showed that ablation results in chronically elevated ecdysteroid levels (McCarthy and Skinner, 1977).

The EcR, calpain and EF2 mRNA levels in thoracic and claw muscles were quantified by real-time PCR. EF2, which is constitutively expressed, was used as an internal standard to normalize the PCR reactions. In thoracic muscle, eyestalk ablation had no significant effect on expression of the Gl-EcR and the three calpains (Fig. 13B). By contrast, Gl-EcR expression in claw muscle increased about 15-fold one day after eyestalk ablation but then decreased to about 2.8-fold above the level in intact animals three days after eyestalk ablation (Fig. 13A). Gl-CalpT mRNA paralleled the expression of Gl-EcR in claw muscle; it increased about 19.3-fold above the level in intact animals one day after eyestalk

```
Gl-CalpT : VFQLMQK---DAREKKQEGMQNLVIGFHIMRVEENRKYRVHRIHDAVATSDYIRTRGIFLREQ-LKQGRY : 468
Calpain5 : LICIQQRPKRSTRERREGKGENLAIGFDIYKVEENRQYRMHSLQHKAASSIYINSRSVFLRTD-QPEGRY : 474
TRA-3    : MFALIQN---DPSEGLKKREPFVTIGMHVMKVENNRQYRVHTAMHPIAISDYASGRSVYLHLQSLPRGRY : 481

Gl-CalpT : VIIPTTFKPDETGEFLLRIFTSKDPDAKEPIRDQP--KSPWYSCFKKAVMVTTITVKCASNLEKQSAFGG : 536
Calpain5 : VIIPTTFEPGHTGEFLLRVFTDVPSNCRELRLDKPPHTTCWSSLCGYPQLVTQVHVLGAAGLK-DSPTG- : 542
TRA-3    : LLIPTTFAPKEQTLFMLRVYSDEHIHFSPLTKHAP---KLGLLKCKSAQSVTRLTIHG---VDFNSASTG : 545

Gl-CalpT : DADAYCIIKCGGETVRTPVKGQQS---YMTPQPFYRAKPEQPIVIEIWNSN-MLVDGFIGRAEVTAPINP : 602
Calpain5 : -ANSYVIIKCEGDKVRSAVQKGTSTPEYNVKGIFYRKKLSQPITVQVWNHR-VLKDEFLGQVHLKADPDN : 610
TRA-3    : THNVYAILKDSRKSFRTKTLSGVKSIQWDEQFLFHKSKNRQQYKIEVWEDRKMARDHLLAQSVIIALIDN : 615

Gl-CalpT : NYTQVQLSLYGRRKEKTVEKQGHLLVQVYSDDDLTSI : 639
Calpain5 : LQALHTLHLRDRNSRQPSNLPGTVAVHILSSTSLMAV : 647
TRA-3    : ENRDTTLQLTDPRG----TVIGTVSVTVSAFDDPMYL : 648
```

Fig. 7. Comparison of amino acid sequences of the domain T in T-type calpains from land crab (Gl-CalpT), nematode (TRA-3) and human (calpain 5). Deduced amino acid sequences were aligned using ClustalW (see Materials and methods). Broken lines indicate gaps for optimizing alignment. Amino acid residues that are identical or similar in all the sequences are highlighted in black; gray background indicates identical or similar amino acids in most sequences. Numbers at the right indicate amino acid positions, numbered from the N-terminus of each protein. Accession numbers: calpain 5, JC5772; TRA-3, NP502751.

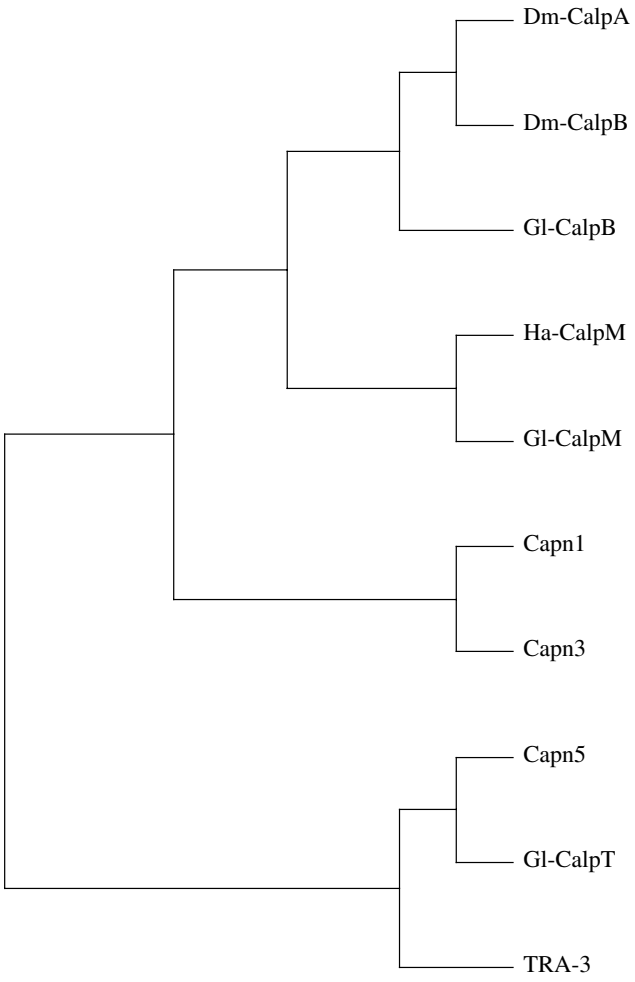

Fig. 8. Phylogenetic relationships of selected calpains from arthropods, nematode and human. The deduced amino acid sequences of the catalytic (II) and C_2-like (III) domains in land crab (Gl-CalpM, Gl-CalpB and Gl-CalpT), lobster (Ha-CalpM), fruit fly (Dm-CalpA and Dm-CalpB), nematode (TRA-3) and human (Capn1, Capn3 and Capn5) calpains were analyzed using ClustalW and Treeview software (see Materials and methods). The calpains cluster into four groups: T-type calpains (TRA-3, Gl-CalpT and Capn5), mammalian 'typical' calpains (Capn1 and Capn3), crustacean M-type calpains (Gl-CalpM and Ha-CalpM) and arthropod A/B-type calpains (Gl-CalpB, Dm-CalpA and Dm-CalpB).

ablation and about 4.3-fold higher three days after eyestalk ablation. Gl-CalpM and Gl-CalpB expression was not affected by eyestalk ablation. The relationship between the three calpain mRNAs and the EcR mRNA is presented in Fig. 14. The expression of Gl-EcR and CalpT was highly correlated in both claw muscle and thoracic muscle from intact and eyestalk-ablated animals (Fig. 14, bottom panels). There was no correlation between EcR expression and either Gl-CalpM or Gl-CalpB in either muscle (Fig. 14, top and middle panels, respectively).

Discussion

cDNAs encoding three full-length calpains were obtained from land crab limb regenerates by PCR cloning. Four calpain genes [*Dm-CalpA*, *B*, *C* and *D* (or *SOL*)] occur in the *Drosophila* genome (Friedrich et al., 2004; Goll et al., 2003). Sequence alignments indicate that the arthropod calpains fall into five categories; a proposed classification is presented in Table 5. Surprisingly, only members of the A/B type occur in both insects and crustaceans. Gl-CalpB, Dm-CalpA and Dm-

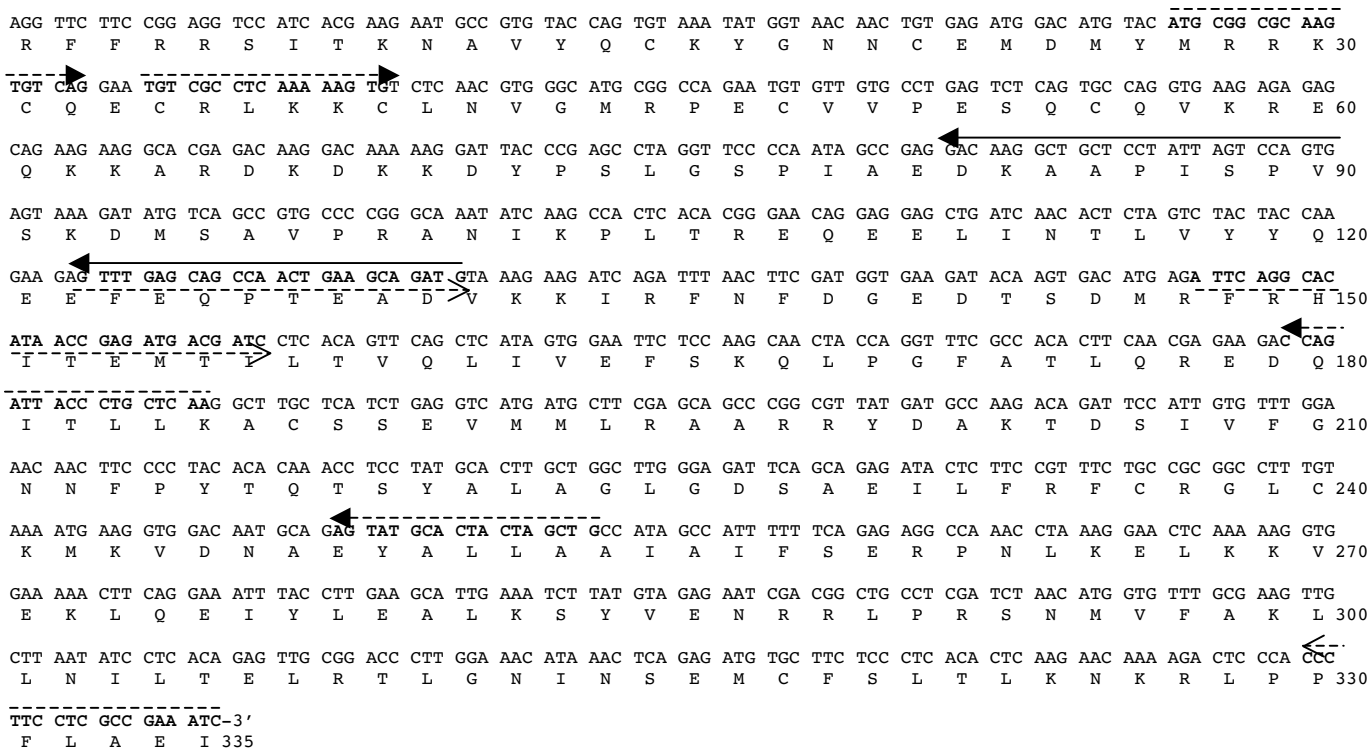

Fig. 9. Nucleotide and deduced amino acid sequence of partial cDNA encoding ecdysone receptor (Gl-EcR) from land crab. The cDNA sequence (1005 bp) encoded a partial protein sequence containing 335 amino acids (GenBank accession #AY642975). Locations of degenerate primers used for nested RT-PCR to obtain the initial cDNA are indicated in bold and with a dashed line with solid arrowhead. Locations of sequence-specific forward primers (cEcR F1 and cEcR F2) and degenerate nested reverse primer (EcR R4) used to obtain more of the 3′ sequence are indicated in bold and with a dashed line with open arrowhead. Solid lines with solid arrowheads indicate locations of sequence-specific primers used for 5′ RACE to obtain additional 5′ sequence in the ORF (Table 1).

CalpB appear to be the arthropod form of typical calpains, as they have the four-domain organization of mammalian calpains 1, 2 and 3 (Figs 2, 6; Goll et al., 2003; Jékely and Friedrich, 1999; Suzuki et al., 2004). Dm-CalpC was initially identified by the genome project as CG3692 and was predicted to have nine transmembrane domains in the N-terminal region. However, a recent study showed that Dm-CalpC actually has a much shorter N-terminal domain that lacks the membrane-spanning segments; it is catalytically inactive because all three catalytic amino acid residues are mutated (Spadoni et al., 2003). Members of the D (SOL), M and T types are atypical calpains, as they lack dIV. Gl-CalpM and Ha-CalpM are truncated at the C-terminal end of dIII (Figs 1, 3), while Gl-CalpT has the T domain in place of dIV in the C-terminus (Figs 1, 4). D calpains have six zinc-finger motifs in the N-terminal 'SOL' domain in place of dIII and dIV in the C-terminal region (Delaney et al., 1991).

Gl-CalpB appears to be the only typical calpain in crustaceans, as it is the only land crab calpain with the C-terminal EF-hand domain IV (Fig. 2). Sequence alignment analysis of domains II and III and its estimated mass (~89 kDa) indicate that Gl-CalpB is most closely related to *Drosophila* calpains A and B (Farkas et al., 2004; Jékely and Friedrich, 1999; Pinter et al., 1992). Gl-CalpB combines features found in Dm-CalpA or Dm-CalpB. It has no insertion sequence in dIV, like Dm-CalpB, but has a dI length similar to that in Dm-CalpA (Fig. 1; Emori and Saigo, 1994; Jékely and Friedrich, 1999; Theopold et al., 1995). These data suggest that Dm-CalpA and Dm-CalpB are derived from duplication of a common ancestral gene and that no such duplication occurred in crustaceans.

Table 5. *Proposed classification of arthropod calpain genes*

Type	Members	Features
A/B calpains	*Dm-CalpA*, *Dm-CalpB*, *Gl-CalpB*	Four-domain structure of typical calpains (domain IV in C-terminus)
C calpains	*Dm-CalpC*	Four-domain structure of typical calpains; all three catalytic amino acid residues mutated (inactive)
D calpains	*Dm-CalpD* (*SOL*)	Zn-finger motifs in N-terminus; domain IV replaced by SOL domain in C-terminus
M calpains	*Ha-CalpM*, *Gl-CalpM*	Truncated (lacks domain IV)
T calpains	*Gl-CalpT*	Domain IV replaced by T domain in C-terminus

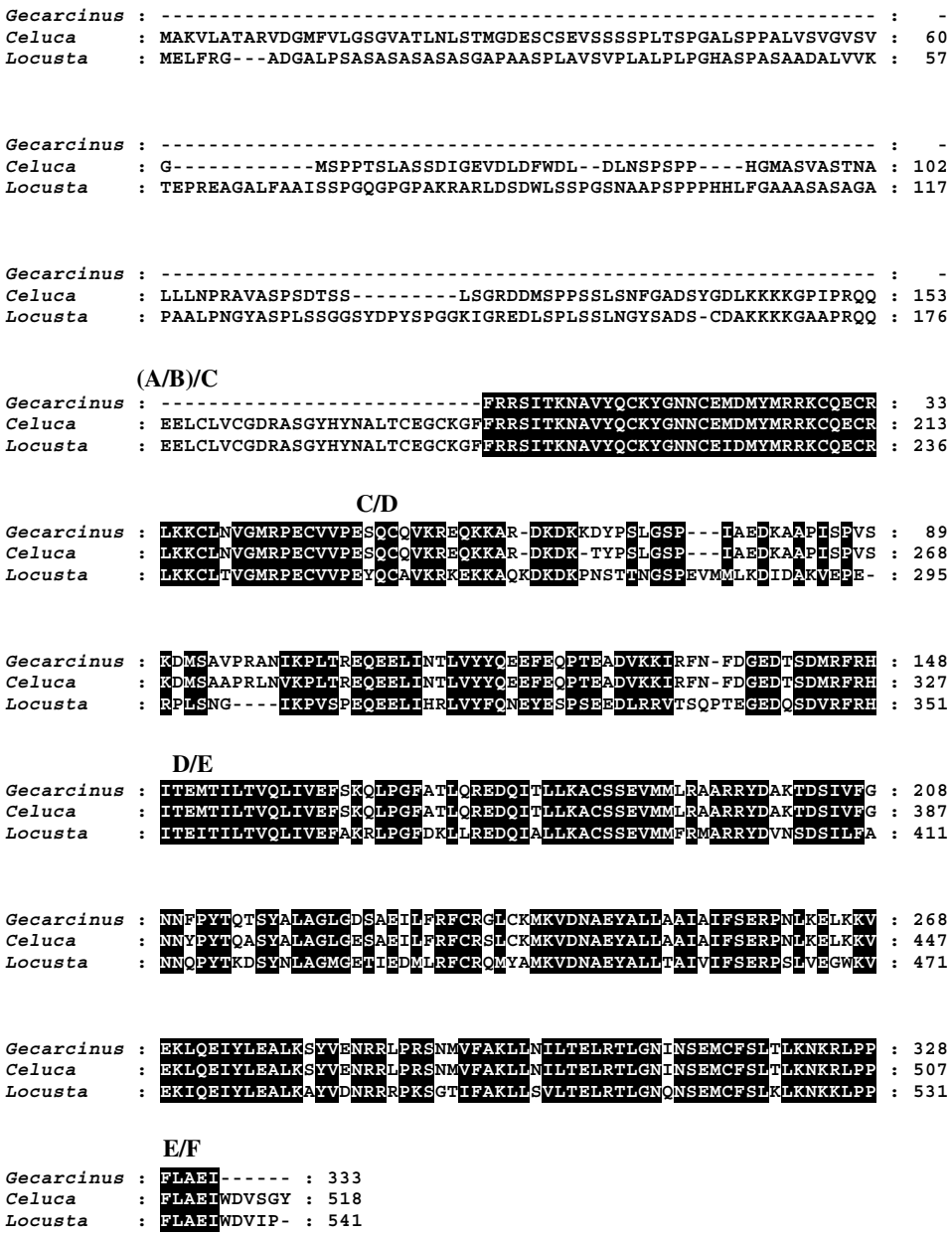

Fig. 10. Comparison of deduced amino acid sequences of ecdysone receptor (EcR) cDNAs from land crab, fiddler crab and locust. The partial Gl-EcR sequence is aligned with EcR sequences from fiddler crab, *Celuca* (*Uca*) *pugilator* (Up-EcR; #AAC33432), and locust, *Locusta migratoria* (Lm-EcR; #AAD19828). Gl-EcR had the highest sequence identity with the other two EcR sequences in the DNA-binding (domain C) and ligand-binding (domain E) domains. Overall identities were 93% between Gl-EcR and Up-EcR and 66% between Gl-EcR and Lm-EcR. Amino acid identities or similarities in all three sequences are highlighted in black. Boundaries between domains are indicated by capital letters.

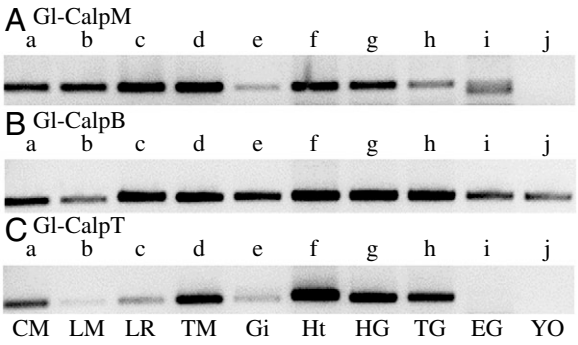

Fig. 11. Tissue expression of land crab calpain B, M and T mRNAs using end-point RT-PCR. Total RNA from each tissue was DNase-treated, reverse-transcribed and PCR-amplified using primers specific for Calpains M, B and T (Table 2). Shown is a reversed image of an ethidium bromide-stained agarose gel of PCR products. The sizes of the products were 310 bp for CalpM, 538 bp for CalpB and 536 bp for CalpT. Gl-CalpB was expressed at varying levels in all tissues; Gl-CalpM was expressed in all tissues except Y-organ; Gl-CalpT was expressed in all tissues except eyestalk ganglia and Y-organ. Lane a, claw muscle (CM); b, leg muscle (LM); c, limb regenerate (LR); d, thoracic muscle (TM); e, gill (Gi); f, heart (Ht); g, hind gut (HG); h, thoracic ganglion (TG); i, eyestalk ganglia (EG); j, Y-organ (YO).

The M-type calpains are unique to crustaceans. Gl-CalpM from land crab has the highest structural similarity with Ha-CalpM, the first calpain cloned from a crustacean (Yu and Mykles, 2003). Both encode polypeptides of about 66 kDa that lack dIV. Unlike other truncated forms, crustacean CalpM does not appear to be produced by alternate splicing of a typical

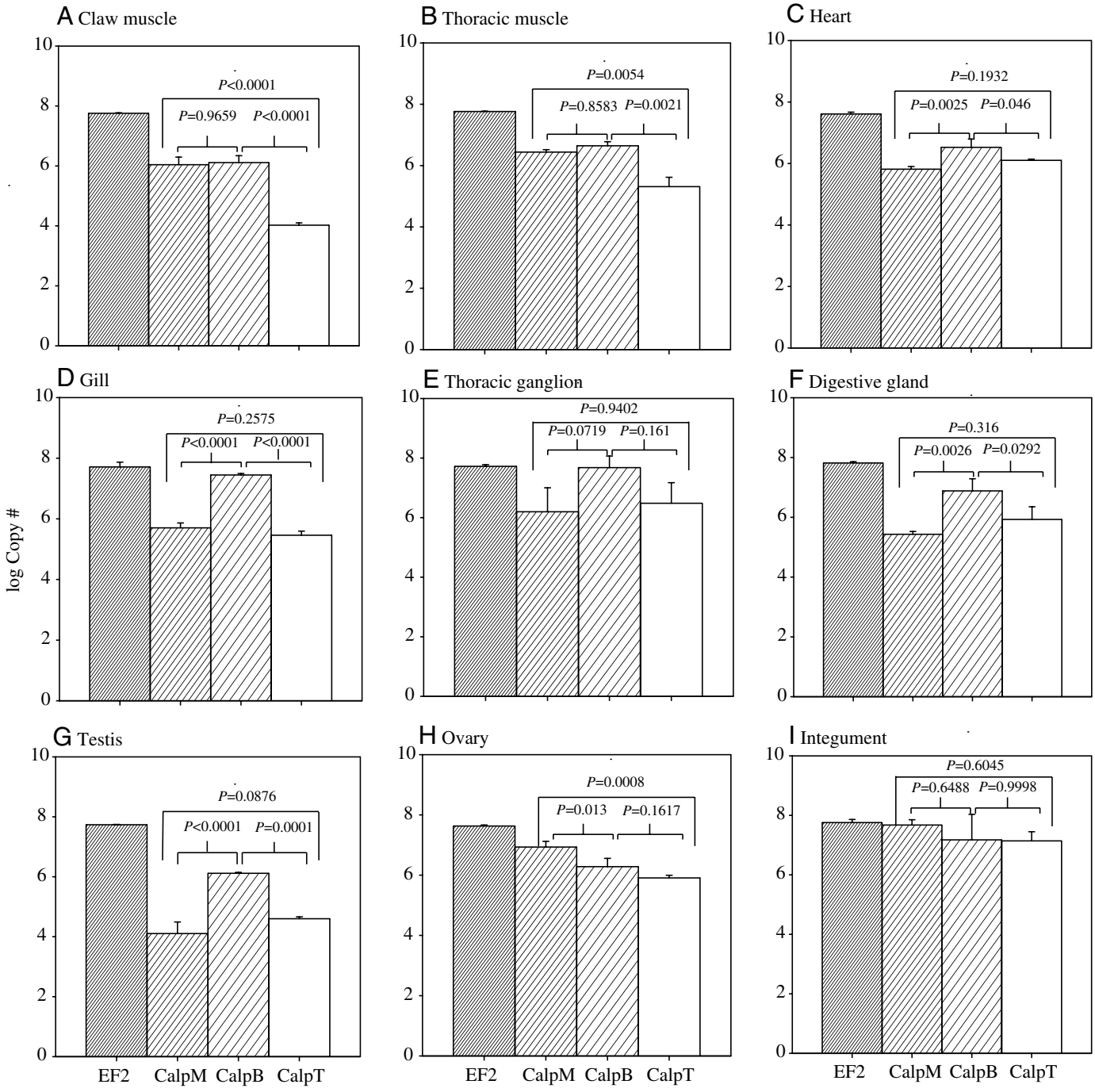

Fig. 12. Quantification of calpain B, M and T mRNAs in tissues from intermolt land crabs using real-time PCR. Transcript levels, expressed as log copy number, were determined in nine tissues. Elongation factor 2 (EF2) mRNA (GenBank accession #AY552550) served as an internal standard to normalize the PCR reactions. *P*-values between means for each calpain within each tissue are given at the top of each graph (see Table 3 for primers and Materials and methods for statistical analysis). There were three general patterns of expression. In skeletal muscle (A,B), CalpM and CalpB were expressed at similar levels, which were at least an order of magnitude higher than CalpT expression. In heart, gill, thoracic ganglion, digestive gland and testis (C–G), CalpB was expressed at higher levels than CalpM and CalpT. In ovary and integument (H,I), CalpM was expressed at higher levels than CalpB, which was expressed at higher levels than CalpT.

calpain gene. Dm-CalpA′, for example, is an alternatively spliced transcript of Dm-CalpA (Theopold et al., 1995). In mammals, nCL-2′, which lacks domains III and IV, is produced by alternative splicing of the *nCL-2* (*Capn8*) gene (Sorimachi et al., 1993). We were not successful in obtaining longer cDNAs containing the dIV region using 3′ RACE PCR with various sequence-specific primer sets. The absence of a longer mRNA is supported by immunoblot results using an

antibody raised against a 28-amino acid N-terminal sequence of Ha-CalpM; only two proteins with masses of 62 and 68 kDa were detected in lobster muscles (Yu and Mykles, 2003). A truncated, alternatively spliced isoform of Gl-CalpM, Gl-CalpM′, was isolated by 3′ RACE. Gl-CalpM′ is likely to be catalytically inactive, as the alternative transcript has a stop codon inserted before the Asn residue of the catalytic triad in dII. As it is expressed at very low levels, it was not characterized further.

The Gl-CalpT is a member of the T domain calpain family. Its amino acid sequence has the highest sequence identity with human Capn5 and nematode TRA-3 (Figs 5, 7). TRA-3 was first isolated as a sex determinant of the soma and germ line in hermaphrodites of *C. elegans* (Barnes and Hodgkin, 1996). TRA-2A is a substrate for TRA-3, and cleavage of TRA-2A by TRA-3 generates a peptide that has feminizing activity (Sokol and Kuwabara, 2000). Two mammalian calpains, Capn5 and Capn6, have the same structure as Gl-CalpT and TRA-3. However, Capn6 is catalytically inactive, as one or two residues of the catalytic triad (C, H, N) are mutated in human (K, H, N) and mouse (K, Y, N) (Dear et al., 1997; Matena et al., 1998). Interestingly, the *Drosophila* genome apparently lacks a CalpT-like gene (Friedrich et al., 2004; Goll et al., 2003).

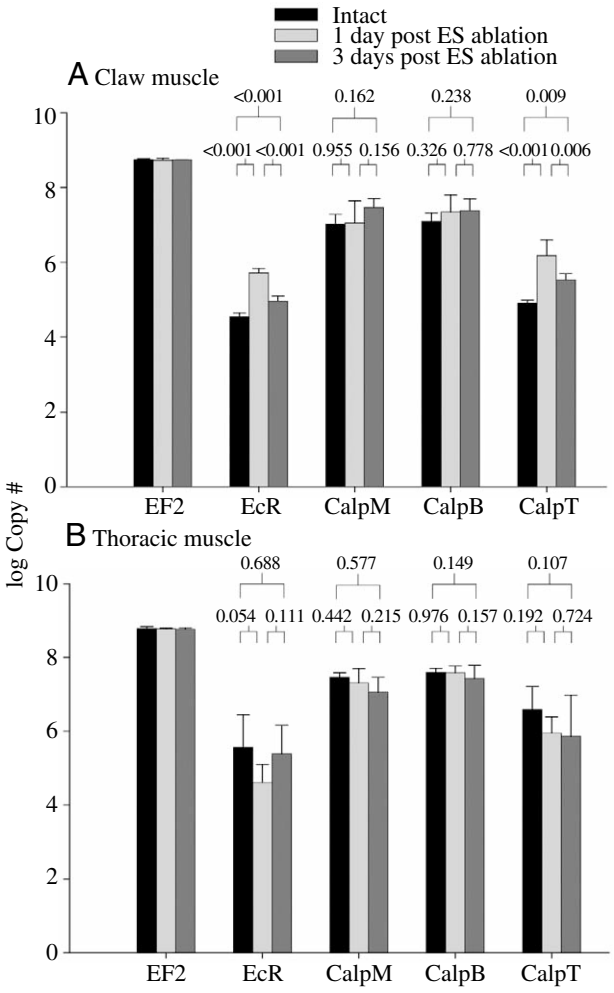

Fig. 13. Effect of eyestalk ablation on expression of land crab ecdysone receptor (EcR) and calpains in skeletal muscles. Transcript levels, expressed as log copy number, in claw (A) and thoracic (B) muscles were quantified using real-time PCR. Elongation factor 2 (EF2) mRNA served as an internal standard to normalize the PCR reactions. *P*-values between means for each gene are given at the top of each graph (*N*=13 for claw muscle; *N*=14 for thoracic muscle; see Materials and methods for statistical analysis). In claw muscle, eyestalk ablation, which increases hemolymph ecdysteroid levels, significantly increased the level of EcR and CalpT mRNAs; CalpM and CalpB mRNA levels were unaffected. In thoracic muscle, there was no significant effect of eyestalk ablation on EcR and calpain expression.

It is likely that all three crustacean calpains have Ca^{2+}-dependent proteinase activity. The deduced amino acid sequences of the protease domain are highly conserved, including the three amino acid residues essential for catalytic activity (Fig. 5). In addition, the three calpains contain two well-conserved non-EF hand Ca^{2+}-binding sites in dII and a C_2-like Ca^{2+}/phospholipid-binding site in dIII (Fig. 5), both of which are important in Ca^{2+}-dependent activation (Alexa et al., 2004; Moldoveanu et al., 2002, 2004). In the absence of Ca^{2+}, the catalytic residues are misaligned and the substrate binding cleft is disrupted; binding of Ca^{2+} to domains II and III activates the enzyme by driving the realignment of the active site residues (Moldoveanu et al., 2002, 2004; Strobl et al., 2000). The interaction of residues R104 and E333 in dII of mammalian calpain provides cooperativity between the two Ca^{2+}-binding sites (Moldoveanu et al., 2004); these two residues are conserved in the crustacean genes. Moreover, calpains lacking the calmodulin-like domain retain Ca^{2+}-dependent activity (Hata et al., 2001; Sokol and Kuwabara, 2000).

Table 6. *Comparison of masses of deduced sequences from land crab and lobster calpain cDNAs with subunit masses of lobster CDPs characterized biochemically*

cDNA	Estimated mass (kDa)	Lobster CDP counterpart (subunit mass in kDa)
Gl-CalpB	88.9	IIb (95*)
Gl-CalpT	74.6	I or IIa (60*)
Gl-CalpM/Ha-CalpM	65.2/66.3	III (59†; 62 and 68*)

A 62-kDa isoform of Ha-CalpM is expressed in claw muscle and a 68-kDa isoform is expressed in abdominal muscle (Yu and Mykles, 2003). The native masses of CDPs I, IIa, IIb and III are 310 kDa, 125 kDa, 195 kDa, and 59 kDa, respectively (Mykles and Skinner, 1986). The putative identities of CalpB with CDP IIb and CalpT with CDP I or IIa have not been established. There is a ~15-kDa difference between the predicted mass of Gl-CalpT and the subunit mass of CDP IIa. The subunit composition of CDP I is not known.

*Mass estimated by gel filtration column chromatography (Mykles and Skinner, 1986).

†Mass estimated by SDS-polyacrylamide gel electrophoresis (Beyette et al., 1997; Beyette et al., 1993; Beyette and Mykles, 1997; Yu and Mykles, 2003).

Four Ca^{2+}-dependent proteinase (CDP) activities have been characterized biochemically in crustacean muscle (Mykles and Skinner, 1986). These activities differ in native mass and subunit composition (Table 6). Ha-CalpM appears to encode CDP III, based on similar masses and chromatography properties (Yu and Mykles, 2003); the native enzyme consists of a single polypeptide (Table 6). Gl-CalpB may encode CDP IIb, as the subunit mass (95 kDa) of the purified lobster CDP IIb (Beyette et al., 1993; Beyette and Mykles, 1997) is similar to the estimated mass (89 kDa) of the deduced Gl-CalpB amino acid sequence. In addition, CDP IIb and the Dm-CalpA 95-kDa gene product share immunological properties. Polyclonal antibodies raised against lobster CDP IIb and Dm-CalpA protein cross-react, while a polyclonal antibody raised against a conserved 20-amino acid sequence around the cysteine residue in the active site of mammalian μ- and m-calpains (GATRTDICQGALGDCWLLAA) does not react with either Gl-CalpB or Dm-CalpA (Beyette et al., 1997). Analysis of this same sequence in Gl-CalpB (GATRFDVKQGELGDCWLLAA) indicates that four residues (Thr, Ile, Cys and Ala) in mammalian calpains are replaced by Phe, Val, Lys and Glu in Gl-CalpB (Fig. 5). The replacement of two uncharged residues with two charged residues may explain why the antibody does not react with lobster CDP IIb. The Gl-CalpB polypeptide probably does not associate with a regulatory subunit, as the sequence in the putative EF-5 is the least conserved with vertebrate heterodimeric calpains (e.g. Capn1; see Fig. 6). This is consistent with the homodimeric structure of the native CDP IIb (Beyette and Mykles, 1997).

The identity of Gl-CalpT with CDP I or IIa is less certain. A polyclonal antibody raised against the 20-residue mammalian active site sequence reacts with a 60-kDa protein

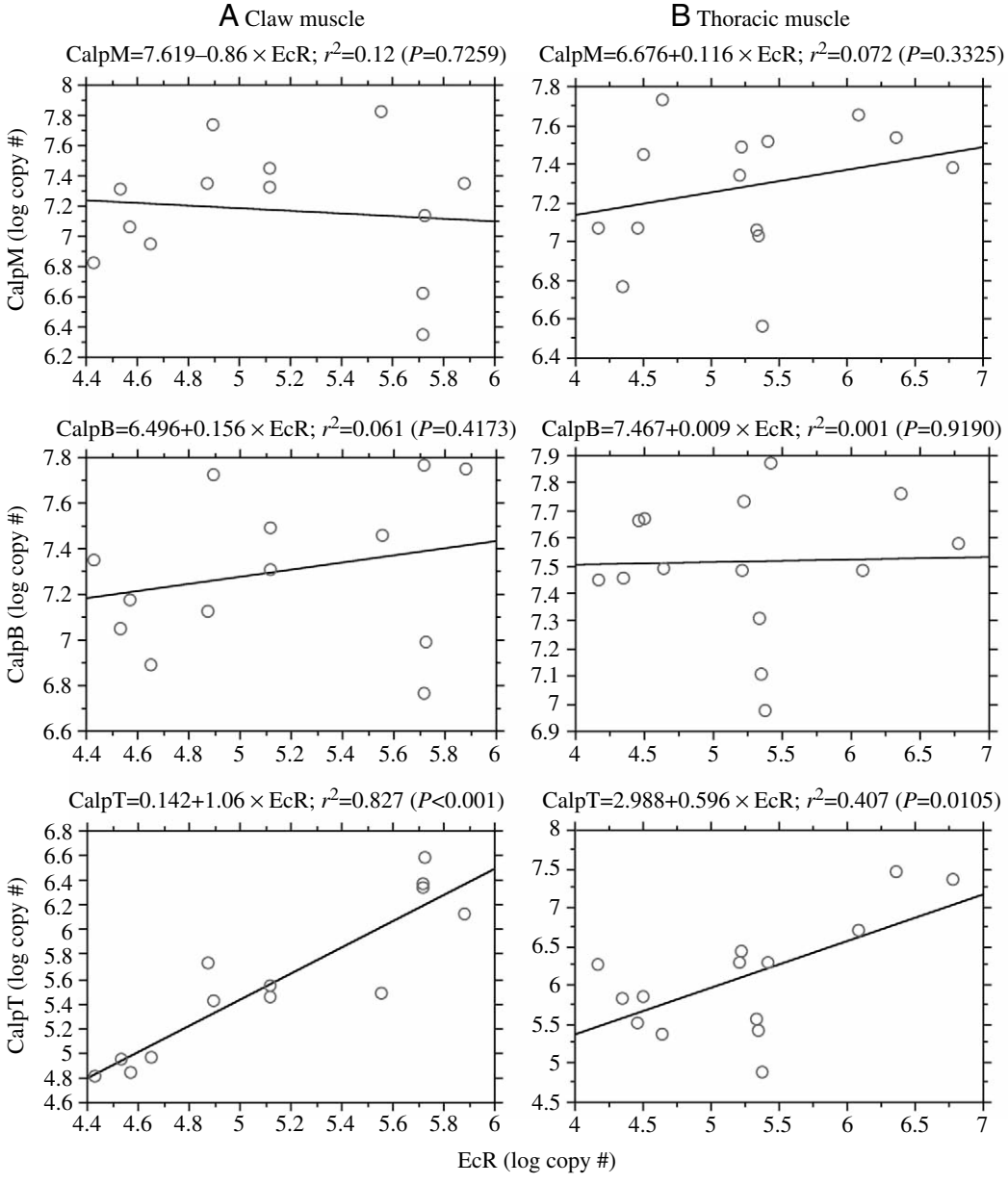

Fig. 14. The relationship between EcR and calpain expression in skeletal muscles from intact and 1- or 3-day eyestalk-ablated land crabs. Correlations between each calpain mRNA and EcR mRNA were determined with Statview (see Materials and methods). The expression of CalpT was significantly correlated with EcR expression in claw (P<0.001) and thoracic (P=0.0105) muscles. CalpB and CalpM mRNA levels were not correlated with EcR mRNA.

in immunoblots of a partially purified preparation of lobster CDP IIa (Beyette et al., 1997). However, eight of the 20 residues around the catalytic cysteine differ between Capn1 and Gl-CalpT (Fig. 5), making it less likely that the antibody would recognize the CalpT sequence. Furthermore, the deduced amino acid sequence of Gl-CalpT has an estimated mass of ~74 kDa, which is significantly greater than the 60 kDa estimated from immunoblots. CDP I is not well characterized. Its large native mass (310 kDa) suggests it is a multimer of the proteins encoded by CalpB, CalpT and/or an additional unidentified calpain gene. CalpM can be excluded as a component of CDP I, as the Ha-CalpM antibody does not react with any proteins in CDP I fractions eluting from a gel filtration column (Yu and Mykles, 2003). Further work is required to reconcile the CalpB and CalpT cDNAs with the calpain activities.

Three general patterns of calpain expression are observed in the nine tissues from adult intermolt land crabs analyzed by real-time PCR. The first pattern, in which both Gl-CalpB and Gl-CalpM are expressed at higher levels than Gl-CalpT, is found in skeletal muscles (Fig. 12A,B). The second pattern, in which Gl-CalpB is dominant, is found in heart, gill, thoracic ganglion, digestive gland and testis (Fig. 12C–G). The third pattern, in which Gl-CalpM mRNA level is highest, is found in ovary and integument (Fig. 12H,I). Differences in calpain expression are also observed in insect. Although *Drosophila* calpains are expressed throughout development, Dm-CalpA, Dm-CalpB and Dm-CalpC differ in their mRNA levels and tissue distribution. In early embryos, Dm-CalpA is localized in the anterior pole and posterior surface regions (Emori and Saigo, 1994), while Dm-CalpB has a uniform distribution (Farkas et al., 2004). During nuclear division, Dm-CalpA is more evenly distributed and becomes associated with the precleavage furrows before cellularization (Emori and Saigo, 1994). In late embryos, Dm-CalpB is preferentially expressed in the trachea and foregut (Farkas et al., 2004). In larvae, Dm-CalpB mRNA and protein levels decline, whereas Dm-CalpC mRNA is moderately elevated (Farkas et al., 2004; Spadoni et al., 2003). Both Dm-CalpB and Dm-CalpC are expressed highly in the salivary glands of third instar larvae (Farkas et al., 2004; Spadoni et al., 2003). In adults, Dm-CalpA mRNA is present in many tissues, such as ovary, brain, ventral ganglion, midgut and heart, but not in the indirect flight muscles (Amano et al., 1997; Theopold et al., 1995). Dm-CalpB is also expressed in ovary (Farkas et al., 2004). The tissue distribution of Dm-CalpC in adults has not been reported. Such dynamic changes in localization and distribution suggest that the different calpains carry out specialized functions in arthropod tissues.

Ha-CalpM was first identified as a muscle-specific calpain, as it is expressed at the highest levels in lobster skeletal muscles (Yu and Mykles, 2003). The analysis of Gl-CalpM, however, shows that it is expressed in ovary and integument at levels higher than that in skeletal muscle. It might first appear that the tissue expression pattern of Gl-CalpM deviates more from that of Ha-CalpM. However, the apparent differences are much less, when one considers the fiber-type compositions of the muscles. The levels of the Ha-CalpM mRNA are about 4-fold higher in fast muscle (cutter claw closer and deep abdominal flexor muscles) than in slow muscle (crusher claw closer) (Yu and Mykles, 2003). The claw closer and thoracic muscles in the land crab are composed of only slow fibers (Mykles, 1988; Mykles and Skinner, 1981). If one excludes the fast muscles, the ratios of Gl-CalpM expression in slow muscle in relationship with that in most of the other tissues are similar to those of Ha-CalpM. An exception is the difference in CalpM expression in the integument. In lobster, Ha-CalpM mRNA level in the integument is about 13% that in crusher claw closer muscle (Yu and Mykles, 2003), whereas Gl-CalpM mRNA level is about 18-fold and 43-fold greater in integument than that in the thoracic muscle and claw muscle, respectively (Fig. 12, compare A, B and I). Gl-CalpM was also highly expressed in ovary. Unfortunately, the lobster ovary was not analyzed for Ha-CalpM expression (Yu and Mykles, 2003).

A cDNA encoding a partial sequence of EcR was obtained from land crab claw muscle mRNA. The domain organization of Gl-EcR is similar to that of fiddler crab EcR (Chung et al., 1998a; Durica et al., 2002), as well as other nuclear steroid receptors (Renaud and Moras, 2000). The N-terminal A/B domain is the least conserved in amino acid sequence and length (Cherbas et al., 2003; Hu et al., 2003; Onate et al., 1998). In insects, receptors differing in A/B domains are generated by alternate transcriptional start sites or alternative splicing (Schubiger et al., 2003; Segraves and Woldin, 1993; Talbot et al., 1993). By contrast, the fiddler crab EcR has one A/B domain isoform but has several alternative spliced isoforms around domain E (Durica et al., 2002). The C domain is highly conserved among different nuclear steroid receptors and primarily serves as a DNA-binding domain (DBD) containing two zinc-finger motifs. The E domain is a moderately conserved region of about 250 amino acids that serves as the ligand-binding domain (LBD) (Billas et al., 2003; Grebe et al., 2003; Wang et al., 2000). Domain E of many nuclear streroid receptors can also interact with numerous proteins, such as homo/heterodimeric partners, co-repressors and co-activators (Hu et al., 2003; Shibata et al., 1997). Both the DBD and LBD of Gl-EcR and Up-EcR are closely related to those domains in insect EcR (Fig. 10) and therefore probably have the same function. The D domain is a hinge region connecting the DBD with the LBD and is important in ligand binding (Grebe et al., 2003).

Gl-EcR and Gl-CalpT expression are upregulated by eyestalk ablation in claw muscle but not in thoracic muscle. Premolt atrophy occurs specifically in the claw muscle (Moffett, 1987; Mykles and Skinner, 1982a), suggesting that sensitivity to ecdysteroid may be important in initiating muscle protein degradation. The expression of Gl-EcR and Gl-CalpT was highly correlated in claw and thoracic muscles in intact and eyestalk-ablated animals (Figs 13, 14). These results suggest that expression of Gl-EcR and Gl-CalpT is linked. One possibility is that the EcR/RXR complex binds directly to the promoter of the *Gl-CalpT* gene, which induces its expression.

Another possibility is that ecdysone early response genes, such as *E75* or *E74* are induced by the EcR/RXR complex, and they, in turn, induce the expression of *Gl-CalpT*. In *Manduca sexta,* the EcR/RXR complex induces *E75* within 30 min (Zhou et al., 1998). The lower expression of Gl-EcR and Gl-CalpT in claw muscle three days after eyestalk ablation (Fig. 13A) suggests a feedback inhibition in response to sustained elevated ecdysteroids that is not mediated by the neurosecretory center in the eyestalk. In insects, 20-hydroxyecdysone inhibits ecdysteroid production in the molting gland (Beydon and Lafont, 1983; Sakurai and Williams, 1989), which is associated with changes in the expression and phosphorylation state of certain USP isoforms (Song and Gilbert, 1998). In crustaceans, ecdysteroid inhibits ecdysteroidogenesis in the Y-organ, but the mechanism is not known (Dell et al., 1999).

The role of each calpain in premolt claw muscle atrophy remains to be established. Unlike mammalian skeletal muscle, in which calpains are restricted to initial disassembly of sarcomeric elements (Goll et al., 2003; Jackman and Kandarian, 2004), crustacean calpains can carry out both disassembly and subsequent degradation of myofibrillar proteins (Mykles, 1998). Only Gl-CalpT is upregulated in response to elevated ecdysteroids, suggesting it is involved in initiating the atrophy program through the proteolytic modification of signaling proteins. This is analogous to the modification of TRA-2A by TRA-3 in mediating feminization of *C. elegans* embryos (Sokol and Kuwabara, 2000). In mammals, limited proteolysis of protein kinases and phosphatases by calpain often alters their biochemical properties (Goll et al., 2003; Mykles, 1998). The ubiquitous tissue expression and lack of ecdysteroid regulation of Gl-CalpB suggests that it has a housekeeping function. There is a preferential degradation of thin filaments during atrophy (Ismail and Mykles, 1992; Mykles and Skinner, 1981). CDP I and CDP IIb more efficiently degrade thin filaments than CDP IIa and CDP III (Mattson and Mykles, 1993). CDP III is encoded by CalpM (Yu and Mykles, 2003). Gl-CalpB probably encodes CDP IIb (Table 6), but Gl-CalpT cannot be identified with either CDP I or CDP IIa without further analysis. Neither Ha-CalpM mRNA nor protein levels in lobster claw muscle change during the molting cycle (Yu and Mykles, 2003). If CalpB and CalpM are involved, their activities are not controlled at the transcriptional level. Calpains are regulated post-translationally by phosphorylation and/or endogenous activators or inhibitors (Friedrich, 2004; Goll et al., 2003; Mykles, 1998). We propose that an increase in the relative activity of Gl-CalpB (CDP IIb) compared with the other calpains mediates the preferential degradation of thin filaments during premolt.

This research was supported by a grant from the National Science Foundation (IBN-9904528). We thank Hector C. Horta (Puerto Rico Department of Natural Resources) and Hector J. Horta for collecting land crabs; Sharon Chang for ecdysteroid radioimmunoassays; Laura Baker, Emma James, Lindsey Progen, Stephanie Spenny and Kristin Van Ort for animal care; and Drs Scott Medler and Kara Lee for critical reading of the manuscript.

References

Alexa, A., Bozoky, Z., Farkas, A., Tompa, P. and Friedrich, P. (2004). Contribution of distinct structural elements to activation of calpain by Ca^{2+} ions. *J. Biol. Chem.* **279**, 20118-20126.

Amano, S., Kawasaki, H., Ishiura, S., Kawashima, S., Suzuki, K. and Emori, Y. (1997). Identification of endogenous substrates for *Drosophila* calpain from a salt-extracted fraction of *Drosophila* ovaries. *J. Biochem. (Tokyo)* **122**, 865-871.

Andresen, K., Tom, T. D. and Strand, M. (1991). Characterization of cDNA clones encoding a novel calcium-activated neutral proteinase from *Schistosoma mansoni*. *J. Biol. Chem.* **266**, 15085-15090.

Barnes, T. M. and Hodgkin, J. (1996). The *tra-3* sex determination gene of *Caenorhabditis elegans* encodes a member of the calpain regulatory protease family. *EMBO J.* **15**, 4477-4484.

Beydon, P. and Lafont, R. (1983). Feedback inhibition of ecdysone production by 20-hydroxyecdysone in *Pieris brassicae* pupae. *J. Insect Physiol.* **29**, 529-533.

Beyette, J. R. and Mykles, D. L. (1997). Autolysis and biochemical properties of a lobster muscle calpain-like proteinase. *J. Exp. Zool.* **277**, 106-119.

Beyette, J. R., Ma, J.-S. and Mykles, D. L. (1993). Purification and autolytic degradation of a calpain-like calcium-dependent proteinase from lobster (*Homarus americanus*) striated muscle. *Comp. Biochem. Physiol. B* **104**, 95-99.

Beyette, J. R., Emori, Y. and Mykles, D. L. (1997). Immunological analysis of two calpain-like Ca^{2+}-dependent proteinases from lobster striated muscles: Relationship to mammalian and *Drosophila* calpains. *Arch. Biochem. Biophys.* **337**, 232-238.

Billas, I. M. L., Iwema, T., Garnier, J. M., Mitschler, A., Rochel, N. and Moras, D. (2003). Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. *Nature* **426**, 91-96.

Chang, E. S. (1993). Comparative endocrinology of molting and reproduction: Insects and crustaceans. *Annu. Rev. Entomol.* **38**, 161-180.

Cherbas, L., Hu, X., Zhimulev, I., Belyaeva, E. and Cherbas, P. (2003). EcR isoforms in *Drosophila*: testing tissue-specific requirements by targeted blockade and rescue. *Development* **130**, 271-284.

Chung, A. C. K., Durica, D. S., Clifton, S. W., Roe, B. A. and Hopkins, P. M. (1998a). Cloning of crustacean ecdysteroid receptor and retinoid-X receptor gene homologs and elevation of retinoid-X receptor mRNA by retinoic acid. *Mol. Cell. Endocrinol.* **139**, 209-227.

Chung, A. C. K., Durica, D. S. and Hopkins, P. M. (1998b). Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-X-receptor mRNA during the molt cycle of the fiddler crab, *Uca pugilator*. *Gen. Comp. Endocrinol.* **109**, 375-389.

Dear, N., Matena, K., Vingron, M. and Boehm, T. (1997). A new subfamily of vertebrate calpains lacking a calmodulin-like domain: Implications for calpain regulation and evolution. *Genomics* **45**, 175-184.

Delaney, S. J., Hayward, D. C., Barleben, F., Fischbach, K.-F. and Gabor Miklos, G. L. (1991). Molecular cloning and analysis of small optic lobes, a structural brain gene of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **88**, 7214-7218.

Dell, S., Sedlmeier, D., Bocking, D. and Dauphin-Villemant, C. (1999). Ecdysteroid biosynthesis in crayfish Y-organs: Feedback regulation by circulating ecdysteroids. *Arch. Insect Biochem. Physiol.* **41**, 148-155.

Denison, S. H., Orejas, M. and Arst, H. N. (1995). Signaling of ambient pH in *Aspergillus* involves a cysteine protease. *J. Biol. Chem.* **270**, 28519-28522.

Durica, D. S., Wu, X. H., Anilkumar, G., Hopkins, P. M. and Chung, A. C. K. (2002). Characterization of crab EcR and RXR homologs and expression during limb regeneration and oocyte maturation. *Molec. Cell. Endocrinol.* **189**, 59-76.

Emori, Y. and Saigo, K. (1994). Calpain localization changes in coordination with actin-related cytoskeletal changes during early embryonic development of *Drosophila*. *J. Biol. Chem.* **269**, 25137-25142.

Farkas, A., Tompa, P., Schad, E., Sinka, R., Jekely, G. and Friedrich, P. (2004). Autolytic activation and localization in Schneider cells (S2) of calpain B from *Drosophila*. *Biochem. J.* **378**, 299-305.

Fernandez-Montalvan, A., Assfalg-Machleidt, I., Pfeiler, D., Fritz, H., Jochum, M. and Machleidt, W. (2004). Electrostatic interactions of domain III stabilize the inactive conformation of mu-calpain. *Biochem. J.* **382**, 607-617.

Friedrich, P. (2004). The intriguing Ca^{2+} requirement of calpain activation. *Biochem. Biophys. Res. Commun.* **323**, 1131-1133.

Friedrich, P., Tompa, P. and Farkas, A. (2004). The calpain-system of *Drosophila melanogaster*: coming of age. *BioEssays* **26**, 1088-1096.

Futai, E., Maeda, T., Sorimachi, H., Kitamoto, K., Ishiura, S. and Suzuki, K. (1999). The protease activity of a calpain-like cysteine protease in *Saccharomyces cerevisiae* is required for alkaline adaptation and sporulation. *Mol. Gen. Genet.* **260**, 559-568.

Goll, D. E., Thompson, V. F., Li, H. Q., Wei, W. and Cong, J. Y. (2003). The calpain system. *Physiol. Rev.* **83**, 731-801.

Grebe, M., Przibilla, S., Henrich, V. C. and Spindler-Barth, M. (2003). Characterization of the ligand-binding domain of the ecdysteroid receptor from *Drosophila melanogaster*. *Biol. Chem.* **384**, 105-116.

Hata, S., Sorimachi, H., Nakagawa, K., Maeda, T., Abe, K. and Suzuki, K. (2001). Domain II of m-calpain is a Ca^{2+}-dependent cysteine protease. *FEBS Lett.* **501**, 111-114.

Hosfield, C. M., Elce, J. S., Davies, P. L. and Jia, Z. (1999). Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. *EMBO J.* **18**, 6880-6889.

Hu, X., Cherbas, L. and Cherbas, P. (2003). Transcription activation by the ecdysone receptor (EcR/USP): Identification of activation functions. *Mol. Endocrinol.* **17**, 716-731.

Huang, Y. H. and Wang, K. K. W. (2001). The calpain family and human disease. *Trends Mol. Med.* **7**, 355-362.

Huet, F., Ruiz, C. and Richards, G. (1995). Sequential gene activation by ecdysone in *Drosophila melanogaster* – the hierarchical equivalence of early and early–late genes. *Development* **121**, 1195-1204.

Ismail, S. Z. M. and 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. and Kandarian, S. C. (2004). The molecular basis of skeletal muscle atrophy. *Am. J. Physiol.* **287**, C834-C843.

Jékely, G. and Friedrich, P. (1999). Characterization of two recombinant *Drosophila* calpains–CALPA and a novel homolog, CALPB. *J. Biol. Chem.* **274**, 23893-23900.

Kamei, M., Webb, G. C., Young, L. G. and Campbell, H. D. (1998). *SOLH*, a human homologue of the *Drosophila melanogaster small optic lobes* gene is a member of the calpain and zinc-finger gene families and maps to human chromosome 16p13.3 near *CATM* (Cataract with microphthalmia). *Genomics* **51**, 197-206.

Kozlova, T. and Thummel, C. S. (2000). Steroid regulation of postembryonic development and reproduction in *Drosophila*. *Trends Endocrinol. Metab.* **11**, 276-280.

Maki, M., Kitaura, Y., Satoh, H., Ohkouchi, S. and Shibata, H. (2002). Structures, functions and molecular evolution of the penta-EF-hand Ca^{2+}-binding proteins. *Biochim. Biophys. Acta* **1600**, 51-60.

Margis, R. and Margis-Pinheiro, M. (2003). Phytocalpains: orthologous calcium-dependent cysteine proteinases. *Trends Plant Sci.* **8**, 58-62.

Matena, K., Boehm, T. and Dear, N. (1998). Genomic organization of mouse Capn5 and Capn6 genes confirms that they are a distinct calpain subfamily. *Genomics* **48**, 117-120.

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

McCarthy, J. F. and Skinner, D. M. (1977). Proecdysial changes in serum ecdysone titers, gastrolith formation, and limb regeneration following molt induction by limb autotomy and/or eyestalk removal in land crab, *Gecarcinus lateralis*. *Gen. Comp. Endocrinol.* **33**, 278-292.

Medler, S. and 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.

Moffett, S. (1987). Muscles proximal to the fracture plane atrophy after limb autotomy in decapod crustaceans. *J. Exp. Zool.* **244**, 485-490.

Moldoveanu, T., Hosfield, C. M., Lim, D., Elce, J. S., Jia, Z. C. and Davies, P. L. (2002). A Ca^{2+} switch aligns the active site of calpain. *Cell* **108**, 649-660.

Moldoveanu, T., Jia, Z. C. and Davies, P. L. (2004). Calpain activation by cooperative Ca^{2+} binding at two non-EF-hand sites. *J. Biol. Chem.* **279**, 6106-6114.

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. (1998). Intracellular proteinases of invertebrates: calcium-dependent and proteasome/ubiquitin-dependent systems. *Int. Rev. Cytol.* **184**, 157-289.

Mykles, D. L. (1999). Proteolytic mechanisms underlying molt-induced claw muscle atrophy in decapod crustaceans. *Am. Zool.* **39**, 541-551.

Mykles, D. L. and Skinner, D. M. (1981). Preferential loss of thin filaments during molt-induced atrophy in crab claw muscle. *J. Ultrastruct. Res.* **75**, 314-325.

Mykles, D. L. and Skinner, D. M. (1982a). Crustacean muscles: atrophy and regeneration during molting. In *Basic Biology of Muscles: A Comparative Approach* (ed. B. M. Twarog, R. J. C. Levine and M. M. Dewey), pp. 337-357. New York: Raven Press.

Mykles, D. L. and Skinner, D. M. (1982b). 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. and Skinner, D. M. (1983). Ca^{2+}-dependent proteolytic activity in crab claw muscle. Effects of inhibitors and specificity for myofibrillar proteins. *J. Biol. Chem.* **258**, 10474-10480.

Mykles, D. L. and Skinner, D. M. (1986). Four Ca^{2+}-dependent proteinase activities isolated from crustacean muscle differ in size, net charge, and sensitivity to Ca^{2+} and inhibitors. *J. Biol. Chem.* **261**, 9865-9871.

Onate, S. A., Boonyaratanakornkit, V., Spencer, T. E., Tsai, S. Y., Tsai, M. J., Edwards, D. P. and O'Malley, B. W. (1998). The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. *J. Biol. Chem.* **273**, 12101-12108.

Pinter, M., Stierandova, A. and Friedrich, P. (1992). Purification and characterization of a Ca^{2+}-activated thiol protease from *Drosophila melanogaster*. *Biochemistry* **31**, 8201-8206.

Renaud, J. P. and Moras, D. (2000). Structural studies on nuclear receptors. *Cell. Mol. Life Sci.* **57**, 1748-1769.

Sakurai, S. and Williams, C. M. (1989). Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* **75**, 204-216.

Salem, M., Killefer, J., Yao, J. and Nath, J. (2004). Identification and molecular characterization of the rainbow trout calpain catalytic subunits (Capn1 and Capn2): Up-regulated expression of the muscle Capn2 during starvation. *FASEB J.* **18**, C138.

Schubiger, M., Tomita, S., Sung, C., Robinow, S. and Truman, J. W. (2003). Isoform specific control of gene activity *in vivo* by the *Drosophila* ecdysone receptor. *Mech. Dev.* **120**, 909-918.

Segraves, W. A. and Woldin, C. (1993). The E75 gene of *Manduca sexta* and comparison with its *Drosophila* homolog. *Insect Biochem. Mol. Biol.* **23**, 91-97.

Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1997). Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. In *Recent Progress in Hormone Research, Proceedings of the 1996 Conference,* vol. **52**, pp. 141-165.

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.

Skinner, D. M. (1985). Molting and regeneration. In *The Biology of Crustacea* (ed. D. E. Bliss and L. H. Mantel), pp. 43-146. New York: Academic Press.

Sokol, S. B. and Kuwabara, P. E. (2000). Proteolysis in *Caenorhabditis elegans* sex determination: cleavage of TRA-2A by TRA-3. *Genes Dev.* **14**, 901-906.

Song, Q. S. and Gilbert, L. I. (1998). Alterations in ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol.* **28**, 849-860.

Sorimachi, H., Ishiura, S. and Suzuki, K. (1993). A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+}-binding domain. *J. Biol. Chem.* **268**, 19476-19482.

Spadoni, C., Farkas, A., Sinka, R., Tompa, P. and Friedrich, P. (2003). Molecular cloning and RNA expression of a novel *Drosophila* calpain, Calpain C. *Biochem. Biophys. Res. Commun.* **303**, 343-349.

Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K., Irie, A., Sorimachi, H., Bourenkow, G., Bartunik, H. et al. (2000). The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. *Proc. Natl. Acad. Sci. USA* **97**, 588-592.

Sun, X., Song, Q. and Barrett, B. (2003). Effects of ecdysone agonists on the expression of EcR, USP and other specific proteins in the ovaries of the

codling moth (*Cydia pomonella* L.). *Insect Biochem. Mol. Biol.* **33**, 829-840.

Suzuki, K., Hata, S., Kawabata, Y. and Sorimachi, H. (2004). Structure, activation, and biology of calpain. *Diabetes* **53**, S12-S18.

Talbot, W. S., Swyryd, E. A. and Hogness, D. S. (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* **73**, 1323-1337.

Theopold, U., Pintér, M., Daffre, S., Tryselius, Y., Friedrich, P., Nässel, D. R. and Hultmark, D. (1995). *CalpA*, a *Drosophila* calpain homolog specifically expressed in a small set of nerve, midgut, and blood cells. *Mol. Cell. Biol.* **15**, 824-834.

Thummel, C. S. (1996). Flies on steroids – *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* **12**, 306-310.

Wang, S. F., Ayer, S., Segraves, W. A., Williams, D. R. and Raikhel, A. S. (2000). Molecular determinants of differential ligand sensitivities of insect ecdysteroid receptors. *Mol. Cell. Biol.* **20**, 3870-3879.

Yao, T.-P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J.-D., McKeown, M., Cherbas, P. and Evans, R. M. (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* **366**, 476-479.

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

Zhou, B. H., Hiruma, K., Jindra, M., Shinoda, T., Segraves, W. A., Malone, F. and Riddiford, L. M. (1998). Regulation of the transcription factor E75 by 20-hydroxyecdysone and juvenile hormone in the epidermis of the tobacco hornworm, *Manduca sexta*, during larval molting and metamorphosis. *Dev. Biol.* **193**, 127-138.