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Molecular cloning and RNA expression of a novel *Drosophila* calpain, Calpain C

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Abstract

The calpains are Ca^{2+}-activated cysteine proteases whose biochemical properties have been extensively characterized in vitro. Less is known, however, about the physiological role of calpains. In this respect, *Drosophila melanogaster* is a useful experimental organism to study calpain activity and regulation in vivo. The sequencing of the fly genome has been recently completed and a novel calpain homologue has been identified in the CG3692 gene product. We embarked on the cloning and characterization of this putative novel calpain. We demonstrate that the actual calpain is different from the predicted protein and we provide experimental evidence for the correction of the genomic annotation. This novel protein, Calpain C, must be catalytically inactive, having mutated active site residues but is otherwise structurally similar to the other known fly calpains. Moreover, we analysed Calpain C RNA expression during *Drosophila* development by RT-PCR and RNA in situ hybridization, which revealed strong expression in the salivary glands.

Keywords: Calpain; Calcium; *Drosophila*; Cloning; RNA; Genomic annotation; RT-PCR; In situ hybridization

Calpains are a family of Ca^{2+}-activated intracellular cysteine proteases present in vertebrates, invertebrates, and lower eukaryotes, implicated in events as diverse as homeostasis [1], development [2,3], cell migration [4], learning and memory [5], necrotic cell death [6], and diseases [7,8].

At least 14 distinct calpain genes exist in mammalian cells [9]. Whereas some calpains appear to be tissue specific, others are ubiquitously expressed. The typical mammalian ubiquitous calpains consist of a large catalytic subunit and a small regulatory subunit, thus forming a heterodimeric molecule. Large subunits comprise four domains: a variable N-terminal domain I which is autolysed upon calcium activation, domain II, a papain-like protease domain containing the active site residues Cys, His, and Asn, domain III which is involved in calcium and phospholipid binding [10], and, finally, domain IV which has a calmodulin-like structure containing EF hand Ca^{2+}-binding motifs. The small regulatory subunit consists of a glycine-rich domain V and a calmodulin-like domain VI similar to domain IV of the large subunit [11]. Calpains are believed to be activated by recruitment to the cell membranes where they may be exposed to local high calcium concentrations, which induce a conformational change in the enzyme, leading to autolysis and activation [12].

In *Drosophila melanogaster*, a useful organism for in vivo functional studies, three calpains have been cloned and characterized: SOL, Calpain A, and Calpain B. SOL, also referred to as Calpain D, is required for normal development of the optic lobes and structurally is atypical since it contains zinc finger motifs in the N-terminal portion. Furthermore, the *Sol* gene has two alternative splicing products, the shorter lacking the calpain-like protease domain [13]. *Drosophila* Calpain A [2,14] and Calpain B [15] have been characterized more extensively. Structurally, they resemble more closely the ubiquitous mammalian calpains, with the typical 4 domains of the large subunits, although they lack the small regulatory subunit. Both Calpain A and Calpain B are active calcium dependent proteases in vitro [15] but the mechanism regulating their proteolytic activity in vivo is still unknown.

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The recent completion of the *Drosophila* genome project [16] revealed that the putative product of gene CG3692 is a protein with high homology to fly calpains. This novel calpain was initially predicted to have mutated active site residues and an extensive N-terminal domain harbouring several transmembrane regions.

We describe in this paper the molecular cloning of the CG3692-derived cDNA encoding a novel calpain, referred to as Calpain C. In the process we demonstrate that this transcript does not encode a calpain with transmembrane regions but, rather, a protein which has a short N-terminal domain and is catalytically inactive. We compare this novel calpain to other family members and we provide a detailed analysis of Calpain C RNA expression during the development of the fly.

Materials and methods

Total RNA extraction. *Drosophila* RNA was prepared from eggs, larvae, pupae, and adult flies. The samples were homogenized and lysed in guanidinium buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% *N*-lauryl sarcosine sodium salt, and 0.1% β-mercaptoethanol; 10 ml buffer/g tissue). Total RNA was then purified by phenol extractions and ethanol precipitation as previously described [17]. Finally, the RNA was resuspended in 100 μl ribonuclease-free water. RNA purity was assessed by agarose gel electrophoresis and ethidium bromide staining (0.5 μg/ml). RNA quantitation was done by spectrophotometric analysis. Total RNA was also prepared from HeLa cells for control experiments using the same protocol.

Genomic DNA extraction. Between 50 and 100 adult flies were homogenized in 4 ml DNA extraction buffer (10 mM Tris–HCl, pH 7.5, 60 mM sodium chloride, 10 mM EDTA, 0.15 mM spermine, and 0.15 mM spermidine). Tissue debris was quickly removed by centrifugation at 800*g* for 1 min. The mixture was further centrifuged at 19,000*g* for 5 min. After removal of the supernatant the pellet was resuspended in 0.5 ml DNA buffer and proteinase K was added to a final concentration of 100 μg/ml. Sodium dodecyl sulphate (SDS) was added to a 0.1% final concentration and the mixture was incubated at 37 °C for 1 h. Genomic DNA was extracted twice with 0.5 ml phenol and once with 0.5 ml chloroform/isoamylalcohol (24:1). The DNA was purified by ethanol precipitation and resuspended overnight in 100 μl TE buffer.

RT-PCR. First strand cDNA was prepared from 2 μg total RNA with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase in the appropriate reaction buffer, using 0.5 mM dNTPs, oligo(dT), and random primers following the protocol recommended by the supplier (New England Biolabs). One-tenth volume of the resulting first strand cDNA mixture was subjected to PCR amplification using the appropriate polymerase buffer at 1× concentration containing 1.5 mM magnesium chloride, 0.5 mM deoxyribonucleotides, 1 μM PCR primers, and 2.5 U Red Taq DNA polymerase (Sigma). The cDNA mixture was heat denatured at 95 °C for 5 min, followed by 30–35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 2 min extension at 72 °C, unless otherwise specified. For genomic amplification 50 ng of DNA was used as template. For analysis of Calpain C RNA expression during development, the quantitative properties of our RT-PCR protocol were verified for all transcripts, as previously described [18,19]. This procedure confirmed that we were able to detect differences in target input over at least a 100-fold dilution range (data not shown). Direct comparisons of transcripts in different samples were always made on the same agarose gel and digital image.

RACE PCR. Rapid amplification of the cDNA ends (RACE) was performed using the 5′ RACE System version 2.0 (Invitrogen) following the procedure recommended by the manufacturer. Briefly, a Calpain C specific reverse oligonucleotide (SD-2556R) was used for cDNA synthesis and the 5′ end of the resulting single-stranded cDNA was poly(dC) tailed. The cDNA mixture was amplified with a nested, calpain specific, reverse primer (SD-1764R) and the 5′ RACE anchor primer supplied by the manufacturer, consisting of a poly(dG) region and a 5′ terminal "adapter" sequence. One-tenth of the PCR product was subjected to a second round of amplification with another nested reverse primer (SD-1265R) and a forward abridged universal amplification primer (AUAP) identical to the adapter sequence (supplied by the manufacturer). RACE 3′ PCR was performed with the same kit with appropriate modifications. cDNA synthesis was achieved using a poly(dT) primer with a 5′ terminal anchor sequence identical to AUAP (3RACE-Rev). LD-specific PCR amplification was performed with 3RACE-Rev and an upstream LD-specific forward oligonucleotide (LD-427F). One-tenth of the resulting product was further amplified with AUAP and three LD-specific forward oligonucleotides (LD-621F, LD-815F, and LD-1016F). The identities of all PCR products were verified by DNA sequencing.

RNA in situ hybridization. The cDNA probe was prepared by PCR amplification of *Drosophila* cDNA with the CALP-C43, SD-2566R oligonucleotides. The 1390 bp PCR product was gel purified and labelled with the Dig DNA labelling kit (Boehringer–Mannheim), following the recommended protocol. In situ hybridization was performed as described in [20]. The hybridization signal was developed by the Dig Detection kit (Boehringer–Mannheim) and analysed by interference contrast microscopy (Zeiss Axioscope II).

RT-PCR oligonucleotide sequences. Oligonucleotides were purchased from MWG-Biotech, Germany. Sequences are 5′ to 3′; "R" in the primer name denotes reverse primers whereas "F" applies to forward primers. LD-621F: GGCAATTCTTTTGCAATCGA, LD-845R: ATACTGG CCACATACGAGAT; LD-1016F: CAACCAAATATAAGTACGAA TT, SD-1265R: TCGCTCAGTATGCGCTCGT A; SD-2319F: TTGT CGCAATCACGAGTCGT, SD-2556R: ATAGCTAACGTGCCGC GT AT; 3RACE-Rev: GGCC ACGCGTCGACTAG TACTTTTTTTTT TTTTTTTTTTTTV (V = A+G+C), LD-427F: TTCGTTCGTCTATT CGACTCG; LD-815F: GAACGCTTTGCA TCGCGTAT; CALP-C43: GGTTAACTTTCACTTCGGCTACG, CALPC-2466R: GAAA CT AAGAAATCTTTCGAGCGA, MED6-F: CATCTGCACAACA TGATCGGTC; MED6-R: ATTGGTGGGGGAAATTTACGC, GA PDH-F: CGACTCGACTCACGGTCGTT, GAPDH-R: GGTCATC AGACCCTCGACGAT, rp49F: CAGTCGGATCGATCTGCTAAG CTGT, rp49R: TAACCGATGTTGGGCATCAGATACT.

Materials and general procedures. All chemicals were purchased from Sigma. PCR products were analysed by densitometry with the Molecular Analyst image analysis software (Bio-Rad Laboratories). PCR products were purified from agarose gel with the Nucleo-Spin extract kit (Macherey–Nagel) following the instructions of the manufacturer. Purified DNA samples were sequenced by MWG-AG Biotech (Ebersberg, Germany). The calpain sequences were aligned by the Clustal W method [21]. The Calpain C amino acid sequence was analysed by searching through the Hits protein motif database [22], available on the Swiss Institute of Informatics website (http://hits.isb-sib.ch/). *D. melanogaster* of the Canton S wild type was kept on standard cornmeal-yeast food at 25 °C. At the appropriate time, eggs, 1st–2nd instar larvae, 3rd instar larvae, pupae, and adult flies were collected, suspended in RNA homogenization buffer, and stored at −20 °C for later RNA extraction.

Results

PCR analysis of the CG3692 gene

Our initial aim was the cloning and characterization of the novel *Drosophila* calpain encoded by the CG3692

gene (Accession No. AE003501), as described in the FlyBase Genomic Annotation database (Release 2) [16]. This gene was localized on the X chromosome and its 3132 bp open reading frame was predicted to consist of seven exons (Fig. 1A). The first five exons (1–5) were found in the EST clone LD13628, which was derived from fly embryos, whereas exons 6 and 7 corresponded to SD01079, an EST clone derived from *Drosophila* S2 Schneider cells. Interestingly, the N terminal extension of this putative novel calpain was predicted to harbour at least 9 transmembrane regions, encoded by LD13628 (hereafter referred to as LD). The portion homologous to calpains, on the other hand, was confined to SD01079 cDNA (SD).

We intended to clone the full length cDNA of this putative novel calpain by RT-PCR. Our amplification attempts, however, were unsuccessful. Although we could amplify shorter cDNA fragments corresponding to either the LD or the SD region using a combination of different primers, we could not amplify the full length cDNA transcript (data not shown).

In view of these negative results and the observation that no LD–SD overlapping sequences could be found in the EST database, we investigated the possibility that the genomic annotation was incorrect and that these two transcripts actually corresponded to two different genes. We compared the amplification of LD- and SD-specific fragments with the amplification of an LD–SD product overlapping the two corresponding sequences, using cDNA and genomic DNA as templates. If LD and SD represented two distinct transcripts, derived from adjacent genes on the same chromosome, amplification of an LD–SD fragment was expected to be achieved only with genomic DNA. The results are shown in Fig. 1B. Whereas the expected LD and SD fragments were amplified from both cDNA and genomic DNA, the LD–SD product was amplified only when genomic DNA was the template. Other LD–SD primer pairs were tested, with essentially the same results (data not shown), strongly suggesting that LD and SD are two unrelated transcripts.

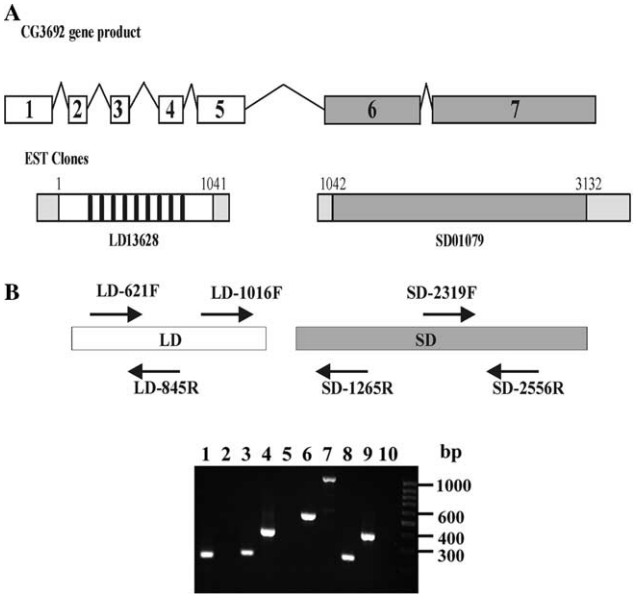

Fig. 1. PCR analysis of the putative CG3692 gene product. (A) The CG3692 gene product (3132 bp) was predicted to consist of 7 exons. Exons 1–5 (bases 1–1041; empty boxes) correspond to the LD13628 EST clone; the predicted 9 transmembrane regions are shown as black bars. Exons 6 and 7 (bases 1042–3132; dark grey boxes) are encoded by the SD01079 EST clone. Both LD13618 and SD01079 are longer than the predicted exons (extra sequence indicated by light grey boxes), according to the genomic annotation (*Drosophila* Genome project; release2). (B) Fragments of the putative CG3692 gene product were amplified by PCR using comparable amounts of cDNA and genomic DNA as templates with primers specific for the LD13268 (LD) sequence (LD-621F and LD-845R), for the SD01079 (SD) sequence (SD-2319F and SD-2556R), and for a region overlapping the two EST sequences (LD-1016F and SD-1265R). The PCR products were separated by agarose gel electrophoresis; the sizes of the expected products are in brackets (please note that some genomic DNA products are longer due to the presence of introns). For cDNA templates: (1) LD-621F, LD-845R (224 bp); (2) LD-1016F, SD-1265R (249 bp); (3) SD-2319F, SD-2556R (237 bp); (4) GAPDH-F/GAPDH-R (400 bp); and (5) GAPDH-F/GAPDH-R, no cDNA. For genomic DNA templates: (6) LD-621F, LD-845R (600 bp); (7) LD-1016F, SD-1265R (1091 bp); (8) SD-2319F, SD-2556R (237 bp); (9) GAPDH-F/GAPDH-R (400) bp; and (10) GAPDH-F/GAPDH-R, no genomic DNA.

RACE PCR and cloning of full length Calpain C

RACE PCR was performed to confirm that the 3′ end of LD and the 5′ end of SD do not overlap, implying

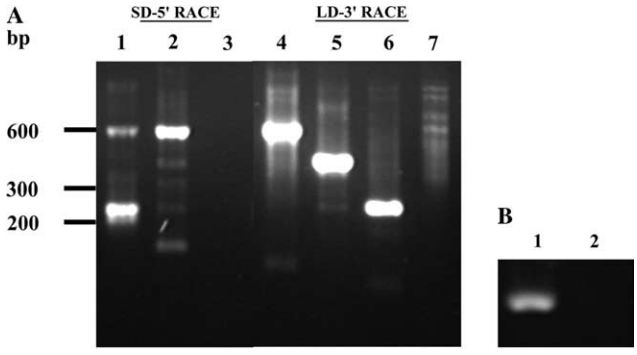

Fig. 2. RACE PCR and cloning of full length Calpain C. (A) RACE PCR amplification was performed as described in the text. For the second and final round of amplification SD-(5′ RACE; lanes 1–3) and LD-specific primers (3′ RACE; lanes 4–7) were used in combination with the standard anchor primer (AUAP). The PCR products were separated by agarose gel electrophoresis. Primer combinations: (1) SD-1265R/AUAP; (2) AUAP alone; (3) SD-1265R alone; (4) LD-621F/AUAP; (5) LD-815F/AUAP; (6) LD-1016F/AUAP; and (7) AUAP alone. Only one specific product was detected for each primer combination, since the 600-bp-long fragment in lane 1 appears to be the product of AUAP mispriming. (B) RT-PCR was performed with total RNA prepared from (1) adult *Drosophila* and (2) Human HeLa cells, as described in the text. The cDNA was amplified with Calpain C specific primers (CAPLC43-CALPC 2466R) designed to amplify a cDNA fragment (2423 bp) containing the entire coding sequence. A PCR product of the expected size can be amplified only from *Drosophila* cDNA but not from HeLa cell cDNA.

that the full length calpain homologue is encoded by SD only. As shown in Fig. 2A, the second round of 5′ RACE amplification was performed with one SD oligonucleotide whereas 3′ RACE amplification was performed with three LD nested oligonucleotides. In each case only one specific product was amplified. The sizes of the amplified fragments are consistent with these target cDNAs being two independent transcripts and this conclusion was further confirmed by DNA sequence analysis of these PCR products, which revealed the correct N-terminus of this novel calpain.

Based on these results, we designed oligonucleotides to amplify a 2423 cDNA fragment containing the complete reading frame of this calpain homologue. The expected fragment was isolated from adult *Drosophila* RNA by RT-PCR and no amplification product was detected in HeLa cell RNA, which was used as negative control for our fly-specific primers (Fig. 2B). The PCR product was analysed by DNA sequence analysis which revealed a 2045 bp open reading frame coding for a calpain-related protein, hereafter referred to as Calpain C (EMBL Accession No. AJ538040).

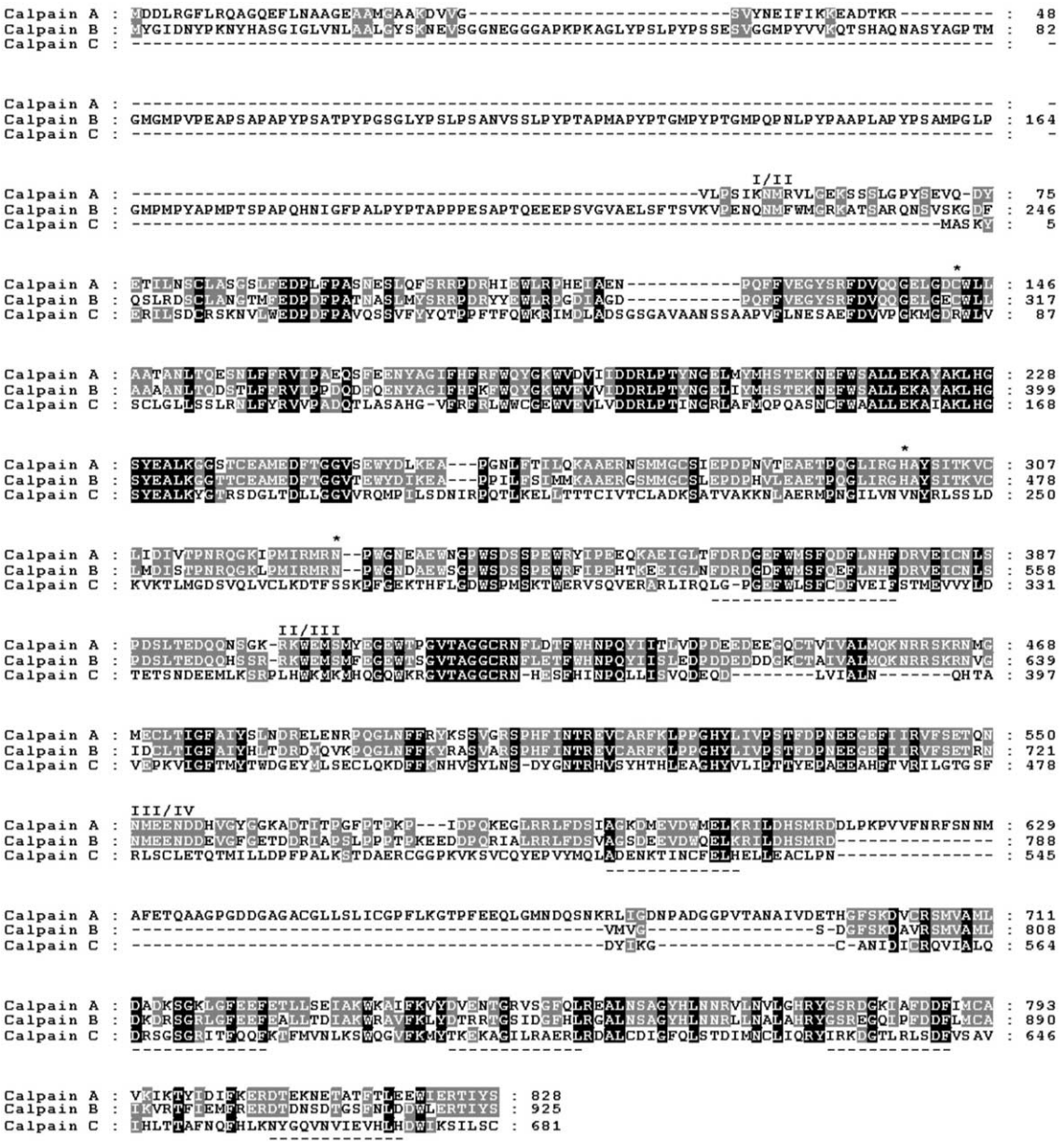

Fig. 3. Protein sequence alignment. Calpain C was aligned with Calpain A and Calpain B by the Clustal W method. Black boxes denote conserved residues in all three calpains, residues identical in two out of three sequences are boxed in grey. The three catalytic site residues, mutated in Calpain C, are marked by asterisks. The roman numerals mark the boundaries of the corresponding calpain domains. The potential EF hand Ca^{2+}-binding domains of Calpain C are underlined.

Protein sequence alignment

The predicted amino acid sequence of Calpain C was aligned with the Calpain A and Calpain B proteins (Fig. 3). Interestingly, all three active site residues are mutated, suggesting that Calpain C is inactive, although it retains structural features of the calpain family. Calpain C lacks an extensive N-terminal domain and is therefore unlikely to be autolysed. It shows significant identity within domains II and III (30% and 35% identity with the corresponding Calpain B domains) and somewhat less in domain IV, which still has predicted EF binding motifs, though.

Developmental RT-PCR

Since Calpain C lacks enzymatic activity, it is interesting to see its expression pattern. To this end, we analysed the RNA expression pattern of Calpain C during development. We prepared total RNA from several fly developmental stages, namely oocytes, the 1st–2nd instar larvae, 3rd instar larvae, pupae, and adult flies. Equal amounts of RNA were reverse transcribed and PCR amplified with primers specific for Calpain C, the transcription factor Med6 and the ribosomal-associated protein rp49. Med6 RNA has been shown to be transcriptionally regulated during *Drosophila* development [23], whereas rp49 is uniformly expressed throughout development and was used to control for equivalent RNA input. We found that Calpain C is detected throughout development with highest overall expression levels in pupa (Fig. 4).

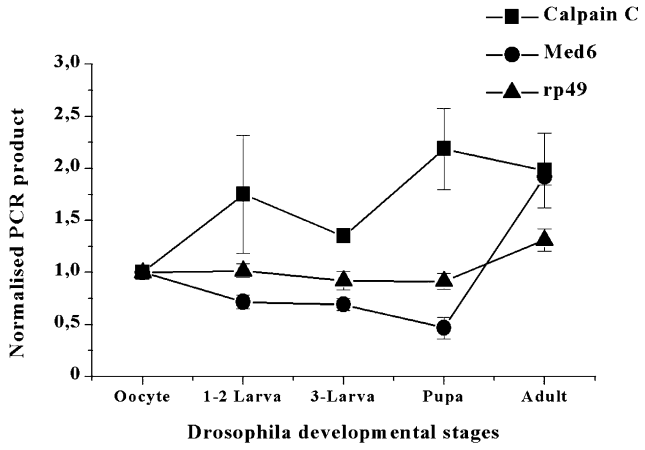

Fig. 4. RT-PCR analysis of Calpain C RNA expression during development. Total RNA was prepared as described in the text from oocytes, 1st–2nd instar larvae, 3rd instar larvae, pupae, and adult flies. The cDNA mixtures were amplified for 30 cycles with specific CALPC (SD-2319F/SD-2556R), Med6 (Med6-F/Med6-R), and rp49 (rp49F/rp49R) primers. The PCR products were separated by agarose gel electrophoresis and the intensity of the PCR signal was measured by densitometry. The Calpain C and Med6 values were normalized to the corresponding rp49 values. The calculated data are plotted as fractions of the initial oocyte values. The average values of four amplifications from two sets of RNA preparations ± SEM are shown.

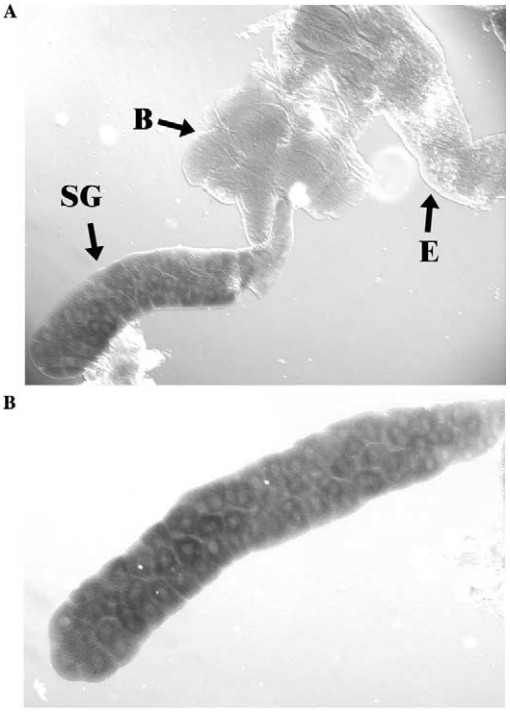

Fig. 5. Expression analysis of Calpain C mRNA by in situ hybridization. (A) The internal organs of 3rd instar larvae were hybridized with a digoxigenin-labelled cDNA probe and the hybridization signal was analysed by interference contrast microscopy. Calpain C mRNA appears to be expressed only in salivary glands (SG) whereas it is not detected in other tissues, such as brain (B) and esophagus (E). (B) Calpain C mRNA is expressed mainly in the cytoplasm of salivary gland cells.

RNA in situ hybridization

Calpain C RNA expression was also analysed by in situ hybridization. Sections prepared from oocytes, embryos, and 3rd instar larvae were hybridized with a cDNA probe labelled with digoxigenin, as described in Materials and methods. Calpain C RNA expression is too weak to be detected in oocytes and embryos by this technique (data not shown) but it is clearly present in the larvae where Calpain C appears to be expressed only in the salivary glands (Fig. 5).

Discussion

We cloned by RT-PCR the cDNA encoding a novel *Drosophila* calpain, Calpain C. This protein had been originally predicted to harbour 9 transmembrane domains within its N-terminal portion. This prediction, based on the provisional annotation of the *Drosophila* genomic database, was shown to be incorrect. We demonstrated this by conventional PCR amplification

and RACE PCR, which allowed us to determine exactly the cDNA termini of this calpain-related transcript.

The initial annotation of newly released genomic sequences is known to contain mistakes which are later rectified when further experimental evidence becomes available. The provisional genomic annotation is performed automatically using gene prediction algorithms. These are normally very efficient in predicting exon sequences but are less dependable when predicting exact splice sites, a frequent problem being that one gene is split into two or that two genes are merged into a single transcript [16,24]. In the case of the CG3692 gene product the coding sequence of Calpain C had been incorrectly merged with the sequence coding for an unrelated protein with transmembrane domains.

Calpain C shows significant sequence identity to the other fly calpains such as Calpain A and Calpain B throughout domains II, III, and IV. Interestingly, all three calpain active site residues (Cys, His, and Asn) are mutated, which renders Calpain C catalytically inactive [25]. Inactive calpains have already been identified in both vertebrates and invertebrates. The product of the nematode *Caenorhabditis elegans capn3* gene has all three active site residues mutated, as with Calpain C. Moreover, in the parasite *Tripanosoma brucei* a family of cytoskeleton-associated calpains lacking the conserved active site cysteine has been identified recently [26]. In vertebrates, mouse Calpain 6 lacks the active site cysteine and histidine residues; human Calpain 6, on the other hand, lacks the active site cysteine but retains histidine [27].

Recent phylogenetic analyses of calpain sequences indicate the existence of at least three groups of calpains: active canonical calpains with the typical four domains, calpains lacking the calcium binding domain IV, and proteolytically inactive calpains. It seems that the appearance of catalytically inactive calpains occurred before the vertebrate and invertebrate lineage diverged, perhaps suggesting a conserved physiological role which is not dependent on proteolytic activity [9,27].

In keeping with this point, the expression of an inactive calpain in the fly is intriguing. It may function by modulating calpain activity by competition for substrate binding or some form of heterodimeric interaction. Such a regulatory mechanism might control *Drosophila* calpains, as calpastatin, the endogenous calpain inhibitor [29], appears to be absent in *Drosophila* [30]. This type of protease regulation has been demonstrated, for instance, with another class of cysteine proteases, the caspases, whereby catalytically inactive homologues modulate caspase activation via binding to upstream regulators [28].

Calpain C RNA is expressed throughout development with the highest level in pupa, indicating a possible role in the development of the fly. Furthermore, we show by in situ hybridization of oocyte, embryo, and larval sections that Calpain C RNA is expressed exclusively in salivary glands. Interestingly, abundant expression in the salivary glands has been observed for Calpain B too (Farkas et al., manuscript in preparation), underlying that these two calpains concur to the same physiological function. *Drosophila* salivary glands consist of secretory cells and duct cells. Salivary glands development occurs rapidly, beginning at 4.5 h after egg laying (AEL) with the formation of the secretory glands, formation of the ducts, and finally the common duct leading to the mouth. The entire process is complete by 10 h AEL during the embryonic stage [31,32]. Calpain C expression becomes evident at the late larval stages whereas it is below the level of detection in oocytes and embryo. It is, therefore, unlikely that Calpain C concurs directly in salivary gland formation and development. It is possible, however, that Calpain C partakes in the secretory activity of mature salivary glands since calpains have been involved in a number of secretory pathways in vertebrates [33–35].

In summary, we have described the molecular cloning of *Drosophila* Calpain C and characterized its RNA expression during fly development. Taken together these data suggest a physiological role for proteolytically inactive calpains and indicate new lines of investigation into the regulation of calpain activity.

Acknowledgments

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