Calpain Localization Changes in Coordination with Actin-related Cytoskeletal Changes during Early Embryonic Development of Drosophila*

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Yasufumi Emori‡ and Kaoru Saigo
From the Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan

Calpain, a calcium-dependent intracellular protease, was identified in Drosophila melanogaster. Drosophila calpain has an amino acid sequence highly homologous to those of mammalian calpains and exhibits a distinct domain structure consisting of cysteine protease and calcium-binding domains. Specific antibodies raised against a recombinant calpain fragment were used to identify the localization of calpain in developing Drosophila embryos. Calpain was first detected near the anterior pole and in posterior region of the embryo just after fertilization. The anterior calpain disappeared during the cleavage cycles. On the other hand, the posterior calpain moved to the posterior pole when polar buds were formed, and condensed just below the pole cells. At cleavage cycles 8 and 9, when nuclei reached the egg surface, calpain was localized between the nuclei at the surface beneath the precleavage furrows. Co-staining experiments with anti-actin antibody revealed that calpain condenses specifically at the edge of and between actin caps that underlie the plasma membrane immediately above each nucleus. These results indicate that calpain is involved in the dynamic changes in the embryonic cytoskeleton, especially actin-related structures, during early embryogenesis prior to cellularization.

Various cellular phenomena involving changes in cell shape such as cell division and differentiation are dependent on cytoskeletal structures. The surface area of the cell is structurally dependent mainly on actin and its associated proteins, which form the so-called actin network (for a review, see Weeds (1982)). On the other hand, internal structures, including the mitotic apparatus, are mainly dependent on microtubules consisting of tubulins and microtubule-associated proteins (for reviews, see Dustin (1984) and Olmstead (1986)). Dynamic changes in actin-related structures are dependent on Ca²⁺ concentration and actin-associated proteins. In addition, proteolytic modification and down-regulation of specific proteins are also considered to play main roles in the reorganization of these structures, especially in irreversible pathways. However, a direct demonstration of proteolytic events and the natures of the responsible proteases have not been demonstrated, probably because of the difficulty in investigating degradative processes inside the cell.

Cytoplasmic polyolysis is believed to be mediated by strictly regulated proteolytic enzymes including calpains (for reviews, see Suzuki et al. (1987) and Murachi (1989)) and other proteolytic species such as proteosome (for a review, see Rivett (1993)). Among these enzymes, calpain is a leading candidate as a key enzyme for the degradation of receptors, cytoskeletal proteins, transcription factors, and other proteins in accordance with intracellular calcium signaling (Beckerle et al., 1987; Harris et al., 1989; Wang et al., 1989; Wiedmer et al., 1990; Hirai et al., 1991; Oda et al., 1993). In particular, many cytoskeleton-associated proteins are known to be degraded by calpain.

Biochemical and molecular biological analyses have shown that the activity of calpain is dependent on calcium ion (for a review, see Murachi (1989)). An affinity of calpain for phospholipids and its translocation to the cell surface have also been demonstrated (for a review, see Suzuki et al. (1987)). In addition, the molecular structures of calpains from vertebrates have also been studied; it has been shown that calpain contains a catalytic domain in its N-terminal part that is similar in sequence to other cysteine proteinases such as papain and cathepsins B, H, and L, and a calcium-binding regulatory domain in its C terminus similar to other E-F hand proteins such as calmodulin and troponin C (Ohno et al., 1984; Emori et al., 1986; Aoki et al., 1986; Imajoh et al., 1988). In mammals, two ubiquitously distributed calpain species showing different calcium requirement, termed μ-calpain and μ-calpain, have been known (Murachi, 1989; Ohno et al., 1984; Emori et al., 1986; Aoki et al., 1986; Imajoh et al., 1988). Recently, two tissue-specific calpains termed μCL-1 and -2, have been identified (Sorimachi et al., 1989, 1993a, 1993b), although their biochemical characteristics are not definitely elucidated. However, physiological studies that demonstrate the in vivo functions of calpain directly have been reported less than studied on its biochemical and structural features.

To resolve these problems, we selected Drosophila melanogaster, for which developmental and genetic approaches are applicable. We targeted here the early Drosophila embryogenesis for the study of calpain functions, because extensive studies about cytoskeletal structures have already been presented. In early Drosophila embryos, it is known that synchronous nuclear division occurs without cytokinesis and that a syncytial blastoderm is formed (for details, see Campos-Ortega and Hartenstein (1995)). Cytokinesis takes place after nuclear division cycle 13, and a cellular blastoderm is formed. Some of the nuclei move toward the posterior pole and become germ line cells (pole cells). During the above processes, both actin-related and tubulin-related cytoskeletons are known to play important roles in nuclear movement and cytokinesis (Warn et al., 1984; Miller et al., 1985, 1989; Kellogg et al., 1989) (for a review, see Schjetner and Wieschaus (1993b)), where protein degradation, possibly catalyzed by proteolytic enzymes such as calpains,
may regulate cytoskeletal structures via irreversible proteolysis against, for example, actin-binding proteins. In this study, we first identified calpain in *Drosophila* at both the DNA and protein levels. Then we showed that calpain is probably involved in early embryogenesis, especially in the organization of the actin-related cytoskeleton.

**MATERIALS AND METHODS**

Isolation of *Drosophila* Calpain cDNA—A *Drosophila* larval cDNA library was constructed in agt10 according to a standard procedure (Emori et al., 1987), and screened with a cDNA fragment encoding chicken calpain (Ohno et al., 1984) under less stringent conditions, in that the final wash was carried out in 2 x SSC containing 0.1% SDS. A clone termed *Dm*-calpain was shown to code for a protein related to the chicken calpain; this clone was then subjected to nucleotide sequencing and other analyses (Emori et al., 1985).

In Situ Hybridization to Polytene Chromosome—Polytene chromosomes were prepared from salivary glands of *D. melanogaster* Canton-S and processed for in situ hybridization using biotin-labeled DNA probe according to the standard method (Engels et al., 1986).

Antibody Preparation—A *Drosophila* calpain cDNA fragment encoding from residue 247 to the C terminus (Fig. 1) was inserted into the plasmid site downstream of the lacZ promoter of pUC18. Production of the recombinant protein was induced with isopropyl-1-thio-β-D-galactopyranoside, and the product was subjected to 5% SDS-polyacrylamide gel electrophoresis (Emori et al., 1988; Laemmli, 1971). The protein band visualized by Coomassie Brilliant Blue staining was excised and crushed to a paste. The paste was sonicated extensively, mixed with an equal volume of complete Freund's adjuvant, and injected into house rabbits. After 3 weeks, booster injections were begun and continued at 2-week intervals; serum was prepared 10 days after each booster. The antibody was affinity-purified as described (Higashijima et al., 1992a, 1992b) and adsorbed with a control extract of *Escherichia coli* not harboring the recombinant plasmid.

The quality of the antibody preparations was confirmed by Western blotting as follows. *Drosophila* extracts were prepared from frozen embryos by homogenizing in five volumes of a buffer containing 20 mM Hepes, pH 7.2, 100 mM KCl, 5% glycerol, 10 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 20 μg/mL leupeptin, and by subsequent centrifugation at 10,000 x g for 5 min. The extracts containing 5–10 μg of protein were electrophoresed in 10% SDS-polyacrylamide gel and blotted onto an Immobilon-P membrane (Millipore); the membrane was immunoreacted with anti-Dm-calpain antibody (1:2000 dilution) using an ABC kit (Vector) and an ECL kit (Amersham Corp.).

Antibodies against actin and tubulin were purchased from ICN.

**RESULTS**

**Drosophila Calpain Is Similar in Overall Structure to Vertebrate Calpains and Contains Distinct Protease and Calcium-binding Domains**—Screening of a *Drosophila* cDNA library with a cDNA probe for chicken calpain (Ohno et al., 1984) yielded several positive clones. One clone was shown to code for a protein with the amino acid sequence characteristics of calpain as shown below; the encoded protein was termed Dm-calpain. As shown in Fig. 1A, the cDNA coded for a protein with 805 residues highly homologous (about 45% identical and about 65% conservative) in sequence to various vertebrate calpains, although an insertion sequence of 76 amino acids was found in the C-terminal region. In particular, the catalytic protease domain containing the putative active site showed the highest similarity (about 65% identity, Fig. 1A). Additionally, in the C-terminal calcium-binding domain, specific residues important for Ca²⁺ binding were also homologous and matched the E-F hand criteria (Emori et al., 1986). The highest similarity in the calcium-binding domain was observed in the first E-F hand region (Fig. 1A); the similarity in the other E-F hand regions was lower. The encoded protein thus appears to be a calcium-dependent proteolytic enzyme.

Regions other than the protease and calcium-binding domains, referred to as domains I and III, also showed significant sequence similarity to vertebrate calpains. Although the functions of these domains are not fully understood, this similarity suggests common functions of domains I and III in *Drosophila* and vertebrate calpains. Together, the total structure and probable biochemical profile of Dm-calpain is probably equivalent to those of vertebrate calpains.

The sequence homology between Dm-calpain and vertebrate calpains were nearly equal, suggesting that Dm-calpain cannot be classified into a subtype of vertebrate calpains as m-calpain and μ-calpain.

Homology search of the amino acid sequences of Dm-calpain using the SwissProt data base (release 25) yielded known vertebrate calpains as closely related proteins. In addition, several cysteine proteinases such as cathepsins B, H, and L, as well as E-F hand calcium-binding proteins such as calmodulin and troponin C, were shown to have significant similarity, although to much lesser extent than in the case of vertebrate calpains.

In situ hybridization to polytene chromosome revealed that Dm-calpain gene maps at position 50D-E of the second chromosome (Fig. 1B).

*Dm-calpain Is Present in Both the Anterior Pole and Posterior Regions of Very Early Embryo*—To know the functional features of Dm-calpain, we prepared and affinity-purified specific antibody against Dm-calpain expressed in *E. coli*. As shown in Fig. 2, this antibody detected a single band with Mr ~ 90,000 in a *Drosophila* extract, consistent with the deduced molecular weight (91,500). Using this antibody as a probe, we investigated the localization of the protein in embryos. As shown in Fig. 3, in the very early embryo, the anterior pole and posterior surface regions of very early embryos show positive signals. Since only a single or a few nuclei (mitotic spindles) visualized by double-staining with anti-tubulin antibody were observed (Fig. 3, A, B, and D), these embryos are believed to represent those just after fertilization.

This anterior expression disappeared rapidly during nuclear division. On the other hand, the posterior signal gradually moved to the posterior pole, and when polar buds can be seen, positive staining was observed around the buds (data not shown). After pole cell formation, the calpain-positive region continues to be observed just below the pole cells (Fig. 3, A and D). However, it should also be noted that calpain does not seem to be located in the pole cells themselves. This posterior signal gradually becomes weak according to the movement of the pole cells in the dorsal direction (data not shown).

**Dm-calpain Co-localizes with Actin-related Cytoskeletal Structure beneath the Preleavage Furrows before Cellularization**—When pole cells were formed, the egg surface began gradually to be stained with anti-calpain antibody in hexagonal lattice or circular forms (Fig. 4, A, B, D, and E). To know the relationship between the positive regions and the nuclei, double-staining with DAPI was carried out. Comparing the two staining patterns shown in Fig. 4, it can be seen that each calpain-positive lattice surrounds a nucleus. During additional...
nuclear cycles, a regular staining pattern was observed in the same manner, but the size of the lattice became smaller according to the cycle (Fig. 4, I and J).

Previously, it was reported that actin filaments form cap structure underlying the plasma membrane immediately above the nuclei (Karr and Alberts, 1986), and some actin-binding proteins have been shown to form a transiently hexagonal framework beneath the plasma membrane, especially just below the precleavage furrow (Miller et al., 1989). Double-staining experiments using anti-actin antibody were carried out in order to examine whether the calpain-positive regions are related to the actin structures. Fig. 5 shows that during cycles 9–13, calpain, shown by black or dark brown, localizes significantly between two adjacent actin caps shown by orange or light brown, where reorganization of actin-related cytoskeletons involving several actin-binding proteins as described by Miller et al. (1989) is thought to take place. In addition, the shape of the calpain-positive regions seems to change according to the nuclear cycle visualized as the staining pattern of actin (Karr and Alberts, 1986). The calpain-positive regions thus seem to be reorganized dependent on nuclear cycles, probably in correlation with actin-related cytoskeletons.

**DISCUSSION**

We report here the first identification of a calpain gene in *Drosophila*. This gene codes for a cysteine protease with a calcium-binding domain that is highly related to known vertebrate calpains. We have also shown that calpain is localized in a specific manner in early *Drosophila* embryos, probably interacting with actin-related cytoskeletal structures.

**Relationship between Dm-calpain and Other Calpains**—The amino acid sequence of Dm-calpain is similar overall to those of previously identified calpains (Ohno et al., 1984; Emori et al., 1986; Aoki et al., 1986; Imajoh et al., 1988; Sorimachi et al., 1989). Although the detailed biochemical characteristics of Dm-calpain have not yet been obtained, Dm-calpain, with a protease domain similar to those of other calpains, should exhibit comparable activity in terms of substrate specificity. On the other hand, the calcium-binding domain shows less similarity to other calpains than the protease domain; thus, the calcium-dependence might be somewhat different. However, special amino acid residues in the E-F hand structures thought to be important for calcium-binding are well conserved, indicating that, like other calpains, Dm-calpain binds calcium ion (Minami et al., 1987).

In addition to domains II and IV, domains I and III, whose functions have not been definitely established, also show sequence similarity between Dm-calpain and mammalian calpains. This suggests that the total structure of Dm-calpain including domains I and III exhibits all of the characteristics observed for calpain molecules.

However, a 76-amino acid insertion is present in domain IV (Fig. 1). A data base search of this sequence yielded no significant similarity to any other known sequences; thus, its function is unclear at present. It is known that two insertion sequences also exist in a mammalian calpain-related protein, p94, also termed nCL-1 (Sorimachi et al., 1989). One of these sequences is located near, but at a point distinct from, the Dm-calpain insertion, although no sequence similarity is observed (Fig. 1). This may mean that the insertion points near the bordering regions between domains III and IV are located on the surface of the calpain molecule. Taken together, the
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**FIG. 4. Localization of Dm-calpain during cleavage cycles 10 and 11 (A-H) and cycles 12-14 (I and J).** Drosophila embryos (0-3 h) were collected and stained with anti-Dm-calpain antibody as described under “Materials and Methods” (A, B, D, E, G, I, and J). Nuclei of the same embryos were also visualized by DAPI (C, F, and H). Panels A-C, D-F, and G-H, respectively, show the results of the same embryos. A and D were focused in the middle (pole cells) of the embryos, and B, E, and G were focused at the surface of the embryos. As shown by arrowheads, positive signals were observed just below the pole cells, but not inside the pole cells (A and D). At the same stage, the surface of the embryo began to be stained, and circular pattern of the staining was observed (E and F). Subsequently, near the surface of the embryos, hexagonal lattices or circles of positive Dm-calpain signals were observed (G), each of which contained a single nucleus as visualized by DNA staining (H). Note that the anterior pole signals observed in very early embryos (Fig. 3) were scarcely detected (A and B). At later cleavage cycles (cycle 12, I; cycle 14, J), hexagon-shaped stainings are clearly observed and the sizes become smaller according to cycle (i.e. the number of nuclei). The bar represents 100 μm.

*Possible Functions of Dm-calpain in the Early Embryo—Immunohistochemical analysis showed Dm-calpain to be localized in the anterior pole region in very early embryos containing few nuclei (Fig. 3). Although direct evidence was not obtained, this expression may be related to invasion of the sperm nucleus or nuclear fusion or early nuclear division. Namely, it is suggested that Dm-calpain is involved in the rearrangement of the cytoskeleton during fertilization.*

On the other hand, the posterior region also contained Dm-calpain from just after fertilization. While the anterior signal structure and biochemical characteristics including proteolytic activity of Dm-calpain should be comparable with those of mammalian calpains.

Recently, two novel calpains have been identified in mammals that show tissue-specific expression (Sorimachi et al., 1989, 1993a, 1993b); these are in addition to conventional calpains (μ- and m-calpains) (Murachi, 1989). Thus, mammals contain ubiquitously distributed μ- and m-calpains and tissue-specific calpains termed nCL-1 and nCL-2. In this respect, Dm-calpain belongs to the former type from its expression pattern as shown in Figs. 4 and 5 and from its ubiquitous distribution in later developmental stages (data not shown).

In Drosophila, two calcium-dependent proteolytic activities, termed calpain I and II activities, have been identified in adult extracts, and some of their properties have been reported (Pinter and Friedrich, 1988; Pinter et al., 1992). At present, the relationship between these activities and Dm-calpain is obscure (Pinter and Friedrich, 1988).
could be still observed, positive signals in the posterior region were vaguely visible at around 60–90% egg length. Since the posterior signal seems to move to the anterior pole during the cleavage cycles, and since it is condensed just below the polar body and pole cells, this expression might be related to the movement of cytoplasm that is later included in pole cells, probably dependent on cytoskeletal structure. It is thus reasonable that the movement of the nuclei and cytoplasm of pole cells depends on calpain-dependent dynamism of the cytoskeleton.

During the cleavage cycles before the nuclei reach the egg surface, no significant localization of Dm-calpain was observed except in the posterior region described above (Fig. 4). At cleavage cycle 9, calpain began to form a hexagonal network beneath the egg surface surrounding the dividing nuclei. At this stage, actin and actin-binding proteins are known to form specific structures that undergo dynamic change dependent on the nuclear stage (Karr and Alberts, 1986). For instance, actin fibers form a cap structure above each nucleus and just below the plasma membrane, and some actin-binding proteins are localized like meshwork in a manner similar to that for Dm-calpain (Miller et al., 1989). Along with the observations in the double-staining experiments showing that calpain is condensed at the edge of the actin cap or between dividing actin caps (Fig. 5), Dm-calpain appears to be involved in reorganization of the cytoskeletal structure related to actin and its binding proteins during cleavage cycles.

In summary, we provide the first description of Drosophila calpain at the molecular level and show that Dm-calpain is closely concerned with the actin-related cytoskeleton. Thus, proteolytic activity of Dm-calpain is probably involved in the dynamism of early embryogenesis occurring immediately after fertilization. Further studies of Dm-calpain localization using various embryos mutated in their cytoskeletal structures (Freeman et al., 1986; Simpson and Wieschaus, 1990; Postner et al., 1992; Sullivan et al., 1993; Ibsouadara et al., 1993; Schejter and Wieschaus, 1993a) would provide more refined results concerning the in vivo functions of Dm-calpain.

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