Expression and immunolocalization of the calpain–calpastatin system during parthenogenetic activation and fertilization in the rat egg

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Abstract

Calpastatin is an intrinsic intracellular inhibitor of calpain, a Ca$^{2+}$-dependent thiol protease. The calpain–calpastatin system constitutes one functional proteolytic unit whose presence and function has already been investigated in various cell types, but not in the egg. We have previously shown that calpain is expressed in rat eggs and is activated upon egg activation. The present study was designed to investigate the calpain–calpastatin interplay throughout the process. Western blot analysis revealed two main calpastatin isoforms, the erythrocyte type (77 kDa) and the muscle tissue type (110 kDa). By immunohistochemistry and confocal laser scanning microscopy, we demonstrated that the 110 kDa calpastatin was localized at the membrane area and highly abundant at the meiotic spindle in eggs at the first and second meiotic divisions. The 77 kDa calpastatin isoform appeared to be localized as a cortical sphere of clusters. The 110 kDa calpastatin and β-tubulin have both been localized to the spindle of metaphase II eggs, both being scattered all through the cytoplasm following spindle disruption by nocodazole treatment, implying a dynamic interaction between calpastatin and microtubule elements. Upon egg activation, membranous calpastatin translocated to the cortex whereas cortical millimolar (m)-calpain shifted towards the membrane. Spindle calpastatin and calpain remained static.

We suggest that calpastatin serves as a regulator of m-calpain. The counter translocation of m-calpain and calpastatin could serve as a means of calpain escape from calpastatin inhibition and may reflect a step in the process of calpain activation, throughout egg activation, that is required for calpain to exert its proteolytic activity.

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Introduction

Sperm–egg interaction elicits a series of cellular events within mammalian eggs, leading to their activation. The initial event is a rapid elevation in intracellular calcium concentration ([Ca$^{2+}$]$_i$) (Miyazaki 1988, Miyazaki et al. 1992), followed by Ca$^{2+}$ oscillations (Miyazaki 1991, Fissore et al. 1992, Kline & Kline 1992, Sun et al. 1992, Ben-Yosef et al. 1993). It had previously been suggested that the initial [Ca$^{2+}$]$_i$ rise triggers completion of the second meiotic division (Ben-Yosef & Shalgi 1998) and cortical granule exocytosis (CGE) (Ducibella 1991, Dandekar & Talbot 1992) which results in modification of the zona pellucida (ZP) and hence in a block to polyspermy (Ducibella 1991, Kline & Stewart-Savage 1994, Ben-Yosef & Shalgi 1998).

Calpains belong to a highly conserved family of intracellular calcium-dependent thiol proteases (Dayton et al. 1976a, 1976b, Goll et al. 2003). There are two ubiquitously expressed isoforms, µ- and m-calpain, that differ significantly in the Ca$^{2+}$ concentration required for their in vitro activation (micro or millimolar for µ- or m-calpain respectively) (Goll et al. 2003). Since Ca$^{2+}$ at a micromolar level is considered a supraphysiological concentration, several cellular mechanisms are suggested for lowering the Ca$^{2+}$ concentration required for activation of calpain. Among these are: binding of calpain to membrane phospholipids; calpain autolysis, though intact calpain may be active as well; escape of calpain from its endogenous inhibitor, calpastatin, protein coactivators and phosphorylation (Michetti et al. 1991, Kuo et al. 1994, Arthur & Crawford 1996, Mellon et al. 1996, Suzuki & Sorimachi 1998, Barnoy et al. 1999, Tullio et al. 1999, Glading et al. 2002).

Calpain-mediated proteolysis of intracellular proteins is a key step in various cellular processes such as cytoskeleton modulation, cell migration, cell cycle progression and apoptosis (Carragher et al. 2002).

Calpastatin is a specific endogenous inhibitor of calpain which regulates calpain activity in vivo (Carragher et al. 2002, Goll et al. 2003) and is known to be restricted to
vertebrates (Goll et al. 2003). There are eight different known isoforms of calpastatin (Geesink et al. 1998, Goll et al. 1999, Takano et al. 2000, Parr et al. 2001, Wei et al. 2002), most of which are ubiquitous while some are tissue specific (Melloni et al. 1998). It is assumed that different calpastatin isoforms inhibit different calpain isoforms within a single cell (Averna et al. 1999). Two main calpastatin isoforms are widely studied: the erythrocyte type (~77kDa) and the muscle tissue type (~110kDa) (Takano et al. 1991, 1993, Barnoy et al. 1999, Tullio et al. 2000).

The calpain–calpastatin system constitutes a functional proteolytic unit (Melloni & Pontremoli 1989) in which calpastatin governs and fine tunes the calpain activity. For calpain to become fully activated, it should escape the inhibitory effect of calpastatin. Furthermore, it has been suggested that subcellular compartmentalization of either calpain or calpastatin may regulate calpain intracellular activity (Lane et al. 1992, Carragher et al. 2002).

Calpain participates in meiosis of Xenopus eggs and starfish oocytes (Watanabe et al. 1989, Santella et al. 1998), as well as of rat eggs (Ben-Aharon et al. 2005), presumably via cytoskeleton remodeling. However, this theory was challenged since it has been implied that calpain is capable of degrading c-mos product only in vitro, at supraphysiological calcium concentrations (Lorca et al. 1991).

The present study was devised to follow calpastatin expression and localization within the rat egg at various developmental phases, and to further elucidate the calpain–calpastatin interplay throughout egg activation.

Materials and Methods

Collection of eggs

For induction of ovulation, 23- to 25-day-old immature Wistar-derived female rats were injected with 10IU human chorionic gonadotropin (hCG; Sigma) 48-52 h after administration of 10IU pregnant mare serum gonadotropin (PMSG; Syncro-part, Sanofi, France).

Eggs arrested at the metaphase of the first meiotic division (MI)

Cumulus-enclosed oocytes at the MI stage were retrieved from antral follicles into Toyoda HEPES (TH) medium (Ben-Yosef et al. 1995) supplemented with 0.4% bovine serum albumin (BSA; Sigma) 8 h after hCG administration. Cumulus cells were removed mechanically. All manipulations were performed at 37°C.

Eggs arrested at the metaphase of the second meiotic division (MII)

Eggs were isolated from the oviductal ampullae into TH medium supplemented with 0.4% BSA for immunofluorescence, or with 0.1% BSA for immunoblotting (Ben-Yosef et al. 1995), 14 h after hCG administration. Cumulus cells were removed by a brief exposure to hyaluronidase (400IU/ml; Sigma).

Fertilized eggs

PMSG–hCG-primed immature females rats were mated with adult males. Eggs at various stages of fertilization were isolated at several time-points after mating. The earliest developmental stage classified was sperm binding (SB), 0–15 min after sperm attachment (Eliyahu & Shalgi 2002), whereas the latest was the mitotic division.

Parthenogenetic activation

MII ovulated eggs were parthenogenetically activated, for 5 min in the dark, by calcium ionophore (2 μM; ionomycin; Calbiochem, San Diego, CA, USA), followed by an additional 0- or 20-min incubation in fresh TH medium lacking the activator, and then fixed.

Meiotic spindle dissociation and recovery

MII eggs were exposed to nocodazole (10 μg/ml; Sigma) for 1 h and were either fixed immediately or rinsed and incubated for an additional 30 min in fresh medium lacking the toxin.

SDS-PAGE and Western blot analysis of proteins

Batches of 500–700 cumulus-free MII eggs were lysed in 7–10 μl lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 1 mM Na 3VO4, 5 mM NaF and 10 μg/ml aprotinin (Sigma)). Proteins were separated by 10% SDS-PAGE at a constant current of 30mA under reducing conditions, alongside marker proteins of known molecular weights (Amersham International plc, Amersham, Bucks, UK). Proteins were electro-transferred for 18 h at 40 mA onto a nitrocellulose membrane (Amersham), by a wet blotting apparatus (Hoeffer, San Francisco, CA, USA). Blots were incubated overnight at 4°C with either monoclonal anti-calpastatin (1:750; Calbiochem) or polyclonal anti-calpastatin (1:100; Santa-Cruz Biotechnology, Santa Cruz, CA, USA) in blocking solution. Primary antibodies were recognized by horseradish peroxidase (HRP) conjugated to goat anti-mouse antibody (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or donkey anti-goat antibody (1:20000; Santa-Cruz Biotechnology) respectively. Detection was performed by enhanced chemiluminescence (ECL; Supersignal; Pierce, Rockford, IL, USA). Myoblast extracts served as a positive control.

Immunohistochemistry and confocal microscopy

Eggs at appropriate developmental stages were fixed for 10 min at room temperature by 3% paraformaldehyde (Merck, Rahway, NJ, USA) in Dulbecco’s phosphate-buffered saline (DPBS; Biological Industries, Beit Ha’emek, Israel), supplemented with 0.01% glutaraldehyde (Polysciences, Warrington, PA, USA). The eggs were then
washed in a solution of 3% fetal calf serum (FCS; Biological Industries) in DPBS (DPBS/FCS), which served as a blocking solution. ZPs were removed by 0.25% pronase (Sigma). ZP-free eggs were permeabilized for 10 min by 0.05% NP-40 (Sigma) in DPBS/FCS and washed with 0.005% NP-40 in DPBS/FCS. Permeabilized eggs were incubated for 2 h at room temperature in the presence of primary antibodies, washed in DPBS/FCS and reincubated for 30 min in the presence of fluorescent-labeled secondary antibodies. Chromosomal stage was detected by a DNA-specific fluorochrome (1 μg/ml Hoechst; Sigma). Eggs stained only with secondary antibody served as controls.

Primary antibodies were anti-77 kDa calpastatin mouse monoclonal antibody (1:200; Calbiochem), anti-110 kDa calpastatin goat polyclonal antibody (1:50; Santa Cruz (R-19); sc-7561), anti-m-calpain mouse monoclonal antibodies (1:50; Calbiochem) and anti-β-tubulin mouse monoclonal antibody (1:7500; Sigma).

Fluorescent-labeled secondary antibodies (Jackson Immunoresearch Laboratories) were donkey anti-goat IgG-Cy3 (1:250), donkey anti-mouse IgG-Cy3 (1:1000 for 77 kDa calpastatin; 1:250 for m-calpain) and donkey anti-mouse IgG-Cy2 (1:5000).

Calpastatin, β-tubulin, calpain and DNA were visualized and photographed by a Zeiss confocal laser-scanning microscope. The Zeiss LSM 410 (Oberkochen, Germany) is equipped with a 25 mW krypton-argon laser, a 10 mW helium-neon laser (488, 543 and 633 maximum lines) and a u.v. laser (Coherent Inc. Laser Group, Santa Clara, CA, USA). A 40 NA/1.2 planapochromat water immersion lens (Axiointer 135 M; Zeiss) was used for all imaging. Co-localization analysis was performed on images of simultaneously detected calpastatin and β-tubulin using the Zeiss co-localization procedure (Talmor et al. 1998).

Results

Expression of calpastatin isoforms in the rat egg

Western blot analysis was preformed on extracts of MII eggs. We employed two calpastatin-specific antibodies which detect either the erythrocyte-type calpastatin isoform at 77 kDa or the muscle tissue-type calpastatin isoform at 110 kDa (Fig. 1). Both calpastatin isoforms were expressed in MII eggs. Extracts of myoblasts served as a positive control.

Localization of the 77 kDa and 110 kDa calpastatin isoforms throughout egg activation

Immunofluorescence confocal microscopy was used for tracking the intracellular distribution of calpastatin throughout egg activation. Eggs parthenogenetically activated by ionomycin were fixed at different time-points during the course of the second meiotic division. Calpastatin was highly abundant at the meiotic spindle (Fig. 2A and B) and at the plasma membrane (Fig. 2A and C) of MII eggs whereas the labeling of the plasma membrane overlying the meiotic spindle was less intense. Immediately after exposure of the eggs to ionomycin, calpastatin displayed a mild translocation to the egg cortex (Fig. 2aF). This trend was further intensified 20 min after activation (Fig. 2aI). Calpastatin labeling intensity at the spindle compartment did not change during all meiotic stages examined (Fig. 2AB, E and H). The 77 kDa calpastatin isoform appeared to be localized as a cortical sphere of clusters (Fig. 2b), which did not change during activation (not shown).

Possible interaction of the 110 kDa calpastatin isoform with the spindle microtubules

Eggs were double stained with anti-calpastatin and anti-β-tubulin prior to immunofluorescence confocal microscopy in order to demonstrate co-localization of calpastatin and microtubules. Co-localization (Fig. 3C) of both calpastatin (Fig. 3A) and β-tubulin (Fig. 3B) to the meiotic spindle was depicted in MII eggs. To further correlate the association of calpastatin with the spindle microtubules, we used 10 μg/ml nocodazole to obliterate the spindle structure. Nocodazole treatment resulted in scattering of both calpastatin and tubulin all through the cytoplasm (Fig. 3D–F). Once the toxin was rinsed off, the spindle was reconstituted with both calpastatin (Fig. 3G) and β-tubulin (Fig. 3H) localized to it (Fig. 3I).

Calpastatin localization at early stages of meiosis and at fertilization

Calpastatin distribution in MI and MII eggs was followed by immunofluorescence confocal microscopy. Calpastatin staining appeared intense at the meiotic spindle of MI and MII eggs (Fig. 4B and E respectively) as well as at the
plasma membrane (Fig. 4C and F respectively). Furthermore, fertilized eggs that had been fixed at an early stage of fertilization (SB stage) exhibited a pattern of distribution resembling that of MI and MII eggs (Fig. 4H and I) respectively. At later stages of fertilization the pattern of calpastatin localization (Fig. 4L) resembled the pattern in parthenogenetically activated eggs (Fig. 2I) where marked cortical staining was demonstrated with no significant change in the meiotic spindle staining (Fig. 4K).

### Intracellular localization of calpain throughout parthenogenetic activation

In order to investigate the interaction between calpastatin and calpain, we immunostained parthenogenetically activated eggs with anti-μ- and anti-m-calpain antibodies. As previously demonstrated (Ben-Aharon et al. 2005), μ-calpain had been localized to the meiotic spindle, as well as to the plasma membrane of MII eggs (not shown) and no change in its localization was observed during any stage of the egg activation process. m-calpain was specifically localized to the meiotic spindle (Fig. 5B) and to the cell cortex (Fig. 5C) of MII eggs. However, mild yet significant translocation of m-calpain to the plasma membrane was observed immediately after exposure to ionomycin (Fig. 5F), which was further intensified at later stages of meiosis (Fig. 5I). No significant alteration in the intensity of m-calpain staining at the spindle was observed throughout egg activation (Fig. 5E and H).

### Discussion

As shown in most of the cells studied, calpain co-exists along with its endogenous inhibitor, calpastatin. Although there is some evidence for a possible role of calpain as a cell cycle regulator in *Xenopus* eggs (Watanabe et al. 1989) or during cytoskeletal remodeling for reinitiating...
meiosis in starfish oocytes (Santella et al. 1998), there are no data regarding either expression of calpastatin or function of a calpain–calpastatin system in mammalian eggs. We therefore aimed, in the present study, to characterize the calpain–calpastatin system in mammalian eggs.

Calpastatin has been documented in various tissues of diverse vertebrates where it is expressed concomitantly with calpain (Goll et al. 2003). Calpastatin is considered to be responsible for monitoring and fine-tuning calpain activity, thus regulating the calpain–calpastatin network (Murachi 1989, Wei et al. 2002). Several studies indicate that calpastatin is comprised of various isoforms with varying molecular weights (Wei et al. 2002). To date, eight different isoforms have been identified (Geesink et al. 1998, Goll et al. 1999, Takano et al. 2000, Parr et al. 2001, Wei et al. 2002). Frequently more than one is expressed in a single tissue (Melloni et al. 1998). We detected the expression of the erythrocyte-type (77 kDa) and the muscle tissue-type (110 kDa) calpastatins in rat eggs, which correlated with the calpastatins reported in other tissue types (Takano et al. 1991, 1993, Barnoy et al. 1999, Tullio et al. 2000).

Calpastatin localization varies in the different tissues and is affected by biochemical modifications such as phosphorylation (Lane et al. 1992, Averna et al. 2001). It has been proposed that \([\text{Ca}^{2+}]\), controls the amount of calpastatin available for calpain inhibition at the different cellular compartments, thus serving as a mechanism for regulating calpain activity (Averna et al. 2001). In the current study, immunofluorescence combined with confocal microscopy revealed a distinct cortical localization of the 77 kDa isoform while the 110 kDa calpastatin was mostly abundant at the meiotic spindle of eggs arrested at the MI as well as the MII stage. The 110 kDa isoform also appeared to be localized to the plasma membrane, but not at the area overlying the MI spindle. This segment of membrane is known to be devoid of microvilli, rich in microfilaments and actin (Eliyahu et al. 2005) and is probably associated with different proteins from the rest of the membrane. Soon after the \([\text{Ca}^{2+}]\) rise during egg activation, either sperm or parthenogenetically induced, calpastatin translocates from the plasma membrane towards the egg cortex, while its localization to the spindle remains unchanged.

In our previous study, we have allocated both \(\mu\)- and \(m\)-calpain to be localized to the egg cortical area as well as to the spindle region (Ben-Aharon et al. 2005). \(m\)-calpain but not \(\mu\)-calpain exhibited a dynamic change in its localization throughout egg activation by translocating from the cortical region to the membrane, shortly after the rise in \([\text{Ca}^{2+}]\). We have determined a correlation between calpain activation and egg activation and inferred a role for \(m\)-calpain in the process of sperm or parthenogenetically induced egg activation. We therefore speculated that

![Figure 3](https://www.reproduction-online.org)

**Figure 3** Co-localization of 110 kDa calpastatin and \(\beta\)-tubulin (\(\beta\)-Tub) in MII eggs before, during and after spindle disruption by nocodazole. MII eggs (A–C), nocodazole (10 \(\mu\)g/ml)-treated MII eggs fixed immediately after treatment (D–F), or after a 30-min recovery period (G–I). Primary antibodies (goat anti-calpastatin (1:50) and mouse anti-\(\beta\)-tubulin (1:7500)) were detected by the two appropriate fluorescent labeled Cy secondary antibodies (Cy-3 (1:250; red) and Cy-2 (1:5000; green) respectively). DNA was labeled by Hoechst (1:1000; blue). Calpastatin (A, D and G; red), \(\beta\)-tubulin (B, E and H; green) and co-localization (C, F and I) and DNA (blue) were visualized by confocal laser-scanning microscopy. Cross-sections through the egg meiotic spindle (A–I). Bar = 10 \(\mu\)m.
m-calpain does not play an active role during this process but rather functions as a bystander or a backup system for m-calpain. As a housekeeping gene product, m-calpain is expressed within the egg. It is quiescent during the early phase of fertilization, but may participate later during early cleavages. Calpain inhibition hinders completion of meiosis and lends credence to a model in which calpain activation is required for the cytoskeletal elements of the meiotic spindle to allow chromatid segregation. Calpain may be involved in the CGE as well, since inhibition of calpain resulted in CGE of a lesser extent (Ben-Aharon et al. 2005). Deducing the possible substrates of calpain upon the localization that has been demonstrated and the functional studies that have been performed using calpain inhibitors, we may imply a role for calpain in mediating cytoskeletal remodeling throughout both major pathways that occur during egg activation—completion of meiosis and CGE. The interaction between calpain and the cell cytoskeletal components has been well established in other cell types, where calpain mediates disassembly of the microtubule lattice (Pettigrew et al. 1996) and interacts with microtubule-associated proteins, two known favorable calpain substrates (Billger et al. 1988, Fischer et al. 1991, Johnson & Foley 1993, Alexa et al. 1996). However, in the mammalian egg, the interplay between calpain and microtubule elements should still be elucidated.

Because of the close proximity of calpastatin and calpain (Goll et al. 2003) and the association of calpain with cytoskeletal structures we further studied the relationship of calpastatin with cytoskeletal components. We double stained MII eggs for calpastatin and tubulin, during spindle disruption by nocodazole and after a recovery period. The results revealed co-localization of both proteins upon depolymerization and repolymerization of the spindle microtubules. These results may imply a dynamic interaction between calpastatin and spindle microtubule elements. Localization of m-calpain and its possible association with cytoskeletal structures in the vicinity of either the spindle or the plasma membrane may provide a

**Figure 4** Localization of 110 kDa calpastatin during various developmental stages. MI (A–C) and MII (D–F) eggs, early (SB; G–I) and late (J–L) fertilization stages. Primary poly antibody (goat anti-calpastatin (1:50)) was detected by a fluorescent labeled Cy-3 secondary antibody (1:250; red). DNA was labeled by Hoechst (1:1000; blue). Calpastatin and DNA were visualized by confocal laser-scanning microscopy. Cross-sections through the egg meiotic spindle (B, E, H and K). Cross-sections at the equatorial plane of the egg (C, F, I and L). Light microscopy (A, D, G and J). Egg meiotic/mitotic spindle (white arrow). Calpastatin translocation from egg membrane towards the cortex (yellow arrow). Bar = 10 μm.
spatial and temporal framework for potential participation in reorganization of cytoskeletal components, as has also been shown in other cell types (Hood et al. 2003). It is well established that unique cellular mechanisms for lowering the supraphysiological requirements of \([\text{Ca}^{2+}]_i\) are involved in the activation of calpain in vitro. Among them are translocation of calpain to the membrane area and the association with phospholipids (Saido et al. 1992) or interaction with protein coactivators (Melloni et al. 1998). Another means of calpain activation that has been previously documented is an escape from calpastatin. It has been suggested, in vivo, calpain eludes calpastatin by associating with the plasma membrane, thus being able to express its proteolytic activity (Tompa et al. 2002, Hood et al. 2003, Todd et al. 2003). The calpain–calpastatin is an intriguing complex in which calpastatin is a negative regulator of the proteinase, but at the same time can be subjected to digestion by calpains (Salamino et al. 1997). We have herein demonstrated a unique counter translocation of m-calpain and calpastatin between the egg membrane and the egg cortex. This observation may reflect an essential step in the process of calpain activation throughout egg activation that is required by calpain to exert its proteolytic activity.

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