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A Physiological Approach to the Study of Pseudopod Extension in the Amoeboid Sperm of the Nematode *Caenorhabditis elegans*

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DEDICATION

I would like to dedicate this work to the memory of my mother who always supported and provided for me even in difficult times. I also dedicate it to Dr. Marco Tulio Gonzalez Mrtinez for being such an inspiration as a scientist. To all those that have less than others, to all those who believed, and finally, to Laura, my love and family.

ABSTRACT OF THE DISSERTATION

A Physiological Approach to the Study of Pseudopod Extension in the Amoeboid Sperm of the Nematode *Caenorhabditis elegans*

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Fertilization is the process in which both the spermatozoon and the oocyte must undergo a myriad of physiological changes resulting in successful fusion to produce progeny. Spermatozoa are highly motile cells that must reach the oocyte in the environment where fertilization takes place and in this regard the study of acquisition of motility in sperm cells is important to understand sperm-egg interactions. In the case of the nematode *Caenorhabditis elegans*, fertilization takes place in the hermaphrodite reproductive tract where sessile spermatids must extend a pseudopod to become motile. The process of pseudopod extension initiates with rearrangement of the plasma membrane and culminates with the extension of a pseudopod by an MSP-based cytoskeleton. Thus, successful fertilization depends on the initiation of a signaling pathway that acts on proteins present on the plasma membrane and has as an ultimate target the MSP filament. A genetic model of pseudopod extension suggests the

interaction of proteins from the SPE group in a multicomponent complex that regulates the timing for pseudopod extension. This complex of proteins resembles membrane microdomains, regions of the plasma membrane enriched in cholesterol and sphingolipids, where membrane and cytosolic proteins from a common signaling pathway interact. In the present work, the presence and involvement of membrane microdomains in *C. elegans* spermatids is tested. This hypothesis is approached by the use of biochemical assays and microscopy techniques that give useful information on the physiological process of motility acquisition. The results suggest that male-derived spermatids are subject to physiological changes that prevent pseudopod extension prior to ejaculation and that membrane microdomains are present in these cells and involved in successful fertilization of the oocyte through the extension of a pseudopod. Using an integrative approach, these results revealed that membrane dynamics are responsible for the signaling cascade that triggers acquisition of motility, complementing the genetic model of pseudopod extension.

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Chapter 1

Physiological steps leading to the acquisition of motility in amoeboid sperm.

A literature review

Abstract

Nematode spermatozoa are highly specialized amoeboid cells that must acquire motility through the extension of a single pseudopod. Despite morphological and molecular differences with flagellated spermatozoa (including a non actin-based cytoskeleton), nematode sperm must also respond to cues present in the female reproductive tract that render them motile, thereby allowing them to locate and fertilize the egg. The factors that trigger pseudopod extension *in vivo* are unknown, although current models suggest the activation through proteases acting on the sperm surface resulting in a myriad of biochemical, physiological, and morphological changes. A compelling amount of evidence shows that pseudopod extension is under the regulation of physiological events that are also observed in other "typical" eukaryotic cells (including flagellated sperm) whereby extracellular effectors induce rearrangements of discrete domains on the plasma membrane where molecular signals are amplified and transduced through ion fluxes and phosphorylation cascades. An integrative approach to the study of non-flagellated spermatozoa will shed light on the identification of unique and conserved processes during fertilization among different taxa.

Introduction

Fertilization is common to all multicellular organisms that reproduce sexually. In this process, two gametes, typically haploid cells, fuse to form a new individual. In order for successful fertilization to occur, two events are necessary. First, the gametes must find one another so that they come into direct physical contact. Second, upon sperm-egg binding, a complex series of biochemical and physiological events must occur, culminating in fusion and egg activation (Yanagimachi, 1994).

The "typical" fertilization process involves a sessile egg that is encountered by a highly motile spermatozoon. Most frequently the spermatozoon is propelled by a microtubule-based flagellum that brings the gametes into close contact ultimately resulting in fertilization. Although this view of fertilization is widespread due to a concentration of investigations on spermatozoa from deuterostomate lineages, a review of sperm from other taxa have shown that non-flagellated sperm are more common than previously realized (Morrow, 2004) (see Fig. 1.1A).

Perhaps the most studied taxonomic group with motile non-flagellated sperm are those in the Phylum Nematoda. This phylum is extremely diverse with both free-living and parasitic representatives comprising gonochoristic, hermaphroditic, and parthenogenetic reproductive strategies (Poinar, 1983). Morphologically, the spermatozoa in the entire group are characterized by the absence of a flagellum and an acrosome as well as the presence of spheroideal membranous vesicles (Justine, 2002). In order to acquire motility, a spherical spermatid must first undergo the process known as spermiogenesis or sperm activation, in which a pseudopod is extended, conferring

motility to the cell in an amoeboid fashion (see Fig. 1.1B). This process of acquisition of motility has been most extensively studied in the nematodes *Ascaris suum* and *Caenorhabditis elegans*, and is currently thought to be the general mode of sperm locomotion in all nematodes. Although, most studies on these sperm have been performed using molecular genetics and biochemistry, an integrative physiological framework is lacking. The present work is intended to summarize the physiological pathways that lead to the *in vitro* extension of the pseudopod in nematode sperm from both *Ascaris* and *Caenorhabditis*.

In vitro Pseudopod extension and crawling of nematode spermatozoa.

Nematode spermatozoa initiate motility subsequent to the extension of the pseudopod (in the case of *C. elegans*, or lamellipod in *A. suum*) allowing the cell to crawl on a substrate. A peculiarity in these amoeboid cells is that they contain non actin-based microfilaments responsible for pseudopod extension (Nelson et al., 1982). Instead of actin, nematode sperm form filaments from the Major Sperm Protein (MSP), a 14 KDa protein that constitutes 15% of the total protein in the sperm (Klass and Hirsh, 1981). In order to polymerize, MSP must be present in dimers to elongate filaments and fibers allowing the cell to crawl (Roberts and Stewart, 2000).

Despite the 3-fold difference in maximum length between the spermatozoa of *C. elegans* (~ 9 μm) *and A. suum* (~ 26 μm) (Royal et al., 1997), the process of pseudopod extension in both nematodes is very similar and initiates with rearrangements of the plasma membrane (Nelson and Ward, 1980) and the extension of transient membrane

protrusions known as “spikes” in *C. elegans* or fillopodia in *A. suum*. Filaments of MSP form these “spikes” which precede the formation of the pseudopod in both *A. suum and C. elegans* (Shakes and Ward, 1989; Rodriguez et al., 2005).

The process of *in vitro* spermatid activation (pseudopod extension) has been studied extensively in both nematode species. In the case of *A. suum*, the sperm extends a crescent-shaped flat pseudopod with distinguishable MSP fiber bundles (Sepsenwol et al., 1989). Spermatids can be activated *in vitro* using either *S. griseus* proteases (25 µg/ml) or a vas *deferens* extract (VDX) from *A. suum* (Sepsenwol and Taft, 1990). Spermatozoa activated using proteases are unable to crawl, while spermatozoa activated by VDX crawl at an average instant velocity of 30.3 ± 16.2 µm/min (Royal et al., 1997). In contrast, spermatozoa from *C. elegans* possess a less flat pseudopod that lacks visible MSP fiber bundles. Spermatids are activated *in vitro* using either *S. griseus* proteases (200 µg/ml), Triethanolamine (TEA), a weak base that promotes an increase in intracellular pH (Ward 1983), or the cationic ionophore Monensin (Nelson and Ward, 1980). *C. elegans* spermatozoa can crawl at an average instantaneous velocity between of 12.0 ± 4.9 µm/min (Royal et al., 1997) and, similar to *Ascaris*, protease-activated spermatozoa are unable to crawl.

Plasma membrane dynamics and microdomains.

The first step in the process of pseudopod extension is the rearrangement of the plasma membrane. Roberts and Ward (1982) observed this dramatic rearrangement by attaching latex beads to the plasma membrane of *C. elegans* spermatids and, using a

microscope, followed their movements throughout spermiogenesis (Roberts and Ward, 1982b). This experiment revealed that the plasma membrane of spermatids undergoes an intermittent nondirected movement at a rate of 10-15 μm/min on discrete portions of the cell surface. This movement, coupled to cell rotation, initiates after treatment with Monensin, stops once the pseudopod is completely formed, and is not present in mutant sperm that fail to extend a pseudopod (Roberts and Ward, 1982b).

The extension of "spike" structures in nematode sperm coincide with membrane rearrangements and a highly fluid cell surface, resulting in the dynamic steps of protrusion, retraction, thickening and fusion of spikes to coalesce into a pseudopod or lamellipod (Shakes and Ward, 1989; Rodriguez et al., 2005). After the extension of the pseudopod and before the acquisition of motility, the nematode-specific Membranous Organelles (MOs) localize to the periphery of the cell body and fuse to the plasma membrane, delivering proteins and membrane material necessary for the total extension of the pseudopod and successful fertilization (L'Hernault 2006; Singson 2008). At this point, all the membrane rearrangements cease in the cell body while a directed membrane flow from the tip to the base of the pseudopod is initiated (Roberts and Ward, 1982a). Protein insertion has been observed at the leading edge of the pseudopod, although it is not clear how this process occurs since there are no organelles found in this region and no endocytosis processes or vesicle trafficking has been reported (Roberts and Ward, 1982a; Pavalko and Roberts, 1987; Shakes and Ward, 1989).

Membrane microdomains

In amoeboid sperm, the plasma membrane is a very dynamic structure that must maintain the integrity of interactions between proteins in common signaling pathways. Thus, we can speculate that the integrity of protein interaction is maintained due to the presence of membrane microdomains. These microdomains have been hypothesized to be cholesterol-enriched dynamic structures that function as signaling platforms sequestering proteins in a common intracellular pathway (Simons and Toomre, 2000; Golub et al., 2004). Recently, this view of "patchy" and regionalized membranes has been discussed (Engelman, 2005) and a compelling amount of evidence supports this hypothesis for *C. elegans* sperm (see Figure 1.2). For instance, the SPE group of proteins, responsible as a trigger for spike formation and further pseudopod extension, functions as a multicomponent complex that involves the interaction of membrane and cytoplasmic signanling proteins (Geldziler et al., 2005) (see Figure 1.2A). Other membrane microdomains may be responsible for the docking and fusion of MOs to the plasma membrane (see Figure 1.2B) since FER-1, a protein involved in vesicle fusion, has a human homologue that interacts with Caveolin-3 (Matsuda et al., 2001), a membrane microdomain marker. FER-1 is hypothesized to promote vesicle fusion through protein-protein interactions with SNARE proteins (Washington and Ward, 2006), a complex localized in cholesterol-enriched microdomains (Lang, 2007).

The most persuasive argument supporting the presence of membrane microdomains is revealed by cholesterol localization in spermatids and the protein redistribution that occurs during spermiogenesis. In this work, (Matyash et al., 2001) used a cholesterol fluorescent analogue, dehydroergosterol (DHE), to localize its distribution

in *C. elegans* during development, showing that males displayed a strong labeling in dispersed cytoplasmic structures (MOs?) of the spermatid. Also in spermatids, proteins that are important for fertilization change their localization subsequent to the extension of the pseudopod (Singson, 2008). For instance, SPE-9 is an EGF repeat transmembrane protein that is localized homogeneously over the spermatid plasma membrane, while after pseudopod extension it localizes exclusively to the pseudopod (Zannoni et al., 2003). Other examples of this reorganization are the proteins SPE-38, a four-pass integral membrane protein (Chatterjee et al., 2005), a TRPC calcium channel known as TRP-3 or SPE-41 (Xu and Sternberg, 2003), and the MO marker 1CB4 (Okamoto and Thomson, 1985). All of these are localized to MOs prior to pseudopod extension and change their distribution upon fusion to the plasma membrane. SPE-38 co-localizes to the pseudopod with SPE-9, TRP-3 redistributes to the plasma membrane in both the cell body and the pseudopod, and the 1CB4 marker is maintained in the MOs bodies (see Figure 1.2B).

In *Ascaris* sperm there is also evidence for membrane microdomains. MSP nucleation and elongation occurs at the leading edge of the pseudopod where the MSP Polymerization Organizing Protein (MPOP) recruits cytosolic proteins to the membrane surface (LeClaire et al., 2003). Elongation and bundling of the filaments into fibers occurs at the leading edge of the pseudopod forming a complex intertwined network of MSP (Roberts 2000), (Bottino et al., 2002). This cytoskeletal network is connected mechanically to the substratum through the membrane, allowing directional movement of the cell. In *C. elegans* sperm, cells crawl by traction exerted through plasma membrane proteins on the substrate. This attachment can be inhibited by antibodies directed against

the membrane proteins TR11 and SP56, but not by the anti MSP antibody, TR20 (Pavalko et al., 1988). Altogether, these results suggest that MSP is localized to the cytoplasmic leaflet of the plasma membrane and other proteins involved in attachment and traction are localized to the extracellular leaflet. If these proteins are colocalized in discrete regions of attachment we can hypothesize that nematode sperm crawling and attachment might be similar to the formation of focal adhesions in cells with an actin-based motility where ordered membrane microdomains play an important role (Wozniak et al., 2004; Gaus et al., 2006), (see Fig. 1.2C).

Collectively, this body of information leads us to hypothesize that membrane microdomains are present in the MOs prior to pseudopod extension and that they are disrupted upon fusion to the plasma membrane. Proteins that are redistributed in the plasma membrane can be released or sequestered within different microdomains and localize to specific morphological regions such as the pseudopod, where they can move from the tip to the base thereby recycling receptors for sperm egg fusion and conferring attachment of the cell to the substrate.

Ion physiology and signal transduction during pseudopod extension

Ionic fluxes during pseudopod extension have been studied to a lesser extent in *C. elegans* sperm. Machaca, *et al* (1996) used the patch clamp technique to investigate voltage-sensitive ion channel activities in these cells and calculated the spermatid's resting potential (V_R = - 8.07 ± 2.26 mV) in Nystatin-perforated whole-cell experiments (Machaca et al., 1996). They discovered several voltage-sensitive ion channel activities in

spermatocytes and residual bodies during spermatogenesis, although only an inward-rectifying chloride channel (Clir) was detected in spermatids upon hyperpolarization. In the same study, the Chloride channel inhibitor 4,4'-diisocyanatostilbene-2,2'-disulfonic acid (DIDS) was found to induce activation in spermatids, suggesting an important role of Clir channels in the process of pseudopod extension. The activation of spermiogenesis by the blockage of Clir is suggested to act by either a change in membrane potential or by a transport of HCO^-_3 ions into the cytoplasm, resulting in an increase of intracellular pH (Machaca et al., 1996). Indeed, the use of ionophores such as Monensin and Valinomycin has provided insight on changes in sperm intracellular pH necessary to maintain pseudopod extension in both *C. elegans* and *A. suum* (Nelson and Ward, 1980; Roberts and King, 1991). However, this cytosolic alkalization is coupled to the exchange of protons with Na^+, in the case of Monensin, and K^+ in the case of Valinomycin. Both Na^+ and K^+ ions are necessary for *in vitro* pseudopod extension. TEA and Monensin spermatid activation are reduced or abolished when these ions are replaced in the medium and activation with Pronase is reduced when K^+ ions are replaced (Nelson and Ward, 1980; Ward et al., 1983). Nelson and Ward (1980) also suggested a trigger for pseudopod extension due to changes in membrane potential based on K^+-dependent mechanisms (Nelson and Ward, 1980). To our knowledge, how nematode sperm membrane potential is affected during pseudopod extension using any *in vitro* activator has not been investigated to date.

Intracellular pH

As mentioned in the previous section, an increase in intracellular pH is necessary and sufficient for pseudopod extension. This was first suggested when spermatids treated with Monensin showed a pH dependence on spermatid activation (Nelson and Ward, 1980). Weak bases, including TEA, also exerted pseudopod extension accompanied by an increase in intracellular pH (Ward et al., 1983). In this work, the authors showed that cytoplasmic alkalization is sufficient to trigger spermatid activation and that removal of TEA did not affect pseudopod morphology. However, in *A. suum* spermatozoa, treatment with weak acids caused loss of motility, disassembly of the MSP cytoskeleton and, consequently, compromised pseudopod integrity (Roberts and King, 1991). Thus, maintenance of a specific intracellular pH, putatively buffered by HCO^-_3, is crucial for pseudopod integrity and crawling of spermatozoa. Further, the MSP cytoskeleton polymerization involved in amoeboid movement (protrusion and retraction) is regulated by an intracellular pH gradient along the pseudopod (Italiano et al., 1999; King et al., 1994). Using the pH-sensitive fluorescent indicator BCECF, King, *et al* (1994), demonstrated an intracellular alkalizaiton of 0.25 pH units during activation of spermatids using *Vas deferens* extract (VDX) (King et al., 1994). The weak bases, TEA and NH_4Cl, induced a similar pH increase of 0.21 and 0.32 units respectively, although, only blebs or "spikes" were formed by the spermatid and no pseudopod was extended. In this study, they also showed an increase of 0.15 pH units between the tip of the pseudopod where alkalization correlates with MSP fibers assembly, and the base of the pseudopod where acidification promotes disassembly of the MSP cytoskeleton. Thus, mechanisms of intracellular pH regulation are involved in the trigger and maintenance of

motility forces in amoeboid sperm (Bottino et al., 2002).

Intracellular Ca^{2+}

The role of Ca^{2+} during nematode spermiogenesis has only been studied in *C. elegans*, however, these studies can give us insights not only on the physiological stages of Ca^{2+} signaling during pseudopod extension but also on the conserved role of this ion during exocytosis and fertilization. In 1980, Nelson and Ward showed that pseudopod extension is not induced with the Ca^{2+} ionophores A23187 nor X537A. External Ca^{2+} is not necessary either for the trigger of spermatid activation with any of the *in vitro* activators, Monensin, TEA nor Pronase, since removal of this ion from the medium has little or no effect on pseudopod extension (Nelson and Ward, 1980; Ward et al., 1983).

Intriguing results from Shakes and Ward in 1989 showed that the Calmodulin inhibitors Trifluoperazine (TFP), Chlorpromazine (CPZ) and Naphtalenesulfonamide (W7) can induce pseudopod extension, although the pseudopod is abnormal in that it lacks villar projections and is devoid of membrane movement (Shakes and Ward, 1989). Once the Calmodulin inhibitor is removed, the pseudopod morphology and movement recovers normally. The Ca^{2+} channel blocker nicardipine was reported in the same paper to have a similar effect. These investigators also observed that spermatids from the *spe-8* and *spe-12* mutants arrest in the "spike" stage after activation with Pronase. This spike arrest can be overcome and pseudopod extension occurs after treatment with TFP, and the other Calmodulin inhibitors, although TEA and Monensin have the same effect. All of these observations are reported to occur in the absence of external Ca^{2+}, suggesting the

involvement of internal Ca^{2+} stores. In contrast, it was shown that motile spermatozoa treated with the Calmodulin inhibitors stop motility and rounding up of the pseudopod. Shakes and Ward (1989) were not able to detect Calmodulin in spermatids using antibodies that cross-react with nematode Calmodulin (Shakes and Ward, 1989). Together, this evidence complemented with the known effects of TFP, CPZ and W7 on processes unrelated to Calmodulin, led the authors to conclude as unlikely the effect of these drugs on the inhibition of Calmodulin, since this would suggest a rather odd role of blocking spermiogenesis by this Ca^{2+}-binding protein.

Subsequently, the Ca^{2+}-binding protein, Calreticulin (CRT-1), was observed to be present in the cytoplasm of *C. elegans* sperm. The *crt-1* mutant spermatozoa appeared to have slightly shorter pseudopods and nuclei that were off center, suggesting a role for CRT-1 in the late stages of spermatogenesis (Park et al., 2001). The same research group discovered the presence of Calcineurin, a Ca^{2+}/Calmodulin-dependent serine/threonine protein phosphatase (PP2B) in *C. elelgans* sperm, with *cnb-1* mutants sharing the same phenotype as *crt-1* mutant sperm - short pseudopod and reduced size (Bandyopadhyay et al., 2002). Recently, Yi, *et al* (2009) identified a serine/threonine protein phosphatase 2A (PP2A) as a key element in the cytoskeletal dynamics that power *A. suum* sperm motility (Yi et al., 2009). Whether, Calmodulin, or a Calmodulin-dependant mechanism, regulates Ca^{2+} dynamics during pseudopod extension is still unclear, although a Ca^{2+} regulated machinery is certainly present in *C. elegans* spermatids. This Ca^{2+}-dependent machinery is complemented by FER-1, a protein of the ferlin family involved in the Ca^{2+}-mediated MO fusion to the plasma membrane (Washington and Ward, 2006). FER-1 is

characterized by multiple C2 domains that function as Ca^{2+} sensors and interact with phospholipids and proteins of the membrane fusion machinery during exocytosis (Bai and Chapman, 2004). It has also been demonstrated that intracellular Ca^{2+} stores are involved in the proper fusion of MOs to the plasma membrane since the membrane-permeable Ca^{2+} chelator BAPTA-AM prevented MO fusion in a concentration-dependent manner (Washington and Ward, 2006).

The final piece of evidence for Ca^{2+} dynamics during spermiogenesis involves a Transient Receptor Potential (TRPC) channel homologue, TRP-3 present in *C. elegans* spermatozoa and required for sperm-egg interaction during fertilization (Xu and Sternberg, 2003). As explained previously, TRP-3 is localized in the MO in spermatids and upon pseudopod extension the channel is translocated to the cell body due to its delivery from MOs fusion to the plasma membrane. Using the fluorescent Ca^{2+} indicator Fura-2, the authors investigated the mechanistic defect of *trp-3* sperm on calcium signaling. In Ca^{2+}-free medium, two types of Ca^{2+} influxes were observed in mature spermatozoa. One influx appeared to be the result of constitutively active Ca^{2+}-permeable channels (CAC) that open immediately upon introduction of extracellular Ca^{2+}. Intracellular Ca^{2+} stores regulated the second influx since treatment with ionomycin and thapsigargin induced a large Ca^{2+} influx after introduction of this ion in the medium. The authors described the latter as a Store Operated Ca^{2+} Entry (SOCE) channel. However, Ca^{2+} release from intracellular stores was not detected during treatment with ionomycin or thapsigargin. The SOCE response was reduced in *trp-3* mutant spermatozoa,

suggesting TRP-3 is necessary for SOCE activity and successful fertilization of the oocyte. In contrast, spermatids show minimal CAC activity and much lower SOCE.

Summarizing this evidence we can hypothesize that extracellular Ca^{2+} is not necessary to trigger pseudopod extension, although a release from intracellular stores is necessary for initiation of spermiogenesis and MO fusion. A Ca^{2+} signaling machinery is present in *C. elegans* sperm that involve Ca^{2+}-binding proteins such as Calreticulin and Calcineurin, a Calmodulin-dependent protein phosphatase homologue to PP2A involved in pseudopod extension in *A. suum*. Thus, Ca^{2+} fluctuations regulated by Calmodulin dependent mechanisms should be investigated in further detail in nematode sperm.

Protein phosphorylation.

Protein phosphorylation cascades are events that orchestrate spermiogenesis in nematode sperm. There is evidence from both *C. elegans* and *A. suum* that these events regulate the extension of a pseudopod. In the case of *C. elegans*, spermatogenesis-enriched genes have a higher presence of kinases and phosphatases than randomly expected (Reinke et al., 2000). The SPE group of proteins, responsible for triggering pseudopod extension, include members that are thought to be involved in protein phosphorylation as concluded by gene sequencing data. SPE-8 is an SH2-domain-containing non-receptor tyrosine kinase with an unknown substrate (WormBase, release WS203). SPE-6 is a serine/threonine Casein kinase 1 that upon inactivation by upstream SPE group members (including SPE-8) allows phosphatases to dephosphorylate the unknown target of SPE-6 (Muhlrad and Ward, 2002). Conversely, SPE-19 is a novel

protein with a predicted single internal transmembrane domain and a large number of serine/threonine phosphorylation sites on the putative intracellular portion (Geldziler et al., 2005). However, changes in protein phosphorylation during spermiogenesis have never been reported in *C. elegans*.

A substantial amount of evidence for signaling cascades involving protein phosphorylation in nematode sperm comes from studies in *A. suum*. The identification of molecular components that regulate polymerization of MSP leading to the acqusition of sperm motility has been facilitated by the *in vitro* reconstitution of fiber assembly (Italiano et al., 1996). These fibers were shown to elongate due to the activity of a cytoplasmic soluble factor and an integral vesicle protein generating the protrusive force necessary for psedupod extension (Roberts et al., 1998). Thus, accessory proteins responsible for MSP fibers elongations were identified in the cytoplasmic fraction and in membrane vesicles of *A. suum* spermatozoa. The first evidence for protein phosphorylation in MSP polymerization was that the *in vitro* reconstitution assay required ATP to elongate fibers (Italiano et al., 1996). Later work showed that the protein tyrosine phosphatase, YOP from *Yersenia enterocolitica*, blocked *in vitro* fiber assembly (Miao 2003), leading to the identification of the MSP Polymerization Organizing Protein (MPOP), an integral membrane phosphoprotein localized at the leading edge of the sperm lamellipod where its tyrosine phosphorylated state can be modulated by intracellular pH (LeClaire et al., 2003). Yi, *et al* in 2007 identified a serine/threonine Casein kinase 1, MSP polymerization-activating Kinase (MPAK), that is recruited by MPOP to the plasma membrane (Yi et al., 2007), regulating MSP polymerization by phosphorylation of the

MSP Fiber Protein 2 (MFP2), a protein that can bind MSP filaments and accelerate their elongation (Buttery et al., 2003; Grant et al., 2005). Curiously, MPAK shares homology with the Casein kinase 1 SPE-6 from C. *elegans* (Yi et al., 2007). Recently, additional proteins that regulate nematode sperm motility have been identified; such is the case of the already mentioned PP2A phosphatase that dephosphorylates MFP3, resulting in the disassembly of MSP filaments and retraction of the cell body (Yi et al., 2009).

Finally, we would like to point out that MPAK, MFP2 and a tyrosine-phosphorylated protein (putatively MPOP) are also involved in the extension of spikes or blebs during spermiogenesis and in the *in vitro* reconstitution of filopodia in *A. suum* (Rodriguez et al., 2005; Miao et al., 2007). The initiation of spermiogenesis, extension of the pseudopod and maintenance of motility are regulated by phosphorylation cascades in *A. suum* and the same might hold true for *C. elegans*. Both nematode spermatozoa possess a Casein kinase 1 that regulates the initiation of spermiogenesis and, putatively, the nucleation and elongation of MSP fibers during acquisition of motility. MFP3 also shows homology with the uncharacterized sperm-enriched proteins SSQ1-4 from *C. elegans*. Whether other proteins from this machinery are conserved between nematode sperm is a question that remains to be answered.

Nematode sperm metabolism, proteases and the connection between *in vitro* and *in vivo* activation of spermiogenesis.

Little is known about changes in cellular metabolism resulting in the extension and maintenance of the pseudopod in nematode sperm. While some nematode

spermatozoa such as *Nipposfrongyfus* (Wright and Sommerville, 1984), and *Nematospiroides* (Wright and Sommerville, 1985) activate and crawl under aerobic conditions, others including spermatozoa from *A. suum* appear to be obligate anaerobes that require high pCO_2 for activation and crawling (Sepsenwol and Taft, 1990). In motile *C. elegans* spermatozoa, Na Azide arrests pseudopod movement (Roberts and Ward, 1982b) and prevents spermatid activation together with other mitochondrial poisons such as Oligomycin and Dinitrophenol (DNP) suggesting that mitochondrial aerobic metabolism is necessary for pseudopod extension and motility (Ward et al., 1983). In contrast, 2-Deoxyglucose prevented pseudopod extension in spermatids activated with TEA but Pronase-activated spermatozoa were insensitive to this treatment. This inhibition could be rescued by the addition of fructose and led the authors to hypothesize a mechanism in which an increase in intracellular pH results in a release of a sperm protease that acts on a surface protein leading to membrane rearrangements and the subsequent initiation of aerobic metabolism. This hypothesis assumes that the pH-dependent release of the sperm protease relies on ATP generated by glycolysis, explaining why Pronase activation overcomes an increase in intracellular pH and is not affected by anaerobic metabolism (Ward et al., 1983). Although no endogenous protease activity has been identified during *C. elegans* spermiogenesis, this hypothesis fits with the recently reported novel function of SPE-4, a member of the spermiogenesis inhibitory pathway and a homologue of the human presenilin gene PS1 that encodes a protein with predicted proteolytic activity and known to perturb Ca^{2+} homoestasis (Gosney et al., 2008). These investigators propose a model in which FER-1 is a target of SPE-4

proteolytic activity, since both are present in MOs and a Ca^{2+} increase is necessary for fusion of these organelles with the plasma membrane. Thus, under this hypothesis, we can assume a role for anaerobic metabolism and intracellular pH in the activation of SPE-4, which ultimately leads to the fusion of MOs based on intracellular Ca^{2+} stores and the initiation of pseudopod extension and acquisition of motility under the control of aerobic metabolism.

To date, the *in vivo* extracellular initiation signals that lead to spermatid activation in nematode sperm are currently unknown. Sepsenwol and Taft (1990) suggested that according to their unpublished results, the *vas deferens* factor responsible for spermatid activation in *A. suum* is a pepsin-sensitive glycoprotein with a molecular mass of approximately 60 kDa (Sepsenwol and Taft, 1990). They further argued that their model is congruent with the inhibition of VDX activation by the serine-protease inhibitor PMSF, although serine proteases fail to initiate spermiogenesis in *A. suum*. In *C. elegans*, spermatids can be activated by Pronase, trypsin and chymotrypsin (Ward et al., 1983) and a recent paper has shown that the serine protease inhibitor SWM-1, which contains two trypsin-like inhibitor domains, is responsible for preventing the initiation of spermiogenesis in male seminal fluid (Stanfield and Villenueve, 2006). Currently, all evidence suggests a role for proteases triggering pseudopod extension, although the connection between this process and the downstream physiological responses that accompany pseudopod extension remain elusive.

Conclusion

The physiological activation of cell motility is a response triggered from cues in the environment. Spermatozoa from the deuterostomate lineage initiate motility due to environmental cues that affect intracellular processes such as cytosolic alkalization, increased ionic influx and increased metabolism, resulting in a modulation of flagellar beat parameters (Darszon 2008; Suarez 2008). Studies on the nematodes *C. elegans* and *A. suum* have revealed that these physiological responses are also true for the acquisition of motility in amoeboid spermatozoa, although the mode of locomotion is a pseudopod, rather than a flagellum. Recapitulating the steps that lead to pseudopod extension and acquisition of motility in nematode sperm, we have that a rearrangement and increased fluidity of the plasma membrane must have consequences in the biophysical properties of the membrane. Thus, cell surface protein redistribution in microdomains might be responsible for the maintenance of quality, duration, and strength of signaling cascades during pseudopod extension as proposed in other cell types (Golub et al., 2004). These rearrangements can be triggered by changes in ionic fluxes involving Na^+, K^+ and Cl^- ions, having a putative effect on membrane potential, and leading to the activation of a Ca^{2+}-dependent machinery necessary for MOs fusion and pseudopod extension. The maintenance of a pseudopod that confers motility to the sperm is achieved by an exquisite regulation and establishment of a pH gradient along the cell, coupled to protein phosphorylation in signaling cascades that orchestrate cytoskeletal dynamics. Altogether, these processes must have as an outcome the successful fertilization of an egg by a highly motile spermatozoon. Despite morphological differences, nematode sperm have recently

been considered as a good model for the development of human male contraceptives, due to the identification of sperm chromatin fertility factors that are evolutionary conserved among mice, humans and nematodes (Chu et al, 2006). Similar arguments may be made when comparing the common physiological factors that lead to sperm activation in these systems.

Thus, this work focuses on an integrative approach using proteomics, biochemistry, genetic and imaging techniques leading to a better understanding on the evolutionary conserved processes that lead to the acquisition of sperm motility in nematode sperm, using *C. elegans* as a model. Broadly, this dissertation is divided in three subsequent chapters that explore, first, the differences in the rate of activation and cellular protein phosphorylation pattern between male and hermaphrodite spermatids from *C. elegans*, second, the effect of cholesterol depletion on the rate of *in vitro* pseudopod extension leading to the acquisition of motility in male spermatids and third, the implication of protein phosphorylation during cytoskeletal dynamics that trigger pseudopod extension.

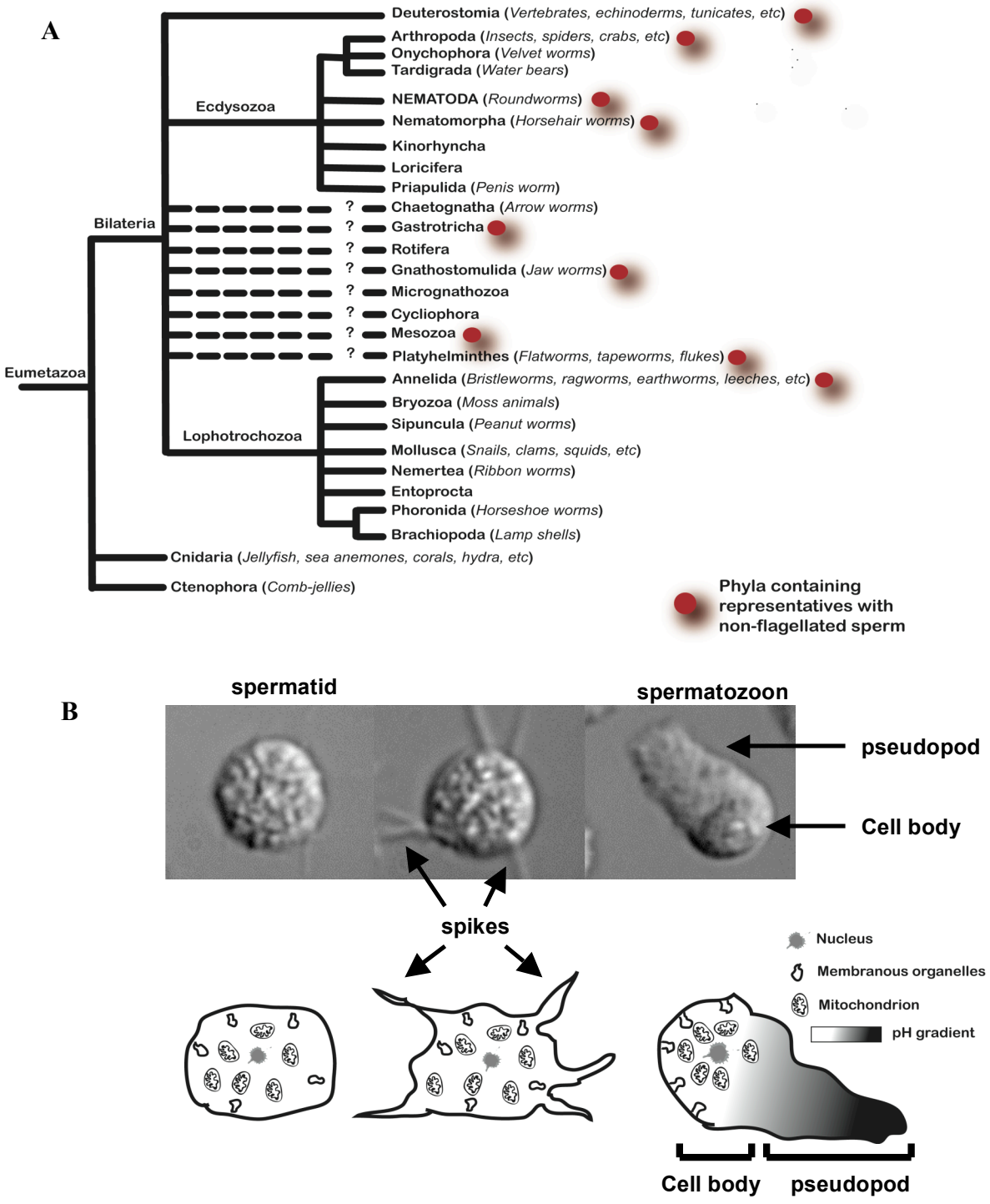
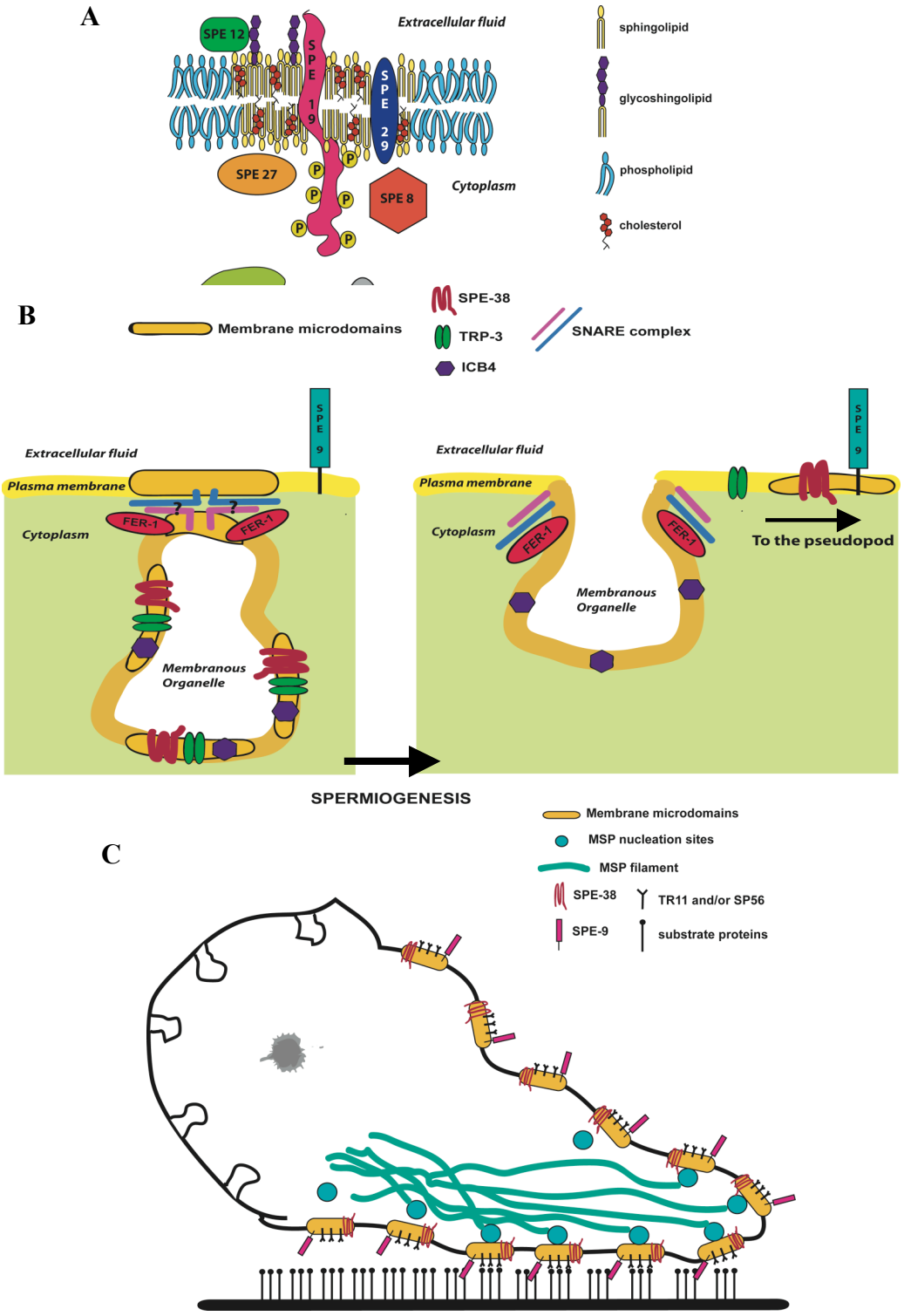


Figure 1.1. Not everyone needs a tail to get around.

Figure 1.2. Hypothetical involvement of membrane microdomains during pseudopod extension.



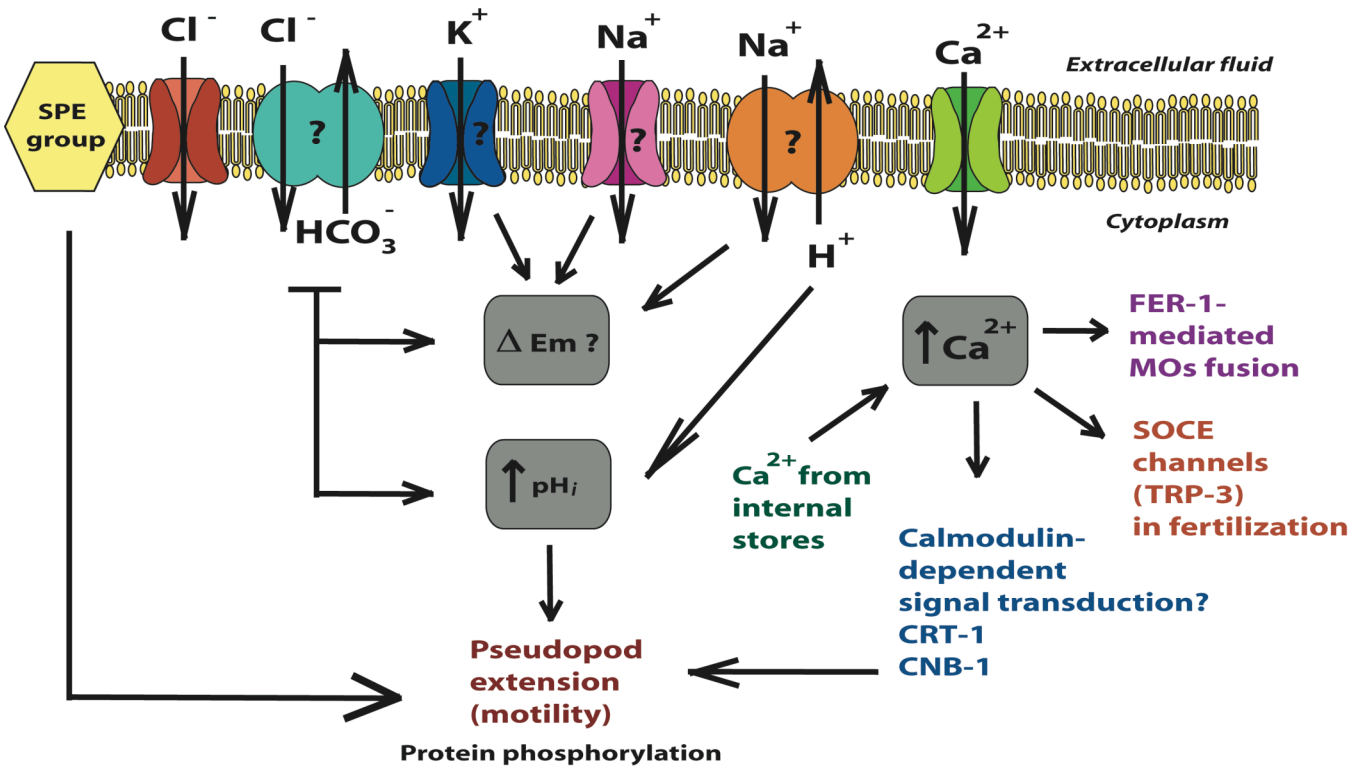


Figure 1.3. Ionic conductance plays an important role in the acquisition of motility in amoeboid sperm.

Figure legends for Chapter 1

Figure 1.1. Not everyone needs a tail to get around. Species with non-flagellated spermatozoa are more abundant in nature than previously realized opening new venues for physiological comparisons among sperm from different taxa. **A.** In the Eumetazoa lineage, non-flagellated sperm is not uncommon. In the phylogenetic tree, red circles represent the Phyla that contain at least one species bearing non-flagellated spermatozoa. (Phylogenetic tree modified from the Tree of Life Web project http://www.tolweb.org/ with information from Marrow, 2004). **B.** The Phylum Nematoda is representative of a taxonomic group where all the members possess motile amoeboid spermatozoa. Male gametes in this group lack a flagellum and an acrosome, and are characterized by the presence of Membranous Organelles that fuse to the membrane during acquisition of motility. Prior to pseudopod extension the cell must undergo physiological and morphological changes that include membrane rearrangements, extension of fillopodia-like projections (spikes) and the confinement of organelles into the cell body, while a pH gradient is formed from the tip to the base of the pseudopod.

Figure 1.2. Hypothetical involvement of membrane microdomains during pseudopod extension. Membrane microdomains ("lipid rafts") are sphingolipid- and cholesterol-enriched signaling platforms involved in the spatial and temporal regulation of processes triggered at the cell surface. **A.** The SPE group of proteins (involved in pseudopod extension) is an agglomerate of membrane and cytosolic proteins present in a multicomponent complex, that effect a signaling response upon an extracellular cue at the cell surface (model modified from Geldziler *et al*, 2004). **B.** The MOs are candidate sites for membrane rearrangements microdomains. The co-localization of putative cholesterol-enriched regions and proteins involved in sperm-egg interaction (that change distribution upon MOs fusion to the plasma membrane) supports the model for protein release and recruitment of microdomain-based rearrangements. **C.** The crawling of nematode spermatozoa involves discrete membrane regions of filament nucleation and substrate attachment that translocate proteins from the tip to the base of the pseudopod, this protein "recycling" could also involve molecules involved in sperm-egg fusion.

Figure 1.3. Ionic conductance plays an important role in the acquisition of motility in amoeboid sperm. Although studied in a lesser extent, ion fluxes are necessary for pseudopod extension and motility maintenance. The blockage of Clir channels induces initiation of spermiogenesis due to changes in the resting membrane potential and/or the accumulation (or transport) of HCO^-_3 into the cytoplasm, causing alkalization. Na^+ and K^+ ions are necessary for spermiogenesis and could also affect membrane potential. An alternative is that the exchange of Na^+ and H^+ (in a manner similar to the *in vitro* activator Monensin) can lead to an increase of intracellular pH, affecting also properties of the plasma membrane. Cytoplasmic alkalization initiates the establishment of of pH gradient that maintains motility in the pseudopod through MSP elongation and protein phosphorylation. Another upstream effector of protein phosphorylation is the SPE group of proteins that initiate spermiogenesis and the Ca^{2+}-dependent machinery that induces MO fusion to the membrane and putatively regulates the MSP cytoskeleton dynamics. The study of ion physiology in nematode sperm will lead us to an integrative understanding of the dynamic processes that lead to acquisition of sperm motility and fertilization in different taxa.

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Chapter 2

Physiological and molecular differences between male and hermaphrodite derived sperm in *Caenorhabditis elegans*

Abstract

Reproductive strategies in nematode species comprise gonochoristic, hermaphroditic, and parthenogenetic forms. In *C. elegans*, hermaphrodites produce sperm in their last larval stage and then switch to the production of eggs allowing for self-fertilization. Hermaphrodites can also mate with males to produce cross progeny. While both sexes produce sperm they do so in different organs, the hermaphrodite in the oviduct and the male in the testis. A signaling pathway that involves proteins of the SPE group triggers sperm motility. This group of proteins is responsible for the extension of a pseudopod that renders the spermatozoa motile and thus enables fertilization. Motility activation is initiated by ovulation in hermaphrodites and by contact with the female oviduct in males. However, little is known about the potential molecular and physiological differences between spermatozoa of males and hermaphrodites. In this chapter, differences in the rate of pseudopod extension and the protein patterns between sperm from males and hermaphrodite worms are presented and discussed.

Introduction

The phylum Nematoda is an extremely diverse group that contains both free-living and parasitic representatives. Reproductive success in this group depends on the number of developing embryos that can be produced by any of their reproductive strategies, comprising gonochorism, hermaphroditism and parthenogenesis (Poinar, 1983). The gonochoristic mode of reproduction (male-female mating system) is thought to be the ancestral state in the family Rhabditidae (Nematoda); and among the *Caenorhabditis* species, hermaphroditism is though to have evolved convergently in *Caenorhabditis elegans* and *Caenorhabditis briggsae* (Kiontke, *et al* 2004).

In *C. elegans*, adult individuals typically occur in two sexual types: hermaphrodites and males (see Figure 2.1A). Hermaphrodites have a karyotype of five autosomes and two sexual chromosomes (5AA, XX). They produce both sperm and eggs in a sequential fashion, starting with the production of sperm and, later on, switching to the production of oocytes. Due to the concomitant presence of sperm and eggs, hermaphrodites can self-fertilize (for review see Singson, 2001). The male form produces only sperm and has a karyotype of five autosomes and a single sexual chromosome (5AA, X0). Males can mate with hermaphrodites and produce cross-progeny. Sexual determination is regulated by a combination of pathways that involve somatic and germ cells sex determination and X-chromosome dosage compensation system (for review see Herman, 2005; Zarkower, 2005; Ellis and Schedl, 2005; Meyer, 2005* Wormbook).

The two sexual types show phenotypic similarities in body anatomy; however, specific sexual dimorphism is evident in their reproductive tissues (Bird and Bird, 1991). For instance, in adult worms, ~30-40% of the cells are specialized in structures and organs involved in mating and reproduction (Sulston and Horvitz, 1977; Herman, 2005). Examples of these are the vulva and spermatheca in hermaphrodites and the seminal vesicle and *vas deferens* in males (see Figure 2.1B).

Regarding sexual dimorphism in the gonads, many similarities and differences can be observed in their structure and function between adult hermaphrodites and males. In both, the reproductive tract is a tube-like structure organized in a distal-to-proximal manner (being distal the tip of the structure and proximal the part closer to where the gametes exit from the organism). Sperm and eggs undergo continuous gametogenesis as they move proximally in the reproductive tract (for review see L'Hernault, 1997; L'Hernault 2005). In the case of the hermaphrodite, the reproductive tract consists of a two-armed U-shaped gonad with each arm ending proximally at a spermatheca. Spermatids are produced at a late larval stage (L4) and completed in the young adult (prior to the production of eggs) when they accumulate in the proximal gonad arm. In the case of the male, the reproductive tract is a J-shaped testis with a seminal vesicle and a *vas deferens*. Spermatid production in the males is also initiated during L4 and continues through adulthood, being the seminal vesicle the site of spermatid storage.

Thus, sperm differ also between the two sexual types. For instance, male-derived sperm are larger than hermaphrodite-derived sperm conferring and advantage to the former during sperm competition (LaMunyon and Ward, 1995; LaMunyon and Ward, 1998). It has also been proposed that there are two sex-specific signaling pathways involved in pseudopod extension through the SPE group of proteins (for review see L'Hernault, 2005 and Singson, 2006). Furthermore, the transition from the sessile spermatid to a motile spermatozoon occurs at different times in the two sexual types. In the case of the hermaphrodite, the first ovulation pushes spermatids from the proximal gonad arm into the spermatheca where they become motile. In the male, spermatids extend their pseudopod after they are mixed with seminal fluid and ejaculated in the hermaphrodite uterus.

Summarizing this information, we have that spermatids from the two sexual types differ in the environment where they are produced (the ovary in hermaphrodites and the testis in males), the cellular size of spermatids (being male-derived sperm larger than hermaphrodite-derived) and the time at which spermatids become motile (during the first ovulation in hermaphrodites and during ejaculation in males). In this regard, the question becomes whether male and hermaphrodite-derived sperm differ in the molecular machinery that triggers pseudopod extension (including the SPE group of proteins) or it is a series of physiological changes what leads to the sex-specific differences in the acquisition of motility. In this chapter, these questions are addressed by comparing the rate of pseudopod extension between hermaphrodite and male-derived spermatids and the

protein composition in subcellular fractions of spermatids from both sexual types using two mutant strains.

Material and methods

Strains and worm synchronization. *Caenorhabditis elegans* strains CB1489: *him-8* (e1489) IV and JK816: *fem-3* (q20) IV, were maintained at 20^{o} C on NGM plates seeded with *Escherichia coli* OP50 as described by Brenner (1974). *him-8* males produce a **H**igh **I**ncidence of **M**ales (~ 40%) compared to ~0.1% males produced by wild type. This is the result of an increased frequency of X chromosome non-disjunctions during spermatogenesis, and thus an increased frequency of genotypically X0 males in self-fertile hermaphrodites populations (Hodgkin et al, 1979). Sperm in this strain are cytologically indistinguishable from wild-type sperm (Ward et al, 1981). The JK816 strain is a gain-of-function (*gf)* of the temperature-sensitive (*ts*) allele q20 of the gene *fem-3* (**FEM**inization of XX and XO animals). The *fem-3* gene encodes a novel protein that promotes male development in all tissues and is involved in the regulation of the sperm to oocyte transition in hermaphrodites (Barton, *et al* 1987; Ahringer and Kimble, 1991; Ahringer, *et al* 1992). Thus, in this strain, the XX germline (hermaphrodites) produces oocytes and excess sperm at 15-20º C (permissive temperature) and only sperm when worms are grown at 25º C. Culture synchronization was performed as described by L'Hernault and Roberts (1995) with some modifications. Briefly, worms were allowed to grow on plates to saturation for three days and the cultures were synchronized using the

alkaline hypochlorite method (35 ml of a bleach/NaOH mixture). Embryos were allowed to hatch for 24 hours in M9 buffer (22mM KH_2PO_4, 22mM Na_2HPO_4, 85mM NaCl, 1mM $MgSO_4$) supplemented with cholesterol and spotted on seeded NGM plates allowing the worms to grow for three days at 20º C (*him-8* strain) or 25º C (*fem-3* strain).

Small-scale sperm isolation method and microscopy. *C. elegans* sperm were isolated using a method described by L'Hernault and Roberts (1995). Synchronized adult male worms from the CB1489: *him-8* (e1489) IV strain, or adult hermaphrodite worms from the JK816: *fem-3* (q20) IV strain were picked from the synchronized plates and placed into a clean agar plate overnight at 20º C or 25º C. Worms were picked and placed in a 10µl drop of Sperm Medium (SM) on a glass slide. SM contained 50 mM HEPES, 25 mM KCl, 45 mM NaCl, 1mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM Dextrose, 10 mg/ml Polyvinylpyrrolidone (PVP) and 200 µg/ml Pronase, pH adjusted at 7.8. Worms were dissected by cutting the posterior end, in the case of males, or the proximal gonad arm close to the spermatheca, in the case of hermaphrodites, using a 20-gauge needle, allowing the release of spermatids. Two pieces of double-sided scotch tape were placed on the opposite edges of the glass slide and spermatids were covered with a 1½, 20x20mm coverslip to avoid evaporation. Nematode sperm activation can be observed using phase-contrast microscopy as suggested by previous studies (Nelson and Ward, 1980). Immediatley after male dissection and spermatid release, the cells were observed using a Nikon Labophot phase contrast microscope using a 40X objective. Time-lapse microscopy of pseudopod extension was performed using a Dage-MTI CDC 100 camera

mounted onto the microscope and using the software Scion Image v. 1.62 from NIH for capture and analysis.

Statistical analysis. After worm synchronization, the L1 worms obtained were divided into 6 different seeded plates. To avoid pseudoreplication and account for spermatids from a population of worms, three individuals form the same plate, were dissected in separate glass slides and pseudopod extension was recorded for each individual in a field of view using a 40x phase contrast objective. The number of spermatids undergoing pseudopod extension from the three individuals from the field of view was added up and the proportion of cells with pseudopod extended was calculated from this total. Each of these combined trials was considered an *n* and a total of *n*=6 were used to calculate the average proportion of pseudopod extension and the associated standard error (SE). The rate of pseudopod extension was calculated from the slope of the activation curve.

Male separation. Plates with adult worms from the CB1489: *him-8* (e1489) IV strain were washed using M9 buffer and filtered using a 35-µm Nitex filter. The filtrate (containing males and hermaphroditic juveniles) was transferred onto a 25-µm Nitex filter and washed using M9 buffer to eliminate juvenile worms. A worm population of ~ 95 % males was collected from the top of the 25-µm filter and poured on a 30 % Hypaque solution to remove bacteria and debris. Worms were centrifuged at 1000*g* for 10 min and recovered from the interface between the Hypaque solution and the M9 buffer.

Hermaphrodite separation. Plates with adult worms from the JK816: *fem-3* (q20) IV strain were washed using M9 buffer and poured on top of a 35-µm Nitex filter to discard any juvenile worm. The resulting hermaphrodite adults were poured on a 30 % Hypaque

solution to remove bacteria and debris. Worms were centrifuged at 1000*g* for 10 min and recovered from the interface between the Hypaque solution and the M9 buffer.

Large-scale sperm isolation method. *C. elegans* sperm were isolated using a method described by L'Hernault and Roberts (1995) with some modifications. Adult male worms from the CB1489: *him-8* (e1489) IV strain, or adult hermaphrodite worms from the JK816: *fem-3* (q20) IV strain were collected and washed in Modified Sperm Medium (MSM). MSM contained 50 mM HEPES, 70 mM Choline Chloride, 5 mM $CaCl_2$, 5mM Dextrose, Polyvinylpyrrolidone (PVP) 10 mg/ml, pH adjusted to 6.5 and supplemented with 10 µl/ml of protease inhibitor cocktail from Sigma (St. Louis, MO), 10 mM Na-Fluoride and 1 mM Na-orthovanadate as phosphatase inhibitors, to prevent activation of spermatids. Worms were centrifuged at 6,000 rpm using a benchtop centrifuge. The pellet of packed males was transferred to the bottom of a glass Petri dish and worms were chopped up using a stainless steel razor blade for approximately 5 min. The chopped worms were then washed from the Petri dish using MSM and passed through a 15µm Nitex filter. The filtrate was then poured through a 5µm Nitex filter and this second filtrate was poured on top of 2ml of Percoll 10 % and centrifuged at 1000*g* for 10 min. The supernatant was discarded and the pellet resuspended in 1 ml of MSM. Spermatids were counted using a hemocytometer and samples were fractionated to maintain a constant number of cells per isolation (~$4x10^6$ cells/ml). Cells were centrifuged again at 1000*g* for 10 min and the pellet was resuspended in 1 ml of Homogenization buffer (20 mM Tris, 20 mM HEPES, 30 mM Mannitol, 1 mM EDTA,1 mM EGTA, pH adjusted to 7.4) and laid on ice for sonication. The sample was sonicated using a Branson Sonifier

250 (VWR Scientific) using 4 pulses of 15 seconds each and resting on ice for 1 min between pulses. To clean the sample from cellular debris, nuclear chromatin and organelles, it was centrifuged at 10 000 *g* for 15 min (P10) and the supernatant (S10) was centrifuged at 100, 000 *g* for 1hr to separate the cytosolic and membrane fractions. The pellet (P100) consisted of the membrane fraction and it was resuspended in 10 µl of PBS and Laemmli sample buffer. The supernatant (S100) consisted of the cytosolic fraction and was concentrated using a microcon tube with a Molecular Weight Cut Off (MWCO) of 3 KDa from Millipore (Billerica, MA). The concentrate was resuspended in 100 µl of PBS and Laemmli sample buffer.

SDS-PAGE and Western blotting. SDS-PAGE was performed using 15 % gels according to the method of Leammli (Laemmli, 1970) and stained with silver. Gels for immunoblotting were transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA) as described by Towbin et al., 1979. Blocking of the membrane was performed in TBS-T (0.1 % Tween-20, 137 mM NaCl, 20 mm Tris, pH 7.6) with 1% bovine serum albumin (BSA) for 1 hr at room temperature and probed with primary antibody for 4 hours. The membrane was washed with TBS-T and probed using a secondary HRP-conjugated antibody for 4 hrs. Bands were observed using the Supersignal West Femto kit (Pierce, Rockford, IL).

Antibodies. Monoclonal antibodies antiphosphotyrosine, clone PY-20 was purchased from Sigma (St. Louis, MO). Secondary antibody used was: a goat anti-mouse IgG, H&L, conjugated to horseradish peroxidase (EMD Biosciences, San Diego, CA).

Results

Pseudopod extension in male and hermaphrodite spermatids

Pseudopod extension in male (*him-8*) and hermaphrodite (*fem-3*) spermatids was recorded using time-lapse microscopy. Unstained images of nematode sperm activation can be observed using phase-contrast microscopy, since samples yield contrast images by altering the amplitude differences between direct and deviated light. The body of the round spermatids, as seen under phase contrast, is bright and the plasma membrane is observed as a crisp dark line surrounded by a bright halo that represents viable cells (Figure 2.2A). In the case of activated spermatozoa, the pseudopod appears as a phase-dark region that protrudes form the cell body and cells that are entirely phase-dark can also acquaint for cell death (see Figure 2.2B). Immediately after worm dissection, images of pseudopod extension were acquired at a rate of 0.2 frames/sec for 100 sec. The proportion of spermatids that extended a pseudopod was plotted for both sexual types (Figure 2.3). A difference in the rate of pseudopod extension between the two sexual types was evident. Approximately, the half-time of pseudopod extension is ~ 30 sec for hermaphrodite (*fem-3*) sperm, while the half-time of pseudopod extension for male (*him-8*) sperm is ~ 50 sec. Thus, the rate of pseudopod extension seems to be slower in male-derived spermatids as compared to hermaphrodite-derived spermatids.

Protein pattern in spermatids from male and hermaphrodite adults

The SPE group of proteins is responsible for the trigger of pseudopod extension in *C. elegans* spermatids. The members of this group are signaling molecules that interact in a common transduction pathway (see Figure 2.4). The molecular weight of each of the known proteins present in the SPE group was calculated based on the predicted gene sequence for each protein (see Table 2.1). The samples resulting from the isolation of ~$4x10^6$ spermatids/ml and subsequent subcellular fractionation were loaded and run on a 15% SDS-PAGE gel to separate the proteins according to their molecular weight (see Figure 2.5). The three subcellular fractions (P100, S100 and P10) differ in their protein pattern and the S100 (putative cytosolic) fraction show a prominent band that corresponds to the molecular weight of the Major Sperm Protein (MSP), suggesting fidelity in the subcellular fractionation process. The fractions of sperm corresponding to *him-8* (male) and *fem-3* (hermaphrodite) samples were compared and the molecular weights of each of the SPE group of proteins were used as a reference for comparison between lanes (see Figure 2.5A). Not many changes are observed in the protein pattern of spermatid subcellular fractions between the two sexual types, except for the subtle change of intensity of three bands in the P100 (putative membrane) fraction, being slightly more prominent in the *fem-3* (hermaphrodite) fraction. This bands correspond to molecular weights of ~22, 35 and 45 kDa (black arrowheads in Figure 2.5A). One of this protein bands is located in the range of migration where SPE-19 should be present (~40 kDa).

In order to have a better understanding of changes in signaling pathways that may exist between spermatids of the two sexual types, the pattern of protein phosphorylation was explored using immunoblotting (see Figure 2.5B). The pattern of tyrosine phosphorylation in the two spermatid sexual types show differences in the P100 fraction in three bands at ~ 45 and 100 KDa (red arrowheads in Figure 2.5B). Interestingly, the male P100 fraction has protein bands with a higher intensity of tyrosine phosphorylation, although, in comparison to the hermaphrodite P100 fraction, the total protein band intensity seems fainter. The S100 (putative cytosolic) fractions didn't seem to have any significant differences in the pattern of tyrosine phosphorylation. However, the prominent band that migrates around the molecular weight of MSP seems to have signal of tyrosine phosphorylation. One band from the hermaphrodite P10 fraction seem to also have a higher intensity in tyrosine phosphorylation.

Discussion

C. elegans spermatids from male and hermaphrodite individuals differ in the environment where they are produced (male and hermaphrodite gonads), in the timing of pseudopod extension leading to the acquisition of motility (first ovulation in hermaphrodite and ejaculation in males), and in their cellular size (male-derived sperm larger than hermaphrodite-derived). These characteristics result in an advantage to male spermatozoa during sperm competition and are critical for male reproductive success and siring cross progeny (Singson, 2006). It is not clear whether two distinct male- and

hermaphrodite-specific *in vivo* activators regulate the difference in timing of pseudopod extension between the two sexual types, or male- and hermaphrodite-derived spermatids have a single or parallel signaling pathways leading to pseudopod extension (Shakes and Ward, 1989; Miniti *et al*, 1996; Nance *et al*, 2000; Muhlrad and Ward, 2002; Geldziler *et al*, 2005).

An alternative consideration is the effect of extracellular signals present in the environment where spermatids are stored. It has been shown that mating males to SPE mutant hermaphrodites promotes pseudopod extension (transactivation) in spermatids that are unable to undergo activation, suggesting that extracellular factors present in the seminal fluid can restore self sterility in this mutants. The results in this chapter show that spermatids produced by male and hermaphrodite individuals differ in the rate of *in vitro* pseudopod extension. Male spermatids responded in a slower fashion to Pronase-induced activation as compared to hermaphrodite spermatids. This might be due to the presence of such extracellular factors in male seminal fluid since spermatids isolated from males are immersed in this fluid in the seminal vesicle and after dissection. On the other hand, spermatids isolated from unmated hermaphrodites do not contain these fluids in their gonads. Stanfield and Villenueve (2006) identified SWM-1, a serine protease inhibitor composed of a signal sequence and two trypsin inhibitor-like (TIL) domains. This molecule is thought to be involved in the block of spermatid activation by regulating the activity of proteases responsible for the cleavage of membrane proteins (putatively members of the SPE group) that initiate a signaling cascade triggering pseudopod extension. Thus, protease inhibitors, such as SWM-1, might be acting on Pronase,

delaying its effect on the *in vitro* activation of male spermatids. In future work, the use of alternative *in vitro* activators (Triethanolamine or Monensin) should be explored to account for the rate of pseudopod extension using non-protease based activators.

To date, no comparison in the protein composition between male- and hermaphrodite-derived spermatids has been done. Here, the results show that spermatids produced by male and hermaphrodite individuals differ only in three bands at 22, 35 and 45 kDa present in the putative membrane fractions. One of these proteins is in the range of electrophoresis migration of SPE-19, a large size (~ 40 kDa) novel protein containing a predicted single internal transmembrane domain with a large number of predicted serine/threonine phosphorylation sites on the putative intracellular portion, making it more likely to be involved in the reception/transduction of activator signals. The pattern of protein tyrosine phosphorylation also showed a difference in band intensity of proteins ~ 40, 45 and 100 kDa, present on the putative membrane fraction between male and hermaphrodite sperm. Male-derived spermatids seem to differ in some proteins present in the putative membrane fraction in both abundance and the state of tyrosine phosphorylation. SPE-8, a member of the SPE group of proteins, is an SH2-domain-containing non-receptor tyrosine kinase with an unknown substrate cytoplasmic tyrosine kinase. SH2 protein domains mediate protein-protein interaction involved in signal transduction pathways by recognizing phosphorylated tyrosine residues and assembling key components of signaling pathways in response to extracellular signals (Schlessinger and Lemmon, 2003).

Altogether, these results suggest minimal differences in the molecular machinery that leads to pseudopod extension in male and hermaphrodite-derived spermatids. Since mature *C. elegans* spermatids are unable to synthesize proteins *de novo* (Pavalko and Roberts, 1989), posttranslational modification of the cytoplasmic pool of proteins must play an important role in controlling the signaling cascades that orchestrate acquisition of motility. In this chapter, the results show evidence for differences between male and hermaphrodite-derived spermatids in the response to *in vitro* activator Pronase and also a difference in posttranslational modification of proteins, in particular tyrosine phosphorylation, between spermatids from the two sexual types. These differences might be the result of extracellular signals that control the physiological response and timing of acquisition of motility in *C. elegans* sperm from both sexual types at the level of plasma membrane. To further explore the initial processes of spermatid activation and plasma membrane dynamics leading to pseudopod extension, the following chapter will focus on the use of imaging techniques to record *in vitro* pseudopod extension under treatments that promote cholesterol depletion on the plasma membrane.

Figures and Tables for Chapter 2

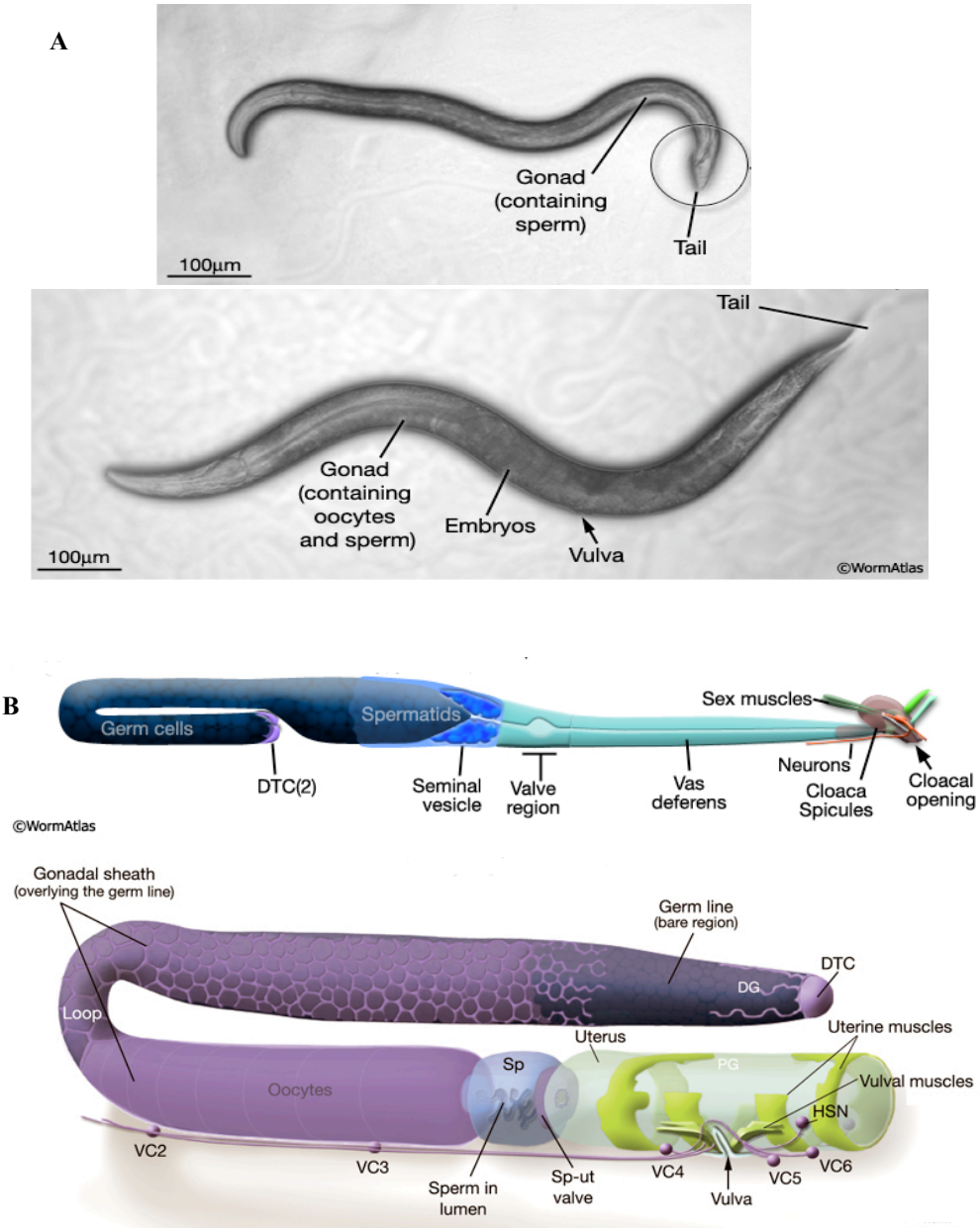

Figure 2.1. Anatomical differences between hermaphrodite and male gonads in adult *Caenorhabditis elegans*

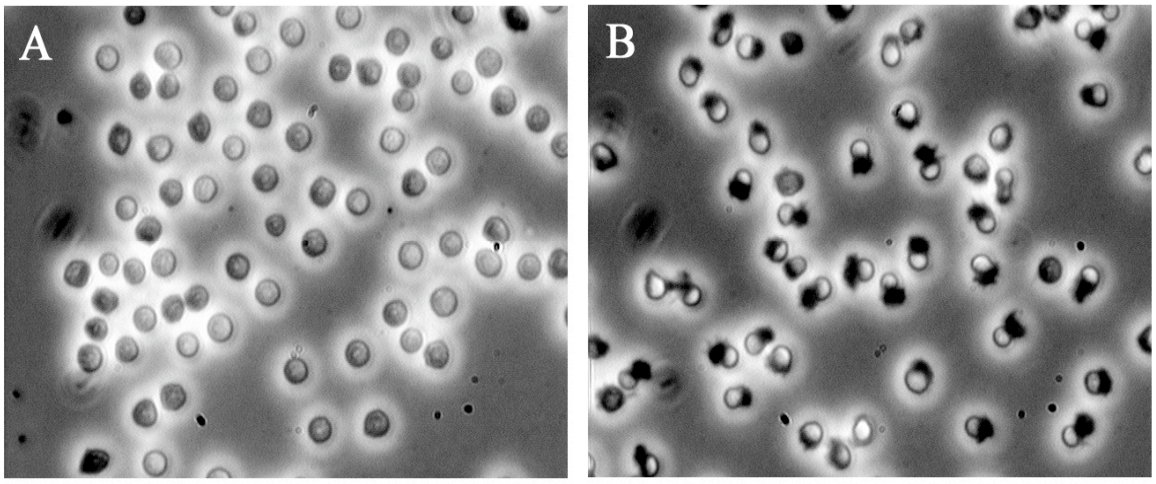

Figure 2.2. Pseudopod extension in *C. elegans* spermatids as seen under phase-contrast microscopy.

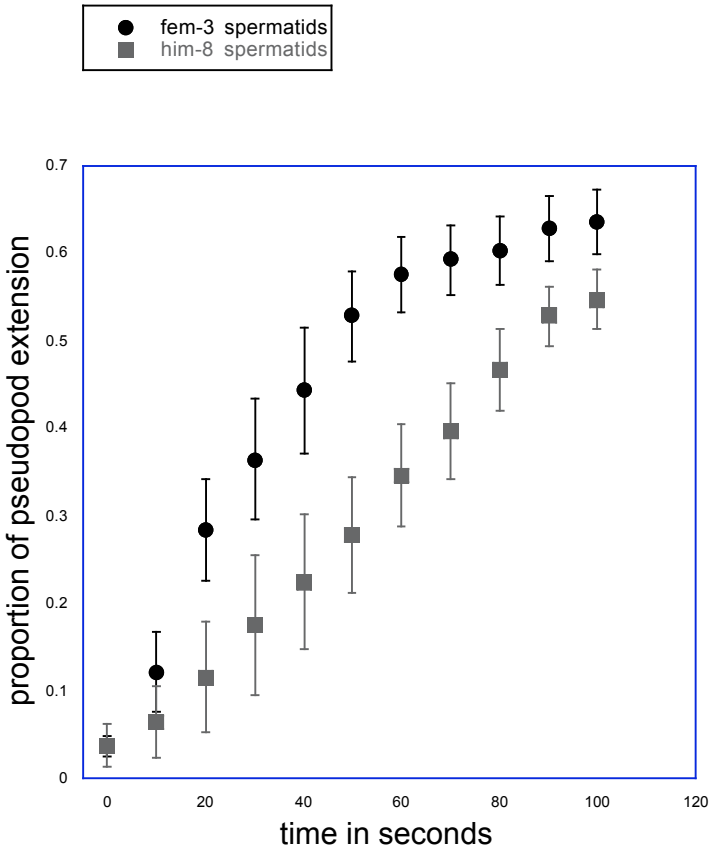

Figure 2.3. Proportion of pseudopod extension in male and hermpahrodite-derived spermatids.

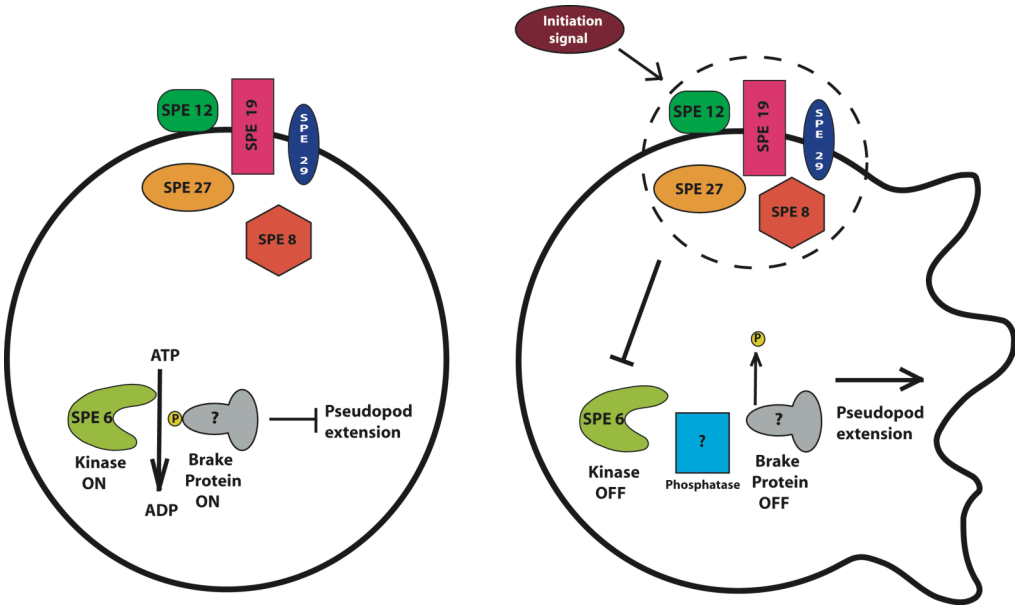

Figure 2.4. Genetic model for pseudopod extension in *C. elegans* spermatids

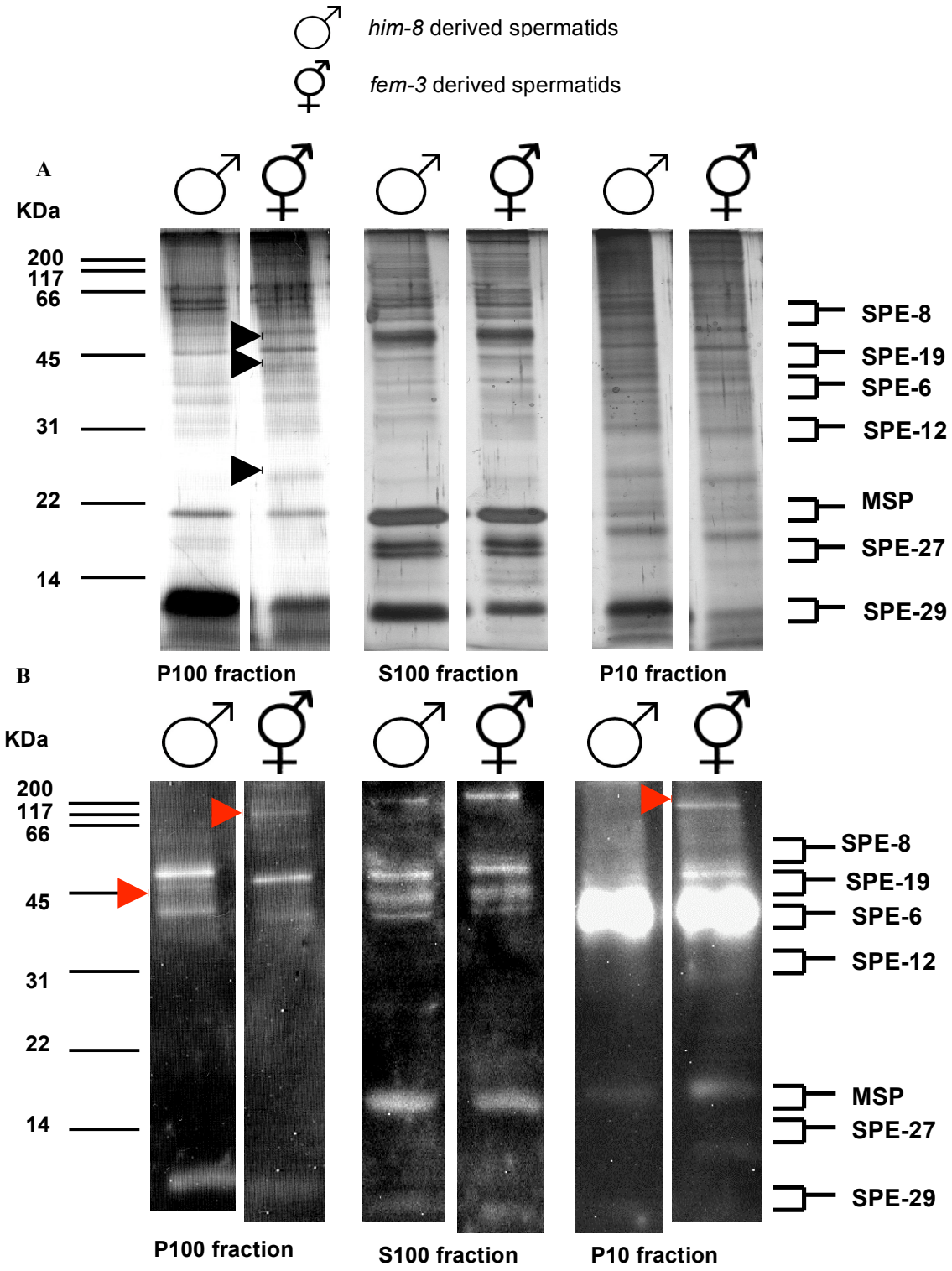

Figure 2.5. Protein pattern differences between male and hermaphrodite derived spermatids.

protein	characteristics
SPE-6 (cytoplasmic)	Putative Casein kinase ~ 379 a.a. MW ~ 45 KDa. Serine/Threonine kinase. Key player in the block of spermatid activation and possible involvement in MSP filament extension.
SPE-8 (cytoplasmic)	Cytoplasmic tyrosine kinase ~512 a.a. MW ~ 60 KDa. (novel protein)
SPE-27 (cytoplasmic)	Mostly hydrophilic with no extended regions of hydrophobicity ~131 a.a. MW ~15 KDa. (novel protein). Possible involvement of C-terminus in signaling.
SPE-12 (membrane)	Localized to the cell surface but does not span the membrane ~255 a.a. MW ~ 30-35 KDa (novel protein). Possible candidate for a protease-dependent activation as in G-protein coupled receptors.
SPE-29 (membrane)	Small size protein with a predicted transmembrane domain ~66 a.a MW ~ 10 kDa. (novel protein)
SPE-19 (membrane)	Large size protein with a predicted single internal transmembrane domain and a large number of serine/threonine phosphorylation sites on the putative intracellular portion ~300 a.a MW ~ 40 kDa. (novel protein)

Table 2.1. The SPE-8 group are signaling proteins involved in pseudopod extension .

Figure legends for Chapter 2

Figure 2.1. Anatomical differences between hermaphrodite and male gonads in adult *Caenorhabditis elegans*. (images taken from Wormatlas www.wormatlas.org) **A.**Two sexual types are found in *C. elegans* adults and both of them produce spermatids. **B.** Male adults produce spermatids in J-shaped testis containing a seminal vesicle, *vas deferens* and sexual muscles that control ejaculation. Hermaphrodite adults produce spermatids in a U-shaped gonad where in early embryonic stages spermatids are produced prior to the production of oocytes (only one arm of the gonad is shown in the picture above).. This gonad is characterized by the presence of a spermatheca where spermatids are stored and where fertilization occurs, the uterus where male spermatozoa are ejaculated and the vulva where fertilized eggs are laid.

Figure 2.2. Pseudopod extension in *C. elegans* spermatids as seen under phase-contrast microscopy. Unstained images of spermatids can be observed using phase-contrast microscopy. The body of the round spermatids is bright and the plasma membrane is observed as a crisp dark line surrounded by a bright halo. In the case of activated spermatozoa, the pseudopod appears as a phase-dark region that protrudes form the cell body and cells that are entirely phase-dark can also acquaint for cell death.

Figure 2.3. Proportion of pseudopod extension in male and hermpahrodite-derived spermatids. Spermatids from male (*him-8* gray squares) and hermaphrodite (*fem-3* black circles) adults were treated with Pronase to induce pseudopod extension and the proportion of cells undergoing this process was counted using time-lapse microscopy. A difference on the half-time of pseudopod extension in hermaphrodite spermatids (~ 30 sec) is different compared to male spermatids (~ 50 sec).

Figure 2.4. Genetic model for pseudopod extension in *C. elegans* spermatids. This diagram modified from (Geldziler *et al*, 2004) explain the model in which proteins from the SPE group act together in a common signaling pathway that promotes pseudopod extension upon an unknown initiation signal.

Figure 2.5. Protein pattern differences between male and hermaphrodite derived spermatids. A. Male (*him-8*) and hermaphrodite (*fem-3*) spermatids differ in very few bands, specifically in the P100 (putative membrane) fraction, where three bands of molecular weights 22, 35 and 45 kDa (black arrowheads). **B.** Consistently, the pattern of tyrosine phosphorylation presents slight changes between the two sexual types, specifically, in the P100 (putative membrane) fraction two bands of ~ 45 and 100 kDa and one band from the P10 fraction (red arrowheads).

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Chapter 3

The effects of cholesterol and membrane microdomains integrity on the dynamics of plasma membrane that lead to motility acquisition in *C. elegans* sperm

Abstract

Plasma membrane dynamics play an important role in the regulation and integrity of physiological signals that are necessary for the initiation of cytoskeletal regulation and motility. In the spermatozoa from the nematode *C. elegans*, the acquisition of motility through pseudopod extension is preceded by spermatid activation and the rearrangement of the plasma membrane. The genetic model for pseudopod extension proposes a multicomponent complex of proteins from the SPE group that interact blocking or triggering motility acquisition. This model can be translated into a physiological context by the involvement of membrane microdomains, which are discrete regions on the plasma membrane that control the temporal and spatial initiation of signaling cascades. Membrane microdomains are structures enriched in cholesterol, and changes in the proportion of this molecule affect the biophysical properties of the membrane. Whether membrane microdomains are present or not in *C. elegans* spermatids, has never been investigated. In this chapter, the effects of cholesterol addition or removal on membrane dynamics, and ultimately on pseudopod extension and the acquisition of motility are explored using *in vitro* activators and time-lapse microscopy together with biochemical techniques for the identification of membrane microdomains.

Introduction

Biological membranes are complex cellular structures that act as selective barriers between the internal and external environment. In particular, the plasma membrane is responsible for regulating homeostasis and the interactions of a cell to its substrate and adjacent cells. This regulation depends on membrane structure, integrity and dynamics due to the heterogeneous composition of lipids and proteins and the process of membrane remodeling (for review see Engelman, 2005; McMahon and Gallop, 2005). The consequences of membrane regionalization and remodeling can affect the quality, duration, and strength of signaling cascades that are important in dynamic responses such as cytoskeletal regulation, cell adhesion and motility (for review see Golub, 2004). The spermatozoa from the nematode *Caenorhabditis elegans* are highly motile amoeboid cells that must undergo a series of membrane rearrangements resulting in the acquisition of motility (Nelson and Ward, 1980; Roberts and Ward, 1982). During this process, a spherical sessile spermatid, with a highly fluid and dynamic cell surface, must extend transient membrane protrusions ("spikes") that can thicken and fuse to coalesce into a pseudopod (Shakes and Ward, 1989). Upon pseudopod extension, membrane flow ceases in the cell body, the nematode specific Membranous Organelles (MOs) fuse to the plasma membrane and pseudopodial membrane flow is initiated from the tip to the base (Roberts and Ward, 1982). This flow, coupled to a dynamic cytoskeleton assembly and disassembly, leads to the maintenance of motility in nematode sperm (Roberts and Ward, 1982b; Roberts and Stewart, 2000). Thus, the plasma membrane of amoeboid sperm is

an extremely dynamic structure that must regulate its integrity and the initiation of signaling cascades effected by protein interactions on the cell surface and the cytoskeleton.

Cholesterol is an important component of the plasma membrane and its structural characteristics enable it to dictate membrane organization and permeability (for review see Maxfield and Tabas, 2005). It has been shown that cholesterol depletion using ß-cyclodextrins can induce plasma membrane lipid reorganization leading to the block or trigger of different signal transduction pathways. These cellular processes include the inhibition of motility in human neutrophils (Pierini *et al*, 2002), T-cells (Gomez-Mouton *et al*, 2001) and breast cancer-derived cells (Mañes *et al*, 1999) and the initiation of a transmembrane signal that results in capacitation of mammalian spermatozoa (Visconti *et al,* 1999).

In the present chapter, the effects of cholesterol uptake and depletion on *in vitro* pseudopod extension in *C. elegans* sperm are explored using Pronase, Triethanolamine and Monensin to induce spermatid activation. Pronase is a mixture of proteases that cleaves proteins from the extracellular surface of the plasma membrane, while the effect of TEA and Monensin results in an increase of intracellular pH (Ward, *et al* 1983). In order to link the effects of cholesterol depletion on pseudopod extension, a biochemical approach was taken to search for the presence of Detergent-Resistant Membrane (DRM) fractions. The high content of cholesterol and sphingolipids present in membrane microdomains render them with two inherent biochemical properties, insolubility at 4º C

in Triton X-100 (TX100) detergent and light buoyant density after centrifugation. With such properties, DRMs can be biochemically isolated as correlates for membrane microdomains (Brown and Rose, 1992). This microdomains could act as the physiological multicomponent complex of proteins from the SPE group that prevent pseudopod extension in the genetic model of *C. elegans*.

Materials and methods

Worm synchronization and male separation. *Caenorhabditis elegans* strain CB1489: *him-8* (e1489) IV was maintained at 20^{o}C on NGM plates seeded with *Escherichia coli* OP50 as described by Brenner (1974). As described in the previous chapter, *him-8* males produce ~ 40% male progeny compared to ~0.1% males produced by wild type (Hodgkin et al, 1979) and their sperm are cytologically indistinguishable from wild-type sperm (Ward et al, 1981). Culture synchronization and male separation were performed as described by L'Hernault and Roberts (1995) with some modifications. Briefly, worms were allowed to grow on plates to saturation for three days and the cultures were synchronized using the alkaline hypochlorite method (35 ml of a bleach/NaOH mixture). Embryos were allowed to hatch for 24 hours in M9 buffer (22mM KH_2PO_4, 22mM Na_2HPO_4, 85mM NaCl, 1mM $MgSO_4$) supplemented with cholesterol and spotted on seeded NGM plates allowing the worms to grow for three days at 20^{o}C.

Small-scale sperm isolation method and microscopy. *C. elegans* sperm were isolated using a method described by L'Hernault and Roberts (1995). Synchronized adult male worms from the CB1489: *him-8* (e1489) IV strain were picked from the synchronized

plates and placed into a clean agar plate overnight at 20º C. Worms were picked and placed in a 10µl drop of either Monovalent Sperm Medium (MSM) or Sperm Medium (SM) on a 1½, 24x60mm coverslip. MSM contained 50 mM HEPES, 70 mM Choline Chloride, 1mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM Dextrose and 10 mg/ml Polyvinylpyrrolidone (PVP), pH adjusted at 6.5. SM contained 50 mM HEPES, 25 mM KCl, 45 mM NaCl, 1mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM Dextrose, and either 10 mg/ml Polyvinylpyrrolidone (PVP) or 1 mg/ml Bovine Albumin Serum (BSA), pH adjusted at 7.8. Worms were dissected by cutting the posterior end of males using a 20-gauge needle, allowing the release of spermatids. Two pieces of double-sided scotch tape were placed on the opposite edges of the 24X60mm coverslip and spermatids were covered with a 1½, 20x20mm coverslip to avoid evaporation. This preparation was mounted on top of an aluminum frame using adhesive tape for attachment. This mounting preparation was performed to achieve high magnification Video-Enhanced Contrast (VEC) microscopy in an inverted microscope and to facilitate *in vitro* activation assays (Weiss, 2006). Nematode sperm activation can be observed using differential Interference Contrast (DIC) microscopy as suggested by previous studies (Ward *et al*, 1983; Shakes and Ward, 1989; Rodriguez *et al*, 2005). After male dissection and spermatid release, cells were observed using a Zeiss Axiovert 10 microscope equipped with DIC optics, using a 100X oil immersion objective with a 1.3 numerical aperture (NA). Live cell time-lapse image acquisition of pseudopod extension was performed using a Hamamatsu C8484-05G digital CCD camera mounted onto the microscope and images were captured, processed and analyzed using the software SimplePCI v. 6.1 from Compix Inc., Smaging Systems.

***In vitro* spermatid activation for live cell imaging and treatments.** Pseudopod extension assays required continuous observation of spermatids using the coverslip preparation described above that consisted of a sandwich of two coverglasses of dissimilar size separated by tape and mounted on top of an aluminum frame the size of a regular slide, 0.8 to 1mm thick and absolutely flat, forming a perfusion chamber (see Weiss, 2006). Image acquisition was initiated and the solutions were perfused using a micropipette at one end of the slide. Spermatids were treated with either 10 mM water-soluble cholesterol in SM, 10 mM 2-OH-propyl-beta-cyclodextrin in SM, or 10 mM methyl-beta-cyclodextrin in SM for 10 min. Activation of spermatids was initiated by perfusing either 25 mM Triethanolamine in SM, 100 nM Monensin in SM, or 200 µg/ml Pronase in SM. In treatments with SM supplemented with 1mg/ml BSA, the cells were washed using SM supplemented with 10 mg/ml PVP and maximum activation was induced using 200 µg/ml Pronase.

Statistical analysis. After worm synchronization, the L1 worms obtained were divided into 6 different seeded plates. To avoid pseudoreplication and account for spermatids from a population of worms, three individuals form the same plate, were dissected in separate glass slides and pseudopod extension was recorded for each individual in a field of view using the 100X objective. The number of spermatids undergoing pseudopod extension from the three individuals from the field of view was added up and the proportion of cells with pseudopod extended was calculated from this total. Each of these combined trials was considered an *n* and a total of *n*=6 were used to calculate the average

proportion of pseudopod extension in each treatment and the associated standard error (SE). Results were compared and analyzed using a *t*-test for significant differences.

Male separation. Synchronized plates with adult worms were washed using M9 buffer and filtered using a 35-µm Nitex filter. The filtrate (containing males and hermaphroditic juveniles) was transferred onto a 25-µm Nitex filter and washed using M9 buffer to eliminate juvenile worms. A worm population of ~ 95 % males was collected from the top of the 25-µm filter and poured on a 30 % Hypaque solution to remove bacteria and debris. Worms were centrifuged at 1000*g* for 10 min and recovered from the interface between the Hypaque solution and the M9 buffer.

Large-scale sperm isolation method and SDS-PAGE. *C. elegans* sperm were isolated using a method described by L'Hernault and Roberts (1995) with some modifications. Male worms were collected and washed in Modified Sperm Medium (MSM) or Sperm Medium (SM). MSM contained 50 mM HEPES, 70 mM Choline Chloride, 5 mM $CaCl_2$, 5mM Dextrose, Polyvinylpyrrolidone (PVP) 10 mg/ml, pH adjusted to 6.5. SM contained 50 mM HEPES, 25 mM KCl, 45 mM NaCl, 1mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM Dextrose, and either 10 mg/ml Polyvinylpyrrolidone (PVP) or 1 mg/ml Bovine Albumin Serum (BSA), pH adjusted at 7.8. MSM or SM were additionally supplemented with 10 µl/ml of protease inhibitor cocktail from Sigma (St. Louis, MO) to prevent protein lysis. Worms were centrifuged at 6 000 rpm using a benchtop centrifuge. The pellet of packed males was transferred to the bottom of a glass Petri dish and worms were chopped up using a stainless steel razor blade for approximately 5 min. The chopped worms were then washed from the Petri dish using MSM or SM and passed through a 15µm Nitex

filter. The filtrate was then poured through a 5µm Nitex filter and this second filtrate was poured on top of 2ml of Percoll 10 % and centrifuged at 1000*g* for 10 min. The supernatant was discarded and the pellet resuspended in 1 ml of MSM or SM. Spermatids were counted using a hemocytometer and cells were centrifuged again at 1000*g* for 10 min. The pellet was resuspended in 1 ml of Homogenization buffer (20 mM Tris, 20 mM HEPES, 30 mM Mannitol, 1 mM EDTA,1 mM EGTA, pH adjusted to 7.4) and laid on ice for sonication. The sample was sonicated using a Branson Sonifier 250 (VWR Scientific) using 4 pulses of 15 seconds each and resting on ice for 1 min between pulses. To clean the sample from cellular debris, nuclear chromatin and organelles, it was centrifuged at 10 000 *g* for 15 min (P10) and the supernatant (S10) was centrifuged at 100, 000 *g* for 1hr to separate the cytosolic and membrane fractions. The pellet (P100) consisted of the membrane fraction and it was resuspended in 10 µl of PBS and Laemmli sample buffer. The supernatant (S100) consisted of the cytosolic fraction and was concentrated using a microcon tube with a Molecular Weight Cut Off (MWCO) of 3 KDa from Millipore (Billerica, MA). The concentrate was resuspended in 100 µl of PBS and Laemmli sample buffer. SDS-PAGE was performed using 15 % gels according to the method of Leammli (Laemmli, 1970) and stained with silver to visualize the pattern of protein migration.

Isolation of Detergent-Resistant Membrane (DRM) fractions. Lipid rafts are isolated by means of their hydrophobic nature. Spermatids were isolated in MSM or SM and were resuspended in 200 µl 0.1% Triton X-100 (in homogenization buffer supplemented with protease inhibitors) at 4º C, sonicated and incubated at this temperature for 30 min. The

sample was mixed with Optiprep® Gradient Density Medium from Sigma (St. Louis, MO) to a 40% final concentration. A continuous gradient was formed by layering on top Optiprep® concentrations of 35, 30, 25, 20, 15, 10, 5 and 0%. Once the gradient was layered, the sample was centrifuged at 200 000*g* for 4 hrs at 4º C in a Beckman TL-100 ultracentrifuge using a fixed-angle TLA100.2 rotor. The gradient was collected by fractions after centrifugation, concentrated using microcon tubes with a Molecular Weight Cut Off (MWCO) of 3 KDa from Millipore (Billerica, MA), and concentrates were resuspended in Leammli sample buffer for SDS-PAGE in 15% gels as described above. Gels were stained with silver to visualize the pattern of protein migration.

Results

In vitro activators of *C. elegans* spermatids.

Pseudopod extension in male (*him-8*) spermatids was recorded using Live-cell time-lapse microscopy. Unstained images of nematode spermatid activation can be observed using DIC microscopy, since samples yield high-contrast optical images of the edges of objects by using a prism to split a polarized light beam into two rays that vibrate perpendicular to each other, the two rays pass through the specimen where the wave paths are altered and interfere with each other when brought back together using a second prism. The variation in optical density of the sample results in one side of the detail appearing brighter and the other side darker, giving a pseudo three-dimensional appearance to the specimen revealing finer details in the image. The body of the round

spermatids, as seen under DIC microscopy, is a granulose 3-D sphere avoiding the bright halo present in phase-contrast microscopy and allowing for more details during "spike" extension (Figure 3.1A, B). In the case of activated spermatozoa, the pseudopod appears as a 3-D region that protrudes form the cell body (see Figure 3.1B, C).

Triethanolamine, Monensin and Pronase are known *in vitro* activators of *C. elegans* spermatids. In this work, pseudopod extension was induced by perfusion of these activators in the microscope chamber described in the materials and methods section. Spermatids were dissected from male worms in Monovalent Sperm Medium (MSM) to prevent activation or in Sperm Medium (SM) to assess the proportion of spermatids that undergo spontaneous pseudopod extension under physiological conditions that promote activation. Pseudopod extension induced with 200 μg/ml Pronase was recorded for a maximum of 15 minutes, using different acquisition times that ranged from 2 to 10 seconds between frames, until maximum proportion of activated spermatids was reached. In the case of the other activators, previously published optimal concentrations were used (Nelson and Ward, 1980; Ward, *et al* 1983). Thus, 25 mM Triethanolamine (TEA) was perfused and recordings were performed for 30 min, with acquisition times between 5 to 20 seconds between frames. Monensin was perfused at a concentration of 100 nM and recordings were obtained for 35 min at an acquisition rate similar to TEA recordings. For both TEA and Monensin-activated spermatids, cells were treated after their activation times with Pronase and recorded for 5 min to ensure cell viability. The proportion of spermatids that reached pseudopod extension in the two different mediums (MSM and SM) was very low (0.6 % ± 0.3 SE for MSM and 1.51% ± 0.4 SE for SM) suggesting

that ionic support and an acidic medium are not sufficient for pseudopod extension. Treatment of spermatids with the *in vitro* activators induced pseudopod extension in a high proportion of cells. The proportion of spermatids undergoing pseudopod extension was different among the three activators (see Figure 3.2). TEA 25 mM induced pseudopod extension in 51.8 % ± 1.1 SE of the spermatids, while Monenisn 100 nM induced pseudopod extension in 84.9 % ± 2.5 SE of spermatids. The most effective activator was Pronase, inducing pseudopod extension in 96.3 % ± 0.76 SE of spermatids. However, when analyzing the time-lapse sequences, a main difference can be seen in the process of pseudopod extension and motility acquisition. When using Pronase to induce activation, the spermatids respond rapidly to the stimulus, and although pseudopods are formed, no motility was acquired by this cells (see Supplemental video SV1). In the case of TEA and Monensin-induced pseudopod extension, spermatids were able to crawl and translocate in different directions, with the only difference that spermatids activated with TEA undergo a process of pseudopod extension and retraction. In the TEA treatment, the pseudopod is eventually formed and the cell can crawl normally (see Supplemental video SV2). In regards of the acquisition of motility by means of pseudopod extension, Monensin resulted the most efficient activator and although, it takes ~30 min for spermatids to extend their pseudopod, this activator induces almost 85% pseudopod extension and a high proportion of motile cells (see Supplemental video SV3).

Role of cholesterol on *in vitro*-induced pseudopod extension.

In order to account for the role of cholesterol dynamics during pseudopod extension, spermatids were treated with either 10 mM water-soluble cholesterol for 10 min, or with known cholesterol acceptors, such as Bovine Serum Albumin (BSA) 1 mg/ml, 10 mM methyl-beta-cyclodextrin (m-beta-cyclo) or 10 mM 2-OH-propy-beta-cyclodextrin (2-OH-p-beta-cyclo). Subsequent activation of cells was induced with TEA, Monensin or Pronase in the concentrations reported above. The acquisition times and the cell viability test were performed as explained above for the previous recordings. The results of these treatments on pseudopod extension are presented for each *in vitro* activator. In general, neither BSA nor the cyclodextrins were sufficient to induce pseudopod extension in spermatids. In particular, Pronase-induced pseudopod extension was affected by the treatment with 10 mM water-soluble cholesterol, although a statistically significant difference was not found ($p = 0.01$). This effect is more evident when observed in the time-lapse sequences. Cholesterol treatment promoted the formation of longer "spike" protrusions and an aberrant pseudopod was extended when activation was successful (see Supplemental video SV4). On the other hand, cholesterol depletion with BSA or cyclodextrins did not have a statistically significant effect on the proportion of pseudopod extension ($p = 0.01$) (see Figure 3.3).

For Monensin-induced pseudopod extension, treatments with cholesterol depletors BSA or cyclodextrins did not have a conspicuous effect in spermatid activation ($p = 0.01$), however, treatment of spermatids with 10 mM water-soluble cholesterol,

greatly prevented *in vitro* activation by reducing the proportion of spermatids that extended a pseudopod to 1.25 % ± 0.51 SE (see figure 3.4). After cholesterol treatment and Monensin-induced activation, the proportion of spermatids that extended a Pronase-induced pseudopod reached 85.06 % ± 4.3 SE.

TEA-induced pseudopod extension was also affected after treatment with 10 mM water-soluble cholesterol, reducing the proportion of spermatid activation to 6.03 % ± 1.56 SE. After cholesterol treatment and TEA-induced activation, the proportion of spermatids that extended a Pronase-induced pseudopod reached 58.14 % ± 6.9 SE. The main effect in TEA activation was seen after cholesterol depletion with 1mg/ml BSA, 10 mM m-beta-cyclo or 10 mM 2-OH-p-beta-cyclo. The proportion of induced pseudopod extension after these treatments increased to 81.97 % ± 2.0 SE, 81.11 % ± 3.3 SE and 78.29 % ± 1.73 SE, respectively (see figure 3.5).

As an indirect way to account for the effect of cyclodextrin treatment on spermatids, the cells were subjected to subcellular fractionation to compare the protein pattern between treatments with BSA and cyclodextrins. In this assay, a change in the protein pattern or band intensity of treated spermatids is expected since cholesterol depletion promotes a reorganization of the plasma membrane. Comparing the P100 (putative membrane) fraction of spermatids between treatments, we can observe a decrease in band intensities and some changes in the protein patters of spermatids depleted of cholesterol through BSA or cyclodextrins. Strikingly, some changes are also evident when cells are incubated in MSM pH 6.5 as compared to SM pH 7.8, suggesting that the presence of Na^+ and K^+ ions coupled to an increase of pH in the medium are

sufficient to induce changes in the protein pattern in the P100 fractions of spermatids (see Figure 3.6). In order to test whether, the treatment was affecting only the plasma membrane, the subcellular fraction S100 (putative cytosolic fraction) was also compared. Interestingly, it can be observed that treatment with Pronase dramatically reduces band intensity in the P100 fraction of spermatids, suggesting a massive loss of proteins from the extracellular leaflet of the plasma membrane, a band that remained present in this fraction corresponds to the molecular weight of MSP, a cytoskeletal protein responsible for pseudopod extension, which is thought to nucleate filaments in the cytoplasmic leaflet of the plasma membrane.

Detergent-resistant membrane (DRM) fractions in *C. elegans* spermatids.

The presence of membrane microdomains in cells can be accounted by the isolation of DRMs due to their insolubility in detergents and light buoyancy. Spermatids isolated in MSM or SM were subjected to detergent extraction of DRMs. Consistent with the differences observed on the P100 fractions isolated in SM and MSM, the DRMs isolated in MSM contained 3 bands that were not present when spermatids were isolated using SM (see Figure 3.7) suggesting also that ionic support and an acidic medium is sufficient for DRMs disruption. The bands present in the DRMs migrated to ~ 40, 30 and 16 kDa. And, at least two of these bands (40 and 30 kDa) are in concordance with the predicted molecular weights of the membrane proteins from the SPE group SPE-19 and SPE-12 (see table I from chapter 2). The ~ 16 kDa band is in the migration range of MSP.

Discussion

The *in vivo* initiation signal that triggers pseudopod extension in spermatids of *C. elegans* is still unknown to date. Previous studies have identified SWM-1, a serine protease inhibitor thought to be involved in the block of spermatid activation by regulating the activity of proteases responsible for the cleavage of membrane proteins (Stanfield and Villenueve, 2006). The target for these proteases could be a protein member of the SPE group that initiate a signaling cascade triggering pseudopod extension. A likely target for the activator signal is SPE-19, a novel signaling protein present in the multicomponent complex of proteins that regulate pseudopod extension (Geldziler, *et al* 2005; Singson, 2006). An alternative hypothesis to the triggering of spermatid activation through proteolytic cleavage of proteins is the disruption of membrane structures that cluster proteins together to block or trigger a signaling pathway. Such hypothesis is explained by the model of membrane microdomains as regions of the plasma membrane enriched in cholesterol and sphingolipids (see Golub *et al*, 2004). Phospholipid bilayers in model membranes can be present in two different phase states, a liquid-disordered (l_d) state in which the phospholipids acyl chains are fluid and disordered, or a liquid-ordered (l_o) state in which increasing amounts of cholesterol confer order to the acyl chains of lipids (for review see Brown and London, 1998). Thus, it can be hypothesized that addition of cholesterol to spermatids separates the l_d phase of the plasma membrane to a l_o phase, changing its biophysical properties and, consequently, the response to pseudopod extension. It has been postulated that changes in

the plasma membrane lipid composition of the of mammalian spermatozoa, can alter the bulk biophysical properties of the membrane by changing membrane fluidity and altering the activity of ion channels and enzymes involved in capacitation, a necessary step for successful fertilization (Kopf *et al*, 1999). In this chapter, *in vitro* activation of spermatids to induce pseudopod extension and further acquisition of motility was studied and the effect of cholesterol addition and depletion from spermatids was analyzed with time-lapse microscopy. It is evident that an increase in the amount of cholesterol in *C. elegans* spermatids disturbed the process of pseudopod extension. In the case of Pronase-induced spermatid activation, cells decreased significantly the proportion of pseudopod extension, this effect was more evident when observing time-lapse sequences and it can be proposed that a l_d state of the membrane is necessary to allow proper extension of the pseudopod. This is corroborated by the total inhibition of TEA and Monensin-induced pseudopod extension by the addition of cholesterol. Although, the different means of action of each *in vitro* activator must also be considered. Pronase is an aggressive activator that cleaves most of the proteins from the extracellular surface of the plasma membrane, putatively initiating pseudopod extension by cleaving also the target of the *in vivo* activator. However, we must also consider that Pronase-induced activation can also be affecting ion cannels and other types of receptors that might be present in *C. elegans* spermatids promoting the physiological trigger of pseudopod extension (see chapter 1). Since Monensin and TEA act by increasing the intracellular pH of the spermatid, and their actions depend on plasma membrane insertion (in the case of the ionophore Monensin) or diffusion through the plasma membrane (in the case of TEA), the possibility of a

decreased effect due to the prevention of insertion or diffusion of these activators through a l_o phase state can not be ruled out.

On the other hand, cholesterol depletion had the opposite effect on spermatid activation. In the case of Pronase and Monensin-induced pseudopod extension, no conspicuous changes were detected between treatments with BSA, m-beta-cyclo or 2-OH-p-□-cyclo, probably due to the fact that these activators induce a very high percentage of activation already. In the case of TEA-induced pseudopod extension, the increase in the proportion of cells extending a pseudopod was evident since the efficiency of TEA reaches only ~50%, thus the effect of BSA, m-beta-cyclo or 2-OH-p-beta-cyclo can be appreciated. It can also be postulated that a l_d phase state increases diffusion and membrane dynamics, promoting pseudopod extension. A remodeling of the plasma membrane in *C. elegans* spermatids, after cholesterol depletion, is suggested by the effect of BSA and cyclodextrins on the P100 (putative membrane) fractions. The presence of membrane microdomains was also corroborated by the extraction of DRMs from spermatids isolated in MSM, although, the presence of Na^+ and K^+ ions and a pH of 7.8 in SM prevented the extraction of these subcellular fractions. It is puzzling to think that the presence of certain ions can remodel the structure of plasma membrane, although, we might also consider this result as a concomitant effect of the physiological changes that occur in the plasma membrane upon the presence of cholesterol. Previous studies have reported modulatory effects of cholesterol on membrane associated ion transporters such as □-aminobutyric acid (GABA) transporters and Na^+, K^+-ATPase (Vemuri and Philipson, 1989; Shouffani and Kanner, 1990). In mammalian spermatozoa, the loss of

cholesterol from the plasma membrane has been correlated with the increase of intracellular pH during capacitation (Cross and Razy-Faulkner, 1997). Thus, it can be proposed that an increases in intracellular pH, and an initiation of ion influxes result in the triggering of signaling events that lead to cholesterol loss from the plasma membrane, facilitating spike protrusion and further pseudopod extension. It is interesting to note that, previous to pseudopod extension, *C. elegans* spermatids bud vesicles that contain the Major Sperm Protein (MSP) and these vesicles promote meiotic maturation in oocytes (Kosinski *et al*, 2005). Vesicles are budded from discrete sites from the spermatid plasma membrane and the signaling for oocyte maturation is reduced when spermatids undergo pseudopod extension.

Summarizing, the results in this chapter support a model in which membrane microdomains play an important role in the block of pseudopod extension by clustering proteins (putatively from the SPE group). Increasing the content of cholesterol in the plasma membrane of spermatids (increasing its l_o state) prevents pseudopod extension, while, depletion of cholesterol (increasing the l_d state of the membrane) promotes a remodeling of membrane microdomains and a trigger of plasma membrane dynamics that might include vesicle budding and further extension of the pseudopod for oocyte maturation and motility acquisition, respectively. The *in vitro*-induced pseudopod extension assays performed in this chapter revealed insights in the role that cholesterol plays during spermatid activation in which membrane microdomains are very likely orchestrating the dynamics that lead to the acquisition of motility. The following chapter will focus on the ultimate molecular target that promotes filament formation and

pseudopod extension, the Major Sperm Protein (MSP) since the results in chapter 2 suggest phosphorylation of this protein and the results in this chapter suggest its localization in the inner leaflet of the plasma membrane where it can be present in membrane microdomains and signaling for either oocyte maturation or filament elongation.

Figures for Chapter 3

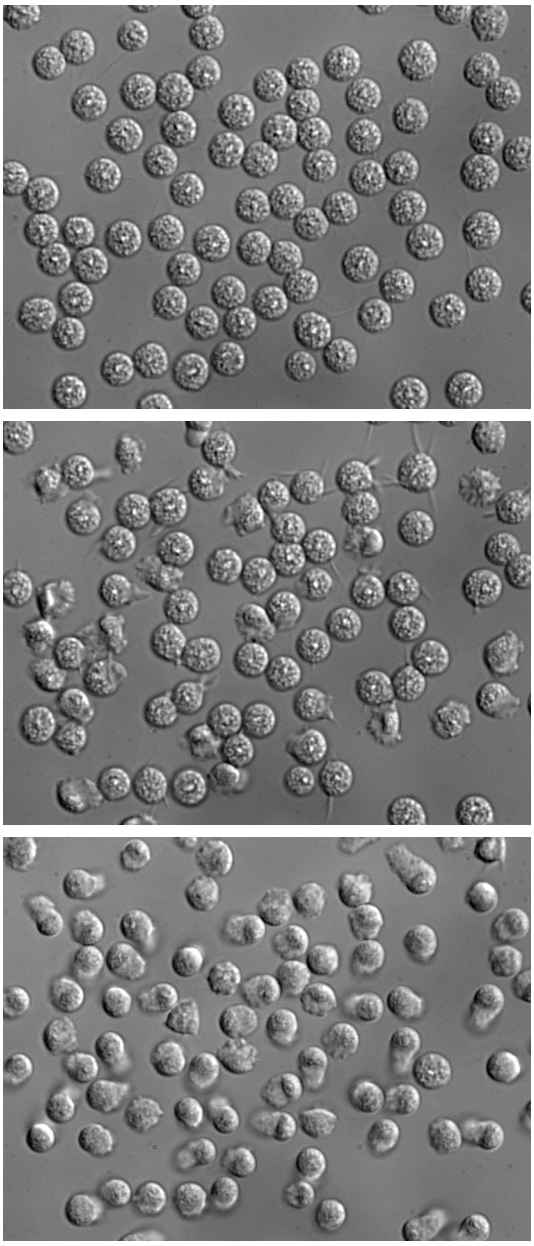

Figure 3.1. Pseudopod extension in *C. elegans* spermatids as seen under DIC microscopy.

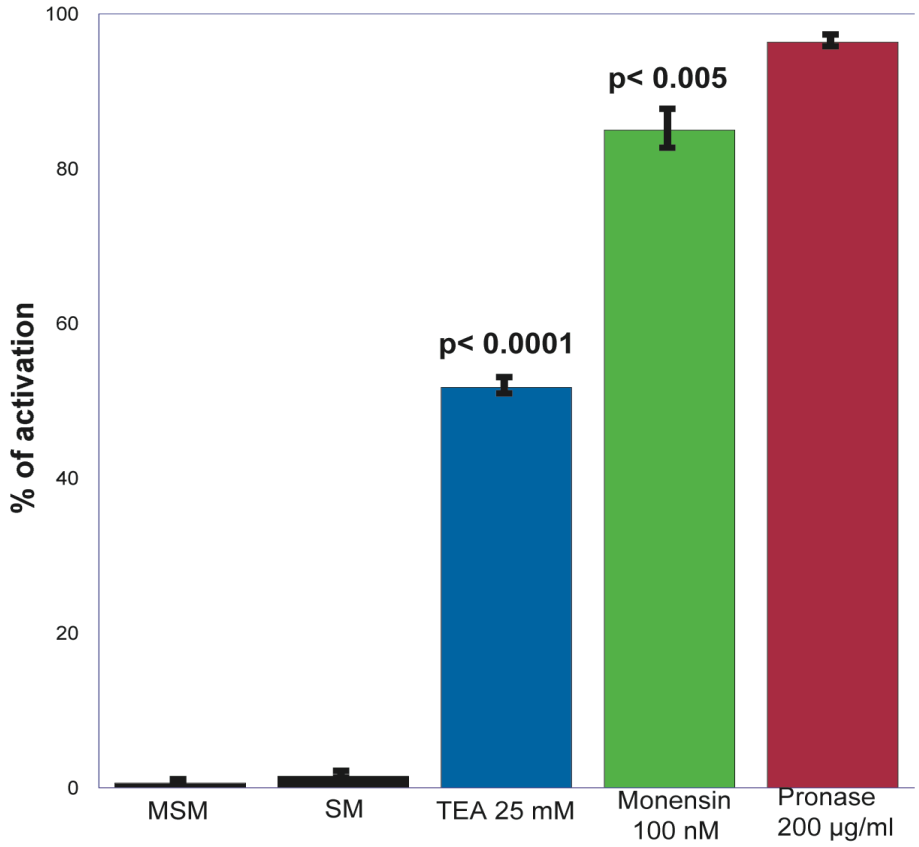

Figure 3.2. Proportion of *in vitro* induced pseudopod extension (activation) in *C. elegans* spermatids.

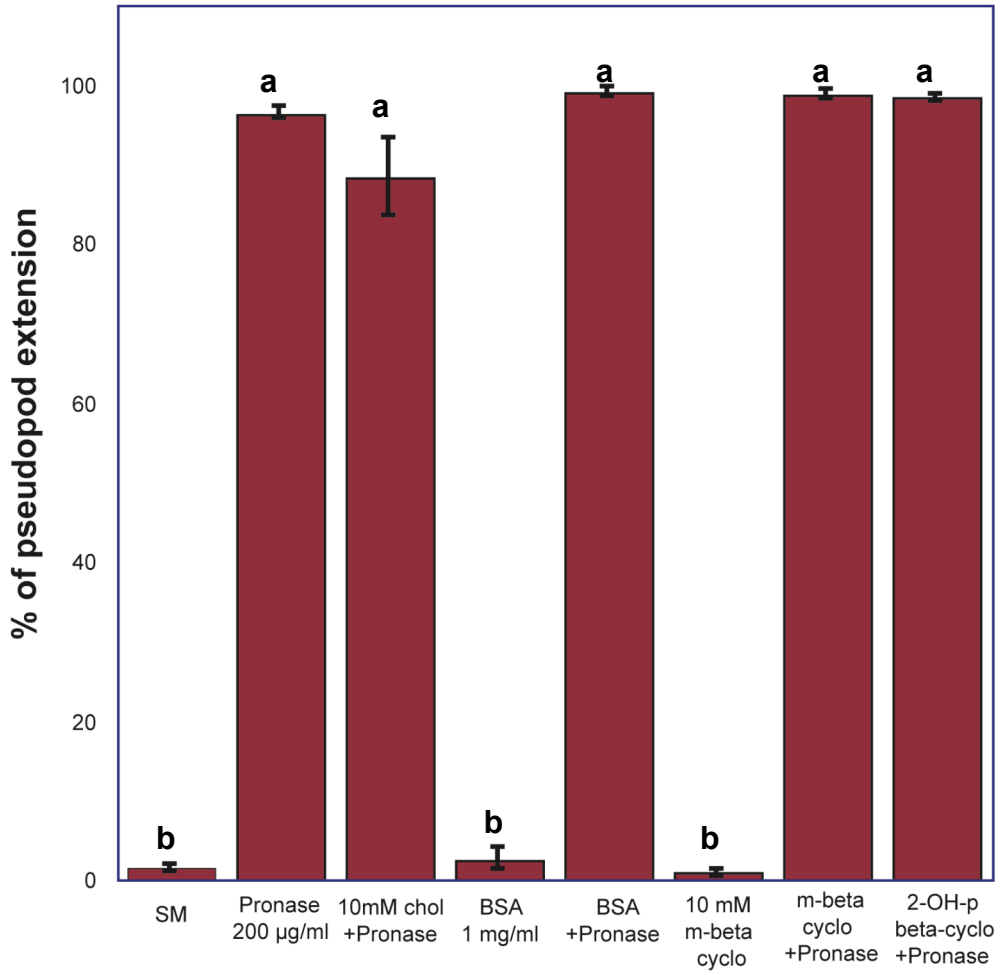

Figure 3.3. The role of cholesterol dynamics on Pronase-induced pseudopod extension in *C. elegans* spermatids

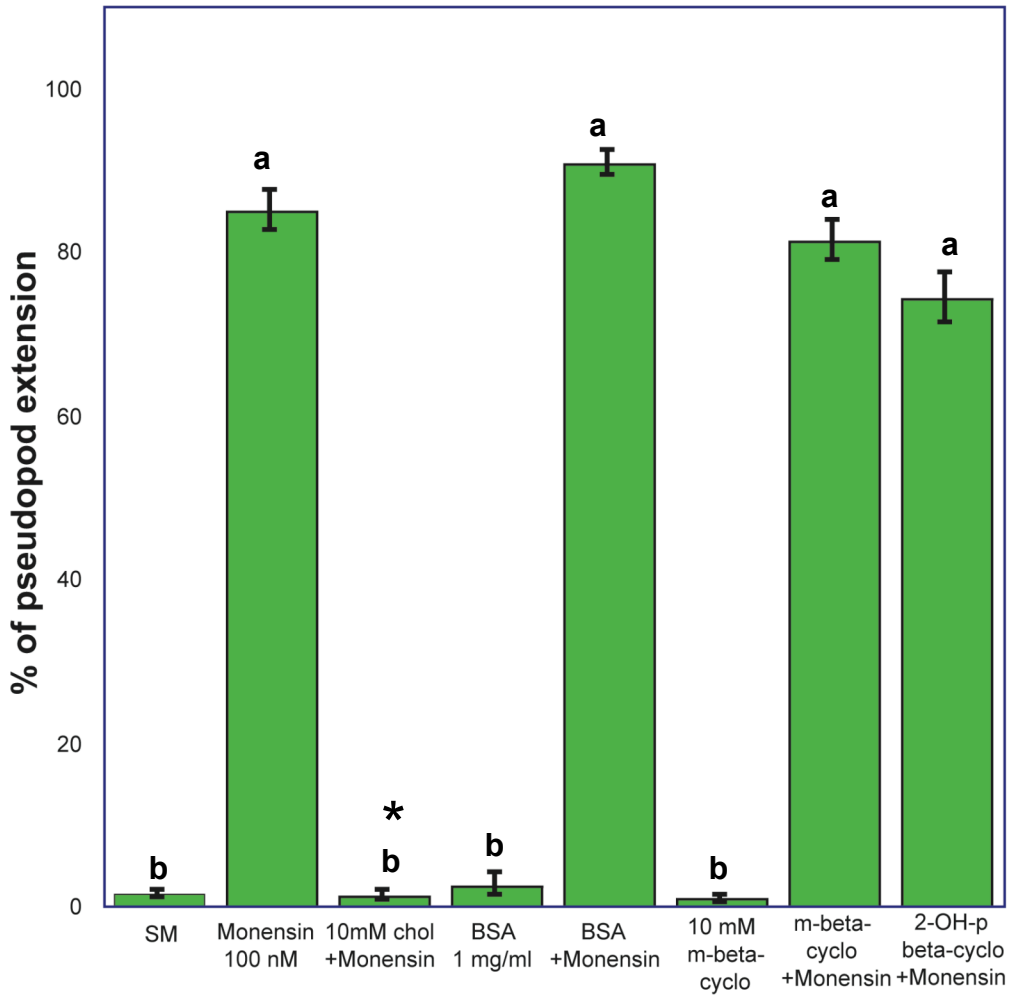

Figure 3.4. The role of cholesterol dynamics on Monensin-induced pseudopod extension in *C. elegans* spermatids

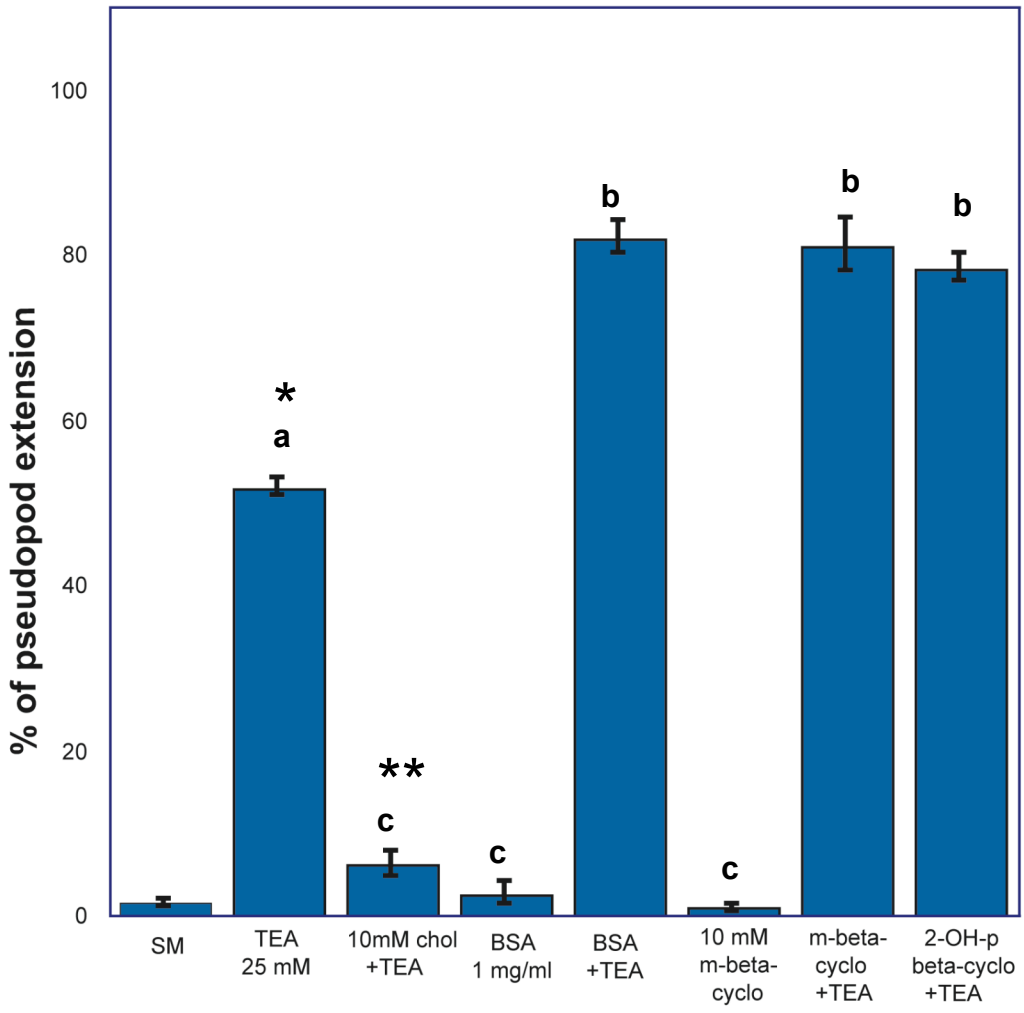

Figure 3.5. The role of cholesterol dynamics on TEA-induced pseudopod extension in *C. elegans* spermatids

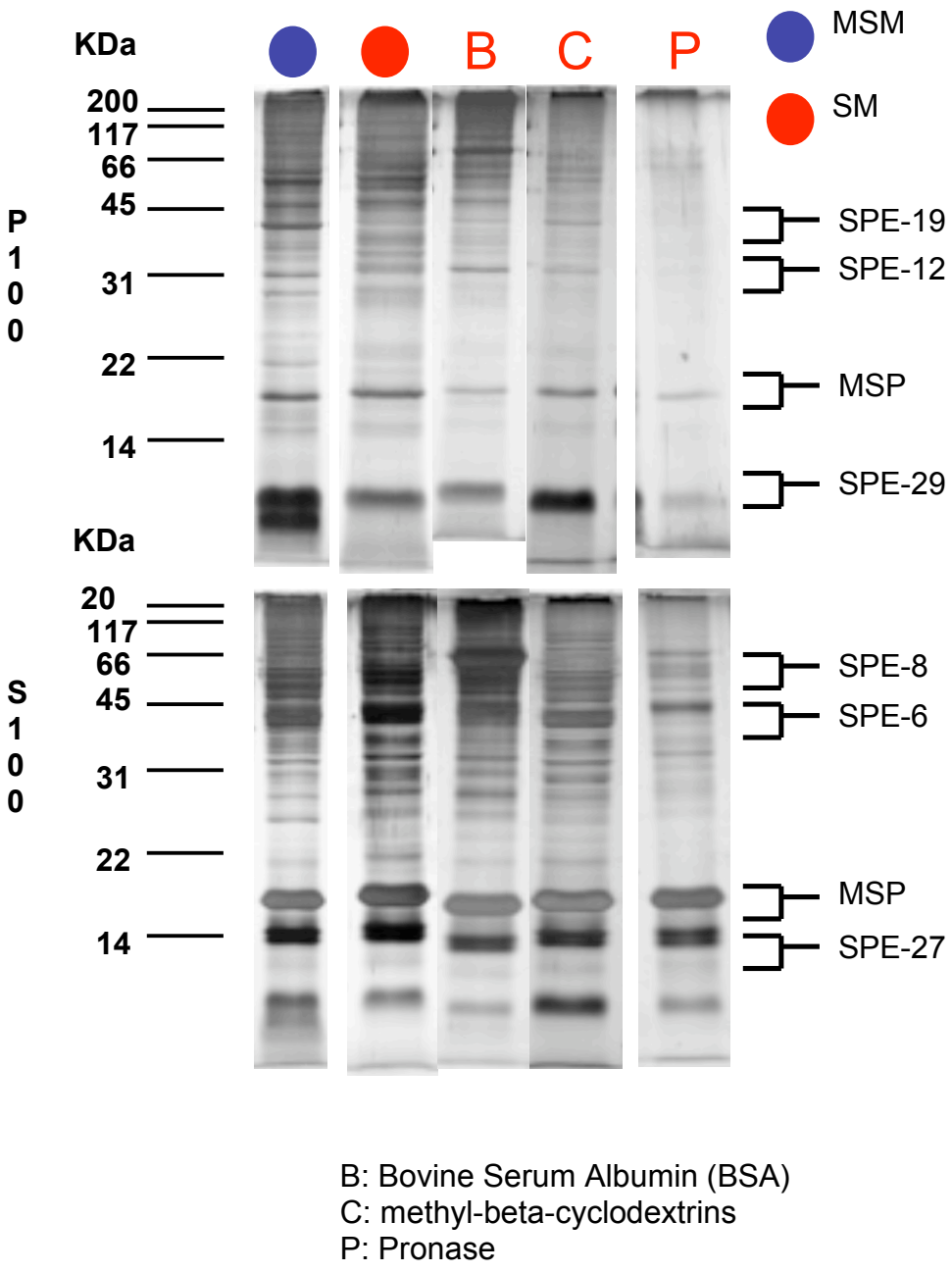

Figure 3.6. Effect of cholesterol depletion on the putative membrane fraction (P100) of *C. elegans* spermatids.

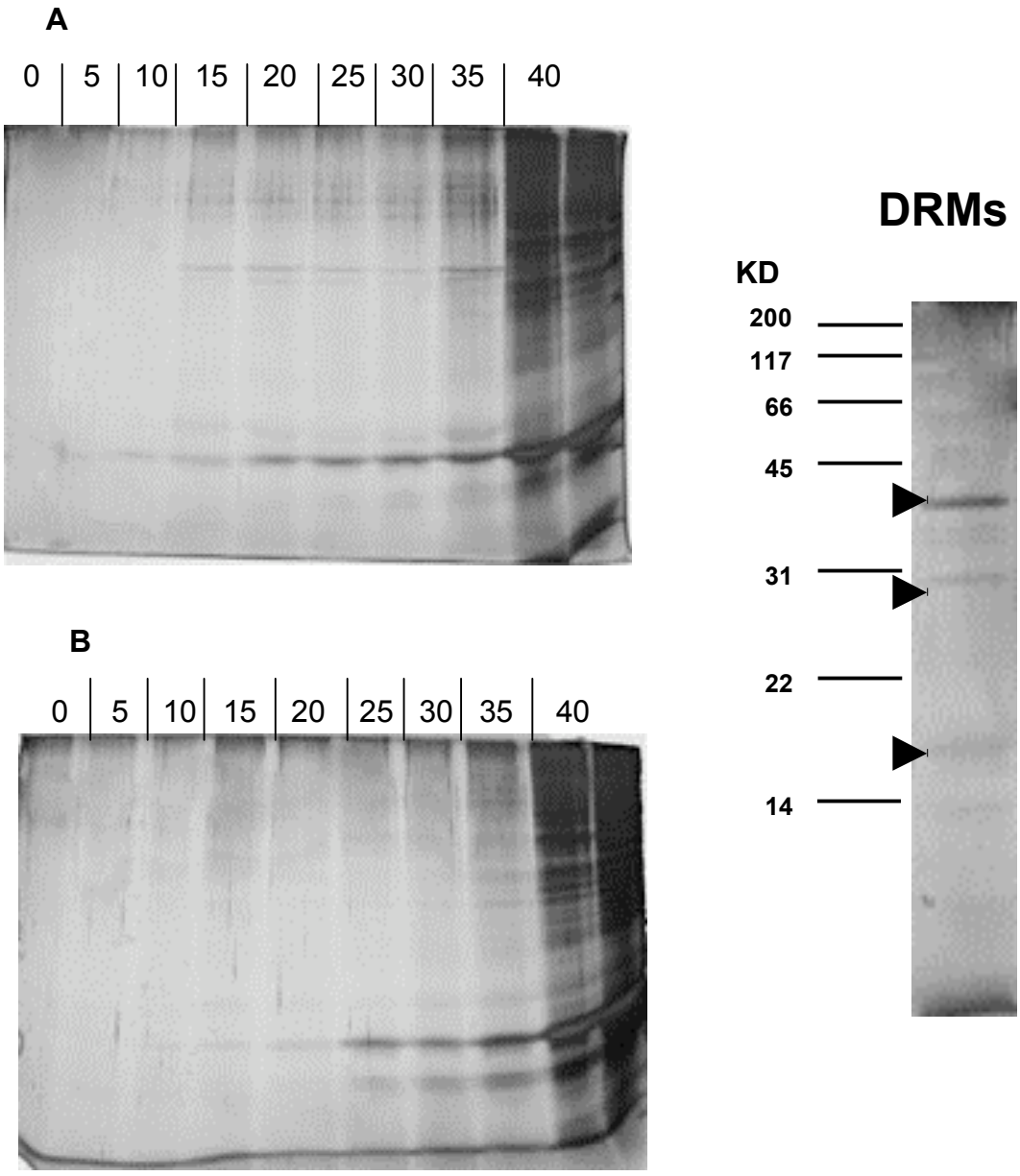

Figure 3.7. Isolation of Detergent-resistant membrane (DRM) fractions from *C. elegans* spermatids

Figure legends for Chapter 3

Figure 3.1. Pseudopod extension in *C. elegans* spermatids as seen under DIC microscopy. Unstained images of spermatids can be observed using DIC microscopy, the image results in one side of the detail appearing brighter and the other side darker, giving a pseudo three-dimensional appearance to the specimen revealing finer details. The body of the round spermatids, as seen under DIC microscopy, is a granulose 3-D sphere avoiding the bright halo present in phase-contrast microscopy and allowing for more details during "spike" extension. In the case of activated spermatozoa, the pseudopod appears as a 3-D protrusion form the cell body

Figure 3.2. Proportion of *in vitro* induced pseudopod extension (activation) in *C. elegans* spermatids. Pseudopod extension was induced by optimal concentrations of known *in vitro* activators of *C. elegans* spermatids. Spermatids were dissected in MSM pH 6.5 and SM pH7.8; Exposure of spermatids to these media did not promote a significant amount of spontaneous pseudopod extension. Treatment of spermatids with the *in vitro* activators induced pseudopod extension in a high proportion of cells, although the efficiency of each activator was different. TEA 25 mM induced pseudopod extension in 51.8 % ± 1.1 SE of spermatids. Monensin 100 nM induced pseudopod extension in 84.9 % ± 2.5 SE of spermatids. Pronase 200 µg/ml, induced pseudopod extension in 96.3 % ± 0.76 SE of spermatids. Statistical differences are shown with respect to Pronase-induced activation.

Figure 3.3. The role of cholesterol dynamics on Pronase-induced pseudopod extension in *C. elegans* spermatids. The effect of cholesterol treatment and depletion in Pronase-induced spermatid activation was assessed. Treatment of spermatids with cholesterol prior to the addition of Pronase 200 µg/ml did not have a statistically significant effect on the proportion of spermatids extending a pseudopod ($p = 0.01$) as compared to Pronase activation alone, this trend was also observed in spermatids treated with the cholesterol depletors BSA (1 mg/ml), 10 mM m-beta-cyclodextrin or 10 mM 2-OH-p-beta-cyclodextrin. The treatment of spermatids by these compounds alone was not sufficient to induce pseudopod extension. Same letters on top of bars show groups that do not show statistical difference.

Figure 3.4. The role of cholesterol dynamics on Monensin-induced pseudopod extension in *C. elegans* spermatids. The effect of cholesterol treatment and depletion in Monenisn-induced spermatid activation was assessed. Treatment of spermatids with cholesterol prior to the addition of 100 nM Monensin prevented pseudopod extension to basal levels as compared to incubation with only SM (* $p < 0.0001$ with respect to Monensin treatment alone). Cholesterol depletion using BSA (1 mg/ml), 10 mM m-beta-cyclodextrin or 10 mM 2-OH-p-beta-cyclodextrin did not have a statistically significant effect on the proportion of spermatids extending a pseudopod ($p = 0.01$). The treatment of spermatids by these compounds alone was not sufficient to induce pseudopod extension. Same letters on top of bars show groups that do not show statistical difference.

Figure 3.5. The role of cholesterol dynamics on TEA-induced pseudopod extension in *C. elegans* spermatids. The effect of cholesterol treatment and depletion in TEA-induced spermatid activation was assessed. Treatment of spermatids with cholesterol prior to the addition of 25 mM TEA prevented pseudopod extension to very low levels (** $p < 0.0001$ with respect to TEA treatment alone). Cholesterol depletion using BSA (1 mg/ml), 10 mM m-beta-cyclodextrin or 10 mM 2-OH-p-beta-cyclodextrin had a statistically significant effect on the proportion of spermatids extending a pseudopod (* $p < 0.0001$ of TEA alone with respect to cholesterol depleted activation). The treatment of spermatids by these compounds alone was not sufficient to induce pseudopod extension. Same letters on top of bars show groups that do not show statistical difference.

Figure 3.6. Effect of cholesterol depletion on the putative membrane fraction (P100) of *C. elegans* spermatids. Large-scale isolation of spermatids in MSM (blue circle), SM (red circle) and treated with either BSA 1mg/ml (B), 10 mM m-beta-cyclodextrin (C), 10 mM 2-OH-p-beta-cyclodextrin (not shown) or 200 µg/ml of Pronase (P) were fractionated using differential centrifugation. The resulting fractions (P100, putative membrane and S100, putative cytosol) were run in a 15 % SDS-PAGE and the protein pattern was compared. The predicted migration ranges of MSP and the SPE group of proteins are shown for reference and comparison. A band in the predicted range of SPE-19 changes localization and/or intensity in each treatment. A band that migrates to the

predicted molecular weight of MSP is present in the P100 (putative membrane) fraction. Changes in the protein pattern in the S100 fraction are not as evident.

Figure 3.7. Isolation of Detergent-resistant membrane (DRM) fractions from *C. elegans* spermatids. DRMs are isolated as biochemical correlates with membrane microdomains. Spermatids were isolated in MSM (A) or SM (B), resuspended and sonicated in 200 µl 0.1% Triton X-100 at 4º C for 30 min. Optiprep® Gradient Density Medium was used to prepare continuous gradient with concentrations of 40, 35, 30, 25, 20, 15, 10, 5 and 0%. After formation of the gradient the sample was centrifuged at 200 000*g* for 4 hrs at 4º. The gradient was collected by fractions, concentrated and run in a 15% SDS-PAGE. **A.** DRMs were isolated from spermatids incubated in MSM pH 6.5 and found in the 15 and 20% fractions. Three bands can be visualized with silver corresponding to molecular weights of ~40, 30 and 15 kDa (black arrowheads). **B.** DRMs could not be isolated from spermatids incubated in SM pH 7.8 from the 15 and 20% fractions.

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Chapter 4

Major Sperm Protein (MSP) phosphorylation and its putative signaling role in cytoskeletal dynamics during pseudopod extension in *Caenorhabditis elegans*.

Abstract

Nematode spermatozoa are highly specialized cells that are aflagellate and extend a pseudopod to initiate motility. Unlike other amoeboid cells, the cytoskeletal elements that are responsible for pseudopod formation are not based on actin microfilaments but are composed of a protein known as the Major Sperm Protein (MSP). In nematode sperm, MSP constitutes 15% of the total proteins and, in addition to its role in pseudopod extension, is also implicated in oocyte maturation and ovulation. To date, no evidence of a direct involvement of MSP in activating intracellular signaling pathways leading to pseudopod extension has been reported and no direct phosphorylation of MSP has been demonstrated in nematode sperm. In this chapter, a proteomic approach is used to report evidence for MSP phosphorylation in *C. elegans* spermatozoa using immunoblot detection coupled to Mass Spectrometry identification, *in silico* prediction of functional domains and *in vitro* filament formation. Concordance of results suggests a direct role of MSP as an intracellular signaling protein involved in the pseudopod cytoskeletal dynamics during sperm activation.

Introduction

Nematode spermatozoa are atypical sperm not only in that they lack a flagellum but also in the protein that forms the cytoskeletal filaments that are responsible for their motility. The Major Sperm Protein (MSP), rather than actin as in other amoeboid cells, is responsible for sperm locomotion in nematodes (Nelson et al 1982; Roberts and Stewart 2000). MSP is an immunoglobulin-like 14KDa protein that forms filaments exclusively involved in locomotion by polymerizing dimers into helical subfilaments [Stewart et al, 1994]; these subfilaments wrap around each other to form helical filaments that ultimately assemble into fibers (King et al, 1994). Polymerization of MSP into filaments and fibers produces a protrusive force along the leading edge of the pseudopod, while depolymerization at the base of the pseudopod promotes retraction of the cell body. These two forces coupled to pseudopod attachment to the substrate confer movement to the sperm cell (for a review, see Roberts and Stewart, 2000).

In addition to its role in conferring sperm motility through the extension of its pseudopod, MSP has also been shown to serve as a signal for oocyte maturation and gonad sheath contraction required for ovulation (Miller et al, 2001). The intriguing roles of MSP as both a signaling and a cytoskeletal protein involved in locomotion has attracted a great deal of attention since proteins carrying the MSP Domain (MSP Domain proteins or MDPs) have been found in taxonomically highly diverse organisms, including protozoans, fungi, plants, the flatworm *Shistososma japonicum*, the mollusk *Aplysia californica,* and a wide variety of vertebrates, including humans (for a review see Tarr

and Scott, 2005). MDPs are proteins that contain the MSP immunoglobulin (Ig)-like domain and can be recognized in two categories: the cytoskeletal MSPs and the VAMP-associated proteins (VAPs), which are integral membrane proteins that contain a MSP domain involved in linking membrane proteins with cytosolic protein complexes (Laurent et al, 2000). Based on the number and relative position of the MSP domains in the protein and the presence or absence of a transmembrane domain, MDPs are organized into five main types (Type I to V) (Tarr and Scott, 2005).

Due to its Ig-like structure, the MSP domain due is thought to be involved in protein-protein interactions, although no intracellular signaling roles have yet been identified in nematode MDPs. To date, the only evidence for intracellular signaling in MDPs is the yeast VAP homologue, SCS2, an endoplasmic reticulum membrane-bound transcription factor involved in inositol metabolism (Kagiwada et al, 1998). In *Ascaris* sperm, previous studies showed that MDPs play a crucial role in the regulation of MSP filament dynamics (Buttery, et al, 2003; Leclaire et al, 2003). However, the molecular machinery that regulates pseudopod extension in *Ascaris* involves the phosphorylation and dephosphorylation of tyrosine and threonine residues in MSP accessory proteins (LeClaire *et al.,* 2003 and Yi, K. *et al.* 2007), while no direct phosphorylation of MSP is known to occur either in *Ascaris* or *Caenorhabditis* sperm.

To address whether MSP is directly involved in the signaling pathway that leads to filament formation and ultimately to the pseudopod extension, we looked for evidence of MSP phosphorylation in *C. elegans* sperm using immunoblot detection coupled to Mass Spectrometry identification, *in silico* prediction of phosphorylation sites and *in*

vitro filament formation. The results suggest a direct role of MSP as an intracellular signaling protein involved in the regulation of cytoskeletal dynamics.

Materials and Methods

Worm synchronization and male separation. *Caenorhabditis elegans* strain CB1489: *him-8* (e1489) IV was maintained at 20^{o}C on NGM plates seeded with *Escherichia coli* OP50 as described by Brenner (1974). *him-8* males produce ~ 40% male progeny compared to ~0.1% males produced by wild type (Hodgkin et al, 1979) and their sperm are cytologically indistinguishable from wild-type sperm (Ward et al, 1981). Culture synchronization and male separation were performed as described by L'Hernault and Roberts (1995) with some modifications as described in the previous chapter. Briefly, worms were allowed to grow on plates to saturation for three days and the cultures were synchronized using the alkaline hypochlorite method (35 ml of a bleach/NaOH mixture). Embryos were allowed to hatch for 24 hours in M9 buffer (22mM KH_2PO_4, 22mM Na_2HPO_4, 85mM NaCl, 1mM $MgSO_4$) supplemented with cholesterol and spotted on seeded NGM plates allowing the worms to grow for three days at 20^{o}C. Plates with adult worms were washed using M9 buffer and filtered using a 35-µm Nitex filter. The filtrate (containing males and hermaphroditic juveniles) was transferred onto a 25-µm Nitex filter and washed using M9 buffer to eliminate juvenile worms. A worm population of ~ 95 % males was collected from the top of the 25-µm filter and poured on a 30 % Hypaque solution to remove bacteria and debris. Worms were centrifuged at 1000*g* for 10 min and recovered from the interface between the Hypaque solution and the M9 buffer.

Large-scale sperm isolation method. *C. elegans* sperm were isolated using a method described by L'Hernault and Roberts (1995) with some modifications as described in the previous chapter. Male worms were collected and washed in Modified Sperm Medium (MSM). MSM contained 50 mM HEPES, 70 mM Choline Chloride, 5 mM $CaCl_2$, 5mM Dextrose, Polyvinylpyrrolidone (PVP) 10 mg/ml, pH adjusted to 6.5 and supplemented with 10 µl/ml of protease inhibitor cocktail from Sigma (St. Louis, MO), 10 mM Na-Fluoride and 1 mM Na-orthovanadate as phosphatase inhibitors, to prevent activation of spermatids. Worms were centrifuged at 6000 rpm using a benchtop centrifuge. The pellet of packed males was transferred to the bottom of a glass Petri dish and worms were chopped up using a stainless steel razor blade for approximately 5 min. The chopped worms were then washed from the Petri dish using MSM and passed through a 15µm Nitex filter. The filtrate was then poured through a 5µm Nitex filter and this second filtrate was poured on top of 2ml of Percoll 10 % and centrifuged at 1000*g* for 10 min. The supernatant was discarded and the pellet resuspended in 1 ml of MSM. Spermatids were counted using a hemocytometer and cells were centrifuged again at 1000*g* for 10 min. The pellet was resuspended in 1 ml of Homogenization buffer (20 mM Tris, 20 mM HEPES, 30 mM Mannitol, 1 mM EDTA,1 mM EGTA, pH adjusted to 7.4) and laid on ice for sonication. The sample was sonicated using a Branson Sonifier 250 (VWR Scientific) using 4 pulses of 15 seconds each and resting on ice for 1 min between pulses. To clean the sample from cellular debris, nuclear chromatin and organelles, it was centrifuged at 10 000 *g* for 15 min and the supernatant was concentrated using a microcon tube with a Molecular Weight Cut Off (MWCO) of 3 KDa from Millipore

(Billerica, MA). The concentrate was either resuspended in 100 µl of PBS and Laemmli sample buffer, or used for crystal precipitation and/or fiber elongation assays.

MSP crystal precipitation and fiber elongation. The concentrated extract isolated from *C. elegans him-8* male spermatids (after sonication and centrifugation) was used for crystal precipitation or fiber elongation assays as described by (King, *et al* 1992; Italiano, *et al* 1996; Miao, *et al* 2007; Olivares-Castillo and Smith, 2008). For crystal precipitation the concentrate was diluted to a protein concentration of 1mg/ml using HKB buffer (50 mM HEPES, 25 mM KCl, 10 mM NaCL, pH 7.2). Aliquots of 10µl were placed in eppendorf tubes and an equal volume of Polethylene glycol (PEG, average M_r 20 000) was added to produce concentrations of 10% after addition to MSP. Crystals were observed using a phase-contrast microscope with a 20X objective. For fiber elongation, the protocol from (Miao, *et al* 2007) was followed. Briefly, the concentrate was diluted in KPM buffer (10 mM potassium phosphate, 0.5 mM $MgCl_2$, pH 6.8) and incubated with either 1 mM Na orthovanadate, 500 nM staurosporine, 150 µM IC261, 10 µM YOP (from *Yersenia enetrocolitica*) and/or 50 mM ATP (all final concentrations after addition to MSP). For fiber elongation at different pH, HKB was used (instead of KPM) at pH 5, 7 and 9. Fibers were observed using a phase-contrast microscope with a 100X objective.

SDS-PAGE and Western blotting. SDS-PAGE was performed using 15 % gels according to the method of Leammli (Laemmli, 1970) and stained with 1% Coomasie brilliant blue. Gels for immunoblotting were transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA) as described by Towbin et al., 1979. Blocking of the membrane was performed in TBS-T (0.1 % Tween-20, 137 mM NaCl, 20 mm Tris, pH

7.6) with 1% bovine serum albumin (BSA) for 1 hr at room temperature and probed with primary antibody for 4 hours. The membrane was washed with TBS-T and probed using a secondary HRP-conjugated antibody for 4 hrs. Bands were observed using the Supersignal West Femto kit (Pierce, Rockford, IL).

Antibodies and antiserum. Monoclonal antibodies antiphosphotyrosine, clone PY-20 and antiphoshoserine, clone PSR-45, were purchased from Sigma (St. Louis, MO) and monoclonal antiphosphothreonine (42H4) from Cell Signaling (Danvers, MA). The monoclonal antibody 4A5 anti-MSP developed by Kosinski et al. (2005) was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The polyclonal anti-MPAK antibody developed by Yi, et al. (2007) was kindly provided by Dr. Thomas Roberts (Florida State University, Tallahasse). Secondary antibodies used were: a goat anti-mouse IgG, H&L, conjugated to horseradish peroxidase (EMD Biosciences, San Diego, CA) and a goat anti-rabbit IgG also conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA)

Mass Spectrometry analysis. Gels were stained with Coomasie Brilliant Blue R250 showing a prominent band at ~ 17 kDa . This band was excised and sent for identification to the W.M. Keck Proteomics Laboratory at the Center for Plant Cell Biology, University of California, Riverside. Peptides were trypsin digested and identified using Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC/MS/MS) and the data generated was submitted to the MASCOT database for protein identification allowing for

variable modifications such as Acetyl (K), Acetyl (N-term), Formyl (N-term), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), Oxidation (M), Phospho (ST).

Prediction of functional domains in MSP. All available *C. elegans* annotated MSP proteins (containing 127 amino acids) were downloaded from Genbank and aligned using ClustalW (Larkin MA, *et al*., 2007). Functional MSP domains were predicted using PROSITE (Bairoch, 1991), available at http://ca.expasy.org/prosite/. Settings for the search included patterns with a high probability of occurrence.

Results

MSP from *C. elegans* sperm extract can form crystal precipitates and elongate fibers.

Following the protocol described in L'Hernault and Roberts (1995) spermatids from *C. elegans* males (*him-8* mutants) were isolated. In order to test whether this extract contained MSP, the extract was treated with Polyethylene glycol (PEG) to precipitate MSP crystals *in vitro* as previously showed with extract from *Ascaris* sperm (King, *et al* 1992) and *C. elegans* MSP expressed in bacteria (Castillo-Olivares and Smith, 2008). Crystal precipitation was induced by the addition of PEG 15% to the sperm extract (1 mg/ml) and was complete within a few seconds as previously reported by (Castillo-Olivares and Smith, 2008). A control using BSA (1mg/ml) and PEG 15% did not result in crystal precipitation, consistently with the previous work (see Figure 4.1A). We tested also if MSP crystal precipitation is reversible by concentrating and diluting the extract

using HKB buffer and adding PEG 15% (see figure 4.1B). All results were consistent with the presence of MSP in the extract and its ability to precipitate into crystals. Fiber elongation was tested in the sperm extract as previously reported by (Italiano, *et al* 1996; Miao, *et al* 2007). Incubation of sperm extract in HKB buffer at pH 5, 7 and 9, did not induce fiber elongation. Incubation with 50 mM ATP alone did not induce fiber formation in the extract. Thus, treatments with 500 nM staurosporine (a general kinase inhibitor), 150 µM IC261 (a casein kinase inhibitor), 10 µM YOP (a tyrosine phosphatase from *Y. enterocolitica*) and 1 mM Na orthovanadate (a tyrosine phosphatase inhibitor) were tested. None of these induced fiber elongation in *C. elegans* sperm extract. It was only after the sperm extract was incubated with 10 µM YOP for 10 min, followed by an incubation of 50 mM ATP for 10 min that fibers elongated (see Figure 4.2), suggesting that a tyrosine dephosphorylatin event followed by phosphorylation due to the addition of ATP are necessary for fiber elongation in MSP from *C. elegans*.

Evidence for MSP phosphorylation using immunoblotting and Mass spectrometry.

Since, putatively, a phosphorylation event can induce MSP fiber elongation, the identification of phosphorylated proteins was performed by immunoblotting. The *him-8* male sperm extract was run on a 15 % SDS-PAGE gel. The gel stained with Coomasie blue contained a prominent band that ran ~18 kDa (a mobility slightly slower than that reported for MSP) although the abundance of the protein strongly suggested it was MSP (Figure 4.3). Identity of this band as MSP was verified by immunoblotting using a specific anti-MSP antibody. Searching for protein phosphorylation, we used specific

antibodies directed against phospho-tyrosine, phospho-serine and phospho-threonine residues, respectively. These antibodies labeled the same band previously identified as MSP. To corroborate the identity of MSP, the band from the acrylamide gel was cut and subjected to mass spectrometry for protein identification. The results from mass spectrometry identified nine significant hits corresponding to MSP isoforms (91 to 93% of amino acid coverage) (see Table 4.1). The best hit was a protein product from the gene Y59E9AR.1 (a 130 amino acid protein that contains one MSP domain) with protein coverage of 93 %.

***In silico* Prediction of MSP Phosphorylation sites.**

Presence of phosphorylation sites in annotated MSP sequences of *C. elegans* was predicted by PROSITE (Bairoch 1991). For all 28 MSP sequences the program returned four predicted functional domains: An N-glycosylation site (NSSA) in amino acids 36-39; a Protein Kinase C phosphorylation site (SaR) in amino acids 38-40; Two N-myristoylation sites (GIktTN) in amino acids 45-50 and (GQedTN) in amino acids 81-86; and a Casein Kinase II phosphorylation site (TnnD) in amino acids 85-88 (see Figure 4.4). The 3-D model of MSP filaments available at the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) [http://www.rcsb.org/pdb/home/home.do] was used to localize the predicted functional sites in MSP-152 (pdb id: 1grw). Using the Molecular Biology Toolkit (MBT) Protein Workshop application (Moreland *et al.*, 2005), it was found that the predicted phosphorylation sites in the MSP filament are not localized near the protein-protein

interaction domain and appear to be available for kinase phosphorylation of MSP (see Fig. 2B). Accordingly, we used a specific antibody against MPAK (a Casein kinase present in *Ascaris* sperm) to look for homologues in *C. elegans* sperm extracts. The antibody recognized a band of ~ 40 kDa (see Figure 4.3) in agreement with the estimated molecular weight of SPE-6 (a Casein kinase involved in pseudopod extension in *C. elegans*), suggesting that a Casein kinase present in the crude sperm extract is involved in the direct phosphorylation of MSP.

The identification of a conserved pattern of predicted phosphorylation sites between the nematodes *C. elegans* and *Ascaris* was investigated. In contrast with results given for all *C. elegans* MSPs, no phosphorylation sites were predicted for the beta-MSP isoform (NCBI accession no. P27440), while the alpha-MSP amino acid sequence (NCBI accession no. P27439) contained a Protein Kinase C (PKC) phosphorylation site (SqK) in amino acids 14-16 and a cGMP-dependent protein kinase (PKA) phosphorylation site (RRlS) in amino acids 51-54 (see Figure 4.5). In addition, Prosite did not return any Casein kinase phosphorylation domains for the *Ascaris* MSP sequences.

Discussion

Amoeboid cell motility has been extensively studied in actin-based systems *in vivo* and *in vitro,* dissecting the molecular pathways that lead to pseudopod extension (for a review see, Pollard and Borisy, 2003). The analogous MSP-based motility system has shown to be very different in terms of molecules involved in filament formation (Rohatgi

et al., 1999 and Yi K. *et al*., 2007). One of the main differences is in the polymerization of the filaments. Unlike actin, MSP does not have any nucleotide binding sites and does not have a plus and minus end (Roberts and Stewart, 2000). Despite these differences, MSP forms filaments and fibers that enable pseudopod extension (King *et al*., 1994). In a previous study, Burke and Ward (1983) labeled sperm *in vitro* using [^{32}P] orthophosphate and did not find [^{32}P] associated with the MSP band before or after activation with Triethanolamine, suggesting that MSP was not directly phosphorylated during pseudopod extension. Here, the evidence that such phosphorylation occurs is reported using *in vitro* fiber elongation assays, immunoblotting coupled to Mass Spectrometry, and *in silico* prediction of phosphorylation sites. The complexity of performing large-scale isolation of *C. elegans* sperm has represented a major impediment to the study of sperm biochemistry as compared to *Ascaris* sperm. In the present chapter, using a large-scale sperm isolation method the ability of sperm crude extract to elongate fibers *in vitro* was tested. A tyrosine dephosphorylation event (as suggested by YOP treatment) is necessary to induce fiber elongation after the addition of ATP to the crude extract (suggesting phosphorylation events). This is in contrast to results from *in vitro* fiber and filopodia elongation in *Ascaris* (Miao, *et al* 2007). Using this sperm extract coupled to immunoblotting, tyrosine, serine and threonine phosphorylated residues were identified in MSP together with a Casein kinase (putatively SPE-6). *In silico* prediction of phosphorylation sites in MSP revealed a Casein kinase phosphorylation site (TnnD) in amino acids 85-88 conserved among *C. elegans* MSP isoforms but not in *Ascaris* MSP isoforms.

Previous studies have shown that a set of accessory proteins such as MDPs, phosphatases and kinases are required for pseudopod extension in the nematode *Ascaris suum* (LeClaire *et al.,* 2003, Buttery *et al.*, 2003 and Yi, K. *et al.,* 2007). One of these accessory proteins is MPAK, a Ser/Thr Casein kinase required for MSP filament formation, and cytoskeletal dynamics (Yi, K. *et al.* 2007). Another Casein kinase (SPE-6) is present in *C. elegans* and is a member of the SPE group of proteins that are thought to play a crucial role in pseudopod extension and transport of MSP into the FB-MOs complex during spermatogenesis (Muhlrad and Ward, 2002). The role of MSP as a structural and extracellular signaling protein has been shown by Miller *et al*, (2001) and in the present study our results suggest that direct MSP phosphorylation by a Casein kinase plays a role in pseudopod extension. Although it is unclear to what extent the intracellular signaling pathways for filament formation are conserved among nematode species, since work on this matter has only been done in *Ascaris* and *C. elegans* spermatozoa.

The lack of a Casein kinase phosphorylation site is in agreement with the fact that MPAK phosphorylates accessory proteins and not MSP directly (Yi, K. *et al.* 2007). In *Ascaris*, only two MSP isoforms (alpha and beta) are known and they differ only in four amino acid residues (King *et al*., 1992). According to King *et al*. (1992), alpha-MSP forms filaments *in vitro* at both lower and precipitant concentrations, allowing the protein to nucleate filament assembly in the pseudopod or to lengthen existing filaments rapidly in the plasmalemmal end of the fiber. These differences might be explained by the amino

acid differences that render the alpha-MSP isoform more basic and with two putative phosphorylation sites.

If these subtle changes in the amino acid sequence could alter the properties of MSP isoforms, and their localization in the cell, then the putative N-myristoylation sites could play a role in localizing MSP to the plasma membrane for filament nucleation or for oocyte signaling. This would be consistent with the structural characteristics of the MDPs as integral membrane proteins and their function in protein-protein interaction. Although additional research in MSP posttranslational modification is needed, it is possible that other phosphorylated isoforms of MSP could be involved in filament formation during pseudopod extension in *C. elegans* sperm.

Given the advantages that *C. elegans* offers as a model organism, there is a great opportunity to elucidate the signaling pathways that lead to the acquisition of motility, complementing the genomic information available with proteomic and biochemical analysis on sperm extract, yielding useful observations in novel and conserved proteins involved in fertilization and pseudopod extension in other organisms.

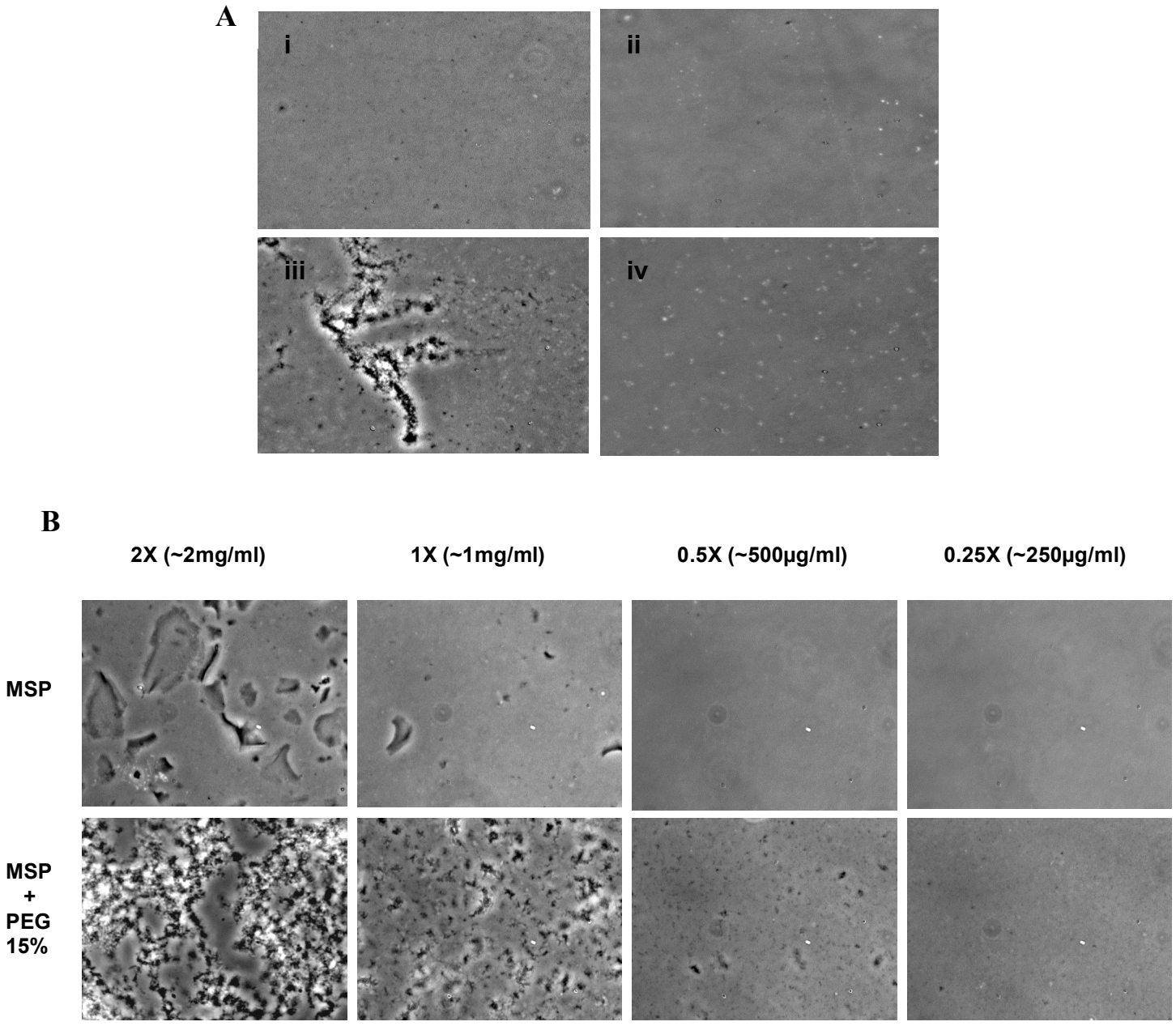

Figure 4.1. Protein extract from *C. elegans* precipitates into MSP crystals after addition of PEG

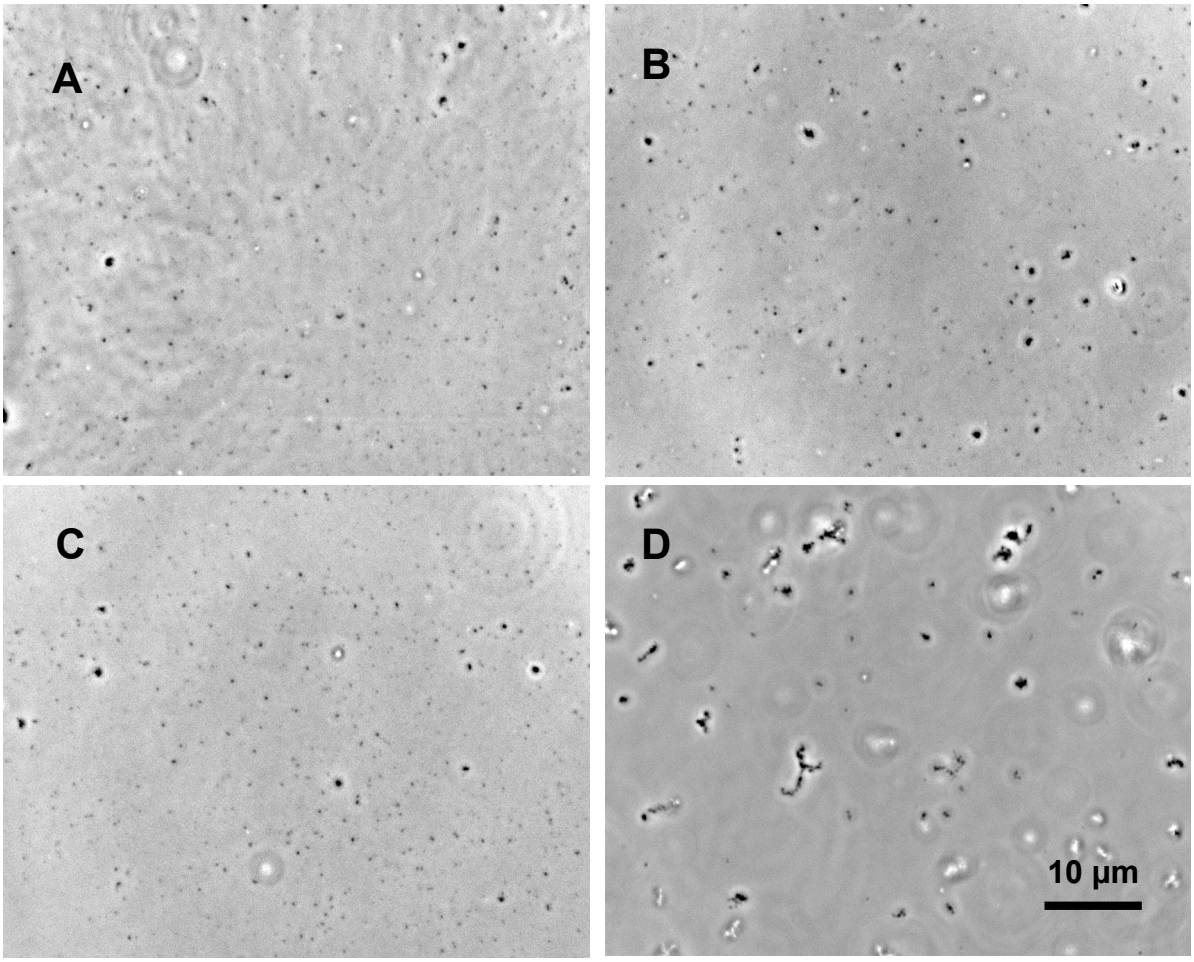

Figure 4.2. MSP fiber elongation is promoted through phosphatase treatment followed by addition of ATP.

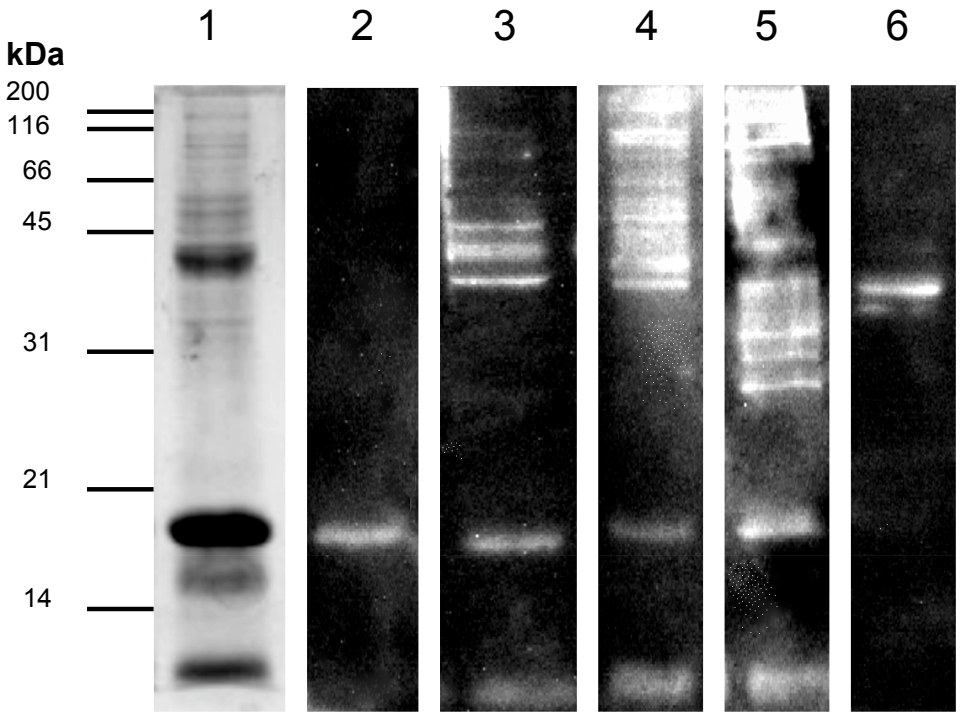

Figure 4.3. Phosphorylation of MSP detected by immunoblotting.

```
MSP-152   Ce MTQSVPPGDIQTQPGTKIVFNAPYDDKHTYHIKVINSSARRIGYGIKTTN 50
MSP-152   Ce MKRLGVDPPCGVLDPKEAVLLAVSCDAFAFGQEDTNNDRITVEWTNTPDG 100
MSP-152   Ce AAKQFRREWFQGDGMVRRKNLPIEYNP 127
```

A

N-glycosylation site (NSSA) aa 36-39
PKC phosphorylation site (SaR) aa 38-40
N-myristoylation sites (GIktTN) aa 45-50
(GQedTN) aa 81-86
Casein kinase II phosphorylation site (TnnD) aa 85-88

B

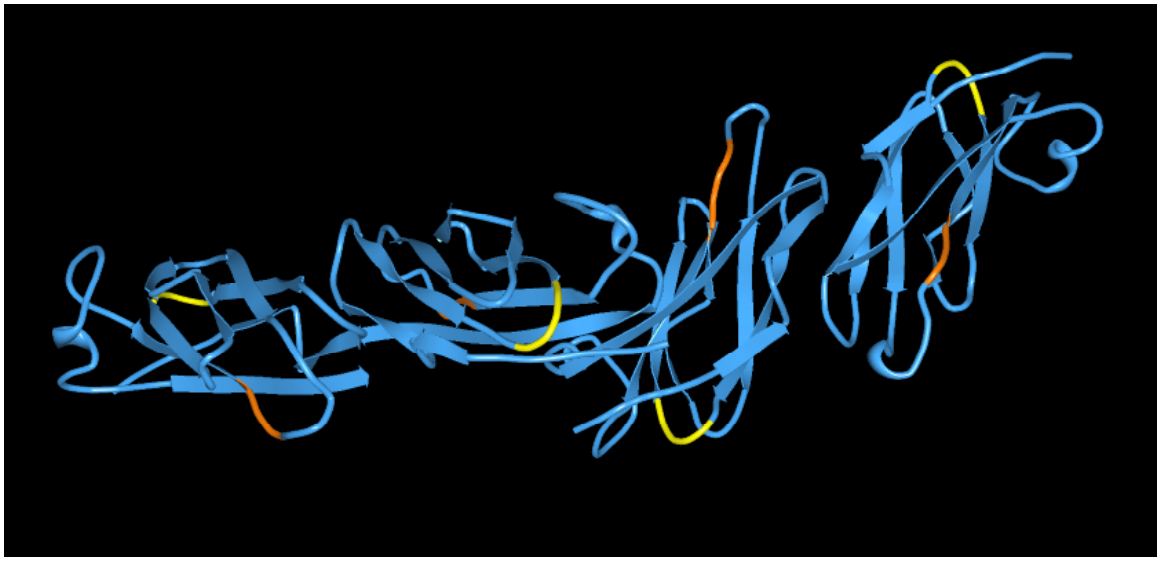

Figure 4.4. *In silico* prediction of functional sites in MSP from *C. elegans*

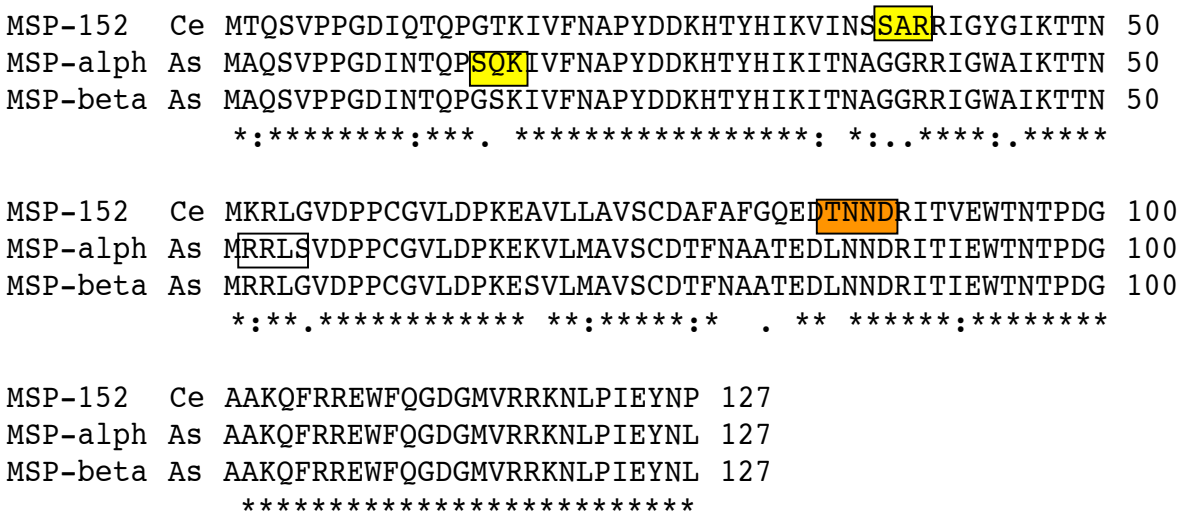

Figure 4.5. Differences on the MSP phosphorylation sites between *Ascaris* and *C. elegans* sperm.

Gene ID	MSP isoform	NCBI Accession No.
Y59E9AR.1	Predicted MSP	NP 500755
F32B6.6	MSP- 77	NP 501781
C34F11.6	MSP- 49	NP 494970
C09B9.6	MSP- 55	NP 500711
R05F9.8	MSP- 33	NP 494888
K07F5.2	MSP- 10	NP 501760
ZK5456.6	MSP- 152	NP 494901
F26G1.7	MSP- 3	NP 494858
T13F2.11	MSP- 78	NP 501742

Table 4.1. List of MSP isoforms identified by MS/MS (91 to 93% of amino acid coverage for each protein) with their respective accession numbers.

Figure legends for Chapter 4

Figure 4.1. Protein extract from *C. elegans* precipitates into MSP crystals after addition of PEG. Sperm extract isolated using a large-scale method was tested for the precipitation of MSP crystals. The sperm extract in a concentration of 1 mg/ml was treated with PEG 15% and precipitated MSP crystals *in vitro*. **A.** The extract itself did not produce crystals (i), the same was true for PEG 15% alone (ii). Addition of PEG 15% to the sperm extract precipitated crystals (iii) and a control using BSA (1mg/ml) and PEG 15% did not result in crystal precipitation (iv). **B.** MSP crystal precipitation is reversible by concentrating and diluting the extract using HKB buffer and adding PEG 15% to each concentration.

Figure 4.2. MSP fiber elongation is promoted through phosphatase treatment followed by addition of ATP. Treatment of sperm extract with YOP and ATP formed fibers *in vitro* observed under phase-contrast microscopy. **A.** Sperm extract itself did not form spontaneous elongation of fibers. **B.** Addition of 50 mM ATP was not sufficient to promote fiber elongation. **C.** Treatment of 10 μM YOP for 15 min did not induce *in vitro* fiber elongation. **D.** Fiber elongation was induced after treatment of the sperm extract with 10 μM YOP for 15 min, followed by addition of 50 mM ATP.

Figure 4.3. Phosphorylation of MSP detected by immunoblotting. The sperm extract was run in a 15% SDS-PAGE and stained with coomasie for protein pattern visualization or transferred into a nitrocellulose membrane for immunoblotting. MSP evident as a prominent band ~ 16 kDa and recognized by a anti-MSP monoclonal antibody. Anti-phosphotyrosine, phosphoserine and phosphothreonine antibodies were used to look for protein phosphorylation in MSP. All three antibodies reacted to the same band identified as MSP. An antibody against MPAK, a Casein kinase from *Ascaris* sperm was used as a control. Lane 1: Coomassie-stained gel of *C. elegans* sperm extract. Lane 2: anti-MSP; Lane 3: antiphosphotyrosine; Lane 4: antiphosphoserine; lane 5: antiphosphothreonine. Lane 6: anti-MPAK for *Ascaris* showing cross reactivity with *C. elegans* sperm. All primary antibodies were diluted 1:1,000 and the secondary antibodies were diluted 1:10,000.

Figure 4.4. *In silico* prediction of functional sites in MSP from *C. elegans.* The sequence of MSP-152 is used as an example for identification of functional sites using PROSITE. **A.** The program returned four predicted functional domains: An N-glycosylation site (NSSA) in amino acids 36-39 (cyan); a Protein Kinase C phosphorylation site (SaR) in amino acids 38-40 (yellow); Two N-myristoylation sites (GIktTN) in amino acids 45-50 and (GQedTN) in amino acids 81-86 (magenta); and a Casein Kinase II phosphorylation site (TnnD) in amino acids 85-88 (orange). The 3-D model of MSP filaments localized the predicted functional sites in MSP (pdb id: 1grw).

B. The predicted phosphorylation sites in the MSP filament are not localized near the protein-protein interaction domain and appear to be available for kinase phosphorylation.

Figure 4.5. Differences on the MSP phosphorylation sites between *Ascaris* and *C. elegans* sperm. Alignment of a representative protein sequence of MSP from *C. elegans* (MSP-152 Ce; NP_494901), and the two *Ascaris* isoforms: alpha (MSP-alph As; P27439) and beta (MSP-beta As; P27440). Predicted phosphorylation sites are shown: PKC phosphorylation sites (yellow); Casein kinase phosphorylation site (orange); and PKA phosphorylation site (white). The most abundant isoform in *Ascaris* sperm, MSP-beta does not contain any phosphorylation sites, while the more basic isoform MSP-alpha contains PKC and a PKA phosphorylation sites.

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General conclusions

During the process of fertilization, a motile spermatozoon must reach the oocyte to fuse with it and produce progeny. A typical image we have of this process is a motile flagellated sperm that "swims" towards the egg in the female reproductive tract or the environment where fertilization takes place. However, non-flagellated sperm are wide spread among the Bilateria and physiological studies on the process through which they acquired motility have been almost neglected. The present work focuses on the Phylum Nematoda, a group in which all the members have non-flagellated spermatozoa. Nematode sperm lacks an acrosome and a flagellum, and motility is acquired through the extension of a pseudopod through an amoeboid locomotion. Thus, rather than "swimming" towards the oocytes this cells "crawl" to the site of fertilization. An advantage offered by this group is to have a species that has been used as a model system, *Caenorhabditis elegans*. These advantages include mutant strains available and the full sequence of the genome. *C. elegans* adults can be present in two sexual types, males and hermaphrodites, both produce spermatids and they differ in the environment where they are produced, the size of the spermatid and on the timing of motility acquisition, conferring an adaptive advantage to the larger male spermatozoa, which are able to outcompete hermaphrodite-derived spermatozoa during fertilization. Genetic screens have identified genes involved in the process of pseudopod extension (also known as spermiogenesis or sperm activation). These genes are part of a group known as the SPE group of proteins and include membrane and cytosolic proteins that act in a common signaling pathway that regulates pseudopod extension. Despite the advantages of *C.*

elegans as a model system, the molecule(s) that promote pseudopod extension *in vivo* have not been characterized to date. A combination of genetic screens and *in vitro*-induced pseudopod extension suggests that a protease is involved in cleaving surface proteins that trigger physiological changes resulting in the acquisition of motility. These physiological changes include plasma membrane rearrangements and an increase of intracellular pH to establish a gradient along the pseudopod. The pH gradient orchestrates the polymerization of filaments formed by the Major Sperm Protein (MSP). In this regard, the initiation signaling for acquisition of motility must act on the surface of the spermatid and have MSP as an ultimate target to promote filament elongation.

The present dissertation focuses on the physiological involvement of membrane microdomains and their disruption promoting MSP filament elongation. A conspicuous amount of evidence supports the presence of these types of domains on the plasma membrane of nematode spermatozoa. However, the question remained unanswered, Are there membrane microdomains involved in the extension of the pseudopod in *C. elegans* sperm?

The results from chapter 2 show differences in the ability of spermatids derived from male or hermaphrodite adults to extend a pseudopod using Pronase, an *in vitro* activator. Male-derived spermatids are slower at the process of pseudopod extension than the hermaphrodite-derived spermatids, this difference might be explained by the presence of seminal fluid in the males that contains molecules such as SWM-1, a serine-protease

inhibitor involved in the regulation of spermatid activation. Thus, a difference in the response of male-derived spermatids to Pronase could be due to the physiological response of these cells to the environment where motility is acquired and not to the presence of alternative pathways between male and hermaphrodite-derived sperm since the protein pattern between these cells does not show striking differences among the two sexual types. Hermaphrodite-derived spermatids must acquire motility during the first ovulation in adult worms, in order to fertilize oocytes and crawl back to the spermatheca where this process takes place. In the case of male-derived spermatids, pseudopod extension must occur after copulation and during the time of ejaculation in the hermaphrodite uterus to enable these cells to crawl to the spermatheca and this work focuses on the male-derived spermatids since it seems to be subject to more physiological changes due to the different environments where it is exposed, the testis prior to pseudopod extension and the uterus after ejaculation.

Pronase, Monensin and Triethanolamine (TEA) trigger pseudopod extension *in vitro* in *C. elegans* male spermatid. The modes of action of these activators are different. Monenisn and TEA, both increase the intracellular pH of spermatids and Pronase cleaves proteins from the cellular surface putatively hitting the actual target(s) that promote pseudopod extension. As observed in chapter 3, Pronase is a very efficient activator regarding the proportion of cells that extend a pseudopod, although it is a very aggressive treatment that cleaves proteins from the cellular surface preventing spermatozoa translocation. In the case of Monensin and TEA, the effect on the proportion of

pseudopod extension in spermatids is lower, however, these activators allow for spermatozoa translocation and attachment to glass slides.

An initial step on the process of pseudopod extension is the rearrangement of the plasma membrane, becoming more dynamic and fluid, allowing for protrusion of spikes that coalesce into a pseudopod. Membrane fluidity, and other biophysical properties, can be regulated through changes in the amount of cholesterol present in the bilayer, by increasing the "ordered" state of the phospholipids that constitute the membrane. This change in the phase of phospholipids can also affect ionic influxes in cells. On the other hand, disruption of the "ordered" state by cholesterol depletion increases the fluidity of the membrane changing its phase to a "disordered" state in which ionic influxes are enhanced and protein interaction is promoted. In the case of *C. elegans* spermatids, these changes affect the ability of the cells to extend a pseudopod. Treatment with cholesterol prevents pseudopod extension in Monensin or TEA-induced activation, and even if no effect is observed in the proportion of cell extending a pseudopod induced by Pronase treatment, the result is a delayed process that culminates in the extension of an aberrant pseudopod. The opposite effect is observed in TEA-induced activation, where cholesterol depletion increases the proportion of cells that extend a pseudopod, suggesting changes in plasma membrane diffusion.

The presence of membrane microdomains in *C. elegans* spermatids is suggested by the isolation of Detergent-resistant membrane fractions. However, the condition for

the isolation of these fractions is the lack of ions and pH necessary for pseudopod extension. The results in this chapter support membrane rearrangements prior to the extension of the pseudopod, including a process of vesicle budding in the signaling of spermatids for oocyte maturation and ovulation. As a result, we can propose that acquisition of motility for successful fertilization in *C. elegans* sperm initiates by the activation of spermatids through an increase of intracellular pH due to ionic exchange. Activation is followed by membrane rearrangements promoted by the loss of cholesterol and the release of membrane vesicles from the plasma membrane. The plasma membrane must become more dynamic for protrusion of spikes, resulting in the extension of a pseudopod after fusion of the membranous organelles to the plasma membrane delivering proteins necessary for fertilization.

The ultimate target of motility acquisition, in the context of success fertilization, is the Major Sperm Protein (MSP). This protein is a cytoskeletal protein that polymerizes to elongate the filaments that cause protrusion and maintenance of the spikes and pseudopod. At the same time, it is the protein responsible for the oocyte maturation signaling in the vesicles released from the spermatid. Thus, MSP is both a structural protein and an extracellular signal that allows the spermatozoa to fertilize an egg. In chapter 4, evidence of the direct role of MSP as an intracellular signaling protein is observed. *C. elegans* MSP has similar characteristic as MSP found in the parasitic nematode *Ascaris*. It precipitates crystals in a concentration-dependent fashion and it elongates fibers *in vitro*. Although, fiber elongation does not follow the same pattern as

MSP from *Ascaris* where addition of ATP is sufficient to promote fiber elongation and a treatment with tyrosine phosphatases stops this process. In the case of *C. elegans*, MSP must undergo a tyrosine dephosphorylation step prior to the elongation of filaments using ATP, suggesting that the characteristics of MSP from these two nematodes differ despite the conserved structure that the proteins show. MSP from *C. elegans* contains putative sites for posttranslational modification such as myristoylation sites and phosphorylation sites. These sites are conserved among the 30 isoforms present in *C. elegans* and not present in the two known isoforms of *Ascaris*, suggesting that subtle changes in the amino acid sequence of this protein can dictate the type of posttranslational modifications that it is subjected to and the role of this molecule in fertilization.

Using an integrative approach that includes the advantage of mutant strains, biochemical assays, pharmacological assays and time-lapse microscopy, this work has explored further the process of pseudopod extension in *C. elegans* spermatozoa including a physiological role for changes in the plasma membrane where the initiation signals must act. Future directions must include the study of sperm translocation, chemotaxis and sperm-egg interaction using this integrative physiological approach. The importance of investigation of motility acquisition in non-flagellated spermatozoa is crucial in the understanding of the fertilization process in all Eumetazoans. *C. elegans* has been used as a model organism to study human-related processes and the spermatozoa from this nematode is not the exception since it seems to be under the same physiological

regulation than its mammalian counterpart and has recently been shown that it contains conserved chromatin proteins that are likely targets for human male contraception.