TRANSPORT AND DISASSEMBLY OF ADHESION JUNCTIONS IN THE TESTIS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Anatomy and Cell Biology; Faculty of Medicine)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December 8, 2003

Co-Authorship statement

Published Manuscript:

Guttman, JA., Janmey, PA and Vogl, AW. 2002 Gelsolin – Evidence for a role in turnover of junction related actin filaments in Sertoli cells. **J. Cell Sci.** 115(3):499-505.

In this manuscript, Julian Guttman designed and performed experiments to test hypotheses that he helped develop. He had a large role in the analysis of all of the data and significantly contributed to the writing of the manuscript. PA Janmey provided numerous reagents and AW Vogl aided in the writing of the manuscript, data analysis and supervision.

Senior Author
A. Wayne Vogl

Accepted/In Press Manuscript:

Guttman, JA., Obinata, T., Shima, J., Griswold, M., Vogl, AW 2003 Non-Muscle Cofilin is a Component of Tubulobulbar Complexes in the Testis. **Biology of Reproduction.**

In this manuscript, Julian Guttman designed and performed experiments to test hypotheses that he developed. He had a large role in the analysis of all of the data and significantly contributed to the writing of the manuscript. T. Obinata provided numerous reagents, J. Shima and M Griswold provided access to their Genechip array data sets. AW Vogl aided in the editing of the manuscript, data analysis and supervision.

Senior Aut
A. Wayne Vogl

Submitted Manuscripts:

Guttman, JA., Takai, Y., and Vogl, AW 2003 Evidence that Tubulobulbar Complexes in the Seminiferous Epithelium are Involved with Internalization of Adherens Junctions. ***(submitted to Biol. Repro. in December)***

In this manuscript, Julian Guttman designed and performed experiments to test hypotheses that he developed. He had a large role in the analysis of all of the data and significantly contributed to the writing of the manuscript. Y. Takai provided reagents and AW Vogl aided in the editing of the manuscript, data analysis and supervision.

Senior Author
A. Wayne Vogl

Guttman, JA., Vaid, KS., and Vogl, AW 2003 Rac1 and Gelsolin Co-localize with Filamentous Actin at Ectoplasmic Specializations Throughout Spermiogenesis ***(submitted to the Anatomical Record in October)***

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Abstract

The cytoskeleton and its associated proteins are involved with numerous cellular activities including intracellular transport, maintenance of cell integrity, and strengthening intercellular junctional attachment. During sperm production, the seminiferous epithelium of the mammalian testis undergoes numerous cyclical changes, which involve cytoskeletal events. This epithelium is composed of the spermatogenic cells and their nurse cells, the Sertoli cells. Intercellular attachment between adjacent Sertoli cells as well as Sertoli cells and the maturing spermatids occurs through specialized actin-rich adhesion junction plaques found within Sertoli cells, termed ectoplasmic specializations. Throughout spermatid maturation, dynamic cytoskeletal-related processes lead to the transport of Sertoli/spermatid associated ectoplasmic specializations along Sertoli cell microtubules. This is followed by junction disassembly at Sertoli/spermatid regions during sperm release and junction turnover at Sertoli/Sertoli sites to allow the next generation of spermatogenic cells into the adluminal compartment of the epithelium. Studying the mechanisms by which these junction plaques are transported and disassembled will increase our knowledge of the processes occurring during spermatid transport within the seminiferous epithelium and spermatid release. In chapter 2, of this thesis I present the first evidence that a kinesin is associated with ectoplasmic specializations and identify two kinesin isoforms potentially involved in the entrenchment of spermatids within the seminiferous epithelium. In chapter 3, I demonstrate that the actin severing and capping protein, gelsolin, is a component of ectoplasmic specializations and show the first evidence that gelsolin may play a role in actin plaque disassembly. In chapter 4, I show that gelsolin and the small GTPase

upstream regulator of gelsolin, Rac1, are both present at ectoplasmic specialization locations throughout spermiogenesis. In chapter 5, I demonstrate that ectoplasmic specialization components are present at tubulobulbar complexes and give the first evidence that tubulobulbar complexes are involved in the internalization of ectoplasmic specialization membrane associated components. In chapter 6, I show that the actin disassembly factor, non-muscle cofilin, is found at tubulobulbar complexes and not at ectoplasmic specializations. These findings, united around the theme of adhesion junction transport and disassembly in the testis, significantly increase our understanding of the molecular events occurring during spermatid entrenchment and release in the seminiferous epithelium.

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List of Abbreviations

°C	Degrees Celcius
1D	One Dimensional
2D	Two dimensional
2DLCMS	Two dimensional Liquid Chromatography Mass Spectrometry
ADP	Adenosine Diphosphate
ARFs	ADP-ribosylation factors
ATP	Adenosine Triphosphate
Auto	Autofluorescence control
BSA	Bovine Serum Albumin
CHAPS	3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate
CIHR	Canadian Institutes of Health Research
cRNA	Complementary RNA
CTP	Cytosine 5'-triphosphate
DAG	Diacylglycerol
ddH20	Distilled, deionized water
DIC	Differential Interference Contrast microscopy
DMEM-F12	Dubecco Modified Eagle's Medium/F12 (Ham's F12)
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EEA1	Early Endosome Antigen 1
EGTA	Ethylene Glycol bis [succinidyl succinate]
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
GRP94	Glucose Regulated Protein 94kD
GTP	Guanosine Triphosphate
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid
HRP	Horseradish Peroxidase
IC74	74kD Intermediate Chain of Cytoplasmic Dynein
IEC	International Equipment Company
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
ILK	Integrin-linked kinase
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
IPG	Immobilized pH Gradiens
JIP	JNK-interacting protein
JNK	c-Jun N-terminal protein kinase
KIF	Kinesin Family member
KIFL	Kinesin-like Family member
LAMP1	Lysosome associated membrane protein 1
LCMS	Liquid Chromatography Mass Spectrometry

LIMK	LIM-kinase
M	Molar
Mab	Monoclomal antibody
MDCK	Madin Darby Canine Kidney
MES	2-[N-Morpholino] ethane sulfonic acid
mRNA	Messenger RNA
NaCacodylate	Sodium Cacodylate
NCIgY	Normal Chicken IgY
NGS	Normal Goat Serum
NHS	N-hydroxysuccinimide
NMC	Non-muscle Cofilin
NMIgG	Normal Mouse IgG
NRS	Normal Rabbit Serum
NSERC	Natural Sciences and Engineering Council
NSIgG	Normal Sheep IgG
OCT	Optimal Cutting Temperature
PBS	Phosphate Buffered Saline
PBS/BSA	Phosphate Buffered Saline/0.1% Bovine Serum Albumin
PEM	PIPES, EGTA, $MgCl_2$
PI(4)P-5 kinase	Phosphatidylinositol-4 monophosphate-5 kinase
PI3-kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid]
PKC	Protein Kinase C
PLCγ	Phospholipase C gamma
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleic Acid
RNAi	RNA interference
RPM	Rotations per Minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SE	Seminiferous epithelium
Sec Ab	Secondary antibody
SGP1	Sulphated glycoprotein1
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline/Tween-20
TESK	Testicular Protein Kinase
TPBS-BSA	Tween/ Phosphate Buffered Saline/0.1% Bovine Serum Albumin
Tris	Tris(hydroxymethanyl)aminomethane
Tween-20	Polyoxylethylenesorbitan Monolautate
UTP	Uridine 5'-triphosphate
UV	Ultra violet
ZO	Zonula Occludens

Acknowledgements

I would like to begin by thanking my supervisor, Dr. A. Wayne Vogl, for his years of guidance, unrelenting determination to ensure our success and friendship throughout my graduate career. I would also like to thank Tara Callan for her encouragement and re-assurances. I must thank the members of my supervisory committee, Dr. Donald Brunette and Dr. Robert Molday without which this thesis could not have been completed, and Dr. Calvin Roskelley for his years of guidance, supervision and critical review of manuscripts.

I would also like to thank Dr. Paul Janmey for his numerous suggestions with regard to the gelsolin project (Chapter 3) and providing the PIP2 binding region of gelsolin peptides and control peptides, Kuljeet Vaid for his help with the Rac1 aspect of this thesis, Dr. Yoshimi Takai for providing the anti-nectin 2, anti-nectin3, and anti-afadin antibodies, Dr. Takashi Obinata for his help with the cofilin aspect of this thesis (Chapter 6) and for providing the Mab-22 anti-cofilin antibody, and the anti-non-muscle cofilin antibody. I would also like to thank Dr. Jim Bartles for providing the anti-espin antibody and Dr. Tama Hasson for providing the anti-kelch/keap1 antibody and the anti-myosin VIIa antibody, Dr. Toshi Azuma for providing the anti-mouse gelsolin antibody, Dr. Carlos Morales for the anti-SGP1 antibody and Dr. Francis Barr for the anti-Rab6KIFL antibody.

Finally I need to thank Dr. Michael Griswold and Jim Shima for scanning their mouse testis and mouse Sertoli cell gene chip array data bases for our genes of interest prior to releasing their database to the public and Derek Smith at the University of Victoria GenomeBC Proteomics Centre for his help with the mass spectrometry analysis.

I was funded through both NSERC and CIHR.

The publication status of the material presented in this thesis is listed below:

Chapter 3
Guttman, JA., Janmey, PA and Vogl, AW. 2002. Gelsolin – Evidence for a role in turnover of junction related actin filaments in Sertoli cells. J. Cell Sci. 115(3):499-505. (published)

Chapter 4
Guttman, JA., Vaid, KS., Vogl, AW 2003. Rac1 and Gelsolin Co-localize with Filamentous Actin at Ectoplasmic Specializations Throughout Spermiogenesis. *(Submitted to the Anatomical Record)*

Chapter 5
Guttman, JA., Takai, Y., Vogl, AW. 2003. Evidence that tubulobulbar complexes in the seminiferous epithelium are involved with internalization of adherens junctions. (*Submitted to the Biology of Reproduction*)

Chapter 6
Guttman, JA., Obinata, T., Shima, J., Griswold, M., Vogl, AW 2003. Non-Muscle Cofilin is a Component of Tubulobulbar Complexes in the Testis. *(Accepted for publication in the Biology of Reproduction)*

CHAPTER1

General Introduction

Introductory Statement

Sertoli cells are the major architectural units of the seminiferous epithelium which play key roles in spermatogenic cell maturation and orchestrate a number of key events during spermatogenesis. These events, which include the positioning of spermatogenic cells in the epithelium, sperm release, and turnover of basal attachments between Sertoli cells, involve elements of the cytoskeletal system and unique intercellular adhesion junctions termed ectoplasmic specializations. The cytoskeleton in Sertoli cells is elaborate, and their ectoplasmic specializations, which undergo a cyclical process of assembly and disassembly during spermatogenesis, are extremely large. Consequently, Sertoli cells are an ideal model *in vivo* system for the study of adhesion junctions. Taking advantage of the various stages of the naturally occurring cyclical process of spermatogenesis, I explore in this thesis the role of intercellular adhesion junctions in microtubule-based spermatid translocation and explore the mechanisms of adhesion junction turnover in the testis. I (1) present evidence that supports the hypothesis that a kinesin motor protein is found associated with adhesion junctions in the testis, a finding consistent with the microtubule-based spermatid translocation hypothesis, (2) demonstrate that gelsolin, a calcium dependent actin severing protein is present at ectoplasmic specializations and present a model for disassembly of the actin plaque in the testis, (3) immunolocalize rac1, an upstream regulator of gelsolin, in the seminiferous epithelium and suggest a possible function for this protein at ectoplasmic specializations (4) explore the hypothesis that specific finger-like structures, called tubulobulbar

complexes, are involved in testis adhesion junction turnover and (5) demonstrate that non-muscle type cofilin is a component of tubulobulbar complexes and is not present at ectoplasmic specializations.

Cytoskeleton

Within cells, protein polymers act as an internal skeleton allowing for numerous events to occur including intracellular trafficking of cargo, mechanical strength and cell movement. This network of filamentous structures is called the cytoskeleton. The cytoskeleton is composed of three major elements; intermediate filaments, microtubules and actin filaments as well as a plethora of associated proteins. Each of the major cytoskeletal structures works to ensure various aspects of normal cellular function.

Intermediate Filaments

Intermediate filaments are strong yet flexible non-polar polymers that confer structural strength to cells and sub-cellular organelles (Coulombe et al., 2000). Their nomenclature refers to their thickness of about 11nm, intermediate between that of actin filaments and microtubules (reviewed by: Strelkov et al., 2003). These polymers are composed of proteins, which are arranged into 6 classes and vary considerably in size and composition. Within the cell cytoplasm, intermediate filaments are often found encircling the cell nucleus and have projections to the periphery (Coulombe et al., 2000).

Actin Filaments

Actin filaments (or F-actin) are composed of globular actin monomers (or g-actin), which non-covalently bind with one another to form polar filamentous structures (reviewed by: Pollard and Borisy, 2003). In cells, the function and maintenance of these 6nm filaments are highly controlled by numerous actin associated capping, severing, and sequestering proteins (reviewed by: Yin and Janmey 2003; Pollard and Borisy 2003). In addition to this there are many actin binding, bundling, membrane-tethering proteins and motor proteins which all play essential roles in actin filament functions (Borisy and Svitkina, 2000; Cooper and Schafer, 2000; Pollard and Borisy 2003).

Microtubules

Microtubules are long, 25nm in diameter, tube-like polymers formed by α and β tubulin heterodimers. They are polar structures with distinct plus (fast growing) and minus (slow growing) ends. In most cells microtubules originate at nucleation sites called the microtubule organizing centers. Microtubules then radiate out to the cell membrane with their plus-ends directed to the cell periphery. Like actin filaments, microtubules have numerous proteins and modifications associated with them which can further stabilize, or disassemble them. Their intrinsic polarity also allows these structures to be used as tracts for directional cargo transport (Goldstein and Yang, 2000; Higuchi and Endow, 2002; Kamal and Goldstein, 2002).

Motor Proteins

Microtubules routinely are used as tracts, for the transport material such as vacuoles, RNA, organelles and other material from one subcellular region to another by microtubule-associated molecular motors (Hirokawa, 1996; Hirokawa, 1998; Brendza et al., 2000). There are 2 major populations of microtubule motors, cytoplasmic dynein and the kinesins. Cytoplasmic dynein is a massive multi subunit protein complex that interacts with another large multi subunit protein complex, the dynactin complex, which activates dynein and allows it to bind to its cargo to transport it (thus far exclusively) towards the minus-ends of microtubules.

Kinesins are a superfamily of microtubule-associated mechanoenzymes comprising over 286 members (last updated March 2001) (The Kinesin Homepage; http://www.blocks.fhcrc.org/~kinesin/index.html) and 45 genes (in mice and humans) (Miki et al., 2001). These molecular motors convert chemical energy from ATP to mechanical energy in order to transport material within cells. They are composed of three main regions, a highly conserved globular head, a very divergent (usually globular) tail and a neck. The neck is the linker of the head to the tail. Kinesins use microtubules as tracts. Because microtubules are constructed with one end (the plus end) growing faster than the other (the minus-end), kinesins take advantage of this polarity to transport their cargo. The majority of known kinesins transport material towards the plus-ends of microtubules, although minus-end moving kinesins also occur. The directional change of kinesins have, thus far, been accomplished through two mechanisms. The first is by moving the location of their motor domain (head). Most kinesin proteins (including

conventional kinesin, also known as KIF5b) transport material towards the plus-ends of microtubules and have their head domain located at the N-terminus of the protein. By moving the motor domain (head) of the kinesin to the C-terminus of the protein an opposite direction of movement occurs (towards the minus-end of microtubules) (Hirokawa 1998). The second method of reversing kinesin's direction is by modifying amino acids in the neck region of the molecule. Experiments showed that through a single mutation of one of the 11 amino acid making up the neck region of one of the kinesin proteins, the directionality of a C-terminal (minus-end directed) kinesin can be reversed to move in the plus-end direction (Endow and Higuchi, 2000).

Kinesins are usually composed of 2 heavy and 2 light chains. The heavy chains contain the motile machinery and the light chains are thought to confer cargo specificities and regulation of the motor protein as a whole. Although the light chains are also thought to participate in cargo binding they interestingly are not found in all species (eg. Fungi) (Steinberg, and Schliwa,1995; Kirchner et al., 1999), suggesting that they are likely not essential for function in all kinesins.

Recently, the regulation and docking mechanisms of kinesins have been the subject of much research. For a number of years, the only docking protein found associated with kinesin was the conventional kinesin docking protein, kinectin. Because this protein is not found in axons, where conventional kinesin is known to be present, the search for other kinesin associated proteins has intensified. To date only a handful of docking/scaffolding proteins have been discovered. These include kinectin (Toyoshima

et al., 1992), the JIP scaffolding proteins (scaffolding proteins associated with the JNK signalling pathway; Verhey et al., 2001) and the amyliod precursor protein (Kamal et al., 2000). Thus far all of these proteins interact with the light chains of the tail of conventional kinesin. To date the one exception is a specific kinesin called KIF 17 which interacts with mLin-10 [a part of a protein complex thought to sort NMDA receptors in neurons (Setou et al., 2000)] through it's heavy chains.

Relatively little is known about the regulation of kinesins and the majority of what is known is based on work using conventional kinesin. Kinesins are thought to be regulated through at least 2 mechanisms, which may work in tandem. The first is by the kinesin tail, possibly through its light chains. Conventional kinesin's tail decreases the motile activity of kinesin, by folding at 2 hinge regions within the kinesin heavy chain (Reilein et al., 2001). It is thought that the head to tail interaction renders kinesin inactive. Phosphorylation (likely the second method of regulation) plays some role in the regulation of kinesin, but its exact role remains to be elucidated (Donelan et al., 2002). The phosphorylation of kinesin, has been shown to decrease the motor's ability to associate with microtubules and decreases the motor's ability to move vesicles *in vitro* (Sato-Yoshitake et al., 1992; Nybakken et al., 2002), but these results have been contradicted by *in vivo* evidence showing that membrane bound forms of kinesin from neurons, presumed to be active are mostly phosphorylated (Lee and Hollenbek 1995).

Morphologically Distinct Intracellular Adhesion Junctions in General

The tissues of the body are composed of cells, which attach to one another usually through morphologically distinct structures called cell-cell junctions and to the substratum by cell-substratum junctions. There are many different forms of these junctions, which confer different functions. Of these, desmosomes, hemidesmosomes, tight junctions, adherens junctions and gap junctions are the most prevalent.

Desmosomes and hemi-desmosomes

Desmosomes (or macula adherens) are intracellular junctions that are confer strong attachements to cells and tissues (Green and Gaudry, 2000). These junctions (often referred to as spot-welds) are composed of protein plaques to which intermediate filaments attach intracellularly (through the use of the linking proteins such as desmoplakin and plakoglobin) and cadherin-like molecules (desmocollin and desmoglein) which attach extracellularly.

Hemidesmosomes appear morphologically as half desmosomes but are molecularly dissimilar. Although both link a protein-rich plaque to the intermediate filament component of the cytoskeleton, hemi-desmosomes attach cells to the extracellular matrix through integrin-based cell adhesion molecules.

Tight Junctions

Tight junctions are cell-cell seals that form selective barriers between different regions of epithelia. They are composed of the calcium dependent transmembrane molecules from the claudin and occludin families which interact with the intracellular

proteins ZO1, ZO2 and ZO3 to attach to the actin cytoskeleton in order to further strengthen the junction's integrity. Tight junctions are usually found in apical regions where 2 cells interact. By linking these junctions cell to cell in a continuous fashion a tight junction barrier (or zone) can be formed called a zonula occludens allowing for the physiological separation of luminal and basolateral regions of epithelia.

Adherens Junctions

Adherens-type junctions are the first junctions to form upon cell-cell interaction (Yeaman et al., 1999). They are usually composed of one of two types of calcium-dependent transmembrane adhesion proteins: the homotypic binding proteins, the cadherins, or the heterotypic interacting proteins, the integrins. Cadherins attach intracellularly with the members of the catenin family. These proteins then connect either directly or through other scaffolding proteins to the actin cytoskeleton. Integrins act similarily, through heterotypic association, one of the 18 α integrins can interact extracellularily with one of the 8 β integrins then to the actin cytoskeleton intracellularly either directly or through scaffolding proteins. The recently discovered nectin family of proteins are a third group of adherens junction proteins, but interestingly these proteins are calcium independent. Like tight junctions, adherens junctions also form morphologically discrete junction zones called zonula adherens. A type of actin-associated intercellular adhesion junction in the seminiferous epithelium is the focus of this thesis.

Gap Junctions

Gap junctions (or connexons) are intercellular channels, which allow hydrophilic molecules up to 1kD in size to selectively pass between adjacent cells. The channels are formed by members of the connexin family of proteins. Six of these proteins make half of the channel in one cell and another six form the channel in the adjacent cell. Gap junctions can be found with few to thousands of channels and the amount of channels open to closed, or the type of connexins forming the connexon can dictate the communication arising from these junctions.

Adhesion Junction Disassembly

Although there is a disproportionately large amount of data on cadherin-based over integrin-based junction disassembly, the endocytosis of some of the adhesion elements of these junctions has begun to be investigated. Thus far, both E-cadherin and β1 intergin have been studied and both have demonstrated the ability to be recycled (Le et al., 1999; Le et al., 2002). Using recycling assays with MDCK cells, Le et al (1999) showed that at least one pool of surface E-cadherin is capable of being actively endocytosed and recycled back to the membrane. This conclusion was further confirmed with the colocalization of E-cadherin with some but not all vesicles when stained with the early endosomal marker rab5, and not with rab7 (a late endosomal marker). Indicating that the internalized vesicles were likely destined for recycling as opposed to late endosomal/lysosomal degradation (Le et al., 1999). Through the use of specific inhibitors, this endocytosis was then shown to be regulated by PKC, a known endocytosis regulator (Le, et al., 2002).

A similar conclusion has been reached with the β1 integrin. Here, Ng et al., (1999) initially found β1 integrin partially colocalized with PKCα in mammary epithelial cells. Following this, they found that the β1 integrin and PKCα coprecipitated. With this interaction in place, Ng et al., (1999) demonstrated that the stimulation of PKCα induced β1 internalization which traffics to the endosomal compartment in a calcium/PI3-kinase dependent manner which is followed by it entering an endocytic recycling pathway (Ng et al., 1999).

Organization of the Testis

The testis is the organ where spermatozoa and androgens are produced. There are two compartments of the testis; the interstitial compartment and the germinal compartment. The interstitial compartment is composed of the material found between the hundreds of small tubules of the testis called seminiferous tubules, in the space of the interstitium. This interstitial compartment has an endocrine function and is the location of a specialized group of cells called the interstitial cells of Leydig that are usually clustered around blood vessles. The function of these cells is to produce testosterone (Clermont and Perey, 1957; Clermont 1972).

The germinal compartment, has an exocrine function and is the site where spermatogenic cells are produced and matured through a 64 day process in humans called spermatogenesis (LeBlond and Clermont, 1952). This germinal compartment is

composed of the seminiferous tubules each lined by a thick seminiferous epithelium separated by a thin tubule wall.

The Seminiferous Epithelium

The seminiferous epithelium is composed of 2 populations of cells, the spermatogenic cells and the Sertoli cells. The spermatogenic cells are the cells of the male germ line that will eventually differentiate into spermatozoa. There are three distinct sub-sets of spermatogenic cells in the seminiferous epithelium; spermatogonia, spermatocytes and spermatids. The most juvenile are the spermatogonia. These proliferative spermatogenic cells are found on the basal lamina and undergo mitosis. Some daughter cells undergo further differentiation to become spermatozoa while others replenish the spermatogonial population. Daughter cells destined for further differentiation are spermatocytes.

Spermatocytes move off of the basal lamina, and undergo meiosis to become the third type of spermatogenic cells, haploid round spermatids. Spermatocytes move through the basal junction complex between adjacent Sertoli cells and complete meiosis in the compartment of the epithelium "above" the junctions.

The spermatids are the cells that undergo the process of spermiogenesis which is the process of morphological differentiation from round through elongate to mature spermatids. This process involves the condensation of the spermatid nucleus, the removal of excess cytoplasm, the production of a flagellum and the production of a large

enzyme-containing secretory vesicle called an acrosome. Upon completion of the process of spermiogenesis, elongate spermatids are released from the seminiferous epithelium and are then referred to as spermatozoa. The process of spermatogenesis occurs in association with and is totally dependent on Sertoli cells.

Sertoli Cells

Sertoli cells are the structural units of the seminiferous epithelium and orchestrate many of the events that occur during spermatogenesis. These tall columnar, highly invaginated cells with many processes, span the entire height of the seminiferous epithelium and are responsible for the positioning of the spermatogenic cells in the seminiferous epithelium and changes in intracellular adhesion. Sertoli cells contain an abundance of cytoskeletal components found generally in cells (intermediate filaments, microtubules and actin filaments).

Sertoli cell cytoskeleton

Unlike most cell types, the cytoskeletal components in adult Sertoli cells are highly segregated. Sertoli cell intermediate filaments (vimentin-type), form a network around the nucleus and have projections to three main areas: 1) hemidesmosome-like junctions located at the base of the Sertoli cell, 2) desmosome-like junctions located along the lateral walls of the basal 1/3 of the the Sertoli cell and 3) certain regions of actin containing intercellular junction plaques found apically.

Unlike in most cultured cells, Sertoli cell microtubules are oriented with their minus-ends directed towards the cell periphery (Redenbach and Vogl., 1991) and are arranged parallel to the long axis of the cell. Sertoli cell microtubules are nucleated from these peripheral sites (Redenbach and Vogl. 1991.) possibly through the use of another tubulin isoform found in Sertoli cells, exclusively at peripheral sites, γ-tubulin (Guttman et al., 2002a). In Sertoli cells few studies have looked at the stability of the microtubules through post-translational modifications. One of these that has been investigated involved tyrosinated α tubulin monomers in Sertoli cell microtubules (Hermo, Oko and Hecht 1991; Wenz and Hess 1998). Tyrosinated tubulin is thought to be associated with less stable microtubules. During the stages of spermatogenesis when the movement of spermatogenic cells is occurring (stages III-V, entrenchment and VI-VII, return to the apex), Sertoli cell microtubules had the least amount of tyrosinated α tubulin, indicating that in this case, the Sertoli cell microtubules may be reasonably stable during spermatid movement as compared to their stability during other spermatogenesis stages, where more extensive microtubule remodelling may occur.

Actin filaments in Sertoli cells are found concentrated in unique adhesion junctions called ectoplasmic specializations

Junctions in the Seminiferous Epithelium

The seminiferous epithelium contains a variety of intercellular junctions. Found towards the base of the epithelium is a junction complex between neighbouring Sertoli cells consisting of tight junctions, unique actin-related intercellular adhesion junctions

called ectoplasmic specializations, gap junctions and desmosomes (Figure 1.1). Desmosomes, gap junctions and apically located ectoplasmic specializations also occur where the Sertoli cells interact with spermatogenic cells at varying stages of maturity.

Tight junctions in the testis

The seminiferous epithelium is divided into two regions, a basal and an apical (or adluminal) compartment. Tight junctions define these seminiferous epithelium compartments thereby forming the blood-testis barrier. These tight junctions are found where two adjacent Sertoli cells interact and are flanked and overlapped by ectoplasmic specialization adhesion junctions at the base of the seminiferous epithelium (Figure 1.1). These junctions physiologically compartmentalize the seminiferous epithelium forming both a hospitable environment as well as an immunological barrier allowing for safe spermatid production. Found in the basal compartment, are the spermatogonia, the spermatogenic cells men are born with. These cells are seen as self and are therefore not attacked by the man's own immune system. Because the cells further along in spermatogenesis are not "self", due to their development at puberty, the apical compartment formed by the tight junctions is the major site of the successive stages of spermatogenesis. In order for certain cells to mature in one compartment while leaving other cells in another, the tight junctions and basally located ectoplasmic specializations disassemble and reassemble in a very controlled manner.

Ectoplasmic Specializations

Ectoplasmic specializations (Figure 1.1) are modified forms of actin-related adhesion junctions found exclusively in Sertoli cells which function in both Sertoli cell/Sertoli cell and Sertoli cell/Spermatid adhesion, spermatid translocation and spermatid release from Sertoli cells. Beginning at the Sertoli cell surface and moving cytoplasmically, these structures are composed of the Sertoli cell plasma membrane, a layer of unipolar hexagonally packed actin filaments and a cistern of endoplasmic reticulum. Molecular components localized at the ectoplasmic specialization include actin (Toyama, 1976; Franke et al., 1978; Vogl et al., 1983; Suarez-Quian and Dym, 1984; Vogl and Soucy, 1985a; Vogl and Soucy, 1985b Vogl et al., 1986; Camatini et al., 1986; Camatini et al., 1987; Masri et al., 1987; Suarez-Quian and Dym, 1988; Fouquet et al., 1989;), α-actinin (Franke et al., 1978; Jockusch and Isenberg, 1981), vinculin (Grove and Vogl, 1989), espin (Bartels et al., 1996), fimbrin (Grove and Vogl, 1989), myosin VIIa (Hasson et al., 1995; Velichkova et al., 2002), α6 β1 integrin (Pfeiffer and Vogl., 1991; Palombi et al., 1992), gelsolin (Guttman et al., 2002b), rac1 (Guttman et al., 2002c), Nectin-2 and afadin (Ozaki-Kuroda et al., 2002) as well as Fyn tyrosine kinase (Maekawa et al., 2002). Interestingly the conventional contractile myosin, myosin II, and the adhesion junction proteins, cadherins and catenins have not been localized to the junction plaque. Other components have been reported but not confirmed (Wine and Chapin, 1999; Cheng and Mruk, 2002).

A large body of work supports the conclusion that ectoplasmic specializations perform primarily an adhesive function. Evidence that these unique plaques are actin-related adhesion junctions includes the following: First, the space between adjacent

membranes where ectoplasmic specializations are present is very small (70-100A) (Russell, 1977) relative to the intercellular space elsewhere, suggesting that the sites are a type of junction. Second, $\alpha 6\beta 1$ integrin and nectin-2, known adhesion molecules, are concentrated at the sites (Pfeiffer and Vogl., 1991; Palombi et al., 1992; Ozaki-Kuroda et al., 2002). Third, when the seminiferous epithelium is fragmented, apical ectoplasmic specializations remain adherent to the elongate spermatid heads (Romrell and Ross., 1979; Vogl, 1996). Finally, the structures contain vinculin (Grove and Vogl., 1989), an adaptor molecule, and a classical marker for actin related adhesion junctions (Geiger et al., 1980). The transport and disassembly of ectoplasmic specializations is the focus of this thesis because both of these processes are fundamental to spermatogenesis.

Junction Transport in theTestis

Throughout spermiogenesis, developing spermatids are found at various levels in the seminiferous epithelium. The transport of ectoplasmic specializations attached to the developing spermatids is thought to be responsible for these differences in height. In order to test this, the microtubule-based spermatid translocation hypothesis was developed. This hypothesis states that spermatids are transported towards the base of the seminiferous epithelium and are returned to the apex by two types of molecular motors. These motor proteins are attached to the cytoplasmic face of the endoplasmic reticulum component of ectoplasmic specializations and use the Sertoli cell's microtubules as tracts.

If this is true, based on the polarity of the Sertoli cell microtubules both plus- and minus-end directed microtubule-based molecular motors should be present on the

ectoplasmic specializations to account for the downward and upward movement of the ectoplasmic specializations and thus the spermatids. Thus far, a variety of evidence supports the hypothesis that ectoplasmic specializations and microtubule-associated motor proteins are responsible for the various spermatid heights in the seminiferous epithelium. Ectoplasmic specializations still attached to their developing spermatids bind more exogenous microtubules in the absence of ATP than in the presence of ATP (Vogl, 1996). This was the predicted result because microtubule-associated molecular motors on the junction plaque should be able to cycle off exogenous microtubules upon motor activation. Further evidence is from *in vitro* motility assays, performed with fluorescently labelled microtubules attached to isolated spermatid/junction plaques. Here microtubules were seen translocating over the junction plaque upon the addition of a motility buffer containing ATP (Beach and Vogl, 1999). In order to test whether the microtubule motility occurred in two directions to account for the entrenchment (down, plus-end directed movement) and return to the apex (up, minus-end directed movement), a polarity marked microtubule motility assay was performed. In this assay, as was predicted, polarity-marked microtubules were recorded moving in both the plus- and minus-end directions upon the addition of an ATP containing motility buffer (Guttman et al., 2000). Other evidence in support of this spermatid translocation hypothesis is from the work by Miller et al., (1999) in which the 74kD intermediate chain of cytoplasmic dynein was localized to ectoplasmic specializations and appeared concentrated at the plaques at the stages when it would be used following the entrenchment process, stages V-VII. Interestingly in the seminiferous epithelium, conventional kinesin has only been found associated with the trans-golgi network and is notably absent from ectoplasmic

specializations. Exploring the hypothesis that a kinesin is associated with ectoplasmic specializations is the focus of chapter 2 of this thesis.

Junction Turnover in the Testis

Virtually nothing is known about the mechanism of adhesion junction turnover in the testis. What is known is that turnover, specifically disassembly, is associated with two major events – movement of spermatocytes through the basal junction complex between Sertoli cells at the base of the epithelium and sperm release. The first event occurs following the spermatogonia's differentiation into a spermatocyte. Here, basally located junction plaques disassemble in front of and re-assemble behind spermatocytes as they move through the junction complex. Other junction types in the basal junction complex undergo a similar turnover. Once in the apical compartment, the spermatocytes are no longer associated with ectoplasmic specializations.

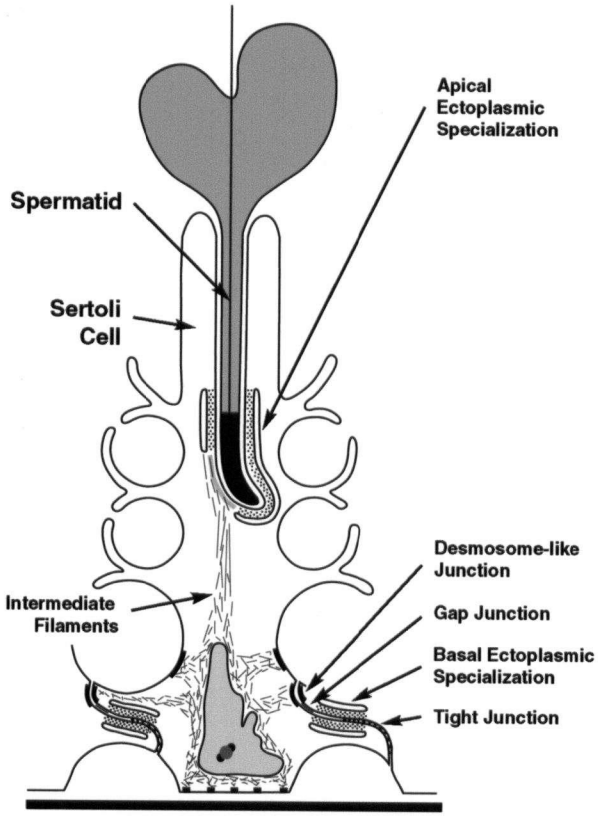
In apical regions, ectoplasmic specializations form adjacent to round spermatids as these cells begin to polarize. Here, the ectoplasmic specializations assemble within the Sertoli cell in regions overlying the site where the acrosome lies, adjacent to the spermatid plasma membrane. As this acrosome continues to develop and elongate in conjunction with the elongation of the spermatid head, so does the apically located ectoplasmic specialization until the ectoplasmic specialization completely overlies the spermatid head. These junction plaques remain attached to the elongate spermatids until the final stages prior to spermatid release. At this point the plaques disassemble and finger-like projections of the spermatid, called tubulobulbar processes develop and protrude into the Sertoli cell cytoplasm forming tubulobulbar complexes (Russell 1979a).

The tubulobulbar complexes are the final structures that anchor the spermatids to the Sertoli cells prior to spermatid release in many mammals. Disassembly of ectoplasmic specializations and the involvement of tubulobulbar complexes in this process are the focus of chapters 3, 5 and 6 of this thesis.

Thesis Problem

In this thesis I will study the processes of ectoplasmic specialization transport and turnover as well as explore the possibility that tubulobulbar complexes are involved with internalization of adhesion junctions during junction turnover. In chapter 2, I will test the hypothesis that kinesin motor protein(s) are present at ectoplasmic specializations by generating pan-specific kinesin antibodies, and identify candidate kinesin isoform(s) using proteomics and other approaches. In the third part of my thesis I report that gelsolin is present at ectoplasmic specializations and based on this finding I generate and test the hypothesis that a gelsolin/PIP_2/PLCγ pathway is involved with ectoplasmic specialization disassembly. In the fourth chapter I follow-up the gelsolin work by investigating the hypothesis that rac1 may be present at the junction plaque and describe the localization of this small GTPase and gelsolin throughout spermiogenesis. In the fifth chapter, I catalogue the location of numerous ectoplasmic specialization molecular components at tubulobulbar complexes and generate a model for how these structures may function to endocytose ectoplasmic specialization components. Finally, in chapter 6 I demonstrate that non-muscle cofilin is a component of tubulobulbar complexes and not ectoplasmic specializations and suggest a possible function for this protein at these structures.

Figure 1.1. Diagram depicting the location of various intercellular junctions in the testis, note that regions of the tight junctions are flanked by the basally located ectoplasmic specializations. From Vogl et al., 2000.


Apical
Ectoplasmic
Specialization
Spermatid
Sertoli
Cell
Desmosome-like
Junction
Intermediate
Filaments
Gap Junction
Basal Ectoplasmic
Specialization
Tight Junction

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CHAPTER 2

Evidence supporting the presence of kinesin-like proteins at unique intercellular adhesion junctions in the testis

Introduction

One of the most dramatic events that occurs during sperm production is the translocation of maturing spermatids from the apex of the seminiferous epithelium to the base and then back up to the apex for release into the lumen of the seminiferous tubule (Figure 2.1). Elongate spermatids are positioned in apical Sertoli cell invaginations termed crypts. Changes in the depth of these apical crypts correlates with changes in the position of the spermatids within the epithelium. Unique intracellular adhesion plaques, called ectoplasmic specializations, occur in Sertoli cells at regions where the plasma membrane of the Sertoli cell attaches to the plasma membrane of the spermatid heads. It has been proposed that, in addition to a primary role in intercellular adhesion, ectoplasmic specializations, at sites of attachment to spermatids, participate in moving and positioning spermatids in the epithelium (Redenbach and Vogl, 1991). This is thought to function through microtubule-associated molecular motors which are attached to the junction plaque (Redenbach and Vogl, 1991; Beach and Vogl, 1999; Miller et al., 1999; Guttman et al., 2000; Vogl et al., 2000). These enzymatic protein machines would use the Sertoli cell's microtubules as tracts for the transport of the junction plaques, and thus the elongating spermatids, towards the base then the apex of the seminiferous epithelium.

Ectoplasmic specializations are found both at basal (Sertoli/Sertoli) and at apical (Sertoli/spermatid) sites of intracellular adhesion in the seminiferous epithelium. They

are tripartite structures composed of the Sertoli cell plasma membrane, a layer of unipolar hexagonally packed actin filaments and a cistern of endoplasmic reticulum. Found on the cytoplasmic side of the endoplasmic reticulum component of the ectoplasmic specialization is the minus-end directed molecular motor cytoplasmic dynein (Guttman et al., 2000).

Sertoli cell microtubules are found mainly in a supranuclear region. These microtubules are arranged parallel to the long axis of the cell and approximately 93% of them have their plus (or fast-growing) ends directed towards the Sertoli cell base and their minus (or slow- growing) ends located at the Sertoli cell apex (Redenbach and Vogl, 1991).

Microtubule-associated proteins are associated with a variety of cellular functions. Some of these proteins, the molecular motors, play a key role in the transport of intracellular components. There are two major populations of microtubule-based molecular motors that are found within cells, the kinesins and cytoplasmic dynein. Cytoplasmic dynein is a massive multi-subunit protein, which transports cargo exclusively towards microtubule minus-ends. One of the subunits of this protein, the 74kD intermediate chain, has been localized to ectoplasmic specializations and is thought to be responsible for the return of the junction plaques, and thus the maturing spermatids, to the apex of the Sertoli cell prior to ectoplasmic specialization disassembly and spermatid release. Although some members of the kinesin family of proteins are also capable of transporting cargo towards the minus-ends of microtubules, the majority of

kinesin's members are plus-end directed transporters. Currently there are well over 264 members of the kinesin superfamily (The Kinesin Homepage: http://www.proweb.org/kinesin//MotorSeqTable.html). Of these, conventional kinesin is the isoform which has been most extensively described. Although there is evidence that conventional kinesin is associated with endoplasmic reticulum structures (Houliston and Elinson, 1991; Henson et al.,1992; Lane and Allan, 1999) in the seminiferous epithelium, conventional kinesin has only been found associated with the trans-golgi network and is notably absent from ectoplasmic specializations. Numerous other kinesins have been identified in the testis through various means (Johnson et al., 1996; Miller et al., 1999; Navolanic and Sperry, 2000; Lai et al., 2000; Junco et al., 2001; Macho et al., 2002; Zou et al., 2002) but again, none of these have been found at ectoplasmic specializations.

Observations that are pertinent to the microtubule-based spermatid translocation hypothesis are the following: (1) there is an abundance of motor proteins in the testis (Sperry and Zhao, 1996; Yamazaki et al., 1996; Henson et al., 1997; Criswell and Asai, 1998; Miller et al., 1999); (2) microtubules are oriented parallel to the direction of spermatid movement (Fawcett, 1975); (3) morphological linkages occur between the endoplasmic reticulum of the ectoplasmic specialization and microtubules (Russell, 1977); (4) in *in vitro* binding assays, isolated junction plaques bind exogenous microtubules in a nucleotide dependent fashion (Vogl, 1996); (5) the 74kD intermediate chain of cytoplasmic dynein is associated with the cytoplasmic face of the endoplasmic reticulum at ectoplasmic specializations (Miller et al., 1999, Guttman et al., 2000), (6)

isolated ectoplasmic specializations support microtubule movement (Beach and Vogl ,1999) and (7) microtubule movement on isolated ectoplasmic specializations is bi-directional (Guttman et al., 2000), in other words, the junctions support movement both in the plus and minus end directions.

If the microtubule-based spermatid translocation hypothesis is true, then based on the polarity of Sertoli cell microtubules, a plus end-directed microtubule-based molecular motor, likely a kinesin, should be involved in entrenching the spermatids deep within the Sertoli cell crypt. The return of the spermatids to the apex of the Sertoli cell (and thus to the apex of the seminiferous epithelium) would be due to the presence of a minus-end directed motor, likely a dynein (Miller et al., 1999; Guttman et al., 2000).

In order to investigate the possibility that a kinesin is associated with ectoplasmic specializations I used a variety of approaches. First, I generated and characterized antibodies to three conserved peptide sequences of kinesin proteins and used them as a screening tool on rat testis material, an approach used by others studying different systems (Sawin et al., 1992). Our results show that these antibodies react with their respective peptides and purified recombinant *Drosophila melanogaster* conventional kinesin protein on western blots as compared to controls. Using immunofluorescent localization, these antibodies labeled areas previously known to contain kinesins (spermatid tails, spermatogenic cell cytoplasm, the manchette and the Sertoli cell cytoplasm) as well as ectoplasmic specializations. Western blots of rat seminiferous epithelium probed with the anti-peptide antibodies showed numerous reactive bands,

some of which disappear upon prior incubation with the antibody's generated peptide. These bands also are not present on normal chicken IgY control blots. 2D western blots of supernatants from concentrated spermatid/ectoplasmic specialization preparations treated with the actin severing protein gelsolin resulted in many reactive protein dots which did not appear on normal chicken IgY control blots. Identical 2D gels were run and correlative protein spots were excised and sent for mass spectrometry analysis resulting in the identification of proteins other than kinesin-like proteins which also contained the generated antibody peptide sequences as well as many hypothetical proteins.

As a second method of identifying kinesin-like proteins at ectoplasmic specializations, gelsolin treated spermatid/ectoplasmic specialization supernatant samples were sent for 2DLCMS analysis which resulted in the identification of kin3, a recently discovered kinesin like protein from the corn smut fungus *Ustilago myadis* (Wedlich-Soldner et al., 2002). A third method, involving the use of GeneChip microarrays also was used. Using mouse testis developmental series GeneChip microarrays, we discovered numerous kinesin and kinesin-like proteins that had increased amounts of mRNA transcripts when spermiogenesis is underway, the time of spermatid entrenchment. Interestingly, only one of these genes, that for Rab6KIFL, had high amounts of transcript in GeneChip arrays screened with Sertoli cell cRNA. Protein expression in the rat testis and rat seminiferous epithelium was confirmed by western blots using an antibody to Rab6KIFL and this protein was detected on supernatant 2D western blots of gelsolin treated spermatid/ectoplasmic specialization complexes. Results

presented here provide the first evidence that kinesin-like proteins may be present at ectoplasmic specializations a finding consistent with the microtubule-based spermatid translocation hypothesis. However, some degree of caution must be taken when interpreting the immunofluorescent results because of the cross reactivity of the anti-peptide antibodies with other proteins.

Materials and methods

Animals

Unless stated otherwise, all animals used in these studies were reproductively active male Sprague Dawley rats. They were obtained from the University of British Columbia animal care colony and maintained according to the guidelines established by the Canadian Council on Animal Care.

Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were obtained from Sigma-Aldrich Canada (Mississauga, Ontario). The paraformaldehyde and NaCl were obtained from Fisher Scientific (Vancouver, BC). All control immunoglobulins (IgG and IgY) as well as all secondary antibodies conjugated to Texas Red or Horseradish Peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pennsylvania)

Antibody generation

Peptides were synthesized to three of the conserved kinesin sequences ('CHIPYRESKLT', 'LNLVDLAGSE' and 'GYNTIFAYGQTG') and coupled to keyhole limpet hemocyanin by Research Genetics. The coupled peptides were mixed with Freunds Complete Adjuvant (500 μg peptide per animal) and each peptides was injected into 3 Leghorn laying hens by QED Biosciences (San Diego, CA). A total of four "boost" injections, consisting of 500μg peptide mixed with Freunds incomplete adjuvant per injection were separated by 7days.

Eggs were collected for 2 weeks pre-injection for isolation of pre-injected control IgY. Both pre- and post-injection IgY were isolated using water separation, centrifugation, filtration and a 12% Polyethylene glycol 6000 cut (to precipitate the IgY out of solution). Post-injected antibodies were affinity purified using their peptide bound to NHS (N-hydroxysuccinimide)-activated Sepharose (Amersham Pharmacia Biotech) columns. To do this, antibodies were initially passed through 0.45μm filters (Osmonics) then loaded onto the columns overnight. Antibodies were eluted with 0.2M NaCl/0.2M Glycein-HCl pH 2.2 (elution buffer) and collected in tubes containing 1.0M Tris pH 9.0 to neutralize the pH.

Dot (line) blots were be used to determine reactivity of the IgY with the peptide sequences. To do this, Immobilon-P Transfer membrane (Millipore; Mississauga, Ontario) was coated in 10 mls of 15μg/ml of peptide solution in TBST for 1hour at room temperature, blocked and reacted with the generated anti-peptide antibodies. After extensive washing and secondary antibody labeling, the line blots were reacted with ECL (Amersham) for chemiluminescent detection.

Other antibodies

Other antibodies used in this study consisted of the following: a mouse anti-gelsolin antibody used at 0.5μg/ml (Transduction Labs), a mouse anti-vinculin antibody used at 2.0μg/ml (Sigma), a rat anti-GRP94 antibody used at 1.33μg/ml (StressGen Biotechnologies Corp., Victoria, BC), a mouse anti-actin antibody used at a 1:15,000

dilution (ICN Biomedicals, Aurora, Ohio), a rabbit anti-rat espin antibody used at 0.2μg/ml (Gift from Dr. Jim Bartles), a rabbit anti-human Keap1 antibody used at 0.444μg/ml (Gift from Dr. Tama Hasson), a mouse-anti IC74 (Dynein) used at a dilution of 1:1000 antibody (Gift from Dr. Kevin Pfister) and a sheep anti-Rab6KIFL antibody used at 1.0μg/ml (Gift from Dr. Francis Barr).

Immunofluorescence

Tissue preparation

Testes were removed from male rats under deep anesthesia. Warm (33°C) PBS (150mM NaCl, 5mM KCl, 0.8mMKH_2PO_4, 3.2mM Na_2HPO_4, pH 7.3) was then perfused through the spermatic artery using a 26G needle attached to a gravity fed perfusion apparatus for 2 minutes to clear the organ of blood. Following this, warm 3% paraformaldehyde (fixative) in PBS was perfused through the testis for 30 minutes. PBS was then re-perfused through the organ to wash out any remaining fixative.

Frozen sections

For frozen sections, the fixed testes were then slowly frozen (using liquid nitrogen) while at the same time being attached by OCT compound (Sakura Finetek USA, Torrance, Ca, USA) to a metal microtome stub. Frozen testis sections were cut and attached to poly-l-lysine coated glass slides. Sections were plunged into –20°C acetone for 5 minutes then allowed to air dry, then processed for immunofluorescence.

Fragmented material

For fragmented material, fixed testes were cut into 1mm sized cubes and transferred into a 15ml plastic Falcon tube along with about 5mls of PBS. The material was gently passed through an 18, then 21 gauge needle for 2-5 passes. This fragmented material was left to sediment by gravity at room temperature for 10-15 minutes at which point the upper most layer was added to poly-l-lysine coated slides and allowed to incubate in a humidity chamber for 10 minutes. All excess PBS was then removed treated with –20°C acetone for 5 minutes and allowed to air dry at which point 5% blocking serum was added.

Antibody labeling and controls

Once the tissue was ready for serum blocking, 5% serum in TPBS-BSA (PBS containing 0.05% Tween-20 and 0.1% Bovine Serum Albumin) from the secondary antibody host was incubated on the testis material for 20 minutes at room temperature. This was used to block the tissue from secondary antibody non-specific binding. After the 20minute incubation, the primary antibody was added to the experimental slides made up in TPBS-BSA with 1% serum from the secondary antibody host and was incubated overnight at 4°C in a humidity chamber. Antibody concentrations consisted of 7.7 μg/ml for the LAGSE antibody, 90 μg/ml for the HIPYR antibody, and 30.45μg/ml for the FAYGQ antibody The material was washed extensively with the TPBS-BSA (wash buffer) then incubated for 60 minutes at 37°C with secondary antibody conjugated to a fluorochrome. After further washing, coverslips were mounted onto the material using

Vectashield fluorescent mounting media (Vector Labs, Burlington, Ontario) and visualized using standard fluorescence microscopy.

Controls consisted of replacing the primary antibody with it's respective immunoglobulin (IgG or IgY) at identical concentrations to the primary antibody, replacing the primary antibody with buffer alone, or replacing both the primary and secondary antibodies with buffer alone.

Filamentous actin labeling was performed using ALEXA FLUOR labeled phallotoxins (Molecular probes, Eugene Oregon).

Imaging

All light microscopy was performed using the Zeiss Axiophot microscope fitted with the appropriate fluorescent wavelength filters to view conventional fluorochromes. All images were recorded directly to T-MAX 400 film that was developed in our lab. Images were scanned into digital format and manipulated using Adobe Photoshop 5.0, 6.0 or 7.0 without compromising the integrity of the data.

Concentration of elongate spermatids with attached ectoplasmic specializations

Spermatid/junction complexes were isolated according to estalished protocols (Miller et al., 1999). Briefly, testes were removed from male rats under deep anesthesia. Once removed, testes were decapsulated and the seminiferous tubule masses were cut into fragments using scalpel blades. The seminiferous epithelium was then squeezed

from individual seminiferous tubules using micro-probes, all visualized using a Zeiss (West Germany) dissecting microscope. The extruded epithelium was then collected, sheared through a fine bore gel-loading pipette tip, loaded onto a 30-60% step sucrose gradient and centrifuged. After centrifugation, testis material was collected from the 40-45% interface, which was enriched for spermatids with attached ectoplasmic specializations.

General 1D electrophoresis and Western blotting

Material was loaded into wells of 1mm thick 10% SDS-PAGE reducing gels and run according to standard protocols (Laemmlli, 1970). Once run, proteins were transferred onto Immobilon-P transfer membrane via wet (Mini Trans-Blot Cell, Bio-Rad Canada) or semi-dry (Nova Blot, Pharmacia) transfer. Proteins transferred to membrane for Western blotting were then washed for 5 minutes at room temperature with TBST (500mM Tris pH 7.5, 150mM NaCl, 0.1% Tween-20) then blocked in order to decrease non-specific antibody binding for 8hours at 4°C using 4% non-fat milk (Blotto, Santa Cruz Biotechnology). Because some antibodies used in this study were raised in chickens, blocking in those cases consisted of a 1:10 dilution of BLOKHEN II, a blocking solution specifically designed for chicken IgY applications. (Aves Labs, Tigard, Oregon) in TBST at room temperature for 8 hours. Following blocking, membranes were washed 3 times, 10 minutes each then incubated with primary antibody (LAGSE used at 0.2μg/ml and HIPYR used at 1.0μg/ml) overnight at 4°C. The following day, blots were washed extensively with TBST followed by a 1-2 hour secondary antibody (conjugated to horseradish peroxidase) incubation. Upon further washing with TBST followed by TBS

(500mM Tris pH 7.5, 150mM NaCl), blots were reacted with ECL (Pharmacia) to visualize the reactive bands on X-OMAT film (Kodak)

Controls consisted of replacing the primary antibodies with IgG or IgY at identical primary antibody concentrations.

2D electrophoresis

Testis material, in solution, was first centrifuged at max speed using an Eppendorf desktop centrifuge for 15 minutes at room temperature. Once completed, the supernatant was collected and treated to acetone precipitation. To do this, three volumes of -20°C acetone were added to the sample and incubated at -20°C for 30 minutes with occasional agitation. The sample proteins were then pelleted and the supernatant was discarded. The resulting protein pellet was allowed to air dry for 10-15 minutes and was re-suspended in 2D-gel sample buffer (8M Urea, 2M Thiourea, 4% w/v CHAPS, 20mM Tris, 0.0025% Bromophenol Blue) at room temperature on a Labquake rotator for 6-8 hours. Once re-suspended, 0.5% of IPG (Immobilized pH Gradient) Buffer (Amersham, Quebec), at the same pH as the IPG focussing strip, was added to the sample. After a 5 minute centrifugation at maximum speed in an Eppendorf desktop centrifuge, the remaining supernatant was added to the IPG strip for a 24hour strip re-hydration. Focussing was then performed using the IPGphor isoelectric focussing machine (Amersham). Focussed IPG strips were loaded onto large format slab gels (Bio-Rad) and run at 50-85 volts.

General Mass Spectrometry methods (Performed by the University of Victoria GenomeBC Proteomics Center)

Protein samples were initially digested into peptides using porcine trypsin. These peptides were then loaded onto C_{18} columns to concentrate and desalt the peptides. The peptides were eluted off the columns using 50% acetonitrile, 0.1% formic acid prior to mass spectrometry analysis using the QStar Pulsar I Quadrupole mass spectrometer. For LCMS, after a 1 second survey scan, the four most intensely charged fragments (excluding those from keratin and porcine trypsin) were selected for fragmentation and analysis. Following the analysis, the selected fragments were excluded from the scan and analysis continued with new selections. Proteins were identified using ProID (Applied Biosystems) with a confidence cut off set to 50 and a minimum ion score of 15. For 2DLCMS, following peptide preparation, the peptides were loaded onto a strong cation exchange column prior to running them through the LCMS. Peptides were eluted off the column by 30 successive salt elutions and peptide fragments from each elution analyzed by LCMS.

Microarray Analysis (Performed by Jim Shima from Dr. Michael Griswold's Laboratory; Washington State University)

Mouse Sertoli cells were isolated from whole testes from 14-16 day old Rosa and B6/129 mice and cultured using standard culture procedures in DMEM/F12 media following the procedure of Karl and Griswold (1990). Cells were incubated at 32°C in

5% CO_2 for 2 days following isolation. The isolation of Sertoli cells was performed on two independent occasions to provide duplicate samples for microarray analysis.

Total RNA from the enriched Sertoli cell culture was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The quality and concentration of the purified RNA was determined using absorbancies at 260 and 280 nm. The standard Affymetrix protocol required 10μg of total RNA with a minimum 260/280 ratio of 1.80.

Ten micrograms of total RNA was used to synthesize the Affymetrix GeneChip® microarray target. Briefly, double stranded cDNA was synthesized from the original RNA using reverse transcription with an oligo-(dT_{17}) primer followed by RNase A treatment and DNA polymerase synthesis of the second strand. The double strand cDNA was used as a template for an *in vitro* transcription reaction using biotinylated CTP and UTP to produce labeled cRNA (MegaScript, Ambion, Austin, TX). The biotinylated cRNA was then fragmented and hybridized to the MGU74Av2 GeneChip® arrays (Affymetrix, Santa Clara, CA). The microarrays were processed with Affymetrix GeneChip® Fluidics Workstation 400 using the Mini_Euk 2v3 protocol, stained with phycoerythrin-coupled streptavidin, and scanned on a Hewlett-Packard Gene Array Scanner (Hewlett-Packard Co., Palo Alto, CA). Microarray data was scaled to a target signal of 125 and analyzed with Microarray Suite 5.0 software (Affymetrix).

Results

In order to investigate the possibility that a kinesin-like protein was associated with ectoplasmic specializations and because there were no pan-kinesin antibodies available, we generated anti-peptide antibodies in chickens against three conserved kinesin head domain sequences (HIPYR, LAGSE and FAYGQ). The main reasons for using chickens instead of rabbits were 2 fold. First, rabbit antibodies routinely non-specifically label spermatid heads when staining testis material. Since ectoplasmic specializations occur within Sertoli cells at the area surrounding spermatid heads, rabbits were not the ideal choice of host in which to generate the antibodies. Second, chickens are know to be capable of producing antibodies against difficult antigens more easily than other species (Stuart et al., 1988). Because kinesins are so ubiquitously expressed, antibody generation against their conserved regions was considered to likely be problematic.

Once the antibodies were generated, the total IgY was isolated from the egg yolks and the antibodies were affinity purified using LAGSE, HIPYR and FAYGQ peptide coated columns. The resulting affinity purified antibodies were characterized initially using line blots, in which the antibodies were reacted against their respective peptides, and compared to both the isolated IgY that did not bind to the column (pre-elution) and commercially available normal chicken IgY (NCIgY), all used at identical concentrations. In all cases, the line blot analysis clearly showed stronger reactivity of the generated antibodies versus the pre-elution IgY and NCIgY (Figure 2.2a). Since the antibodies were generated against regions that were conserved amongst members of the

kinesin superfamily, I was interested in seeing if they reacted with kinesin protein. On blots using commercially purchased recombinant kinesin protein, the anti-LAGSE, anti-HIPYR and anti-FAYGQ antibodies reacted more intensely with the kinesin protein (Figure 2.2b) than did NCIgY used at identical concentrations (Figure 2.2c).

Once the antibody characterization was complete, the antibodies were reacted on blots of rat testis material. Here, two different probing methods were performed resulting in similar conclusions. First, the generated antibodies were reacted against rat testis Western blots and compared against NCIgY (Figure 2.3a, 2.3b, 2.3e, 2.3f). Although NCIgY reacted non-specifically with numerous protein bands in the testis on its own, many other bands appeared in the anti-peptide antibody blots, which were absent in the NCIgY blots (Figure 2.3 arrows). The reactive bands that were seen on the real antibody blots were expected since the testis is known to house many kinesin proteins. As a second control, when the anti-peptide antibodies were pre-incubated with their respective peptides prior to their Western blot incubation (Figure 2.3c and 2.3g) and compared to the generated peptide antibodies, when equivalent amounts of peptide carrier solution were included (Figure 2.3d and 2.3h), the reactive bands which were previously absent (or had a decreased intensity) when compared to the NCIgY, were also absent in the peptide pre-incubation experiment (Figure 2.3 arrows). These results indicated that NCIgY was an appropriate control for the generated anti-peptide antibodies. Unfortunately, the anti-FAYGQ antibodies did not react well on blots using rat testis material, a result that was anticipated due to difficulties reported by others (Sawin et al., 1992).

I then used these anti-LAGSE, anti-HIPYR and anti-FAYGQ antibodies to stain both rat testis sections and rat testis fragments and compared them to controls. All three antibodies reacted in similar manners. The antibodies clearly labeled the Sertoli cell cytoplasm (Figure 2.4a and 2.4b) and were found concentrated at areas consistent with the location of ectoplasmic specializations (Figure 2.4a arrows). Controls, represented here by the LAGSE controls, were devoid of specific staining although a haze of fluorescence appeared within the epithelium on primary antibody controls (Figure 2.4a). The location of ectoplasmic specializations was confirmed on the sectioned material by double labeling the sections with phalloidin to stain filamentous actin (Figure 2.4c). I use filamentous actin as an ectoplasmic specialization marker because it is highly concentrated at these sites in the testis.

It has been well documented that upon fragmentation of the seminiferous epithelium, spermatids retain their associated ectoplasmic specializations (Miller et al., 1999; Guttman et al., 2002b). I stained fixed fragmented testis material in order to more clearly determine where the LAGSE, HIPYR and FAYGQ antibodies were staining (Figure 2.5a). Antibody labeling of this material clearly showed staining at three main areas; spermatid tails (Figure 2.5a), manchettes (Figure 2.5a and 2.5b) and ectoplasmic specializations (Figure 2.5a, 2.5b, and 2.5c). Filamentous actin staining again co-localized with the anti HIPYR and anti-LAGSE and anti-FAYGQ labeling at ectoplasmic specializations (Figure 2.5a).

In order to try to identify the exact kinesin isoform found at ectoplasmic specializations, a proteomics-based approach was used. Spermatids with their overlying ectoplasmic specializations were concentrated and treated with the actin severing and capping protein gelsolin, under severing conditions. This severed the ectoplasmic specialization associated actin filaments, thereby releasing the actin, actin-associated proteins and overlying endoplasmic reticulum as well as its associated proteins, into the supernatant (Figure 2.6a). The samples were probed with phalloidin both before and after treatment to ensure that the filamentous actin of the junction plaque had been disassembled (Figure 2.6b). The resulting supernatant, containing the actin and endoplasmic reticulum associated proteins, were either subjected to 2D gel analysis followed by either Western blotting or gel extraction for mass spectrometry analysis. Alternatively the supernatant samples as a whole were subjected to two dimensional liquid chromatography mass spectrometry (2DLCMS).

Supernatant samples were initially analyzed by 2D Western blots to ensure that the known junction components: gelsolin, espin, GRP94 (an endoplasmic reticulum marker), keap1, 74kD intermediate chain of cytoplasmic dynein (IC74), vinculin and actin were present (Figure 2.6c). Two-dimensional blots were also probed with the anti-LAGSE and anti-HIPYR antibodies and yielded numerous reactive dots (Figure 2.7). Reactive dots which were reactive with both the LAGSE and HIPYR antibodies or those appearing intensely reactive in one or the other and not with NCIgY probed blots were excised from duplicate SYPRO Ruby stained 2D gels and sent for mass spectrometry analysis. Analysis using this technique did not yield any kinesin proteins likely for two

reasons. First the excision of proteins dots could have been impure due to overlapping dots and accuracy of dot comparison from blot to gel and second, some of the proteins identified, for example the Y-Box protein, had contained multiple peptide sequence fragment copies identical to those used for the anti-peptide antibody generation (Figure 2.7).

In order to circumvent the problems from the previous proteomics approach, we used another mass spectrometry method, 2DLCMS. With this technique, soluble samples were loaded onto a strong cation exchanger and eluted off prior to LCMS analysis. Using this technique, a 2 peptide identification consisting of the peptides TVAATNMNETSSR and ANSTGATGARLK appeared corresponding to single kinesin protein termed kin3 with a confidence score of 90 (a 90% probability that each peptide was from that protein). These identified peptides appear in the head region of the kin3 molecule (Figure 2.8a). Upon sequence analysis it was discovered that this molecule did not contain the full peptide sequences that were used for the antibody generation (Figure 2.8a).

As a final method of identifying a kinesin that may be present for spermatid entrenchment during spermiogenesis, a kinesin mRNA transcript screen was performed using mouse GeneChip arrays. Known kinesins were identified whose transcripts were increased in the testis at a gestational age of 30-35 days, the age when condensing spermatids and spermatozoa appear in the mouse as compared to day 20 arrays (Kramer and Erickson 1981). These included Kif9, Kif17, Kinesin 7 and Rab6KIFL. Assay abundances from day 20 to day 30 data sets were the following: Kif9, 444-764; Kif17,

52-1441; Kinesin 7, 280-366 and Rab6KIFL, 46-212. The minimum confident abundance limit is 70, results below that result are suspect. When a Sertoli cell screened GeneChip array database was used to again look for kinesin transcripts, only the Rab6KIFL transcript had appreciable transcript levels at 498. Results of the other 3 kinesins in Sertoli cells were the following: Kif9, 25; Kif17,41; and kinesin 7, 34. Surprisingly, the protein corresponding to this transcript again contains only fragments of the synthetic peptide regions used for the generation of the antibody (Figure 2.8b). Based on the mRNA findings we focused our study on Rab6KIFL and looked for its expression in the seminiferous epithelium. Using antibodies described elsewhere (Hill et al., 2000) we probed 1D western blots of rat seminiferous epithelium and found that a single band migrated to the appropriate molecular weight for Rab6KIFL, about 100kD (Figure 2.9a). Duplicate blots probed with normal sheep IgG were negative (Figure 2.9a). Because this antibody did not react immunocytochemically on rat testis material, we pursued our study by probing 2D western blots of gelsolin-treated concentrated rat spermatid/junction complexes with the anti-Rab6KIFL antibody and interestingly found that a protein dot reacted in the region expected for this protein.

Discussion

Results presented here support but do not prove the hypothesis that a kinesin-like protein is likely associated with apically located ectoplasmic specializations. Through the use of antibodies raised against peptide sequences known to be conserved against most kinesins, we characterized the antibodies and stained testis sections and fragments ensuring that the antibodies 1) reacted with kinesin, 2) labeled areas known to contain kinesins and as predicted 3) also stained areas consistent with the location of ectoplasmic specializations. This preliminary screen guided us to try to identify candidate kinesin-like proteins that may be at the junction plaque. Through the use of both 2DLCMS and microarray analysis at least 2 candidate proteins were identified kin3 and Rab6KIFL, which are candidate plaque motors.

The choice to generate polyclonal anti-peptide antibodies in chickens rather than in rabbits was mainly due to the well known fact that many rabbit antibodies react non-specifically around spermatid heads, the area of apically located ectoplasmic specializations. Although normal chicken IgY did react with multiple non-specific bands on 1D western blots, it did not react non-specifically at spermatid heads on immunofluorescent controls. While the generated antibodies did also react with the non-specific bands on 1D western blots (accounted for by the control blots), other bands also appeared which we determined to be specific due to their disappearance upon peptide pre-incubation with their generated peptide. When both the NCIgY and pre-incubated peptide control blots were compared, their similarity suggested to us that NCIgY was in fact an appropriate control for the generated anti-peptide antibodies.

Immunofluorescently, all three antibodies reacted similarly and were markedly different than NCIgY controls. Both NCIgY and the pre-elution IgY had identical staining patterns, again suggesting to us that NCIgY was an appropriate control for these antibodies.

The important finding that the generated antibodies reacted with recombinant kinesin as well as at ectoplasmic specializations further suggested to us that a kinesin or kinesin-like protein was likely a component of the junction plaque.

It is generally accepted that gelsolin can disassemble the actin zone of ectoplasmic specializations (Miller et al., 1999; Guttman et al 2000; Guttman et al 2002b). Until now, known ectoplasmic specialization components have not been confirmed from samples following the gelsolin treatment of concentrated spermatid/ectoplasmic specialization complexes. Following staining of 2D blots of these preparations and confirming the presence of these known components, we took advantage of this preparation technique to attempt to identify the kinesin isoforms at the apically located ectoplasmic specializations.

Using duplicate SYPRO Ruby stained 2D gels and 2D western blots of gelsolin treated spermatid/ectoplasmic specialization supernatants stained with the generated antibodies, standard mass spectrometry was performed on hand excised protein 2D gel dots. Numerous difficulties arose from this technique. First the correlation of reactive blotted proteins to the correct dot on the 2D gel was quite difficult. Once accomplished,

complicating factors included being able to visually see the protein of choice and being capable of excising it cleanly from the gel (illuminated with UV) without inadvertently excising other proteins which in some instances overlapped. Once these factors were all accomplished and the protein plugs were sent out for mass spectrometry analysis the resulting proteins were mainly other proteins containing the generated peptide sequence (which were not kinesins) or hypothetical protein fragments. Based on these findings I decided to abandon this approach in favor of another proteomics method, 2DLCMS. This technique abolishes the need to run 2D gels or blots and instead uses a type of shotgun approach. Here the entire supernatant of the gelsolin-treated spermatid/ectoplasmic specialization mixture was loaded on to a strong cation exchange column then slowly eluted off prior to it running through an LCMS. Although in actuality this method did not identify all of all of the proteins that are known to be present at ectoplasmic specializations (which were confirmed by our previous 2D western blotting) it did identify with high confidence 2 peptide fragments from the kin3 molecule. This molecule is known to transport endosomes (in *Ustilago maydis*) in the plus end-direction (Wedlich-Soldner et al., 2002), the same direction that the entrenching spermatids are transported. The two peptide fragments were found in the head region of the molecule leaving the strong possibility that differences in this protein's tail region may impart different functions of this protein in different systems. Interestingly, this protein does not contain any of the complete peptide sequences that the peptide antibodies were generated against. The most complete fragments were that of the FAYGQ and LAGSE peptides.

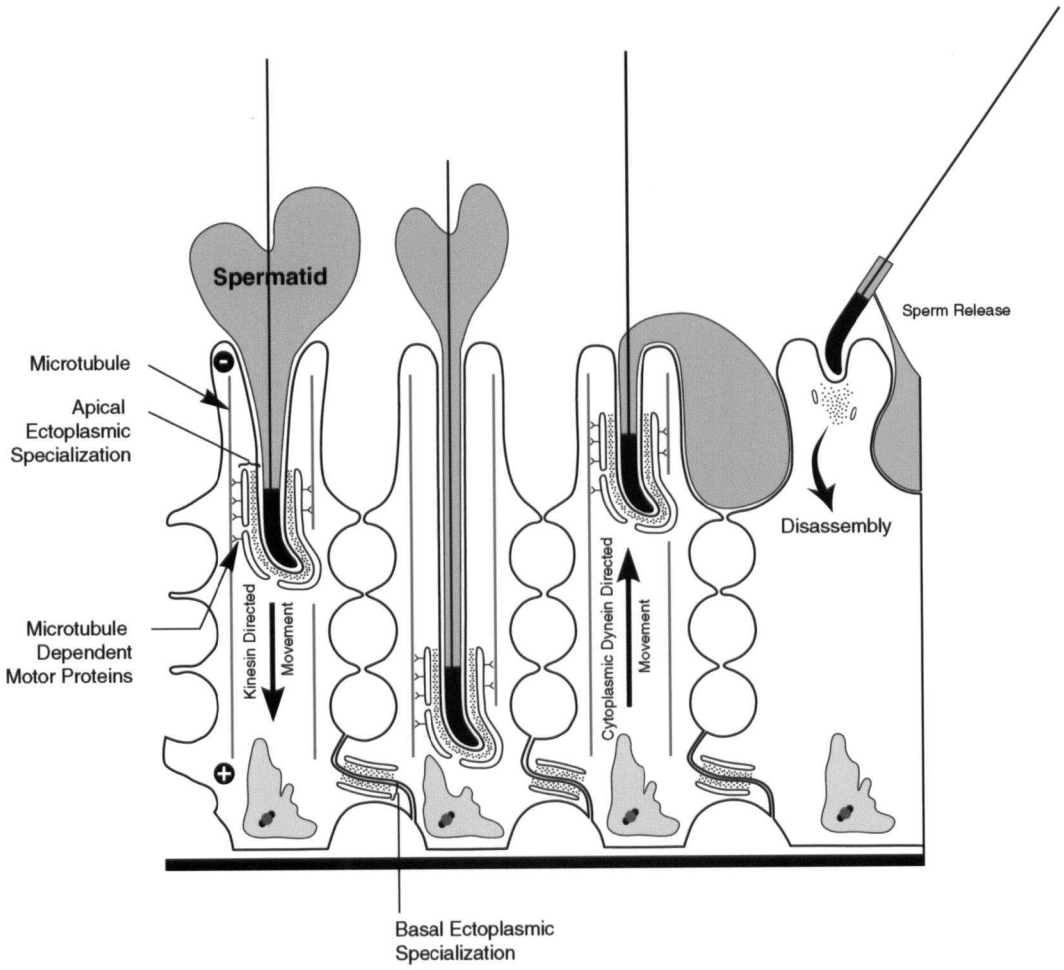
To further identify kinesin-like proteins associated with ectoplasmic specializations, we used a microarray approach. Using both the recently developed mouse testis developmental series and mouse Sertoli cell databases, Jim Shima (from Dr. Michael Griswold's laboratory; Washington State University, Pullman, Washington) scanned the data sets for kinesins, then I focused on those that had increased mRNA transcripts at developmental days 30-35, the time of spermiogenesis and thus spermatid entrenchment. I then looked to see if any of the resulting transcripts levels were comparably high in the mouse Sertoli cell transcript database and found a single transcript that met both criteria, Rab6KIFL. Following this we found Rab6KIFL expression in rat seminiferous epithelium and interestingly also in the supernatant of gelsolin-treated spernatid/ectoplasmic specialization preparations using western blots. Unfortunately the antibody used for the western blotting did not work for immunolocalization on testis material.

Rab6KIFL [also known as kinesin family member (KIF) 20a and MKLP2 (mitotic kinesin-like protein 2)] has thus far been implicated in two functions, intracellular trafficking (Echard, et al., 1998) and cytokinesis (Hill et al., 2000). This protein is so named due to its ability to interact with the GTP bound form of the small GTPase rab6. Interestingly, this protein has very few amino acids in common with the generated peptides used in the antibody generation, further complicating our study of kinesins at this site. Although studies have just begun on this protein, our identification of it in the testis suggests that it may have another function, possibly spermatid translocation.

Other interesting findings in this study include the fact that the 2DLCMS technique did not identify Rab6KIFL and the microarray technique did not identify kin3. This suggests that even though both are powerful techniques, false negatives and possibly false positives arise, opening the door for further experimentation. Also, based on the finding that the generated anti-peptide antibodies react with proteins other than kinesins on western blots, this suggests that the immunofluorescent staining seen associated at areas known to contain ectoplasmic specializations may not be exclusively labeling kinesin motors.

The work presented here does not exclude the possibility that other kinesin-like proteins may also be present at ectoplasmic specializations nor does it conclusively prove that kin3 and Rab6KIFL are at the plaque and are responsible for spermatid entrenchment. What it does do is further corroborate previous data supporting the microtubule-based spermatid translocation hypothesis and suggests that kin3 and Rab6KIFL are candidate proteins that should be further pursued.

Figure 2.1. Diagrammatic representation of the motility events occurring in the seminiferous epithelium. Elongate spermatids attached to apically located ectoplasmic specializations are transported towards the base of the Sertoli cell and are returned to the apex along Sertoli cell microtubule tracts. The movement initially is in the plus-end direction (entrenchment) then followed by their return to the apex of the Sertoli cell, and thus the seminiferous epithelium, prior to spermatid release (Modified from Vogl et al., 2000).


Spermatid
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Kinesin Directed
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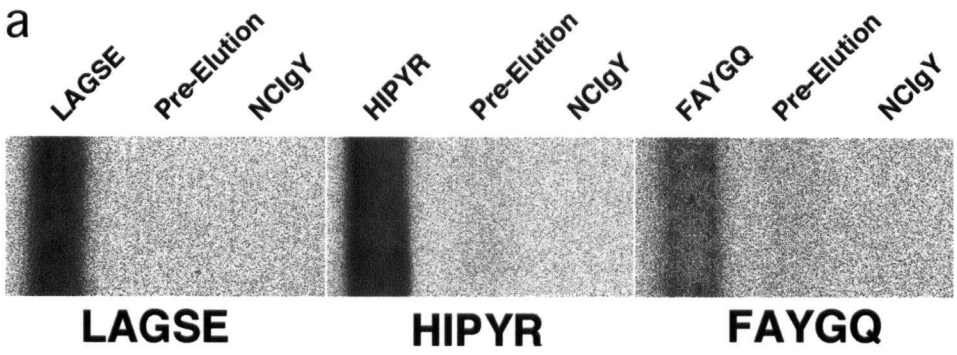
Movement
Sperm Release
Disassembly
Basal Ectoplasmic
Specialization

Figure 2.2. (a) Dot (line) blots of the generated pan-specific anti-kinesin antibodies, pre-elution control IgY and normal chicken IgY (NCIgY) used at identical concentrations for each antibody. (b) Immunoblots demonstrating that the three anti-peptide antibodies (HIPYR, LAGSE, FAYGQ) react with recombinant kinesin supplied by Cytoskeleton Inc. (residues 1-379 of human kinesin heavy chain motor domain + GST-Tag at the amino terminal end). This kinesin is not full length and therefore migrates at a lower molecular weight than full length conventional kinesin. (c) NCIgY probed control blots of kinsin used at identical concentrations.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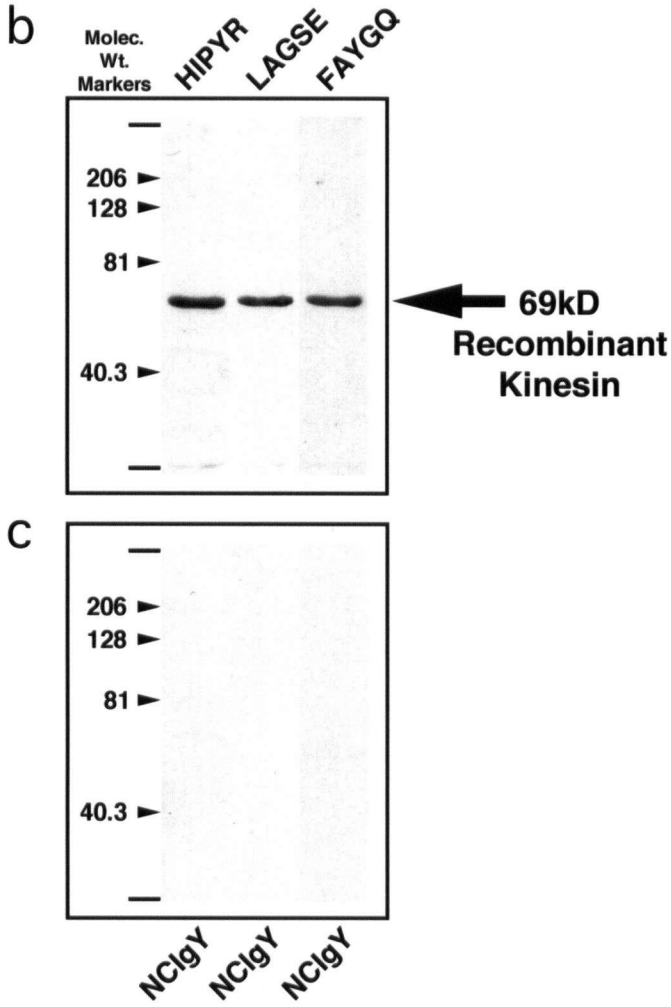
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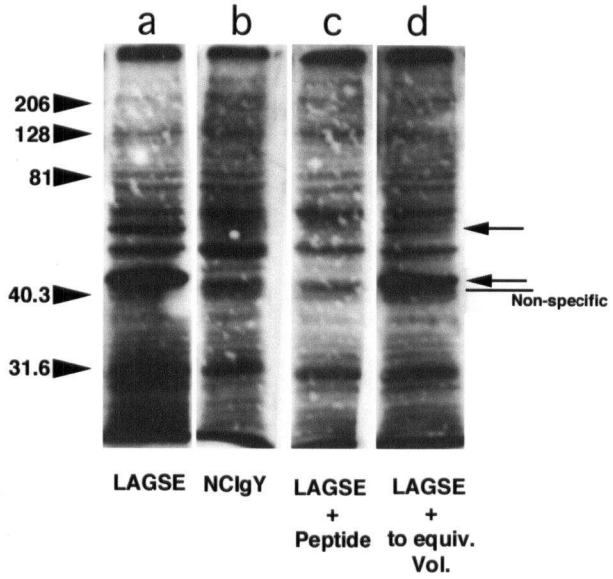
Figure 2.3. Anti-LAGSE, anti-HIPYR and control western blots of rat seminiferous epithelium. (a) Anti-LAGSE probed blot, (b) normal chicken IgY probed blot used at identical concentrations to (a), (c) seminiferous epithelium probed blot using the anti-LAGSE antibody pre-mixed with the LAGSE synthetic peptide, (d) seminiferous epithelium probed blot using the anti-LAGSE antibody used at identical concentration to (c) pre-mixed with the LAGSE synthetic peptide carrier buffer used at identical volume to (c). (e) Anti-HIPYR probed blot, (f) normal chicken IgY probed blot used at identical concentrations to (e), (g) seminiferous epithelium probed blot using the anti-HIPYR antibody pre-mixed with the HIPYR synthetic peptide, (h) seminiferous epithelium probed blot using the anti-HIPYR antibody used at identical concentration to (g) pre-mixed with the HIPYR synthetic peptide carrier buffer used at identical volume to (g). Arrows indicate specific reacting bands that are not present in control blots. Non-specific bar represents a secondary band below the specific band marked by the arrow.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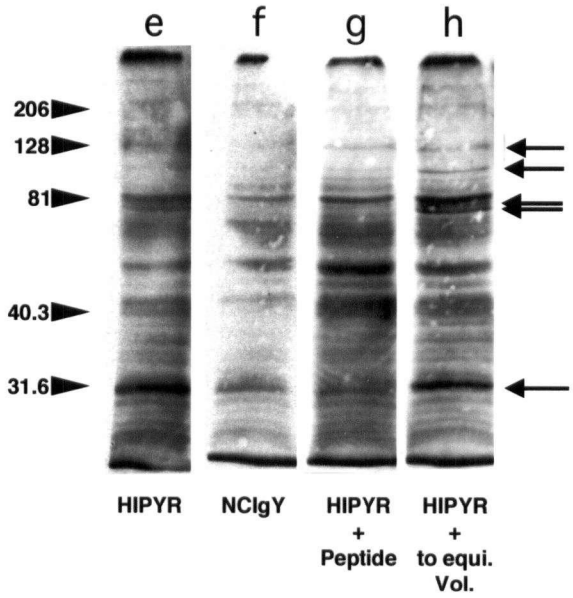
LAGSE + to equiv. Vol.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206
128
81
40.3
31.6
HIPYR
NCIgY
HIPYR + Peptide
HIPYR + to equi. Vol.

Figure 2.4. (a) LAGSE probed seminiferous epithelium frozen sections and controls. LAGSE antibody, purified IgY from the identical chicken (pre-immunization), normal chicken IgY control, secondary antibody control and autofluorescent controls. Bar = 10μm. (b) Frozen sections of rat testis labeled using the three pan-specific anti-kinesin antibodies. Bar = 10μm. (c) LAGSE and phalloidin labeled rat seminiferous epithelium frozen sections. Arrows indicate the location of apical ectoplasmic specializations. Bar = 10μm.

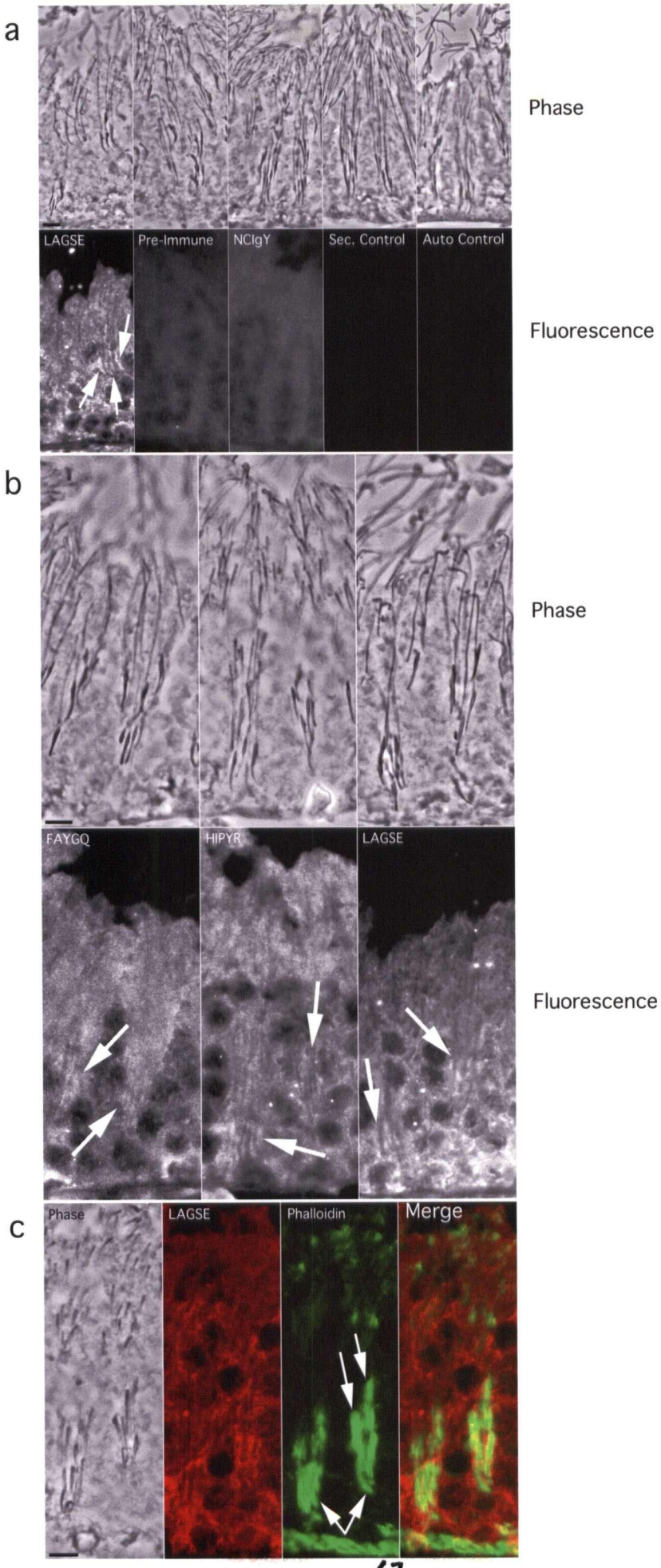
a
Phase
LAGSE
Pre-Immune
NCIgY
Sec. Control
Auto Control
Fluorescence
b
Phase
FAYGQ
HIPYR
LAGSE
Fluorescence
c
Phase
LAGSE
Phalloidin
Merge

Figure 2.5. Fixed fragmented rat testis material. (a) Rat testis fragments double labeled with the anti-peptide antibodies and phalloidin. Note the staining of the manchette (M), spermatid tails (T) and ectoplasmic specializations (ES). Bar = 5μm. (b and c) LAGSE antibody and controls on fragmented testis material. Note the specific staining at ectoplasmic specializations and the manchette in the LAGSE micrographs and the lack of staining in all controls. Bars = 5μm.

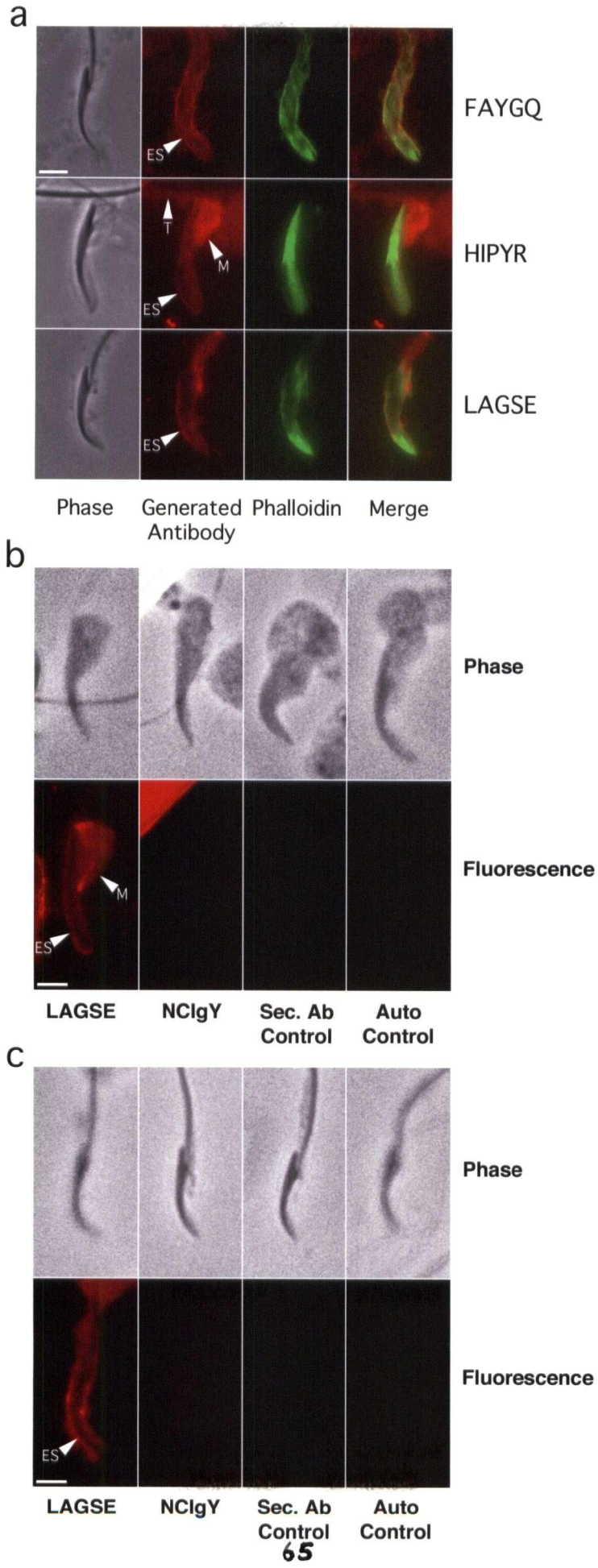
a
FAYGQ
HIPYR
LAGSE
ES
T
M
Phase
Generated Antibody
Phalloidin
Merge
b
Phase
Fluorescence
LAGSE
NCIgY
Sec. Ab Control
Auto Control
c
Phase
Fluorescence
LAGSE
NCIgY
Sec. Ab Control
Auto Control

Figure 2.6. (a) Diagram of the theory behind the gelsolin treatment of spermatid/ectoplasmic specialization complexes. (b) Micrographs of elongate spermatids pre- and post-gelsolin treatment. Bar = 10μm. (c) SYPRO Ruby stained 2D gel of supernatants from gelsolin treated spermatid/junction complexes. Known components of the junction plaques were verified on the gels by 2D immunoblots of paired gels.

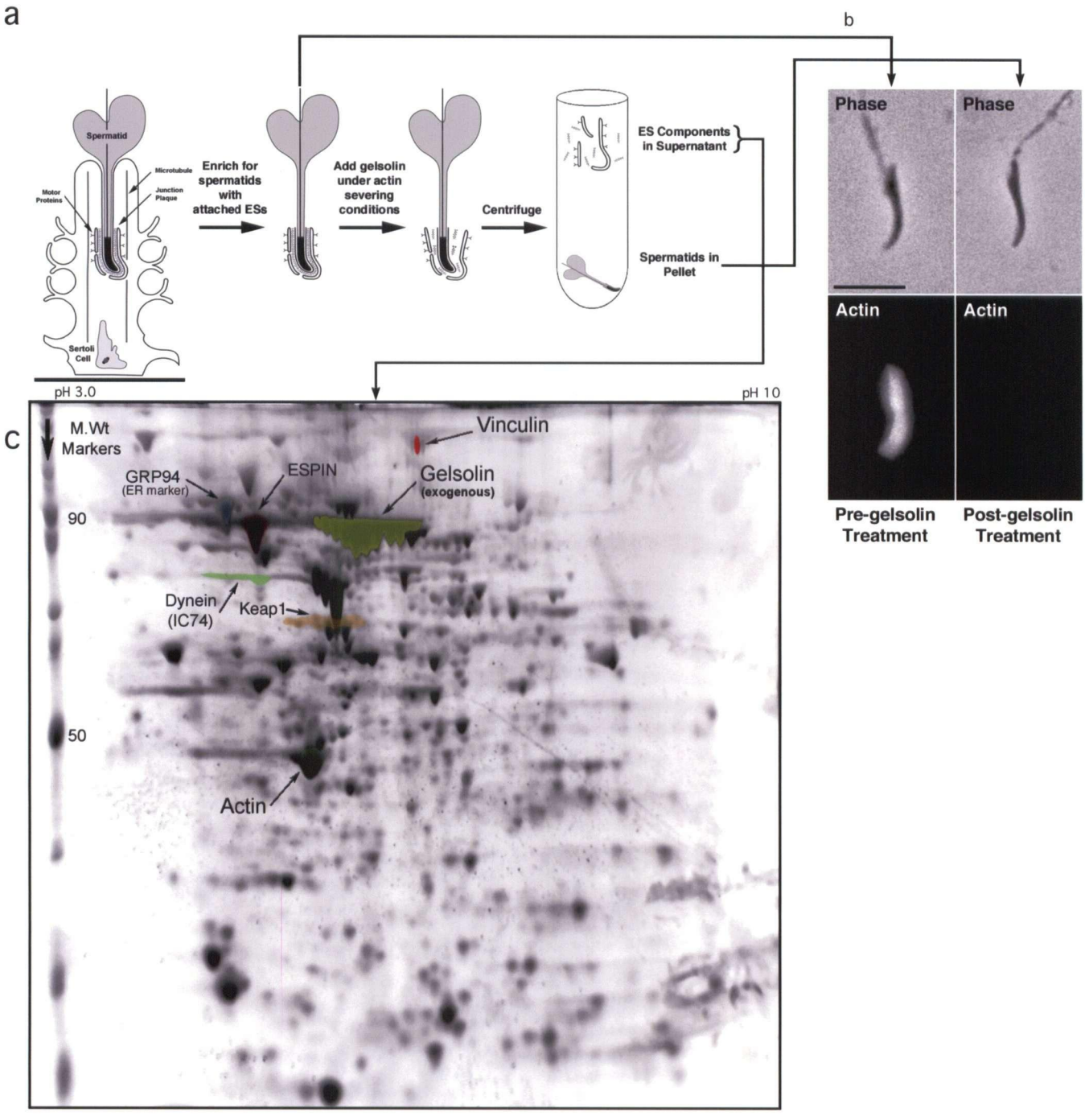
a
b
Spermatid
Microtubule
Junction Plaque
Motor Proteins
Sertoli Cell
Enrich for spermatids with attached ESs
Add gelsolin under actin severing conditions
Centrifuge
ES Components in Supernatant
Spermatids in Pellet
Phase
Phase
Actin
Actin
Pre-gelsolin Treatment
Post-gelsolin Treatment
pH 3.0
pH 10
c
M.Wt Markers
Vinculin
GRP94 (ER marker)
ESPIN
Gelsolin (exogenous)
90
Dynein (IC74)
Keap1
50
Actin

Figure 2.7. 2D Western blots of gelsolin treated ectoplasmic specializations probed with the pan-specific kinesin antibodies "LAGSE" and "HIPYR". Numerous reactive spots are detected using both antibodies. The spot labeled Y-Box protein has been identified by mass spectrometry and contains numerous peptide sequences found within the synthetic LAGSE peptide.

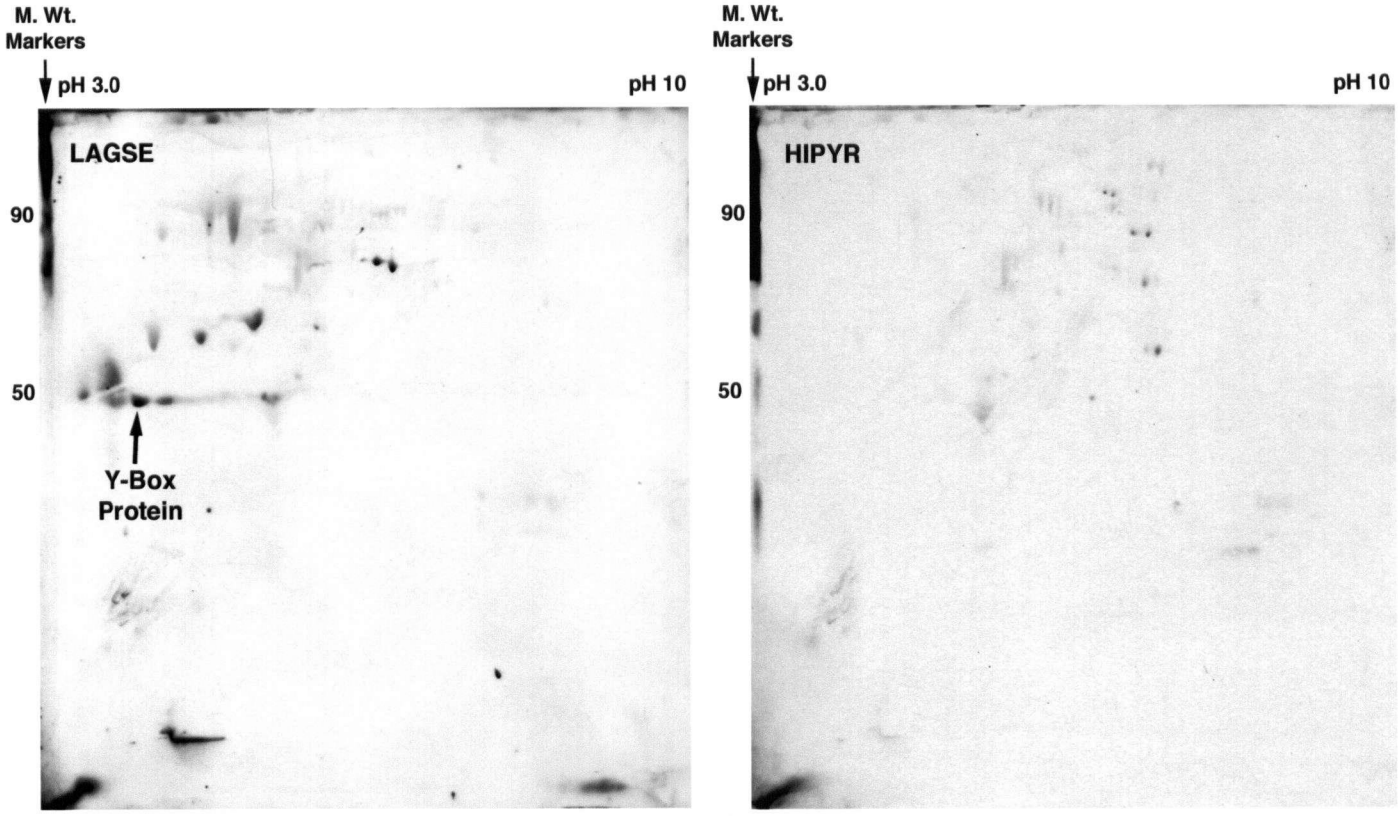
M. Wt.
Markers
pH 3.0
pH 10
LAGSE
90
50
Y-Box
Protein
M. Wt.
Markers
pH 3.0
pH 10
HIPYR
90
50

Figure 2.8. (a) Protein sequence of kin3 from Wedlich-Soldner et al. (2002). Marked in blue are the amino acids present this protein that correspond to the synthetic amino acids used to generate the anti-FAYGQ antibody, red represents the anti-LAGSE antibody and green represents the anti-HIPYR antibody. The peptide fragments identified by mass spectrometry are in yellow. (b) Protein sequence of Rab6KIFL from Echard et al. (1998). Marked in blue are the amino acids present this protein that correspond to the synthetic amino acids used to generate the anti-FAYGQ antibody, red represents the anti-LAGSE antibody and green represents the anti-HIPYR antibody.

a

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   1 madsgnikvv vrcrpmnsre rnrgasnlie fvdqhqlils ppneadtken skatkkksmp
  61 fsfdraydeh teqddlfqyi gvellqhafn gfntcvfayg qtgsgkshsm vgyaqakgii
 121 pltcarlfed inqktaadpn lkisvevsyi eiynekvrdl lnpknkgnlk vrehpslgpy
 181 vedlsklvva sypdimnlmd egnkartvaa tnmnetssrs havftlvltq krfdvqtkle
 241 aekvsrismv dlagserans tgatgarlke ganinrsltt lgkviaalai assavepvkg
 301 akkpktasld sfvpyrdsvl twllkdslgg nsktamiaai spadyeetls tlryadqakk
 361 iknkavvned pnaklirelk eelellrtrv sggggadges nwdpsippdk qvvryqtktg
 421 eiktvtkael qeqleqseki msslneswee kltktqeiqk erekaleelg isvdkgnvgv
 481 htpkklphlv nlnedplmse cliyqikpgh tlvgnldsgp dvhiklsgtk ilnkhcmfdh
 541 qdglvtvtam pdsmtmvngk rlapdepkrl rsgyrvilgd fhvfrfnhpe evrkardrvr
 601 stlalstgea hnetlidgdl pstrpdspas gdvdwtyarr eytmaklngq nvnfdnlnee
 661 dleklfedis rarskksmgs vlgrpesras lfddnasesa ssvirpyshg altddtsidp
 721 wsqagsemgs mrfsagtpik enaytgagas spalvaashr eteslrakvr eyeekltrma
 781 ngsprladep ieysdtqkal lrkvlvkwka htkvsmaeda lckavlvkea nviskelakr
 841 vtyqytivdd fplavptsgv eaiagltefd dvsdpdlasc akpcmgikvl dylhstcyvw
 901 smpkfeqrlq kmrnlytfvd kpeyskhlnw sdpfyeaphp tyafvastlv pltplsrqls
 961 akyrlplhdr htakqigwcs vsvkfvslsp vpvsaraggt alpapsgsrn pssptsssct
1021 ngivnplvgq klgfqilvda isgissddfa sihlqvklss fagnelgkde iytsipvdlv
1081 nqeslaevrl rrtlsfvltp etiqwlrtga apievyaklr phylvaleqh dsaresegqq
1141 haaafvplhd dsrsltagqq ktmsntiker lsenemrnee rhfilasvqi celdasgeym
1201 pvpvrassal dpgsfflrqg lqrklvlqla hdsgrqflws rvtkleladv rlldsrgrvh
1261 ggkasdavql ktplkqqsve fanngtsqle lwawwdssvh dslhlnrtts aghrvlirls
1321 feiqvdrcsa paafsmdlav singrdakpp grlmsfiegs tsmtktsaif evrlvppmmk
1381 rpcelwrldt gskyvrgqem lggwkargvs lvgdhaalvq rerrraeveg vratlkgrsa
1441 mmrnvddana eskeelaarv vavwqravrd skvgvvigvq pstnaasagg ttcktgadgl
1501 vgmfaapsav dgtnglgidn lsassspaki ertrstwsst apapapapsa paapaaltai
1561 vallprtatt shrgylwipl etitdtwvrr flvlrrpflh iyesnaqvde vmvinveavr
1621 veydenterl lgkqnvfavy tannsyffqa dsdkdrqvwm klldgsyngd aghvfc
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b

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   1 mshrilsppa gllsdedvvd spilestaad lrsvvrkdll sdcsvisasl edkqalledt
  61 sekvkvylri rpfltseldr qedqgcvcie ntetlvlqap kdsfalksne rgvgqathkf
 121 tfsqifgpev gqvaffnltm kemvkdvlkg qnwliytygv tnsgktytiq gtskdagilp
 181 qslalifnsl qgqlhptpdl kpllsneviw ldskqirqee mkklslligg lqeeelstsv
 241 kkrvhtesri gasnsfdsgv aglsstsqft sssqldetsq lwaqpdtvpv svpadirfsv
 301 wisffeiyne llydllepps hqhkrqtlrl cedqngnpyv kdlnwihvrd veeawkllkv
 361 grknqsfast hmnqqssrsh sifsirilhl qgegdivpki selslcdlag serckhqksg
 421 erlkeagnin tslhtlgrci aalrqnqqnr skqnlipfrd skltrvfqgf ftgrgrscmi
 481 vnvnpcasty detlhaakfs alasqlvhap pvhlgipslh sfikkhspqv gpglekedka
 541 dsdledsped eadvsvygke ellqvveamk alllkerqek lqleiqlree icnemveqmq
 601 qreqwcserl dnqkelmeel yeeklkilke slttfyqeqi qerdekieel etllqeakqq
 661 paaqqsggls llrrsqrlaa sastqqfqev kaeleqckte lssttaelhk yqqvlkpppp
 721 akpftidvdk kleegqknir llrtelqklg qslqsaerac chstgagklr qaltncddil
 781 ikqnqtlael qnnmvlvkld lqkkaaciae qyhtvlklqg qasakkrlga nqenqqpnhq
 841 ppgkkpflrn llprtptcqs stdsspyari lrsrhspllk spfgkky
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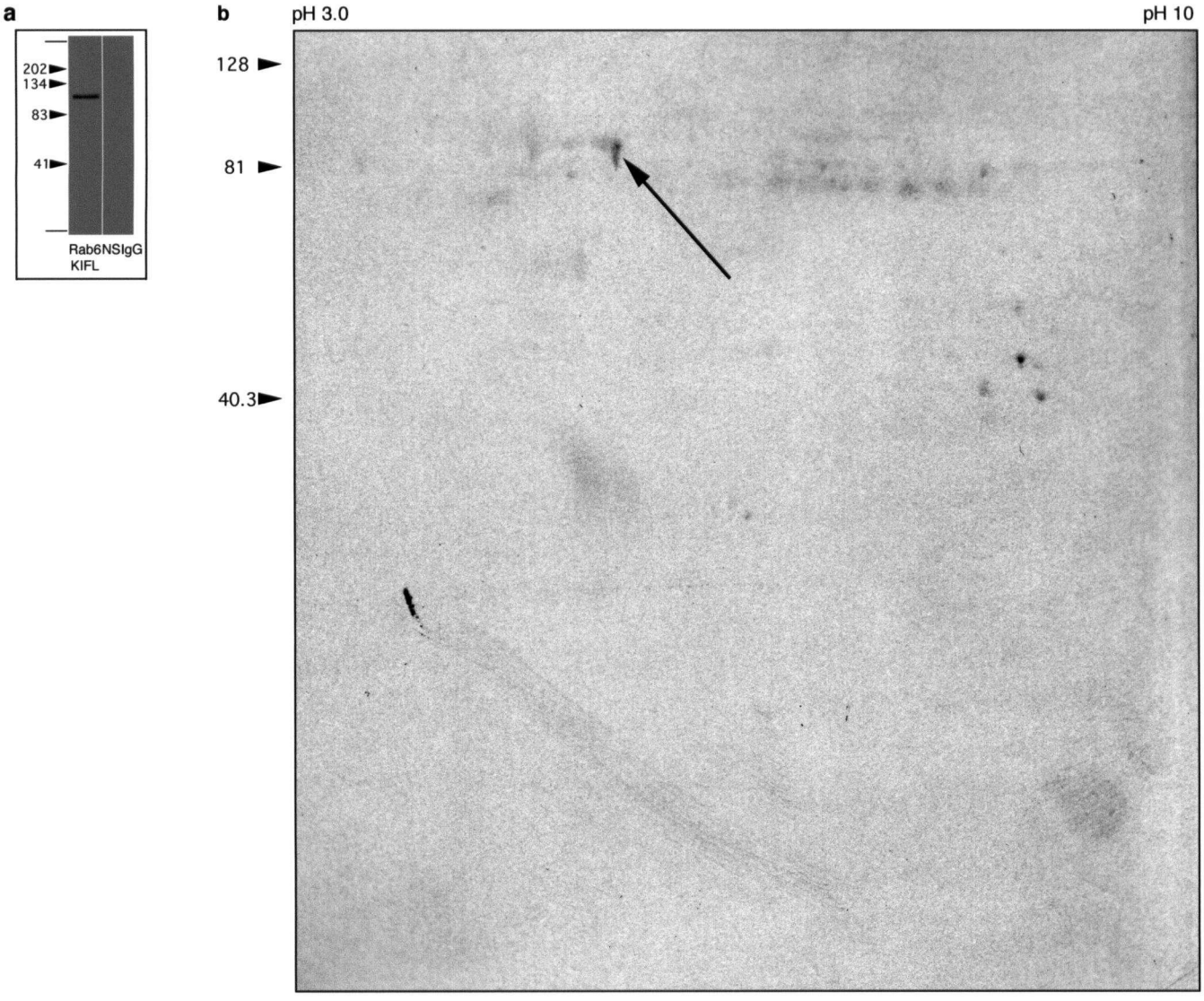
Figure 2.9. (a) Western blots of rat seminiferous epithelium probed with the sheep anti-Rab6KIFL antibody and it's appropriate control, probed with normal sheep IgG (NSIgG) at identical concentrations. Bars indicate the top and bottom of the gel. (b) 2D Western blot of gelsolin treated ectoplasmic specializations probed with the anti-Rab6KIFL antibody. Arrow marks the location of the protein of interest.


a
202
134
83
41
Rab6NSIgG
KIFL
b
pH 3.0
pH 10
128
81
40.3

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Chapter 3

Gelsolin – Evidence for a Role in Turnover of Junction Related Actin Filaments in Sertoli Cells

Introduction

Ectoplasmic specializations are actin filament-containing adhesion complexes found at sites of intercellular attachment in the seminiferous epithelium of the testis. They are present only in Sertoli cells and occur at sites of attachment to spermatids in apical regions of the epithelium, and as part of the junction network between neighboring Sertoli cells in basal regions of the epithelium (Fig. 3.1A). Tight junctions within this basal junction network form the blood-testis barrier. Ectoplasmic specializations are morphologically characterized by the Sertoli cell plasma membrane, a sub-membrane plaque of actin filaments and an attached cistern of endoplasmic reticulum (Fig. 3.1B,C). The actin filaments are hexagonally packed into bundles oriented parallel to the plasma membrane.

Turnover of ectoplasmic specializations is related to two changes in intercellular adhesion that are fundamental to the process of spermatogenesis. At basal sites, turnover is correlated with the loss of attachment between adjacent Sertoli cell plasma membranes and the movement of spermatocytes from basal to adluminal compartments of the epithelium (Russell, 1977). At apical sites, disassembly is associated with sperm release (Russell, 1984). Little is known about how the structures are regulated or how the three elements (plasma membrane, actin filaments, endoplasmic reticulum) of the structures are functionally interrelated.

A version of this chapter has been published. Guttman, JA., Janmey, PA and Vogl, AW. (2002) Gelsolin – Evidence for a role in turnover of junction related actin filaments in Sertoli cells. J. Cell Sci. 115(3):499-505.

Here I report that gelsolin is a component of ectoplasmic specializations. In addition, I report that phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphoinositide-specific phospholipase C (PLCγ) also are present in the structures. Treatment of isolated ectoplasmic specializations with exogenous PLCγ or with a synthetic peptide of the PIP_2 binding region of gelsolin results in the release of gelsolin and loss of filamentous actin from the adhesion junctions. These results support a model for the disassembly of junction-related actin filaments during sperm release and turnover of the blood-testis barrier that involves the gelsolin-phosphoinositide pathway. Moreover, I include in our model the possibility that the endoplasmic reticulum component of ectoplasmic specializations may participate in gelsolin-mediated filament disassembly by regulating Ca^{2+} levels within the filament layer.

Materials and Methods

Chemicals and Reagents

Unless otherwise indicated, most reagents used in the study were from Sigma Chemical Co. (St Louis, MO, USA).

Animals

Animals used in this study were reproductively active Sprague-Dawley rats and New Zealand white rabbits. They were acquired and maintained in accordance with guidelines established by the Canadian Council on Animal Care.

Immunofluorescence

For immunolocalization of gelsolin, testes were perfusion (rat) or immersion (rabbit) fixed with 3% paraformaldehyde in PBS (150 mM NaCl, 5 mM KCl, 0.8 mM KH_2PO_4, 3.2 mM Na_2HPO_4, pH 7.3) and then cryosectioned. Sections were single or double labeled with Alexa 488 phalloidin (Molecular Probes, Eugene, OR, USA) for filamentous actin and with mouse monoclonal antibodies generated against gelsolin (0.0625 μg/ml Sigma antibody; 0.0049 μg/ml Transduction Laboratories antibody) (Sigma, St Louis, MO, USA; BD Transduction Laboratories, Mississauga, ON, Canada). Secondary antibodies consisted of goat anti-mouse IgG conjugated to Texas Red. Controls included replacing primary antibodies with equivalent concentrations of normal mouse IgG, replacing primary antibody with buffer alone, and replacing both primary and secondary antibodies with buffer alone.

For immunolocalization of PLCγ, fixed frozen sections were single or double labeled with 50 μg/ml mouse anti-Phospholipase Cγ IgG (Transduction Laboratories, Mississauga, ON, Canada) and with Alexa 568 phalloidin (Molecular Probes, Eugene, OR, USA). Secondary antibodies consisted of goat anti-mouse IgG conjugated to Alexa 488 (Molecular Probes, Eugene, OR, USA). Controls were similar to those described for gelsolin immunostaining.

To immunolocalize PIP_2, rat testes were perfusion fixed with 3% paraformaldehyde in PBS containing 2 mM EGTA. The tissue was cut into small pieces and then spermatids with attached junction plaques were mechanically dissociated from the epithelium by asperating the pieces through a graded series of syringe needles. Large fragments were allowed to settle and spermatids with attached ectoplasmic specializations that were still in suspension were removed and attached to polylysine coated slides. These spermatid/junction complexes were then single or double labeled with 10μg/ml of purified mouse monoclonal anti-phosphatidylinositol-4,5-bisphosphate (PIP_2) IgM (Echelon Research Laboratories, Salt Lake City, Utah, USA) and with Alexa 568 phalloidin (Molecular Probes, Eugene, OR, USA). Secondary antibodies consisted of goat anti-mouse IgM conjugated to Alexa 488 (Molecular Probes, Eugene, OR, USA). Controls (not shown) included replacing the specific antibody with the equivalent concentration of normal mouse IgM, replacing the primary antibody with buffer alone, or replacing both primary and secondary antibodies with buffer alone.

Immunoelectron Microscopic Localization of Gelsolin

Testes were perfusion (rat) or immersion (rabbit) fixed with 3% paraformaldehyde in PBS. The tissue was cut into small pieces and then spermatids with attached junction plaques were mechanically dissociated from the epithelium by asperating the pieces through a graded series of syringe needles. Large fragments were allowed to settle and then were removed. Cells remaining in solution were concentrated by centrifugation, treated with 50 mM glycine, and then labeled first with a primary antibody to gelsolin (167 μg/ml Sigma antibody; 23 μg/ml Transduction Laboratories antibody) and then with a secondary goat anti-mouse antibody (1:40 dilution) conjugated to nanogold (1.4 nm) (Nanoprobes, Yaphank, NY, USA). Controls were similar to those for immunofluorescence. The material was embedded in Unicryl (British BioCell International, Cardif, UK) and all sections treated with a silver enhancement system (HQ SILVER, Nanoprobes, Nanoprobes, Yaphank, NY, USA).

Peptide Competition Experiments

Testicular fractions enriched for spermatids with attached ectoplasmic specializations were isolated generally as described elsewhere (Miller et al., 1999). The method involved manually collecting epithelia from seminiferous tubules in buffer (PEM) (80 mM PIPES, 1.0 mM EGTA, 1.0 mM $MgCl_2$, pH 6.8) containing 250 mM sucrose, 10 μg/ml soybean trypsin inhibitor, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.1 mM PMSF, and then mechanically fragmenting the material by asperation through syringe needles. The fragments were loaded onto three step sucrose gradients (30 - 60%

sucrose in PEM buffer) and then the gradients were centrifuged. The fractions were resuspended in cold buffer (3 mM EGTA, 25 mM HEPES, 80 mM KCl, 0.5 mM DTT, pH 7.0) and then the suspension was divided into 4 equal volumes that were incubated on ice for 10 minutes. Following this, the cells were pelleted by centrifugation and then resupended in carrier buffer alone, or carrier buffer containing 40 μM of control peptides ["QRLFGKDEL" or "FRVKLKQGQR"] or the PIP_2 binding region of gelsolin ["QRLFQVKGRR"] directly conjugated to rhodamine B (Cunningham et al, 1996). The control peptide "QRLFGKDEL" consisted of the first four residues of the specific peptide followed by residues thought to localize the peptide to the ER. The other control peptide had the same sequence as the specific peptide, but in random order. The specific peptide consisted of residues 160-169 of human plasma gelsolin with rhodamine B conjugated to the amino terminus. $CaCl_2$ was adjusted in each tube to result in a calculated 10 μM free Ca^{2+}. The suspensions were incubated at 37°C for 15 minutes with gentle agitation every 30 seconds. Cells were pelleted by centrifugation and equivalent volumes of supernatant collected from each tube and relative actin concentrations compared qualitatively by SDS reducing immunoblots using a monoclonal anti-actin antibody (Sigma, St Louis, MO, USA) used at 0.01 mg/ml. To access qualitatively the amount of gelsolin in the supernatants, we stripped the blots probed for actin and re-probed them using a polyclonal rabbit anti-mouse gelsolin antibody (Gift from Dr. Toshi Azuma) used at 1:2000. Unlike the antibodies used for the morphological work, this probe reacted with rat gelsolin on Western blots.

To determine if the three synthetic peptides could bind to ectoplasmic specializations, spermatids with attached ectoplasmic specializations were treated for 30 minutes with buffer alone (3 mM EGTA, 25 mM HEPES, 80 mM KCl, 0.5 mM DTT, pH 6.5) or with buffer containing 20μM synthetic peptides. The cells were washed and then examined with a fluorescence microscope.

PLCγ Experiments

Testicular fractions enriched for spermatids with attached ectoplasmic specializations were obtained as described for the peptide competition experiments, pooled, and then diluted in 1 ml of buffer (3 mM EGTA, 25 mM HEPES, 80 mM KCl, 0.5 mM DTT) not containing Ca^{2+}. Equal volumes of suspensions were added to the required number of treatment tubes and then the cells pelleted by centrifugation. The supernatants were discarded and the cells in each of four tubes were resupended in 500 µl buffer containing a calculated 11 µM free Ca^{2+} (3 mM EGTA, 25 mM HEPES, 80 mM KCl, 0.5 mM DTT, 2.92 mM $CaCl_2$). Cells to be used in the no Ca^{2+} control were resuspended in 500 µl buffer containing no Ca^{2+} (3 mM EGTA, 25 mM HEPES, 80 mM KCl, 0.5 mM DTT). The cells were allowed to sit on ice for 10 minutes. Following this, the tubes again were centrifuged and the pellets resuspended in 100 µl of treatment buffers containing the appropriate calculated amounts of Ca^{2+} (11 µM or 1.5 mM) and PLCγ or buffer alone. The reaction mixtures were incubated for 15 minutes at 37°C with gentle agitation every 30 seconds. Following incubation, cells were pelleted by centrifugation and equivalent volumes of supernatant were removed from each tube and

assayed, by SDS reducing immunoblot, for actin and gelsolin as described above for the peptide competition experiments.

Results

While doing a screen for a number of candidate actin-binding proteins that could be involved with actin dynamics in ectoplasmic specializations in the rat, I found that two different monoclonal antibodies to gelsolin reacted with apical and basal sites known to contain the unique adhesion complexes (Figure 3.1D). Staining was dramatically co-distributed with actin filaments, as indicated by fluorescent phallotoxin staining, in double-labeled material. Results were confirmed at the ultrastructural level where staining was restricted to the region of the adhesion complex containing the actin layer (Figure. 3.1E). Staining in this region was present both on the plasma membrane and endoplasmic reticulum side of the actin layer, as well as within the layer itself. Although both antibodies reacted in a site specific fashion on fixed frozen sections, neither reacted with a band corresponding to purified bovine gelsolin, nor with any other bands, on immunoblots of rat testis (data not shown). Using native gels did not improve immunoreactivity, nor did mild protease treatment or fixation of the blots. We repeated all the experiments using rabbit testis. Immunolocalization results at the light (Figure 3.2A) and the electron microscopic (Figure 3.2B) levels were identical to those obtained using rat tissue. Significantly, the two monoclonal antibodies reacted with a single band that migrated slightly ahead of purified bovine plasma gelsolin on immunoblots of rabbit testis (Figure 3.2C). Rabbit cytoplasmic gelsolin is known to be 25 amino acids smaller than human plasma gelsolin (Kwiatkowski et al., 1986). This result, together with the immunolocalization data, indicated to us that the antibodies were monospecific. Our results are generally consistent with a previous report of gelsolin in the apical cytoplasm of human Sertoli cells (Rousseauz-Prevost et al., 1977). We conclude that gelsolin is a

major component of Sertoli cell ectoplasmic specializations, and that it is localized to the actin-containing region between the plasma membrane and the endoplasmic reticulum.

The presence of gelsolin in ectoplasmic specializations has significant implications for the assembly and disassembly of the actin plaques. Gelsolin is a potent Ca^{2+} dependent actin severing and capping protein (Sun et al., 1999). The presence of gelsolin within the actin containing region of ectoplasmic specializations, structures that are stable during most of the long process of spermatogenesis, indicates to us that much of the protein may be 'inhibited' until it is required for actin disassembly or reorganization. In addition to the lack of Ca^{2+}, the only known inhibitors of the severing and capping functions of gelsolin are certain phospholipids (Janmey and Stossel, 1987; Meerschaert et al., 1998; Sun et al., 1999), the most notable of which is phosphatidylinositol 4,5- bisphosphate (PIP_2).

To determine if PIP_2 is present in ectoplasmic specializations, I treated isolated spermatids, to which the adhesion complexes remained attached, with an antibody to the phospholipid. The antibody positively reacted with regions that also labeled with probes for junction related actin filaments (Fig. 3.3a). Similar staining was not observed when normal mouse IgG was substituted for primary antibody. I also labeled fixed sections of rat testis with antibodies to phosphoinositide-specific phospholipase C ($PLC\gamma$). This probe specifically labeled regions of the seminiferous epithelium known to contain both basal and apical ectoplasmic specializations, and was stage specific. At stages when the junctions are stable, staining at the junctions was weak (Figure 3.3b). Significantly,

staining was most intense during the period of spermatogenesis (Stage VII in rat) when the adhesion complexes are disassembling apically and turning over basally (Figure 3.3c). The antibody reacted specifically with one band on blots of rat testis and rat seminiferous epithelium (Figure 3.3d).

As an alternative approach to verifying the presence of PIP_2 at the junction plaque, I labeled unfixed spermatid/junction complexes, in the absence of Ca^{2+}, with a synthetic peptide of the PIP_2 binding domain of gelsolin directly conjugated to rhodamine B (Cunningham et al., 1996). Staining of regions known to contain ectoplasmic specializations in controls was weak or absent (Figure 3.4 A-C, A'-C') whereas staining with the specific peptide was relatively intense (Figure 3.4 D,D').

To test the hypothesis that gelsolin in ectoplasmic specializations may be bound to PIP_2, I mechanically dissociated rat spermatids, to which ectoplasmic specializations of Sertoli cells remained attached, from the seminiferous epithelium and incubated the cells with a synthetic peptide of the PIP_2 binding region of gelsolin or with PLCγ. In the first set of experiments, I predicted that the specific peptide would compete with endogenous gelsolin for binding to PIP_2 and, in the presence of Ca^{2+}, would result in increased actin disassembly when compared to controls. Relative to supernatants collected from cells treated with buffer alone or with two control peptides, more actin was present in blots of supernatants collected from cells treated with specific peptide (Figure 3.4E). Importantly, when blots were re-probed with an antibody that recognizes rat gelsolin on Western blots, more gelsolin was detected in supernatants collected from

cells treated with specific peptide than in blots of supernatants treated with control peptides or buffer alone.

In the second set of experiments, I predicted that exogenously added PLCγ would hydrolyze PIP_2 to inositol triphosphate (IP_3) and diacylglycerol, thereby releasing gelsolin. In the presence of Ca^{2+}, actin in the adhesion complexes associated with spermatid heads should disassemble and the amount of actin in solution should increase relative to controls. The buffer systems used contained calculated micromolar and millimolar levels of Ca^{2+}. Millimolar levels were included in the design to swamp any effect of PLCγ treatment on Ca^{2+} release from junction related ER that would indirectly activate any gelsolin not bound to PIP_2. Following incubation, spermatid/junction complexes were pelletted by centrifugation and equivalent volumes of supernatants from experimental and control cells collected and analyzed, by immunoblotting, for actin. In some experiments, an equivalent volume of cells was removed from each tube prior to centrifugation and stained with fluorescent phallotoxin to label filamentous actin. Generally, less actin was visible in the adhesion complexes associated with cells treated with PLCγin the presence of Ca^{2+} than in control cells. At mM levels, Ca^{2+} alone increased actin in supernatants relative to other controls. Significantly, treatment with PLCγ in the presence of μM and mM Ca^{2+} increased the amount of actin in supernatants collected from spermatid/junction complexes relative to supernatants collected from all control cells (Figure 3.5E). When the same blots of supernatants were re-probed for gelsolin, PLCγ in the absence of Ca^{2+} increased the level of gelsolin in supernatants without a corresponding increase in the amount of actin. At mM levels, Ca^{2+} alone

increased the amount of gelsolin in supernatants. At μM levels, the effect was reduced and the amount of gelsolin present in supernatants was less than when samples were treated with PLCγ in the presence or absence of Ca^{2+}.

Discussion

The results presented here provide insight into the molecular mechanism of junction related actin filament disassembly related to sperm release and turnover of basal junction networks between Sertoli cells. The mechanism may involve, at least in part, gelsolin and the phosphoinositide pathway. Gelsolin and PLCγ are concentrated in ectoplasmic specializations, and PIP_2 also is present in the structures. In addition, treatment of isolated ectoplasmic specializations either with the PIP_2 binding domain of gelsolin or with PLCγ in the presence of Ca^{2+} increases the disassembly of the junction related actin filaments relative to controls. Significantly, both of these treatments also result in an increase in the amount of free gelsolin suggesting that gelsolin is functionally linked to PIP_2. Although these results do not rule out the possibility that related proteins, such as scinderin, also may be involved with turnover of cortical actin in Sertoli cells, as has been suggested (Pelletier et al., 1999), gelsolin is the first member of this group of actin severing proteins to be localized specifically to ectoplasmic specializations.

The fact that the severing and capping functions of gelsolin are Ca^{2+} dependent (Yin and Stossel, 1979) together with the general finding that IP_3 stimulates the release of Ca^{2+} from intracellular stores (Berridge, 1993) may account for the presence of a cistern of endoplasmic reticulum as an integral part of the adhesion complex. The endoplasmic reticulum of this complex is suspected to regulate Ca^{2+} levels (Franchi and Camatini, 1985; Pelletier et al., 1999); however, strong experimental evidence that the cistern actually can sequester and release the cation locally is still lacking. It is possible that, in stable plaques, the ER may function to maintain low levels of local Ca^{2+} within the actin

layer thereby inhibiting the severing function of gelsolin, and perhaps of related proteins. The capping function of gelsolin can occur at lower Ca^{2+} concentrations than the severing function (Janmey et al., 1985). This observation may account for the presence of gelsolin amongst actin filaments in stable actin plaques, in addition to being located on either side of the actin layer where presumably the gelsolin could be bound to PIP_2 in adjacent membranes. Gelsolin caps on actin filaments also may account for the effect of increased Ca^{2+} alone noted in our experiments. During plaque disassembly, hydrolysis of PIP_2 by PLCγ would not only release gelsolin bound to PIP_2, but may generate a local surge in Ca^{2+} release from the ER, through the action of IP_3 (Figure 3.6). This Ca^{2+} surge would stimulate the severing function of gelsolin within the actin plaque.

How actin filament disassembly, involving gelsolin, is initiated and coupled to signaling pathways associated directly with intercellular adhesion molecules in the plasma membrane remain to be determined. In addition to a role in filament disassembly, it also is possible that gelsolin is involved with nucleation and elongation of actin filaments during junction assembly and during the changes in actin filament rearrangement that occur within ectoplasmic specializations during spermatogenesis. The finding that gelsolin is an integral component of structures related to sites of intercellular adhesion in Sertoli cells may have general implications for the molecular mechanisms underlying assembly and disassembly of actin complexes associated with intercellular adhesion junctions generally in cells.

Figure 3.1. Position of ectoplasmic specializations in the seminiferous epithelium of the testis and immunolocalization of gelsolin to the actin component of the structures. (A) Ectoplasmic specializations are present only in Sertoli cells and occur apically, at sites of adhesion to spermatids, and basally, at sites of adhesion to neighboring Sertoli cells. (B and C). Typical appearance of ectoplasmic specializations in transmission electron micrographs. The structures consist of the plasma membrane of the Sertoli cell, a layer of actin filaments and a cistern of endoplasmic reticulum. The junctions shown here are from sites of Sertoli cell attachment to spermatids in the ground squirrel testis. Bars = 100 nm (D) Immunofluorescence localization of gelsolin to ectoplasmic specializations in frozen sections of perfusion fixed rat testis. Sections were treated with a primary monoclonal antibody to gelsolin and with a secondary antibody conjugated to Texas Red. Actin filaments were labeled with fluorescent phalloidin. Within the seminiferous epithelium, gelsolin and actin are co-localized at ectoplasmic specializations. Sites of apical and basal ectoplasmic specializations are indicated by the 'a' and 'b' respectively in the panel labeled for actin. Specific staining for gelsolin was not observed in any of the controls (not shown). Bar = 10 μm. (E) Immunoelectron microscopic localization of gelsolin to the actin zone of ectoplasmic specializations. Spermatids with attached ectoplasmic specializations were mechanically dissociated from perfusion fixed testes and treated with a primary antibody to gelsolin and a secondary antibody conjugated to nanogold. The material was embedded and sectioned, and then the sections were silver enhanced and stained. Shown here is an ectoplasmic specialization attached to a spermatid head. Notice that silver grains (small arrows) are associated with the actin zone of the junction plaque. Bar = 500 nm.

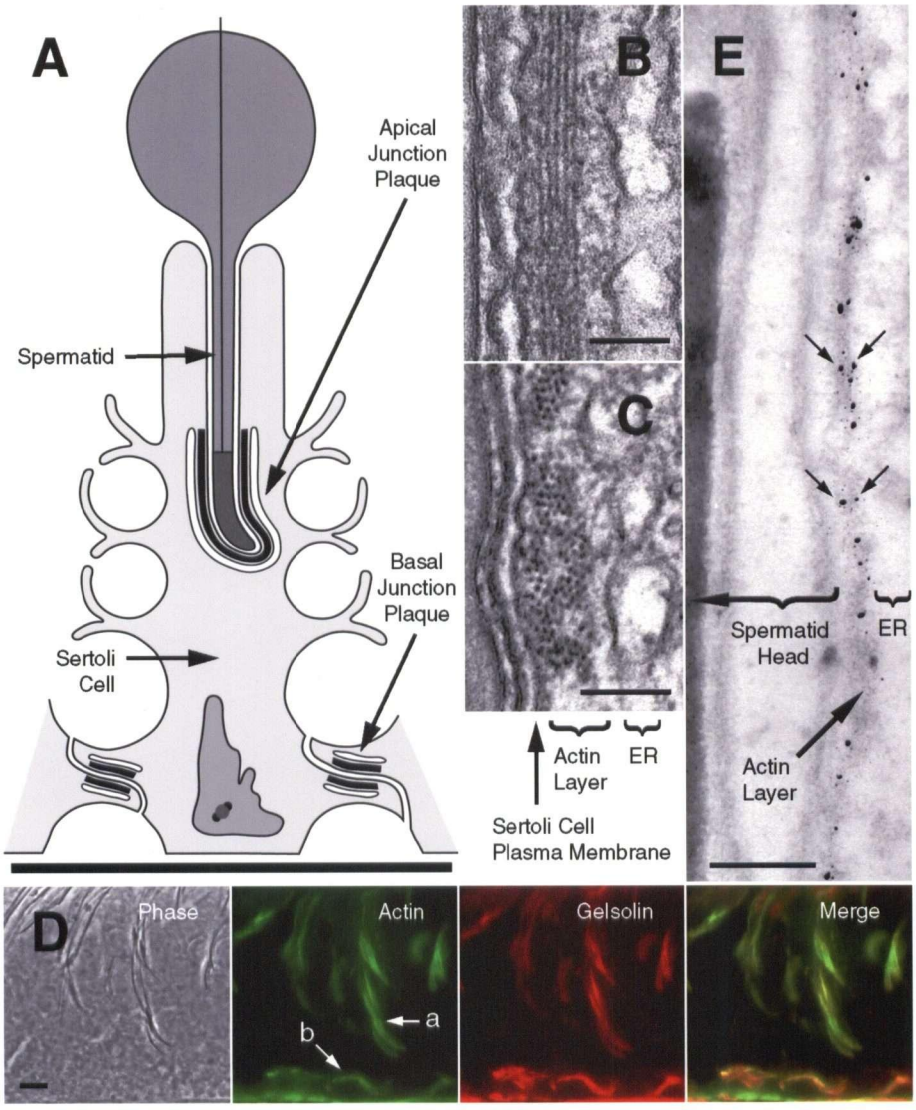

A
Apical
Junction
Plaque
Spermatid
Basal
Junction
Plaque
Sertoli
Cell
B
C
Actin
Layer
ER
Sertoli Cell
Plasma Membrane
E
Spermatid
Head
ER
Actin
Layer
D
Phase
Actin
a
b
Gelsolin
Merge

Figure 3.2. Immunolocalization of gelsolin to ectoplasmic specializations in rabbit testis. (A) Fluorescence of fixed-frozen sections of rabbit testis labeled for actin and gelsolin. Apical and basal ectoplasmic specializations are indicated by the 'a' and 'b' respectively in the actin panel. Bar = 10 μm. (B) Immunoelectron microscopic localization of gelsolin to the actin filament containing region of an apical ectoplasmic specialization. The material was processed and labeled exactly as described in the legend to Figure 3.1. As in the rat, the silver grains (small arrows) are associated with the actin zone of the junction plaque. Bar = 250 nm. (C) Immunoblot of rabbit testis and purified bovine gelsolin (Sigma). The antibodies from Sigma and from Transduction Laboratories reacted with a single band on immunoblots of rabbit testis, and this band migrated slightly ahead of purified bovine plasma gelsolin. A similar band was not present on control blots in which the primary antibody was replaced with a similar concentration of normal mouse IgG (not shown). The lines indicate the top and bottom of the gel. Loading densities were 0.05 μg of purified gelsolin and 100 μg of decapsulated testis homogenate.

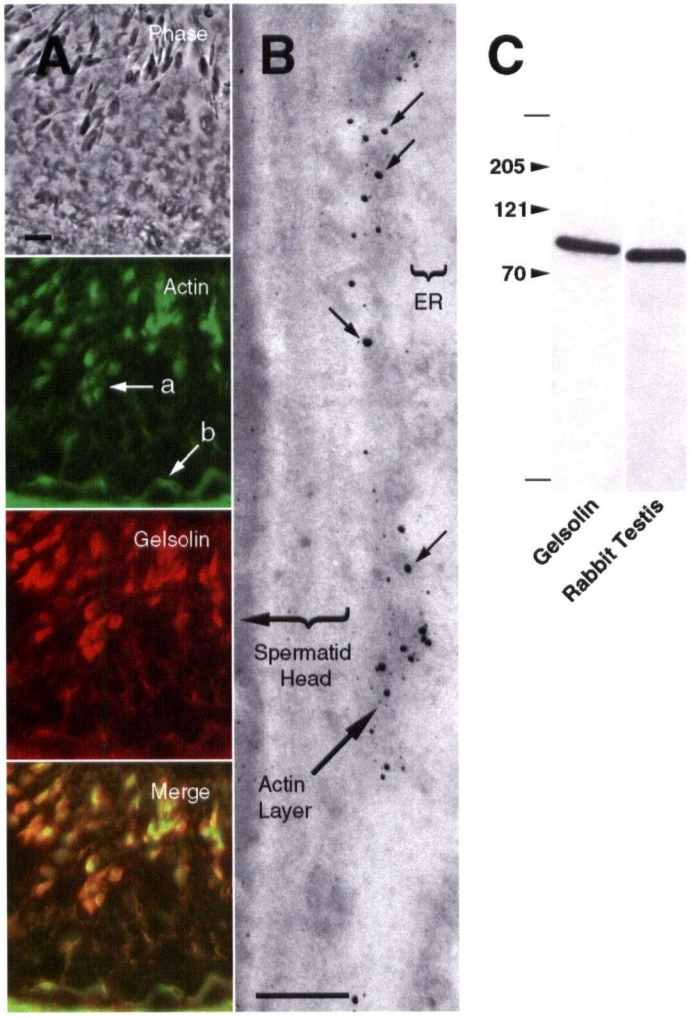

A
Phase
Actin
a
b
Gelsolin
Merge
B
ER
Spermatid
Head
Actin
Layer
C
205
121
70
Gelsolin
Rabbit Testis

Figure 3.3. Immunolocalization of PIP_2 and phosphatidylinositide specific phospholipase C (PLCγ) in rat ectoplasmic specializations. (A) Phase and fluorescence images of isolated spermatids with attached ectoplasmic specializations that have been labeled with an antibody raised against PIP_2. The spermatids have also been treated with fluorescent phallotoxin to label actin. Notice that the probe for PIP_2 stains the region surrounding the head containing an ectoplasmic specialization that labels with the probe for actin. Specific staining for PIP_2 was not observed in any of the controls (not shown). Bar = 5 μm. (B and C) Immunofluorescence localization of PLCγ in fixed frozen sections of rat seminiferous epithelium at Stage V (B) and Stage VII (C) of spermatogenesis. The locations of apical and basal ectoplasmic specializations are indicated by the 'a' and 'b' respectively in the actin panels. At Stage V, the probe for PLCγ reacts weakly at ectoplasmic specializations. The situation is much different at stage VII when apical ectoplasmic specializations are disassembling as part of the sperm release process and basal ectoplasmic specializations are turning over to allow the next generation of spermatocytes through junction complexes between Sertoli cells. At this stage, apical and basal regions containing ectoplasmic specializations, indicated by the actin staining, also react with the probe for PLCγ. Specific staining was not observed in any of the controls (data not shown). The intense staining of the tubule wall (asterisk) is due to non-specific staining by the secondary antibody. Bar = 10 μm. (D) Immunoblot of rat testis (lane 1) and rat seminiferous epithelium (lane 2). The PLCγ antibody reacts specifically with a single band in each lane (~148 kD). The minor bands indicated by the asterisk are non-specific and are present in blots treated with normal mouse IgG instead of primary

antibody. The lines indicate the top and bottom of the gel. Loading densities were 80μg of testis homogenate and 80μg of seminiferous epithelium.

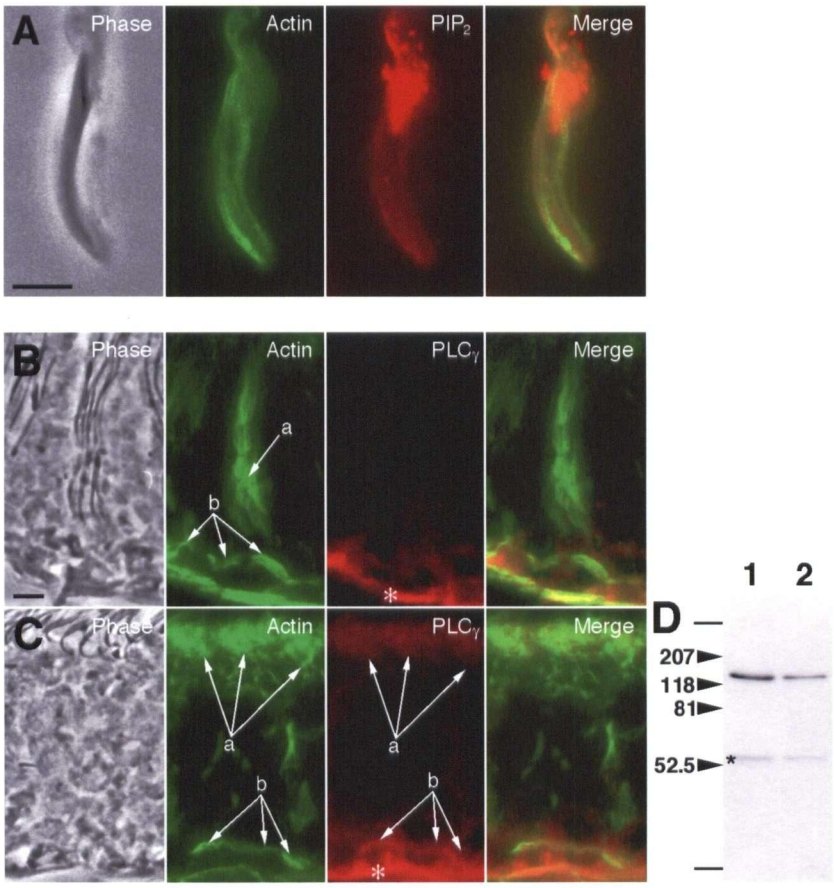

A
Phase
Actin
PIP2
Merge
B
Phase
Actin
PLCγ
Merge
a
b
*
C
Phase
Actin
PLCγ
Merge
a
b
*
D
1
2
207
118
81
52.5
*

Figure 3.4. Peptide binding and competition experiments. Paired phase (A-D) and fluorescence (A'-D') micrographs of spermatids with attached ectoplasmic specializations that were mechanically isolated from rat seminiferous epithelium and treated for 30 min with buffer (A,A') or with buffer containing 20μM synthetic peptides directly conjugated to rhodamine B. The peptides consisted of two control sequences ["QRLFGKDEL" (B,B') and "FRVKLKQGQR" (C,C')] and the PIP_2 binding region of gelsolin ["QRLFQVKGRR" (D,D')]. Note that the PIP_2 binding region of gelsolin labels regions surrounding the spermatid head more strongly than the control peptides or buffer alone. Bar = 5 μm. (E) Shown here are the results of a peptide competition experiment. From left to right, the lanes are of supernatants collected from spermatids with attached ectoplasmic specializations treated with buffer alone, sequence "QRLFGKDEL" (control Peptide 1), sequence "FRVKLKQGQR" (control Peptide 2), and sequence "QRLFQVKGRR" (Gelsolin Peptide). The blots were probed with antibodies to actin and gelsolin. Notice that the amount of actin and gelsolin present in the supernatant from material treated with the PIP_2 binding region of gelsolin ("QRLFQVKGRR") is greater than in supernatants treated with control peptides or buffer alone.

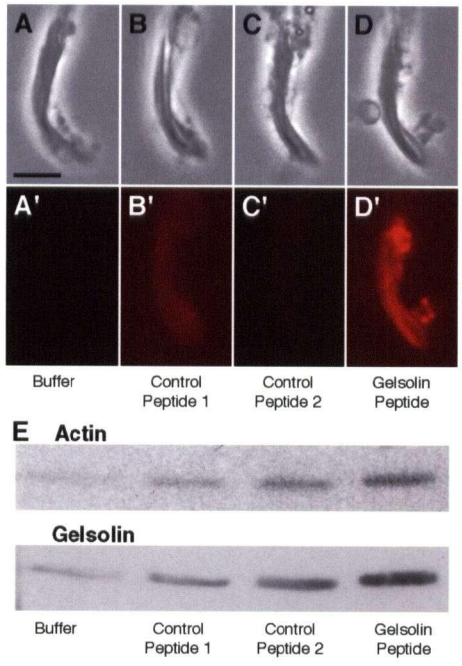

A
B
C
D
A'
B'
C'
D'
Buffer
Control Peptide 1
Control Peptide 2
Gelsolin Peptide
E
Actin
Gelsolin
Buffer
Control Peptide 1
Control Peptide 2
Gelsolin Peptide

Figure 3.5. PLCγ in the presence of Ca^{2+} results in the loss of filamentous actin from rat ectoplasmic specializations. In the experiment shown here, spermatids with attached ectoplasmic specializations were incubated in the presence or absence of either phosphoinositide specific phospholipase C (PLCγ) in the presence and absence of Ca^{2+}. Shown in panels A,A' to D,D' are paired phase and fluorescence micrographs of spermatids with attached adhesion complexes fixed and labeled with fluorescent phalloidin for filamentous actin immediately after isolation (A,A') or after incubation with or without PLCγ in the presence or absence of a calculated 11μM free Ca^{2+}. Bar = 5μm. (B,B' to D,D'). The least amount of filamentous actin is associated with cells treated with PLCγ in the presence of Ca^{2+}. (E) Treatment of spermatid/adhesion complexes with PLCγ in the presence of Ca^{2+} resulted in increased levels of actin and gelsolin in supernatants, relative to controls, as assessed by immunoblot. In these experiments, spermatids with attached junction complexes were incubated in buffers with or without PLCγ in the presence or absence of a calculated 1.5 mM (upper two blots) or 11 μM free Ca^{2+} (lower two blots). Cells were removed from solution by centrifugation and equivalent volumes of supernatants assessed for actin and gelsolin on immunoblots. The amount of actin and gelsolin are greatest in supernatants from spermatid/junction complexes treated with both PLCγ and Ca^{2+}. Significantly, treatment with PLCγ in the absence of Ca^{2+} results in increased gelsolin in supernatants, but not in increased of actin.

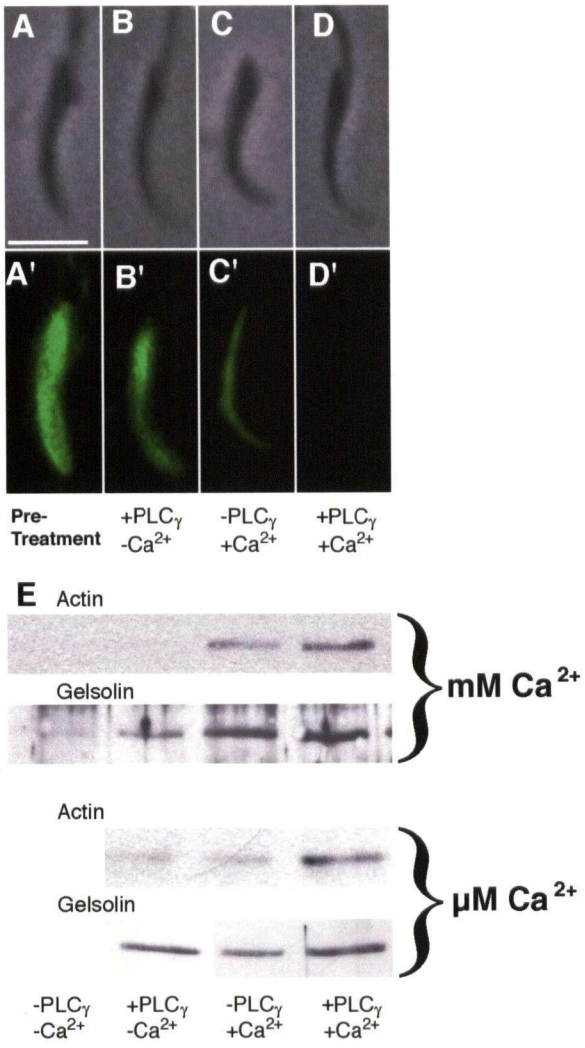
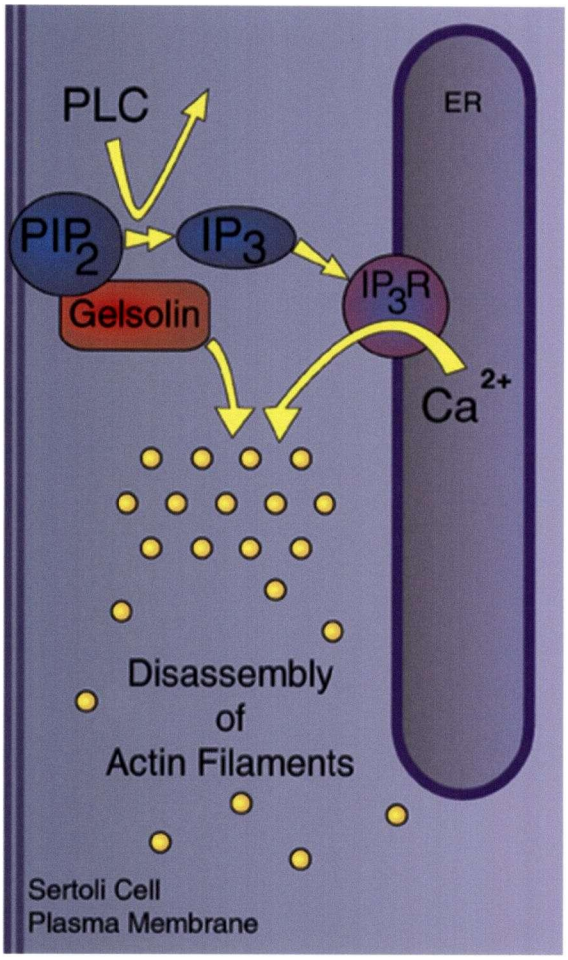

A
B
C
D
A'
B'
C'
D'
Pre-Treatment
+PLCγ -Ca2+
-PLCγ +Ca2+
+PLCγ +Ca2+
E
Actin
Gelsolin
mM Ca 2+
Actin
Gelsolin
μM Ca 2+
-PLCγ -Ca2+
+PLCγ -Ca2+
-PLCγ +Ca2+
+PLCγ +Ca2+

Figure 3.6. Putative model for gelsolin activation at the time of ectoplasmic specialization disassembly in Sertoli cells. The model involves hydrolysis of PIP_2 resulting both in the release of gelsolin and a surge in local levels of Ca^{2+}. Although PIP_2 is diagrammed in association with the plasma membrane, the model is not meant to exclude the possibility that PIP_2 also is present in the membrane of the ER.


PLC
ER
PIP2
IP3
IP3R
Gelsolin
Ca2+
Disassembly
of
Actin Filaments
Sertoli Cell
Plasma Membrane

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CHAPTER 4

Rac1 and Gelsolin Co-localize with Filamentous Actin at Ectoplasmic Specializations Throughout Spermiogenesis

Introduction

The seminiferous epithelium of the mammalian testis is a dynamic tissue composed of two major cell populations, spermatogenic cells and the Sertoli cells. Actin related adhesion junction plaques called ectoplasmic specializations are found at certain sites of intercellular attachment. These junctions occur where two Sertoli cells interact with each other at the base of the epithelium and where the Sertoli cell comes in contact with the heads of developing spermatids. Ectoplasmic specializations are tripartite structures, composed of the Sertoli cell plasma membrane, actin filaments arranged in paracrystalline arrays and a cistern of endoplasmic reticulum.

Ectoplasmic specializations are disassembled and reassembled at two specific times. One occurs when spermatocytes move from basal to adluminal compartments of the epithelium. Here, ectoplasmic specializations disassemble above the spermatocytes and assemble below. The second is during sperm release and polarization of the new generation of spermatids. Ectoplasmic specializations disassemble as part of the sperm release process at the apex of Sertoli cells, and new ectoplasmic specializations assemble as the next generation of spermatids begin to elongate. There currently is great interest in the molecular architecture and control of ectoplasmic specializations because they may

A version of this chapter has been submitted for publication. Guttman, JA., Vaid, KS., and Vogl, AW (2003) Rac1 and Gelsolin Co-localize with Filamentous Actin at Ectoplasmic Specializations Throughout Spermiogenesis. *(submitted to the Anatomical Record)*

contain molecular targets for male contraception (Cheng and Mruk, 2002).

Recently the protein gelsolin has been implicated in playing an important role in disassembling ectoplasmic specializations. Gelsolin is a calcium dependent actin severing and capping protein. Other than the absence of calcium, the only known naturally occurring inhibitor of gelsolin's actin severing capability is the phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2). It has been proposed that actin plaque disassembly at ectoplasmic specializations is part of a gelsolin/PIP_2/PLCγ cascade. In this model, gelsolin is functionally linked to the Sertoli cell plasma membrane through PIP_2 and is released by PIP_2's conversion to inositol 3,4,5 tris phosphate (IP_3) by Phospholipase C γ (PLCγ). This IP_3 would trigger IP_3 receptors on the endoplasmic reticulum cistern of the ectoplasmic specialization thereby releasing calcium and activating the unbound gelsolin to disassemble the junctional actin filaments. Although not yet explored in the testis, work in other systems has indicated that small GTPases, in particular rac1, also may be upstream regulators of gelsolin.

Rac is involved in numerous cellular functions including the formation of lamellipodia (reviewed by Kaibuchi et al., 1999a) and the regulation/breakdown of cadherin-based cell-cell adhesion junctions (Braga et al., 1999; reviewed by Kaibuchi et al., 1999a), but it's cell junction disruption activity does not extend to integrin based cell-cell junctions (Braga et al., 1999, 2000). Among Rac's other functions is its ability to interact with and recruit PI(4)P-5 kinase, the enzyme that produces PIP_2 (Tolias et al.,

1995; Honda et al., 1999). This recruitment involves the use of another family of small GTPases, the ADP-ribosylation factors (ARFs).

The activity of Rac1 is mediated through its association with either GTP (active) or GDP (inactive) (Kaibuchi et al., 1999). The regulation of these activity states is controlled through guanine nucleotide exchange factors (GEFs) and the GDP dissociation inhibitor (GDI). GEFs aid in the release of GDP, allowing for the incorporation of GTP. GDI inhibits the dissociation of GDP. Another mechanism of regulating Rac1 activity is through the Rho-GTPase activating proteins (GAPs). These proteins convert the Rho-GTPases to their inactive forms through the stimulation of the GTPase activity.

Although the relationship between PIP_2 and gelsolin has been well established (Lin et al., 1997; Sun et al., 1999; Yin and Janmey, 2003), the exact relationship between the small GTPase, rac1, and gelsolin is unclear. This is due to evidence in neutrophils, that demonstrated that activated, GTP loaded rac can disassociate gelsolin from actin in the absence of phosphoinositides (Arcaro, 1998). Other evidence using gelsolin null dermal fibroblasts, detected a marked overexpression of rac, which was returned to normal levels upon gelsolin re-expression (Azuma et al., 1998). This coupled with the finding of rac and gelsolin's localization to the leading edge membrane of fibroblasts lead Azuma et al., (1998) to be the first to hypothesize that upon receptor activation, rac is recruited to the membrane at which point it recruits the PIP_2 synthesis enzyme PI(4)P-5 kinase to synthesize PIP_2 which would then control gelsolin and influence calcium levels.

Even though PIP_2 and gelsolin have been strongly implicated in the turnover of ectoplasmic specializations, no small GTPases have yet been localized to these structures.

In this chapter I demonstrate that gelsolin is co-localized with actin throughout spermatogenesis and rac1 has a similar but more diffuse pattern of distribution. The results are consistent with the possibility that gelsolin may play a role at ectoplasmic specializations other than during disassembly, and that rac1 is in a morphological position to be involved.

Materials and methods

Chemicals and reagents

Unless indicated otherwise, reagents used in this study were from the Sigma-Aldrich (Oakville, ON, Canada). Paraformaldehyde was obtained from Fisher Scientific. Glutaraldehyde, OsO4, Unicryl and sodium cacodylate were all obtained from British BioCell International (Cardif, UK)

Antibodies raised against gelsolin were from Sigma-Aldrich Co. and BD Trandsuction Laboratories (Mississauga, ON, Canada). The antibody raised against Rac1 also was obtained from Transduction Laboratories. Fluorescent phalloidin as well as Alexa Fluor conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA). All normal IgG, serums, Texas Red and horseradish peroxidase (HRP) labeled secondary antibodies came from Jackson ImmunoResearch (West Grove, PA, USA). Secondary antibodies conjugated to 1.4nm nanogold clusters and the HQ silver enhancement kit, were obtained from Nanoprobes Inc. (Yaphank, NY, USA).

Animals

Animals used in this study were male reproductively active, Sprague-Dawley rats and New Zealand White rabbits. They were all obtained from colonies at the Animal Care facility at the University of British Columbia and maintained and used in accordance with the guidelines of the Canadian Council on Animal Care.

Immunofluorescence

Rat tissue used for immunofluorescent labeling was prepared in the following fashion. Testes were removed while the animals were under deep anesthesia. Once removed, the testicular artery was cannulated and warm (33°C) PBS (150mM NaCl, 3.2mM $NaHPO_4$, 0.8mM KH_2PO_4, 5.0mM KCl, pH 7.3) was perfused through the organ for about 2 minutes to rid the testis of blood. Following this, warm fixative (3% Paraformaldehyde in PBS pH 7.3) was perfused through the organ for 30 minutes at which point PBS was passed through it for 30minutes, to wash out any excess fixative.

Rabbit testes were removed from New Zealand White rabbits euthanized with carbon dioxide. Testes capsules were punctured using a 25G needle and submersion fixed for 2 hours in room temperature 3% paraformaldehyde in PBS (pH 7.3). Following the 2hour incubation the fixative was removed and replaced by PBS for 3 ten minute washes before processing for cryosectioning.

Rat and rabbit tissue for frozen sectioning was mounted onto aluminum stubs using Histo Prep (Fisher Scientific), frozen with liquid nitrogen, then cryosectioned to 8-10 μm sections. Sections were then attached to poly-lysine coated glass slides.

Slides with attached sections were immediately submerged into –20°C acetone for 5 minutes and then allowed to air dry. Once dry, the material was re-hydrated for 20 minutes at room temperature using 5% normal goat serum (NGS) in TPBS/BSA (PBS pH 7.3 with 0.05% Tween-20, and 0.1% bovine serum albumin (BSA)). The 5%NGS was

then removed and replaced with primary mouse monoclonal antibodies raised against either gelsolin (used at 0.47 μg/ml), or Rac1 (used at 0.47 μg/ml) and incubated overnight at 4°C. Once completed the primary antibodies were washed off and replaced with goat anti-mouse secondary antibodies conjugated to either Texas Red or Alexa Fluor 488 for a 60 minute incubation at 37°C. Secondary antibodies were then washed off and either Alexa Fluor 568 or 488 phalloidin was added to double label the tissue for filamentous actin in order to identify the location of the junction plaques.

Controls included replacing primary antibody with normal mouse IgG (NMIgG), carrier buffer, or replacing both primary and secondary antibodies with buffer.

Immunoelectron microscopy

Once fixed (as described for the immunofluorescent section) the testes were minced with 2 scalpels into 1-2mm pieces, transferred to a 50ml Falcon tube, then gently passed (2-3 times each) through first an 18G needle and then through a 21G needle. The material was then allowed to stand for 10 minutes at room temperature. The suspended material was removed and centrifuged at setting 6 in an IEC tabletop centrifuge. The supernatant was discarded and the pellet was treated with 5% NGS in PBS/BSA before dividing the sample equally into 4 tubes for antibody processing. Rac1 antibodies (0.228mg/ml) were added to the experimental tubes and incubated at room temperature for 1hour under constant rotation, followed by a 4°C overnight incubation. The samples were then placed back on the rotator for 1hour at room temperature then washed twice with PBS/BSA. The samples were then treated with a goat anti-mouse secondary

antibody conjugated to 1.4nm nanogold (Nanoprobes, Yaphank, NY) at a concentration of 0.2mg/ml and placed on a rotator for 1 hour at room temperature. The antibody labeled material was washed twice then fixed with 1% glutaraldehyde (EM Sciences) in PBS. The tissue was washed twice with ddH2O then dehydrated through a series of increasing ethanol solutions (30%, 50%, 70%, 90%, 95%, 100%). Once dehydrated, the tissue was embedded in increasing concentrations of Unicryl (Cedarlane Laboratories) before being exposed to UV light overnight to polymerize. Following the polymerization the blocks were sectioned and silver enhanced according to the manufacturers instructions using the HQ Silver Enhancement Kit (Nanoprobes).

Western Blots

In order to investigate the specificity of the antibodies used for immunostaining (mouse anti-gelsolin and mouse anti-rac1), SDS reducing western blots were loaded with 80μg of rat testis homogenate or rat seminiferous epithelium into individual lanes and run with the Bio-Rad Protean II Mini Gel apparatus. In the case of both gelsolin antibodies, each reacted with rat and rabbit tissues with a similar staining pattern on tissue, but neither reacted with rat testis on Westerns (Guttman et al., 2002). To verify the specificity, 100μg of rabbit testis homogenate as well as 0.05μg purified bovine plasma gelsolin (Sigma-Aldrich) were used. After protein transfer onto Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) blots were blocked using 4% Blotto (non-fat dry skim milk from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)) in TBS (500mM Tris pH 7.5, 150mM NaCl) for at least 8 hours at 4°C. Blots then were probed overnight at 4°C with each antibody (anti-gelsolin from Sigma at 3.33μg/ml, anti-gelsolin from Transduction laboratories at 1μg/ml or anti-rac1 at 0.625μg/ml) in TBST (TBS + 0.1% Tween-20) containing 1%BSA. After excessive washing, secondary antibodies

consisting of goat anti-mouse antibodies conjugated to HRP in TBST with 1%BSA was incubated for 60 minutes then washed again. Blots were then reacted with ECL solution (Amersham Pharmacia Biotech, Baie d'Ufre, PQ, Canada), exposed onto X-Omat film and developed using GBX developer.

Results

Gelsolin is present at ectoplasmic specializations throughout spermiogenesis

The immunofluorescent localization of two antibodies raised against gelsolin from two different suppliers (Sigma and Transduction Labs) showed identical staining at areas consistent with the location both of apical and of basal ectoplasmic specializations in rat testis material (Figure 4.1 A''-E''). Apical and basal junction staining co-localized with actin, but was notably decreased, as was actin, during stages of ectoplasmic specialization disassembly. The gelsolin antibodies also stained filamentous actin containing fingerlike-projections called tubulobulbar complexes (Figure 4.1 E'' arrows). These complexes arise just before spermatid release and are composed of spermatid projections into corresponding Sertoli cell invaginations, which are surrounded by filamentous actin (Vogl et al., 1985). Interestingly, labeling was not evident at intercellular bridges located between adjacent elongate spermatids, even though filamentous actin was present at these sites. (data not shown).

Specific staining was not present in primary antibody, secondary antibody, or autofluorescent controls (Figure 4.1F). Some staining was evident in the tubule wall, due to non-specific reactivity with the secondary antibody.

Problems arising from testis western blots using these gelsolin antibodies have been previously described and results using the Sigma antibody presented (Guttman et al., 2002). Using rabbit testis homogenate and purified bovine plasma gelsolin for western blotting and probing the blots with the Transduction Labs gelsolin antibody, a

clear single band at about 93kD appeared on the purified gelsolin lane and as predicted one a little further down (at about 90kD) was evident in the rabbit testis homogenate lane (Figure 4.2 E). These bands did not appear on control blots (Figure 4.2E lanes 3 and 4)

Gelsolin staining at ectoplasmic specializations using rabbit testis tissue

Because both antibodies reacted on blots of rabbit material, all immuno experiments initially performed using rat tissue were replicated with rabbit material. The staining patterns in rabbit were similar to those in rat. That is, gelsolin staining co-localized with actin at areas consistent with the location of both apical and basal ectoplasmic specializations (Figure 4.2 A-D''').

Rac1 is present at ectoplasmic specializations

Although more diffuse in nature, rac1 specific staining appeared at locations consistent with both apical and basal ectoplasmic specializations (Figure 4.3A'-E'), as indicated by phalloidin labeling (Figure 4.3A''-E'''). This localization was confirmed by immunoelectron microscopy, where labeling was detected in the actin zone of ectoplasmic specializations (Figure 4.4A). Interestingly, rac1 staining appeared to remain in regions previously occupied by actin filaments of ectoplasmic specializations during sperm release (Figure 4.3F-F''').

Controls for antibody labelling, for both immunofluorescence and immunelectron microscopy, included replacing the primary antibody with identical concentrations of normal mouse IgG (NMIgG), or buffer alone, and replacing both the primary and

secondary antibodies with buffer alone (Figure 4.4A and 4.4C). All resulted in an overall lack (immunofluorescence) or reduction (in normal mouse IgG electron microscopy) of labeling (Figure 4.4A). Western blots of rat testis homogenate and rat seminiferous epithelium showed that the antibody reacted monospecifically with a single band at the appropriate molecular weight of about 20kD (Figure 4.4B lanes 1 and 2 respectively) and did not react with normal mouse IgG (Figure 4.4B lanes 3 and 4).

Discussion

In this study, evidence is provided that gelsoin and rac1 are present in ectoplasmic specializations at all stages of spermatogenesis.

Gelsolin, an actin capping and severing protein, depolymerizes actin filaments in the presence of μM levels of calcium. The finding that gelsolin co-localizes with filamentous actin at basal and apical ectoplasmic specializations supports our previous hypothesis that gelsolin is a candidate protein involved with the disassembly of the actin component of the junction plaque. Recent evidence demonstrated that PIP_2 and PLC γ are associated with the junction plaque and that PLC γ is found more intensely in apical regions during sperm release (Guttman et al., 2002) supports our disassembly model. Phospholipase C γ (PLC γ) is a protein capable of releasing gelsolin from PIP_2 in the plasma membrane by converting PIP_2 to inositol triphosphate (IP_3). This inositol triphosphate could act on inositol triphosphate receptors on the junctional endoplasmic reticulum to release calcium, thus triggering the released (formerly membrane-bound) gelsolin to disassemble the actin filaments of the plaque. Although gelsolin null mice from a pure strain background die before birth, those from a mixed strain background are viable and reproduce normally (Witke et al., 1995). One interpretation of the latter result is that an alternative or redundant mechanism of plaque disassembly may be present to compensate for the loss of gelsolin in the mixed strain mice.

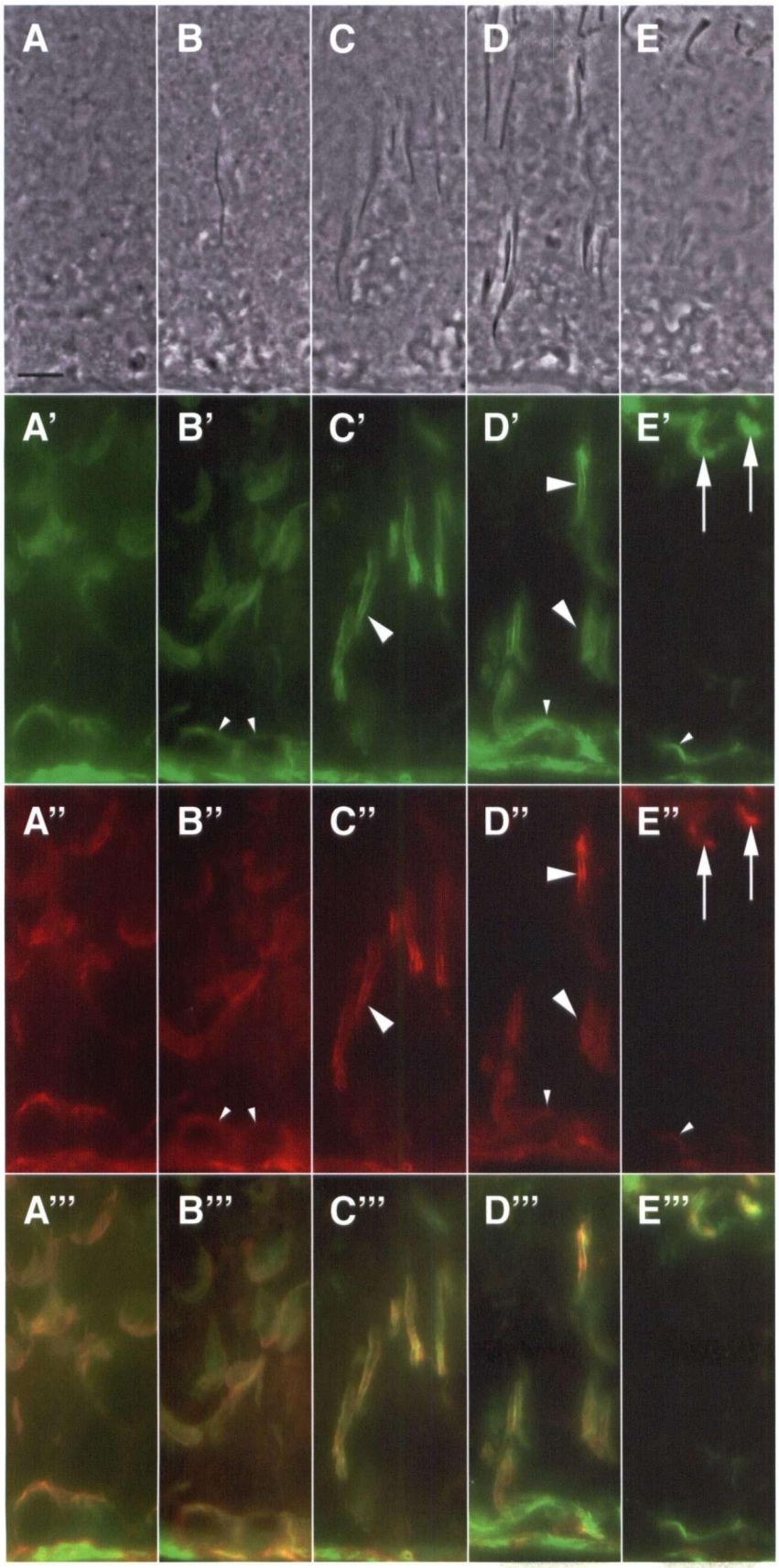
The presence of gelsolin at all stages of spermiogenesis suggests that in addition to its actin severing function, gelsolin also may play additional roles in controlling

filament number and length by its ability to cap and uncap barbed ends thereby regulating assembly/disassembly kinetics at this site. It is known that the pattern of actin filaments in the junction plaques change during spermatogenesis (Vogl et al 1985; Vogl et al., 2000).

The presence of rac1 at ectoplasmic specializations is consistent with reports in other systems that GTPases may be involved with regulating gelsolin/phosphoinositide pathways. Apart from rac's numerous functions generally in cells (Fukata and Kaibuchi, 2001; Dickson 2001; Wittmann and Waterman-Storer, 2001; Kaverina et al., 2002), it is thought to be an up-stream regulator of gelsolin possibly by recruiting the PIP_2 synthesis enzyme PI(4)P-5 kinase (Azuma et al.,1998). This would stabilize the plaque. Alternatively, rac1's role at the junction plaque could be linked to it's ability to dissociate gelsolin from actin filaments (Arcaro et al., 1998). Activated rac would dissociate gelsolin from the actin filaments leaving exposed barbed ends, allowing for the filaments to assemble or disassemble. Therefore, it is possible that rac1 presence at the junction plaque could do at least two things; 1) it may recruit PIP_2 synthetic machinery to ensure that gelsolin can remain bound to the Sertoli cell plasma membrane and 2) it may regulate the on/off rate of any gelsolin that may be bound to the actin filament barbed ends. At stages VII to VIII rac1 could be inhibited at the junction plaque thereby decreasing the amount of PI(4)P-5 kinase recruited to the plaque and therefore PIP_2 at the plaque. At this point PLCγ could hydrolyze the PIP_2 bound gelsolin, releasing gelsolin and triggering the actin plaque disassembly cascade.

Our results indicate that gelsolin and rac1 are present at ectoplasmic specializations throughout spermiogenesis and are strong candidates for a role in actin plaque dynamics during spermatogenesis.

Figure 4.1. Stage progression of gelsolin and actin in the rat seminiferous epithelium and controls. Actin (green A'-E') and gelsolin (Transduction Laboratories) (red, A''-E'') dual labeled sections of rat seminiferous epithelium. Co-localization of both signals is apparent during all stages of apical ectoplasmic specialization (large arrow heads) and basal ectoplasmic specialization (small arrow heads) presence (A'''-E'''). The location of tubulobulbar complexes are shown in panel E' and E'' (arrows). (F) Shows normal mouse IgG (NMIgG), secondary antibody and autofluorescent controls. Bar in A and F = 10μm.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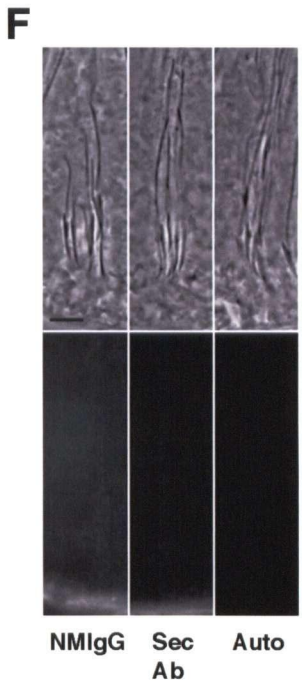
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Figure 4.2. Stage progression of gelsolin and actin in the rabbit seminiferous epithelium (A-D'''). Actin (green, A'-D') and gelsolin (red, A''-D'') dual labeled sections of rabbit seminiferous epithelium. Co-localization of both signals is apparent during all stages of apical ectoplasmic specialization (large arrow heads) and basal ectoplasmic specialization (small arrow heads) presence (A'-D'''). (E) Gelsolin western blot of purified bovine plasma gelsolin (lane 1 and 3) and rabbit testis homogenate (lane 2 and 4). Bovine plasma gelsolin and rabbit testis were reacted with the anti-gelsolin monoclonal antibodies (Sigma supplied antibody presented here). A single band at approx 93kd, the expected molecular weight for plasma gelsolin (lane 1), was apparent, as well as an expectedely slightly lower molecular weight band at about 90kD in the rabbit protein lane (lane 2). When control normal mouse IgG blots were run, no specific bands appeared in the plasma gelsolin lane (lane 3) or rabbit testis lane (lane 4) although a few non-specific bands were present at molecular weights different than those for gelsolin. Straight bars indicate the top and bottom of the blot. Bar in A = 10μm.

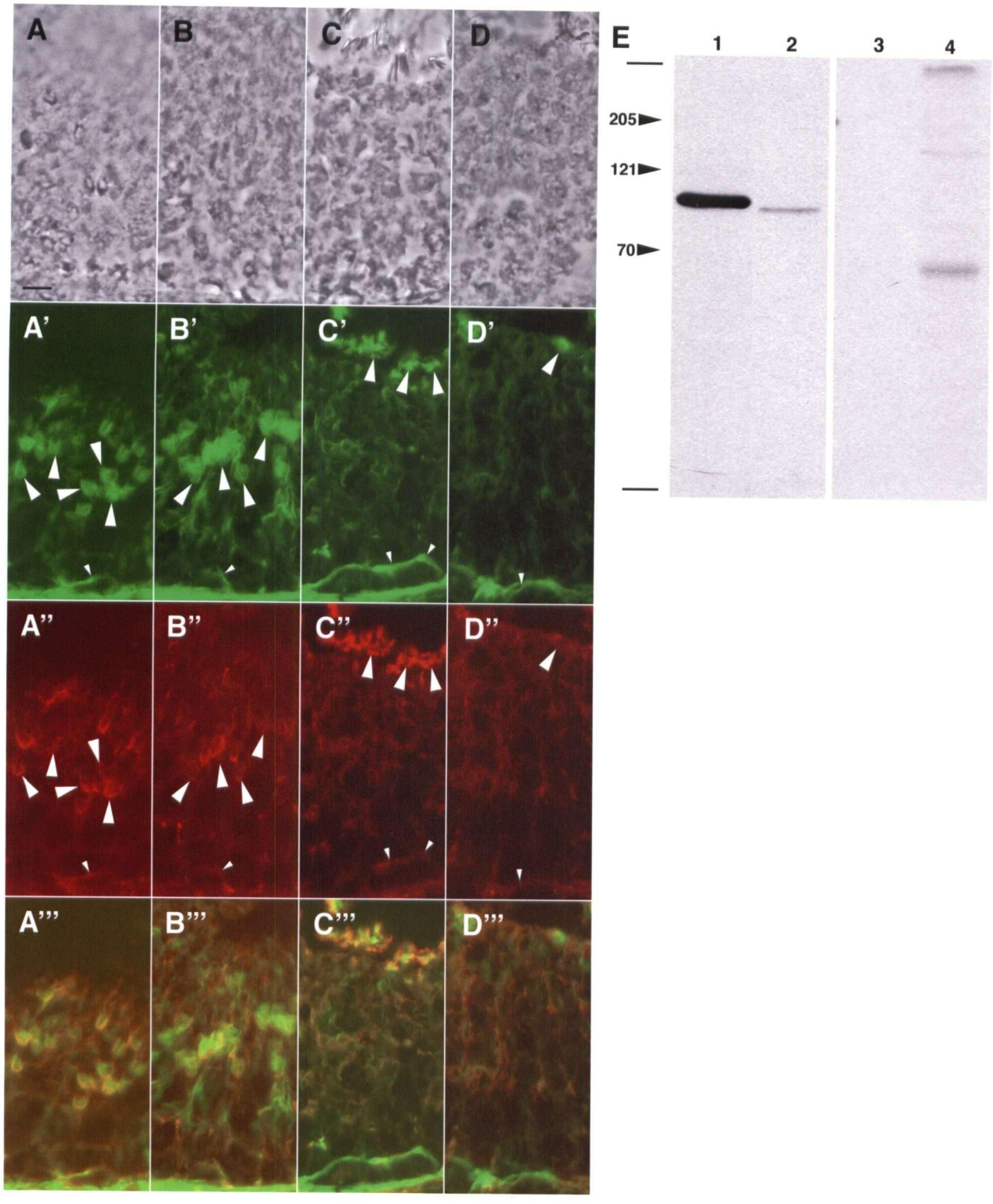
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Figure 4.3. Stage progression of rac1 and actin in the rat seminiferous epithelium (A-F'''). Rac1 (red, A'-F') and actin (green, A''-F'') dual labeled sections of rat seminiferous epithelium. Co-localization of both signals is apparent during all stages of apical ectoplasmic specialization (large arrow heads) and basal ectoplasmic specialization (small arrow heads) presence (A'''-E'''). Upon apical ectoplasmic specialization disappearance (F'', arrowhead) rac1 remained within the seminiferous epithelium within the concaved area of the spermatid heads (F', arrowhead). F''' shows the merged filamentous actin and rac1 micrographs of F' and F''. Bar = 10μm.

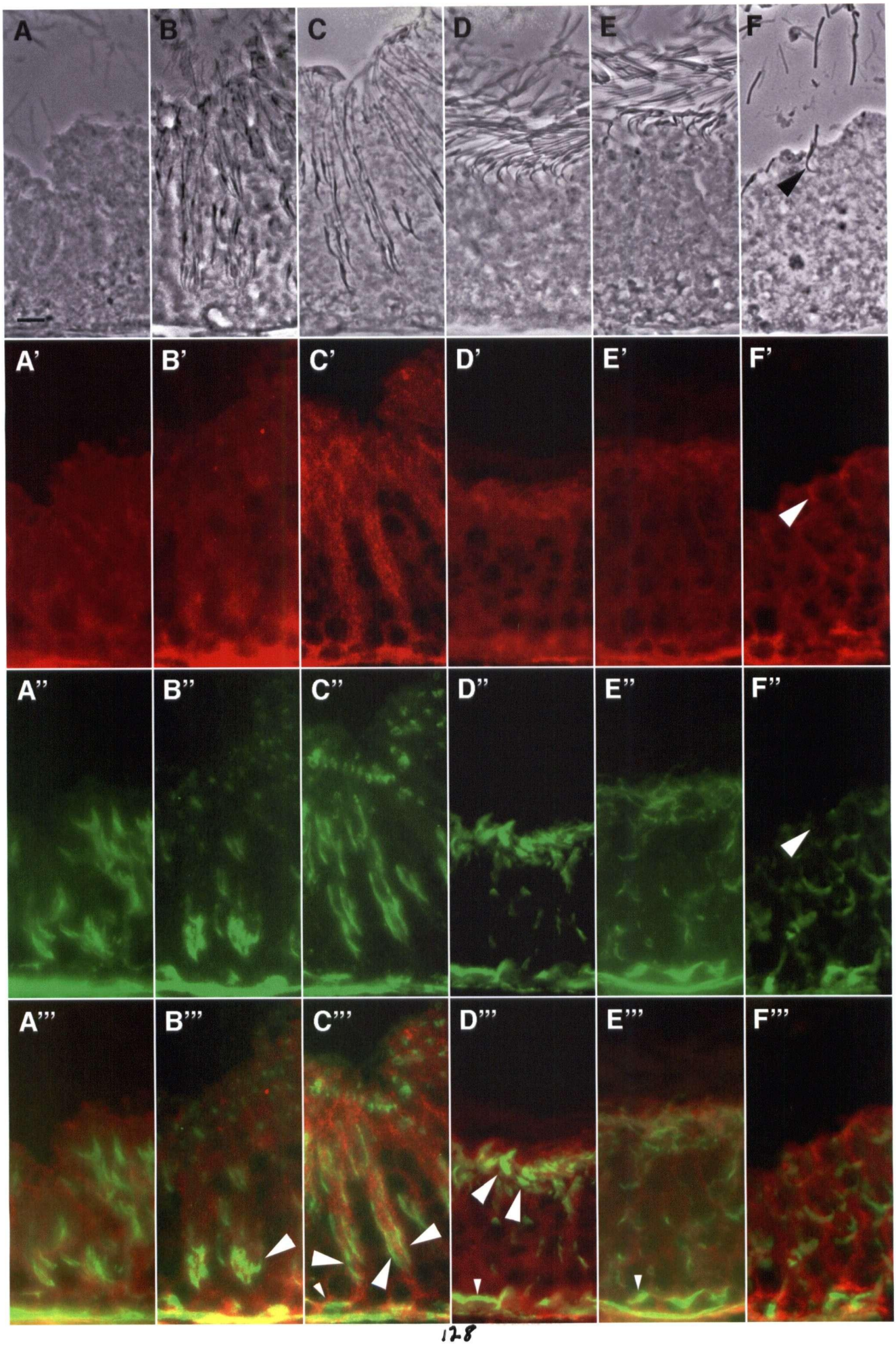
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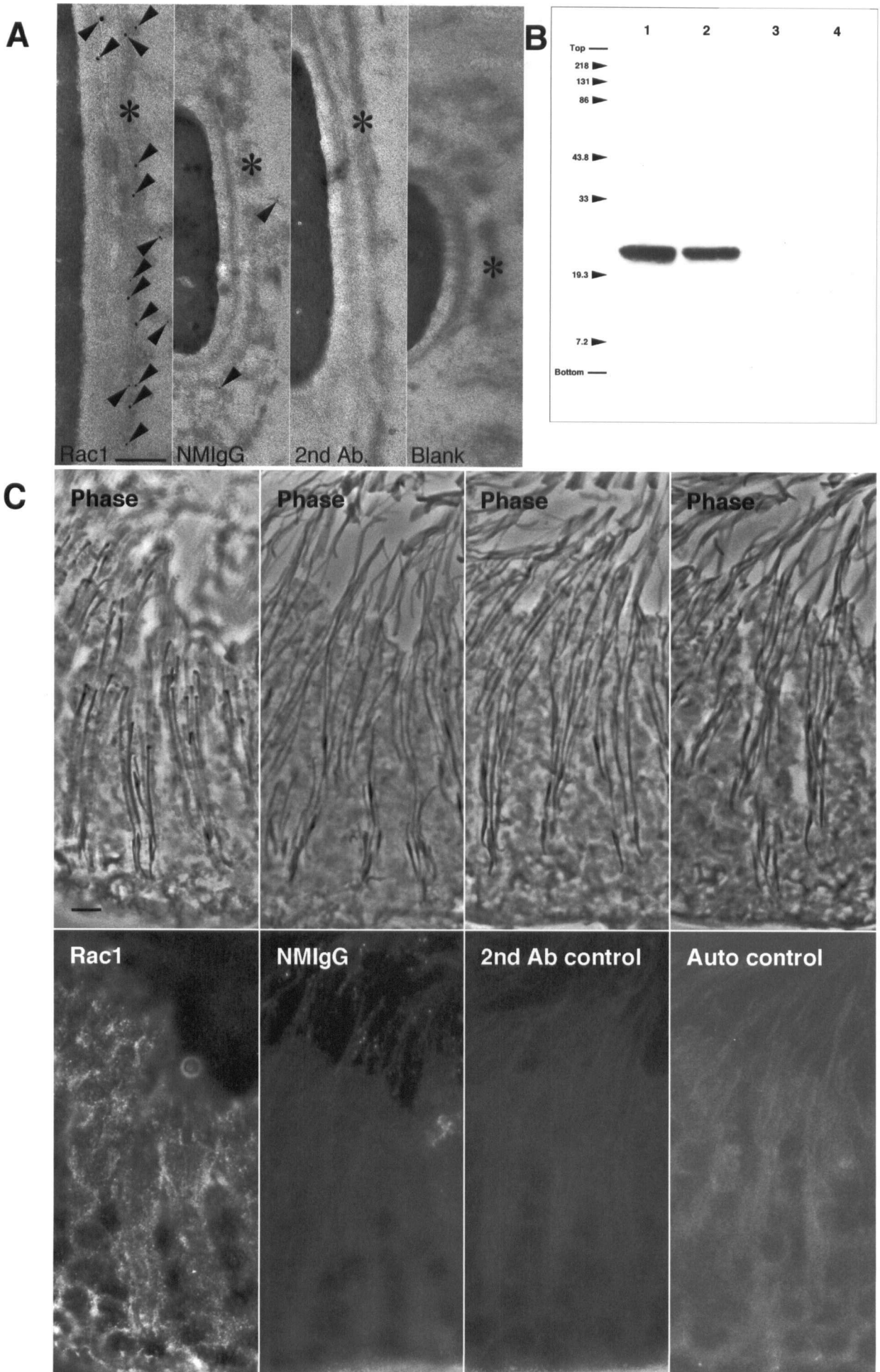
Figure 4.4. Rac1 immuno electron microscopic analysis at ectoplasmic specializations, rac1 western blots and rac1 immunofluorescence controls. A) Rac1 immunoelectron microscopic labeling (arrowheads) appeared at ectoplasmic specializations primarily within the actin layer of the plaque. Few gold particles appeared in the normal mouse IgG controls and all gold particles were absent in secondays and blank controls. The * represents the actin layer of the ectoplasmic specialization. B) Rac1 western blot of rat testis and rat seminiferous epithlium. Rat testis (1) and rat seminiferous epithelium (2) were reacted with the anti-rac1 monoclonal antibody. A single band at approx 20kd, the expected molecular weight, was apparent. Straight bars indicate the top and bottom of the blot. Primary antibody (NMIgG) controls showed no specific reactivity with rat testis (3) or rat seminiferous epithelium (4). C) Rac1 immunofluorescent control images. Rac1 labeling appeared as expected in rat seminiferous epithelium and was notably absent in all controls including the primary antibody controls (NMIgG), secondary antibody controls, and autofluorescent controls. Bar in A = 250nm, bar in C = 10μm.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Phase
Rac1
NMIgG
2nd Ab control
Auto control

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CHAPTER 5

Internalization of Adhesion Junction Components: A Possible Role for Tubulobulbar Complexes in the Testis

Introduction

Turnover of actin-related intercellular adhesion junctions in the testis are associated with two events fundamental to male fertility, sperm release and the movement of spermatocytes from basal to adluminal compartments of the seminiferous epithelium. During disassembly of adhesion junctions known as ectoplasmic specializations, finger-like structures termed tubulobulbar complexes form (Figure 5.1). These tubulobulbar complexes in the rat are usually 3-5μm in length and consist of projections from the developing spermatid heads (tubulobulbar processes) that extend into corresponding invaginations in the Sertoli cells (Russell and Clermont, 1976; Russell and Malone, 1980). Each tubulobulbar complex, which consists of both spermatid and Sertoli cell components, is composed of three structural segments; a large proximal tube, a bulbous region at the distal end of the proximal tube and another small distal tube region (distal to the bulbous region) (Russel 1979a) (Figure 5.2). Tubulobulbar complexes occur in two regions of Sertoli cells; 1) where two Sertoli cells come in contact with one another at the base of the seminiferous epithelium and 2) where the Sertoli cell interacts with the head of the maturing spermatid, in more apical regions of the epithelium. Tubulobulbar complexes at both sites form in regions previously occupied by ectoplasmic specializations.

A version of this chapter has been submitted for publication. Guttman, JA., Takai, Y., and Vogl, AW (2003) Evidence that Tubulobulbar Complexes in the Seminiferous Epithelium are Involved with Internalization of Adherens Junctions. *(submitted to Biol. Repro.)*

Ectoplasmic specializations are unique junction plaques that have a tripartite structure. They are composed of the Sertoli cell plasma membrane, a layer of hexagonally packed actin filaments and a cisternae of endoplasmic reticulum. Molecular components that are found within the actin layer of ectoplasmic specializations include: the actin bundling protein, espin (Bartles et al.,1996), the actin severing and capping protein, gelsolin (Guttman et al., 2002b), the actin dependent motor protein, myosin VIIa (structural,non-motile) (Hasson et al., 1997a; Velichova et al., 2002), the actin binding proteins kelch/keap1 (Velichova et al., 2002) and vinculin (Grove and Vogl., 1989).

Intercellular adhesion elements found at ectoplasmic specializations include the $\alpha 6\beta 1$ integrin (Palombi et al., 1992; Salanova et al.,1998), and the recently localized nectin family protein, nectin-2 (Ozaki-Kuroda et al., 2002). Nectin-2's binding partner in the germ cell plasma membrane is nectin-3 (Ozaki-Kuroda et al., 2002). The nectins are a family of calcium independent immunoglobulin-like adhesion elements which interact with the actin cytoskeleton through a linking protein, afadin, a protein also found at ectoplasmic specializations (Ozaki-Kuroda et al., 2002).

Tubulobulbar complexes contain two major structural components that are also found at ectoplasmic specializations; actin filaments, found surrounding the proximal tubular segment of the tubulobulbar complex and elements of the endoplasmic reticulum surrounding the bulbous regions. In addition, at the end of the distal tubular segment is a double membrane coated pit (Russell and Malone 1980). Also within the Sertoli cell, are numerous vesicles some which are double membrane bound (Figure 5.1, 5.2 and Russell

and Malone 1980). The coated pit is the first part of the complex to form (Russell 1979a). Phagocytotic vacuoles and a population of lysosomes are found within the Sertoli cell, adjacent to the tubulobulbar complexes (Russell 1979b).

Although numerous labs are currently investigating the regulators of cadherin-based adherens junction disassembly, only recently has the endocytosis of adherens junction proteins begun to be examined. Thus far both E-cadherin and β1 intergin have been studied in cell culture, and both have demonstrated the ability to be endocytosed and recycled through PKC and PKCα based pathways (Le et al.,1999; Ng et al., 1999; Le et al., 2002). Apart from actin, molecular components of tubulobulbar complexes have not been defined.

Tubulobulbar complexes associated with spermatids have been proposed to have three major functions: (1) to act as attachment devices between spermatids and Sertoli cells (Russell, 1979a and b; Russell and Malone 1980), (2) to remove excess spermatid cytoplasm and acrosomal contents prior to spermatid release (Tanii et al., 1999; Russell and Malone 1980`), and (3) to internalize and degrade junction components as part of the disassembly process (Russel, 1979b; Vogl, 1989).

If tubulobulbar complexes are involved with adhesion junction turnover and junction protein internalization, then (1) they should contain similar components to ectoplasmic specializations and, (2) adhesion molecules should be internalized through an endocytic pathway from tubulobulbar complexes.

In order to explore these predictions I probed testis fragments with antibodies to the ectoplasmic specialization adhesion associated proteins nectin-2, nectin-3 and the adaptor molecule afadin as well as the ectoplasmic specialization actin associated proteins espin, kelch/keap1, myosinVIIa, and gelsolin. As predicted, espin, kelch/keap1, myosinVIIa, and gelsolin were all found associated with tubulobulbar complexes, the area where filamentous actin is located. Interestingly, nectin 2,3 and afadin also were all found associated at tubulobulbar complexes. The nectins were clearly present at the most distal tubulobulbar structures, (the vessicular-like structures). Nectin-3, the nectin associated with the spermatogenic cells, was absent from both testis and epididymal spermatozoa. PKCα, LAMP1 [lysosome associated membrane protein 1 (a lysosomal marker)] and SGP1 [sulphated glycoprotein1 (a marker of secondary lysosomes in Sertoli cells)] also were found associated with tubulobulbar complexes and not with ectoplasmic specializations. These observations are consistent with the hypothesis that tubulobulbar complexes are associated with junction turnover and support the idea that tubulobulbar complexes are distinct structures involved in the internalization of ectoplasmic specialization components.

Materials and Methods

Animals

All animals used in these studies were reproductively active male Sprague Dawley rats. They were obtained from the University of British Columbia animal care colony and were maintained according to the guidelines established by the Canadian Council on Animal Care.

Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were obtained from Sigma-Aldrich Canada (Mississauga, Ontario). The paraformaldehyde and NaCl were obtained from Fisher Scientific (Vancouver, BC). All control immunoglobulins (IgG) as well as all secondary antibodies conjugated to Horseradish Peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pennsylvania). Secondary antibodies conjugated to ALEXA fluorochromes were all purchased from Molecular Probes (Eugene, OR).

Immunofluorescence

Tissue preparation

Testes were removed from male rats under deep anesthesia. Warm (33°C) PBS (150mM NaCl, 5mM KCl, 0.8mM KH_2PO_4, 3.2mM Na_2HPO_4, pH 7.3) was perfused through the spermatic artery using a 26G needle attached to a gravity fed perfusion apparatus for 2 minutes to clear the organ of blood. Following this, warm 3%

paraformaldehyde in PBS was perfused through the testis for 30 minutes. PBS was then re-perfused through the organ to wash out any remaining fixative.

Frozen sections

For frozen sections, the fixed testes were frozen (using liquid nitrogen) while at the same time being attached to an aluminum stub by OCT compound (Sakura Finetek USA, Torrance, Ca, USA). Frozen testis sections were cut, attached to poly-l-lysine coated glass slides, immediately plunged into –20°C acetone for 5 minutes allowed to air dry, then processed for immunofluorescence.

Fragmented material

For fragmented material, fixed testes were de-capsulated, cut into 1mm sized cubes and transferred into a 15ml plastic Falcon tube along with about 5mls of PBS. The material was gently passed through an 18, then 21gauge needle for 2-5 gentle passes. This fragmented material was left to sediment by gravity at room temperature for 10-15 minutes at which point the upper most layer was added to poly-l-lysine coated slides and allowed to incubate in a humidity chamber for 10 minutes. All excess PBS was then removed and immediately treated with –20°C acetone for 5 minutes and allowed to air dry at which point 5% blocking serum was added.

Antibody labelling and controls

Once the tissue was ready for serum blocking, 5% normal goat serum (NGS) in TPBS-BSA (PBS containing 0.05% Tween-20 and 0.1% Bovine Serum Albumin) from the secondary antibody host was incubated on the testis material for 20 minutes at room

temperature. This was used to block the tissue from secondary antibody non-specific binding. The primary antibodies consisted of rat anti-mouse nectin2 used at a dilution of 1:1 (gift from Dr Yoshimi Takai (#502-57 Takahashi et al., 1999)), rat anti-mouse nectin3 used at a dilution of 1:1 (gift from Dr Yoshimi Takai (#103-A1 Satoh-Hirokawa et al., 2000)), mouse anti-rat–l-afadin used at a dilution of 1:1 (gift from Dr Yoshimi Takai (#3 Sakisaka et al., 2001)), mouse anti-gelsolin used at 2.47μg/ml(Transduction Labs), mouse anti-PKCα used at 2.47μg/ml (Transduction Labs), rabbit anti-rat espin used at 10μg/ml (gift from Dr. Jim Bartles (Bartles et al., 1996)), Rabbit anti-human myosin VIIa used at 10μg/ml (gift from Dr. Tama Hasson (Hasson et al., 1995, 1997)), Rabbit anti-kelch/keap1 used at 10μg/ml (gift from Dr. Tama Hasson)(Velichkova et al., 2002)), rabbit anti-SGP1, used at a 1:100 dilution (gift from Carlos Morales) and mouse anti-LAMP1 used at 0.94-1.88μg/ml were added to the experimental slides made up in TPBS-BSA with 1% NGS and incubated overnight at 4°C in a humidity chamber. The material was washed extensively with the TPBS-BSA (wash buffer) then incubated for 60 minutes at 37°C with secondary antibody conjugated to a fluorochrome (goat anti-mouse ALEXA 488, goat anti-rabbit ALEXA 568 or goat anti-rat ALEXA 546). After further washing, coverslips were mounted using Vectashield (Vector Labs, Burlington, Ontario) and visualized by standard fluorescence microscopy using a Zeiss Axiophot microscope or by confocal microscopy using a Bio-Rad Radiance Plus attached to a Zeiss Axiovert microscope.

Controls consisted of replacing the primary antibody with it's respective immunoglobulin (IgG) or serum at identical concentrations to the primary antibody

(when the concentration was known or with identical dilutions of 1mg/ml stock IgG solutions when the antibody concentrations were unknown), replacing the primary antibody with buffer alone, or replacing both the primary and secondary antibodies with buffer alone.

Phalloidin/phallotoxins

Filamentous actin labeling was performed using ALEXA 488 or ALEXA 568 phalloidin (Molecular probes, Eugene Oregon).

1D Western blotting

Material was loaded into wells of 1mm thick 10% SDS-PAGE reducing gels and run according to standard protocols (Laemelli, 1970). Proteins were transferred onto Immobilon-P transfer membrane (Millipore) then washed for 5 minutes at room temperature with TBST (500mM Tris pH 7.5, 150mM NaCl, 0.1% Tween-20). The blots were then blocked in order to decrease non-specific antibody binding for 8 hours at 4°C using 4% non-fat milk (Blotto, Santa Cruz Biotechnology). Following blocking, membranes were washed 3 times, 10 minutes each then incubated with primary antibody overnight at 4°C. The following day, blots were washed extensively with TBST followed by a 1hour secondary antibody (conjugated to horseradish peroxidase) incubation at room temperature. Upon further washing with TBST followed by TBS, blots were reacted with ECL (Pharmacia) to visualize the reactive bands on X-OMAT film (Kodak).

Controls consisted of replacing the primary antibodies with IgG or serum at identical primary antibody concentrations or identical serum dilutions.

Electron Microscopy

Testes were removed and perfusion fixed for 30 minutes with 0.1M NaCacodylate pH 7.3; 1.5% paraformaldehyde; 1.5% Glutaraldehyde. The testis was then cut into small pieces and immersion fixed for an additional 90 minutes. The testis material was then washed with 0.1M NaCacodylate for 3 ten minute washes then further fixed on ice for 60 minutes in a 1:1 mixture of 3% ferrocyanide: 2% osmium fixative. Following the incubation the material was washed 3 times with ddH2O, ten minutes each wash, then stained for 1 hour with 0.1% uranyl acetate. The material was then washed another three times in ddH2O then dehydrated in an ascending alcohol series (50%, 70%, 95%, 100%), for 10 minutes each step). Propylene oxide was then infiltrated for two 15 minute rounds of infiltration then left in a 1:1 solution of propylene oxide: polybed overnight. The material was then embedded and incubated at 60°C for 24 hours.

Immunoelectron Microscopy

Immunoelectron microscopy was performed according to the techniques used in chapter 3.

Results

Tubulobulbar complexes form in areas previously occupied by ectoplasmic specializations

Tubulobulbar complexes are formed in regions previously occupied by ectoplasmic specializations. In order to further understand this relationship, immunofluorescent and electron microscopy was used to visualize tubulobulbar complexes in association with ectoplasmic specializations. Using DIC and phalloidin labelling, a progression of tubulobulbar complex maturation was assembled (Figure 5.3). Tubulobulbar complexes were easily identified by their finger-like structures. Ectoplasmic specializations, also visualized by phalloidin labeling, appeared throughout the progression of tubulobulbar complex formation. On electron micrographs, actin filaments were identified as an electron-dense region surrounding the proximal tube of the complex and the endoplasmic reticulum at tubulobulbar complexes was clearly visualized around the bulbous region (Figure 5.2A. Found distal to the bulbous region, clearly identifiable bristle coated pits were visualized (Figure 5.2C). A more detailed relationship between tubulobulbar complexes and ectoplasmic specializations was observed on the electron microscopy micrograph in Figure 5.3 where the tubulobulbar complex appears between two ectoplasmic specialization regions.

Tubulobulbar complexes contain molecules also found at ectoplasmic specializations

To see if proteins known to exist in the actin layer of ectoplasmic specializations also were present at tubulobulbar complexes, I immunolocalized the known actin related proteins; espin, kelch/keap1, and myosin VIIa on fixed fragmented rat testis material

(Figure 5.4). The fragmentation of the seminiferous epithelium resulted in elongate spermatids retaining their associated ectoplasmic specializations and tubulobulbar complexes, allowing for easy morphological and immunocytochemical identification. Apically located ectoplasmic specializations have a characteristic staining pattern, labelling the area surrounding the developing spermatid heads, and tubulobulbar complexes are identified by their unique "finger-like" staining projections (Figure 5.3). In all cases staining was found at areas known to contain tubulobulbar complexes, in the concave area of the spermatid head where tubulobulbar complexes occur in the rat (Figure 5.3, 5.4). The staining routinely also appeared at the late stage ectoplasmic specializations and electron microscopic images found both ectoplasmic specializations and tubulobulbar complexes present concurrently (Figure 5.3, 5.4). The calcium-dependent actin disassembly protein gelsolin, has been well documented as a component of ectoplasmic specializations and was found at the tubulobulbar complex area using standard fluorescence microscopy (Figure 5.4D, D') and was more clearly imaged through confocal microscopy (Figure 5.4E). Immunoelectron microscopy confirmed this staining and further found the labelling localized to the actin zone at tubulobulbar complexes (Figure 5.4F,G). β1 integrin, found on the Sertoli cell plasma membrane of ectoplasmic specializations, and vinculin, a protein known to occur within the actin zone of ectoplasmic specializations were identified from previously published articles at tubulobulbar complexes (Salanova et al.,1995 Figure 4C and 5D; Grove et al., 1990). β1 integrin staining is absent from released spermatozoa (Palombi et al., 1992).

Nectin-2 and 3 are present in distal regions of tubulobulbar complexes and in adjacent vesicles

I continued this investigation by looking for evidence of internalization of both spermatid and Sertoli cell adhesion elements. To do this I stained fixed fragmented mouse testis tissue with anti-nectin-2 and 3 antibodies. Mouse was used for these studies because the antibodies did not react with rat tissue. Upon labelling this fragmented material with the integral membrane adhesion proteins nectin2- and nectin-3, staining was found both at ectoplasmic specializations and tubulobulbar complex regions (Figure 5.5). This tubulobulbar complex labelling clearly appeared as finger-like staining and interestingly antibodies to both nectin-2 and 3 also appeared to label vesicular-like structures found distal to the projections (Figure 5.5A',B'). This staining notably also maintained it's localization at ectoplasmic specializations (Figure 5.5). It was also noted that when gelsolin was investigated at these vesicular-like structures, gold particles were absent (Figure 5.4G).

Afadin, the nectin and actin binding protein, had a similar yet distinct immunocytochemical localization. It too was found at ectoplasmic specializations as well as at tubulobulbar complexes (Figure 5.5C-E'). Interestingly, there was a distinct decrease in staining along the dorsal part of the ectoplasmic specializations, attached to the late step spermatids (Figure 5.5E-E'). When a step progression of afadin staining was investigated, staining along the dorsal part of the ectoplasmic specialization gradually appeared to decrease in intensity whereas an increase in staining intensity was observed

in the tubulobulbar complex area resulting in a finger-like staining pattern (Figure 5.5C-E').

Double membrane vesicles associated with tubulobulbar complexes

Electron microscopy was used to investigating whether double membrane bound vesicles were present in the tubulobulbar complex region (to account for both internalized Sertoli cell and spermatid membrane). Upon electron microscopic analysis, numerous of these structures were identified as well as large vesicular double membrane structures (Figure 5.6). Double membrane vesicles and multi vesicular bodies were also found within the stalk of Sertoli cells undergoing sperm release (Figure 5.7).

Lysosomal and endosomal markers are found in the vesicular region of tubulobulbar complexes

To study the possibility that an endocytic pathway may be involved with the internalization of tubulobulbar complex-associated proteins, I used antibodies to the endocytosis marker PKCα, the late endosome/lysosomal marker LAMP1 and the lysosomal marker (known to label Sertoli cell secondary lysosomes) SGP-1 for immunolocailzation. Here, labelling of all of the proteins was clearly found in the vicinity of tubulobulbar complexes (Figure 5.8). Staining was also present at the tail regions of the spermtids.

Nectin-3 is absent from released sperm cells

I also looked at the functional distribution of nectin-3 on the spermatogenic cells at steps of spermatid release. If tubulobulbar complexes are involved with the internalization of membrane associated adhesion elements, then nectin-3 (the nectin isoform associated with the spermatogenic cells which interacts with the nectin-2 of the Sertoli cell ectoplasmic specialization) should be present on spermatids and not on released spermatozoa. Nectin-3 labelling was detected only when the spermatids were associated with the Sertoli cells and was notably absent from both testicular and epididymal spermatozoa (Figure 5.9). As a confirmation of spermatid release, phalloidin was used to double label the testicular material (Figure 5.9A'', 5.9B''). Released sperm are those without associated ectoplasmic specialization actin.

Discussion

Given the great interest in intercellular adhesion in general and the fundamental importance of this adhesion in male fertility, it is somewhat surprising that tubulobulbar complexes have been largely ignored by researchers in male reproduction (Cheng and Mruk, 2002). Tubulobulbar complexes are essentially the last structures to link the maturing spermatids to the Sertoli cell prior to their release into the lumen of the seminiferous tubules. Hypotheses of their function include that they are a mechanism of cytoplasmic or acrosomal elimination from the spermatids (Russell, 1979a and b; Russell and Malone 1980; Tanii et al., 1999), that they serve as attachment devices and that they eliminate membrane domains involved with intercellular adhesion (Vogl, 1989). In this chapter I provide data consistent with the latter function.

One of the most important observations in this study is that the intercellular adhesion proteins found at ectoplasmic specializations (nectin-2 and 3) also are found along the tubulobulbar complex tubular segments and at vesicular-like structures, a location consistent with internalization of junction molecules, whereas the actin associated protein gelsolin is not found associated with vesicles. The nectin vesicular staining pattern suggests that the nectin proteins (and possibly other integral membrane adhesion elements) may be undergoing an internalization process in order to degrade or recycle the junction complexes. In addition to this, the significant finding that nectin-3 (the nectin found on the spermatid plasma membrane) is absent on spermatozoa, suggests that spermatid as well as ectoplasmic specialization components may be internalized by the Sertoli cell prior to spermatid release. Another interesting observation is the staining

pattern of the nectin scaffolding protein afadin. The staining along the dorsal part of the ectoplasmic specialization eventually becomes less intense or disappears completely in the areas of ectoplasmic specializations whereas the staining at tubulobulbar complexes intensifies prior to sperm release. It has been hypothesized that the cytoplasm within the developing spermatid may be transported from a dorsal region around the spermatid head and towards the tubulobulbar processes (Russell 1979b). If this is true then a similar transport event may be occurring with the junctional afadin staining. One would predict that if the afadin staining is transported in this manner, then nectin-3 and especially the nectin-2 (the one found on the Sertoli cell side of the ectoplasmic specialization) should behave similarily. This may be the case but unfortunately was not observed in our study because the anti-nectin-2 and 3 antibodies only reacted on mouse material, not rat, where the tubulobulbar complexes are visualized more easily.

The presence of PKCα in the region of tubulobulbar complexes also may prove to be a significant finding. In the model of ectoplasmic specialization actin plaque disassembly, presented elsewhere (Guttman et al., 2002b), PLCγ converts PIP_2 to inositol triphosphate (InsP3) and diacylglycerol (DAG). The hydrolysis of PIP_2 releases gelsolin from the Sertoli cell plasma membrane and the resulting InsP3 would trigger InsP3 receptors in the membrane of the endoplasmic reticulum of the ectoplasmic specialization. This would in turn activate the released gelsolin to disassemble the actin of the ectoplasmic specialization plaque. The DAG part of the pathway has not been addressed in previous works with regard to ectoplasmic specialization disassembly, but may play a role in the formation and function of tubulobulbar complexes. In general,

DAG participates in endocytotic events through its association with protein kinase C (PKC). There are numerous forms of PKC, most of which have been localized in the testis with varying degrees of certainty to specific structures (Um et al., 1995; Zini et al., 1997). In our study we have successfully localized PKCα to areas where other researchers have not, in regions known to contain tubulobulbar complexes. PKCα is the most widely expressed PKC isoform and is calcium dependent. The fact that a cisternae of endoplasmic reticulum is also found at more distal tubulobulbar complex areas provides a potential calcium store, although its function at this point has not been proven.

Once internalized, the membrane bound vesicles may be destined for either degradation or recycling. Based on the layout of the system it is convenient to hypothesize that ectoplasmic specialization-associated proteins may be recycled to the next generation of ectoplasmic specializations juxtaposed to the newly polarized round spermatids (Figure 5.1, 5.10). Whereas the spermatogenic cell associated proteins, such as nectin-3, are degraded by the Sertoli cell (Figure 5.1, 5.10).

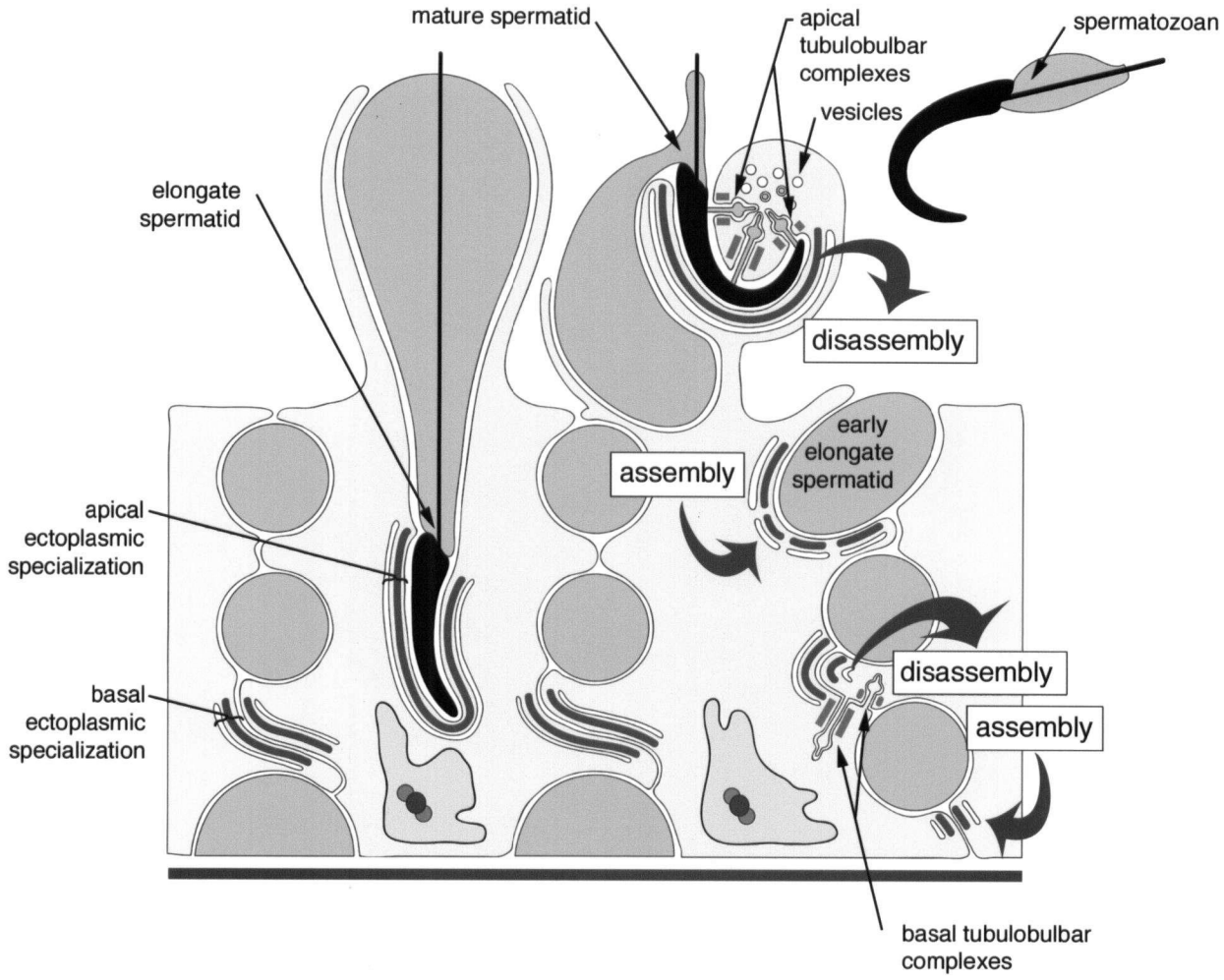
One of the major components of ectoplasmic specializations are actin filaments. They are found packed in paracrystalline arrays, which is in stark contrast to the filamentous actin arrangement at tubulobulbar complexes. At tubulobulbar complexes, actin filaments appear as a filament network. This filament difference may be the result of morphological changes to the areas surrounding the spermatid heads or may be the result of gain or loss of certain proteins at these newly formed structures. Because spermatid release from the seminiferous epithelium and thus Sertoli cells is such an

important event, the rearrangement and disassembly of actin filaments around the complexes may prove to be important.

The interesting finding of a lack of gelsolin labelling in vesicles in the tubulobulbar complex area suggests that at tubulobulbar complexes, gelsolin is not associated with the Sertoli cell plasma membrane, but rather the actin filaments. This is significant since gelsolin, a major component of ectoplasmic specializations, has been shown to be associated with membranes when bound to PIP_2 and in our system has been demonstrated to be released by peptides consisting of the gelsolin binding region of PIP_2. Therefore at tubulobulbar complexes gelsolin may provide a capping function on the associated actin filaments rather than being bound to the plasma membrane through PIP_2.

The presence of both membrane- and actin-associated junction components as well as endocytic pathway components distal to these structures supports the hypothesis that tubulobulbar complexes are unique structures involved in internalizing ectoplasmic specialization components.

Figure 5.1. Diagrammatic representation of various stages of the seminiferous epithelium. The diagram shows the location of both the basal and apical ectoplasmic specializations as well as the tubulobulbar complexes that arise both basally and apically in the seminiferous epithelium. Note the presence of vesicles at regions near the apically located tubulobulbar complexes, this does not exclude their presence at the basal tubulobulbar complex sites. Assembly and disassembly of ectoplasmic specializations occur at both the apical and basal regions of the seminiferous epithelium.


mature spermatid
apical
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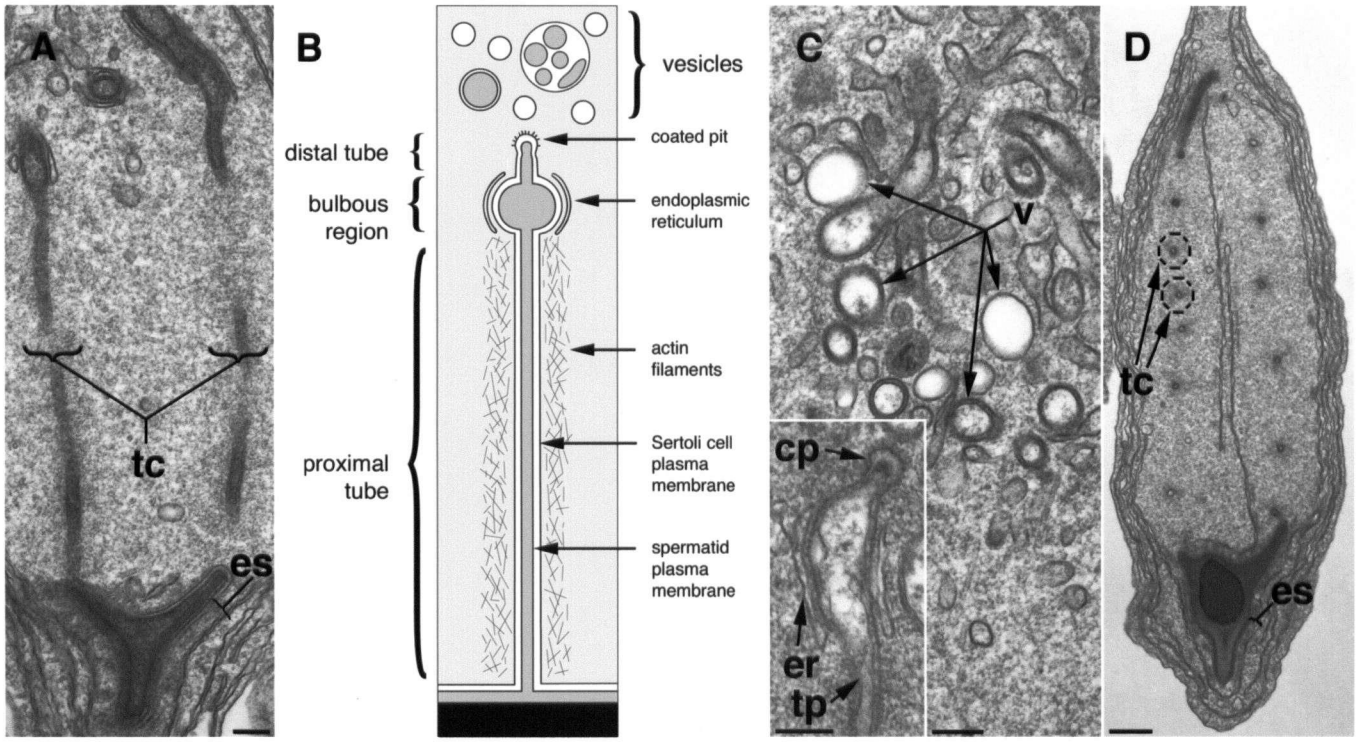
ectoplasmic
specialization
disassembly
basal
ectoplasmic
specialization
assembly
basal tubulobulbar
complexes

Figure 5.2. Electron microscopy and diagram of tubulobulbar complexes. (A) Longitudinal section through a spermatid with both ectoplasmic specializations (es) and tubulobulbar complexes (tc), Bar = 200nm. (B) Diagram of the structure of a single tubulobulbar complex and its associated vesicles distal to the complex. Note that actin filaments surround only the proximal tube and endoplasmic reticulum surrounds only the bulbous region. (C) Electron micrographs of vesicles found in the region of tubulobulbar complexes and an example of a coated pit (cp) distal to the bulbous region of the complex surrounded by endoplasmic reticulum (er). The spermatid tubulobulbar process (tp) is also labeled, Bar = 200nm, Bar in inset = 100nm. (D) Tubulobulbar complexes viewed in cross section with associated ectoplasmic specialization. Dashed line marked by (tc) demarcates the actin surrounding the proximal tube of the complex. The center of the complex is the tubulobulbar process of the spermatid. (es) marks the ectoplasmic specialization, Bar = 500nm.


A
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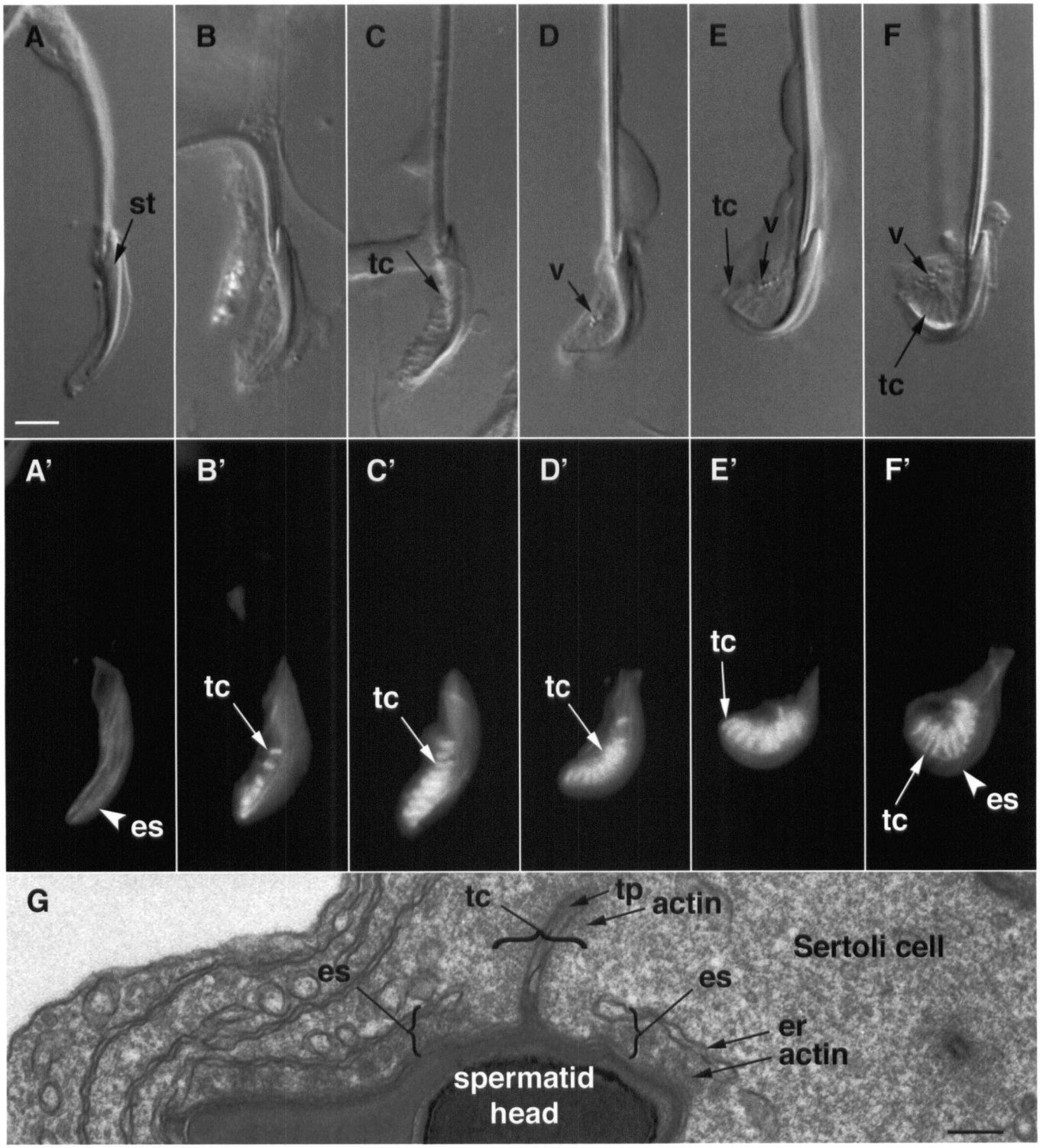
es
B
vesicles
distal tube
coated pit
bulbous region
endoplasmic reticulum
actin filaments
proximal tube
Sertoli cell plasma membrane
spermatid plasma membrane
C
v
cp
er
tp
D
tc
es

Figure 5.3. Tubulobulbar complex association with ectoplasmic specializations. (A-F') A paired DIC and phalloidin stained stage progression of tubulobulbar complex formation. Bar = 5μm. Ectoplasmic specialization (es) and tubulobulbar complexes (tc) are marked as are the actin, endoplasmic reticulum (er) and tubulobulbar process (tp) in (G). Note that the tubulobulbar complexes (B-F) and vesicles (D-F) are able to be seen by DIC imaging and the tubulobulbar complex is flanked on either side by an ectoplasmic specialization in (G), Bar = 200nm.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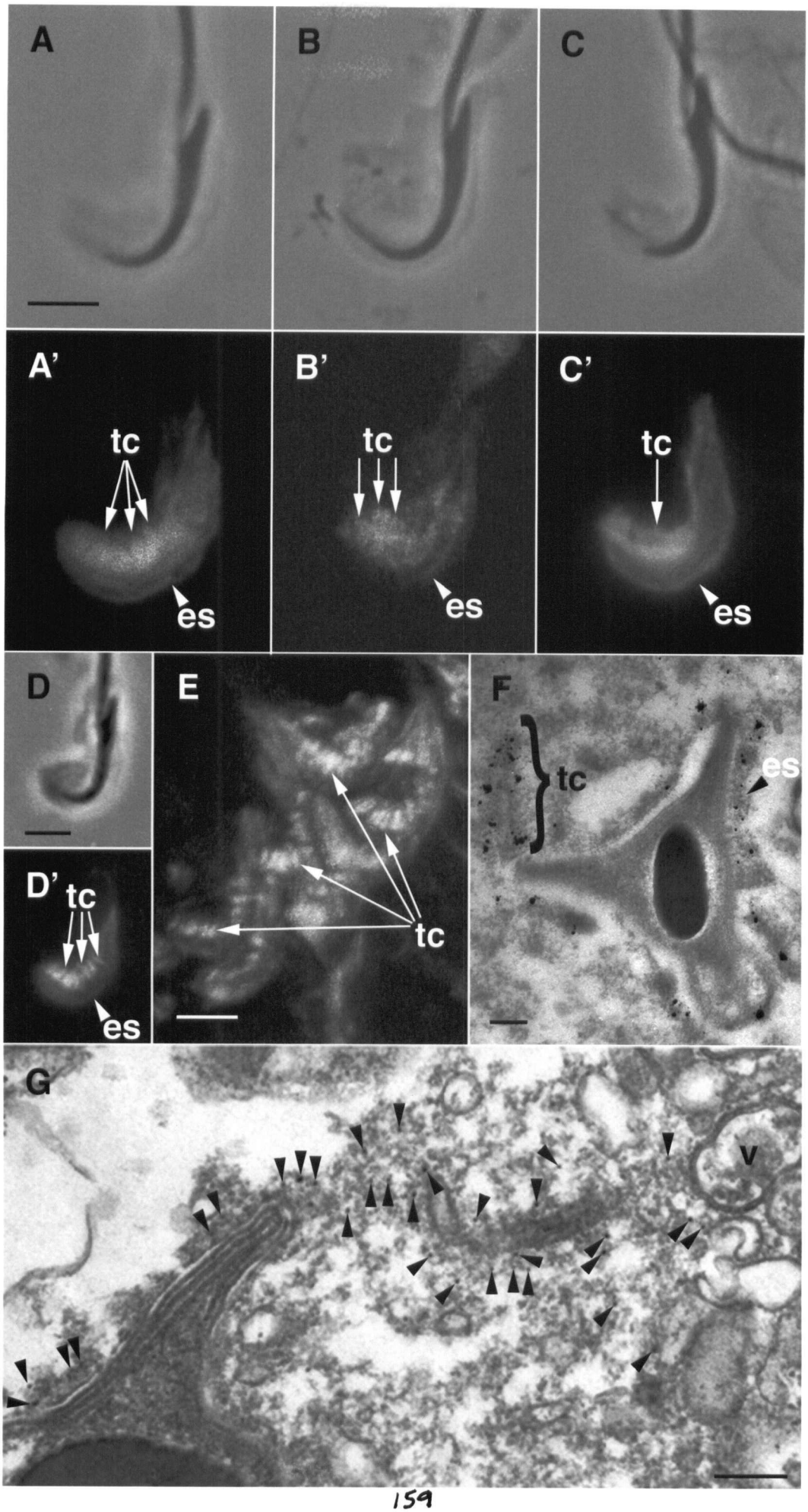
es
G
tc
tp
actin
Sertoli cell
es
es
er
actin
spermatid
head

Figure 5.4. Actin associated ectoplasmic specialization components found at tubulobulbar complexes. (A-D') Paired phase and fluorescent micrographs of spermatid/tubulobulbar complexes labeled with antibodies to espin (A'), kelch/keap1 (B'), myosin VIIa (C'), and gelsolin (D') Bar in A and D = 5μm. (E) is a confocal slice of gelsolin stained material showing it's labelling at tubulobulbar complexes (tc), Bar = 5μm. (F) Immunoelectron micrograph of gelsolin. Labelling is found at both tubulobulbar complexes (tc) and at ectoplasmic specializations (es). (G) Immunoelectron micrograph of gelsolin probed testis material. Arrowheads point to silver clusters labelling the tubulobulbar complex. Note the vesicle (V) does not have any labelling associated with it, Bar = 200nm.


A
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C
A'
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es
B'
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es
C'
tc
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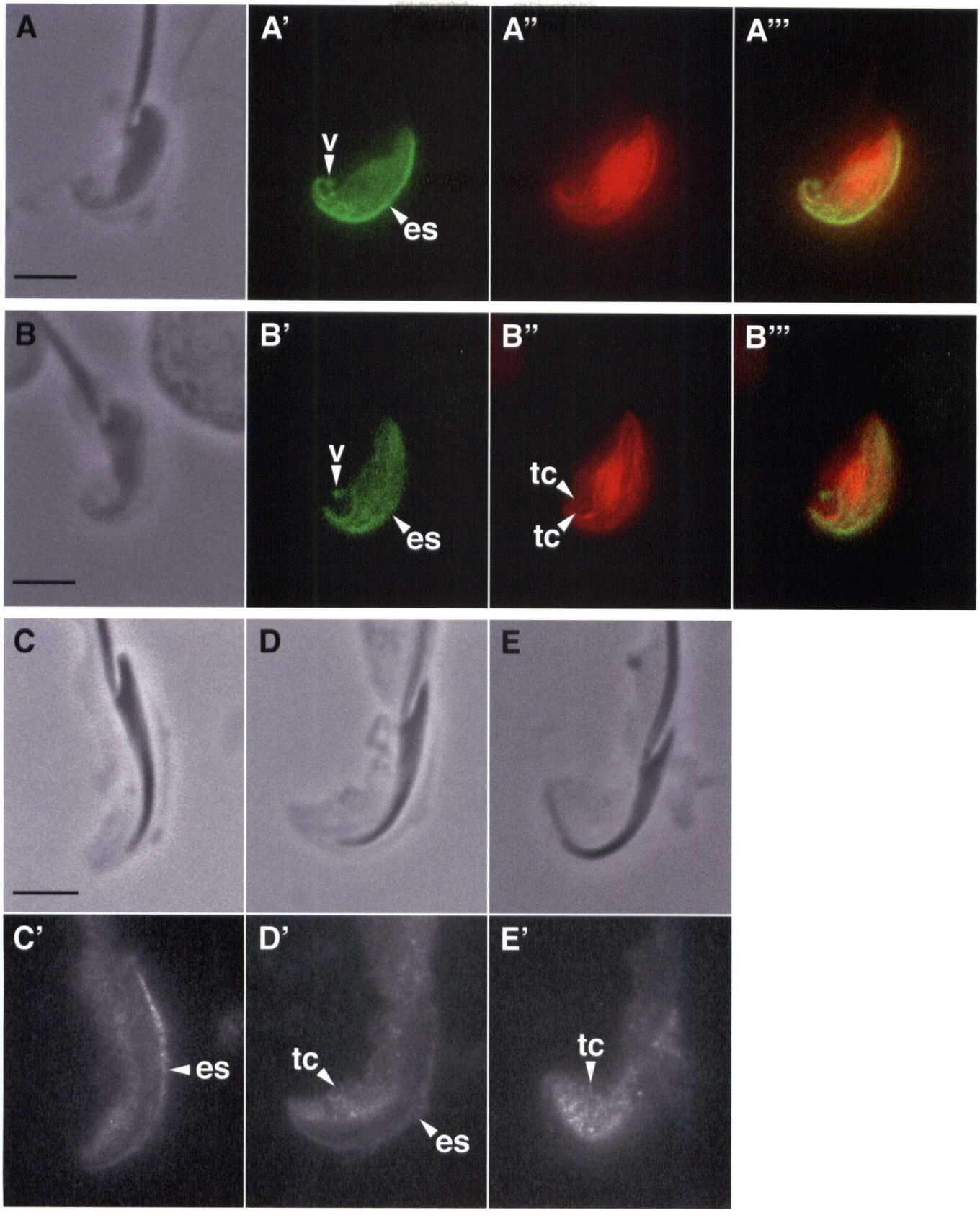
D
D'
tc
es
E
tc
F
tc
es
G
v

Figure 5.5. Intercellular adhesion elements are found at ectoplasmic specializations, tubulobulbar complex and vesicular regions. (A-A''') Grouped phase (A), nectin-2 (A'), filamentous actin (A'') and merged images of mouse spermatids with associated tubulobulbar complexes (A'''), Bar = 5μm. (B-B''') Grouped phase (B), nectin-3 (B'), filamentous actin (B'') and merged images of mouse spermatids with associated tubulobulbar complexes (B'''), Bar = 5μm. Note in both A' and B' the presence of a vesicular staining pattern (v) and labelling at ectoplasmic specializations (es). Filamentous actin staining identifies ectoplasmic specialization presence and the tubulobulbar complex (tc) location. (C-E') A stage progression of paired phase and fluorescent images of anti-afadin labeled rat spermatids with associated tubulobulbar complexes. Prior to tubulobulbar complex formation afadin is present at ectoplasmic specializations (C') during tubulobulbar complex formation, afadin's presence at tubulobulbar complexes increases and fluorescent labeling appears to decrease (D') until it is barely detectable at ectoplasmic specializations and is strongly labeled at mature tubulobulbar complexes (E').


A
A'
v
es
A''
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es
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D
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C'
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D'
tc
es
E'
tc

Figure 5.6. Vesicles found in the tubulobulbar complex region. Electron micrographs showing single membrane, double membranes (Arrowheads) and large-vesicular structures (*). Arrows point to large vesicular structures containing areas of double membranes. (}) Spans a tubulobulbar complex. Bar in A = 200nm, Bar in B = 250nm.

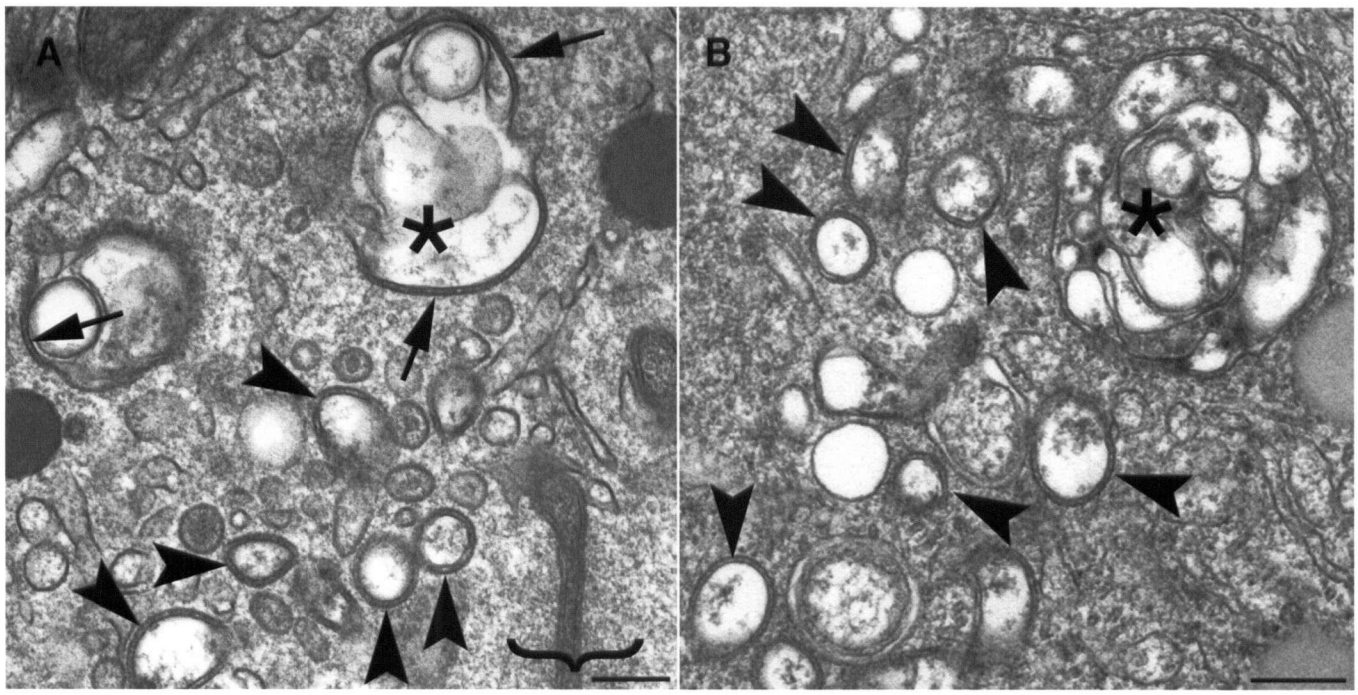

A
B

Figure 5.7. Electron micrographs of a section of late stage seminiferous epithelium. (A) Electron micrograph of a late stage (VIII) spermatid at sperm release. Note the absence of tubulobulbar complexes and ectoplasmic specializations. Bar = 250nm (B) Apical stalk supporting the spermatid in (A) with structures containing structures resembling multi-vesicular bodies (mvb). Bar = 200nm. (C) Double membrane vesicle (arrowhead) in the apical stalk of the Sertoli cell of another section of late stage epithelium. (rl) refers to the residual lobe of spermatid cytoplasm being phagocytosed by the Sertoli cells. Bar = 200nm.

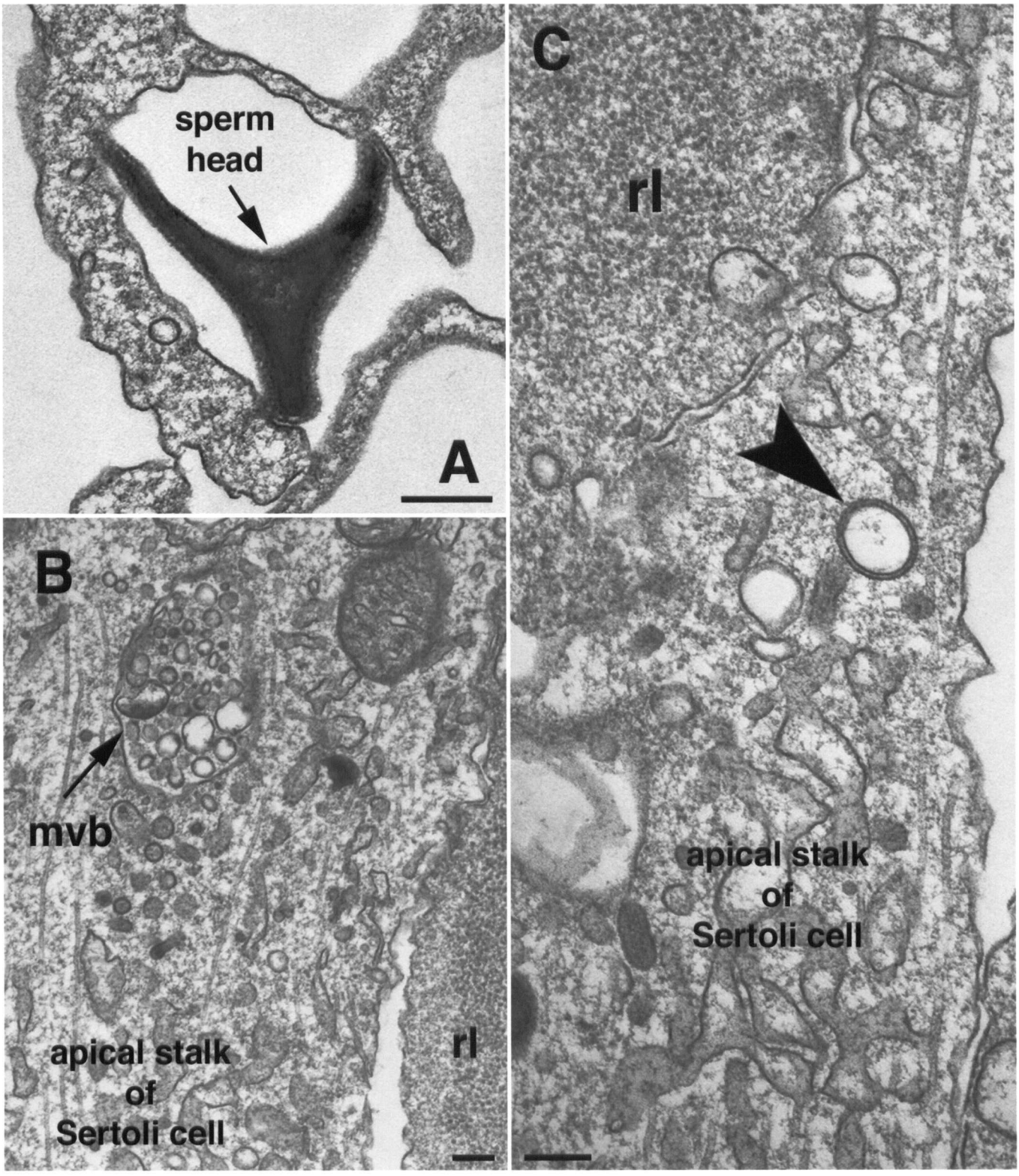

sperm
head
A
B
mvb
apical stalk
of
Sertoli cell
rl
C
rl
apical stalk
of
Sertoli cell

Figure 5.8. Paired phase and fluorescent micrographs of endocytosis and lysosomal markers. (A-C') paired phase and fluorescent images of mouse testis material stained with anti-PKC α antibodies and controls, Bar = 5 μm. The Anti-PKC α antibody labeled areas known to contain tubulobulbar complexes (tc) in the mouse. Both primary antibody NMIgG controls (B') and secondary antibody controls (C') showed a distinct lack of staining at these structures. (D-D''') Phase (D), anti-PKC α (D') and filamentous actin (D'') labeled and merged (D''') mouse material micrographs. Filamentous actin (Phalloidin) staining was used to ensure the presence of the ectoplasmic specialization Bar = 5 μm. (E-E''') Phase (E) and double labeled anti-LAMP1 (E') and filamentous actin (E'') images. Note the LAMP1 labeling stains circular structures and for the most part is distal to and does not co-localize with filamentous actin. (F-I') Anti-SGP1 labelling of rat spermatids with associated tubulobulbar complexes and controls. (F') Anti-SGP1 stained material specifically labels punctate structures in the region of lysosomes associated with the tubulobulbar complex region (arrowheads). Numerous regions of non-specific staining occur in the primary antibody control normal rabbit serum micrographs (G'), but increased labeling in the lysosomal region is absent. Secondary antibody (H') and autofluorescente antibody (I') controls are both devoid of staining. Bar in F= 5 μm.

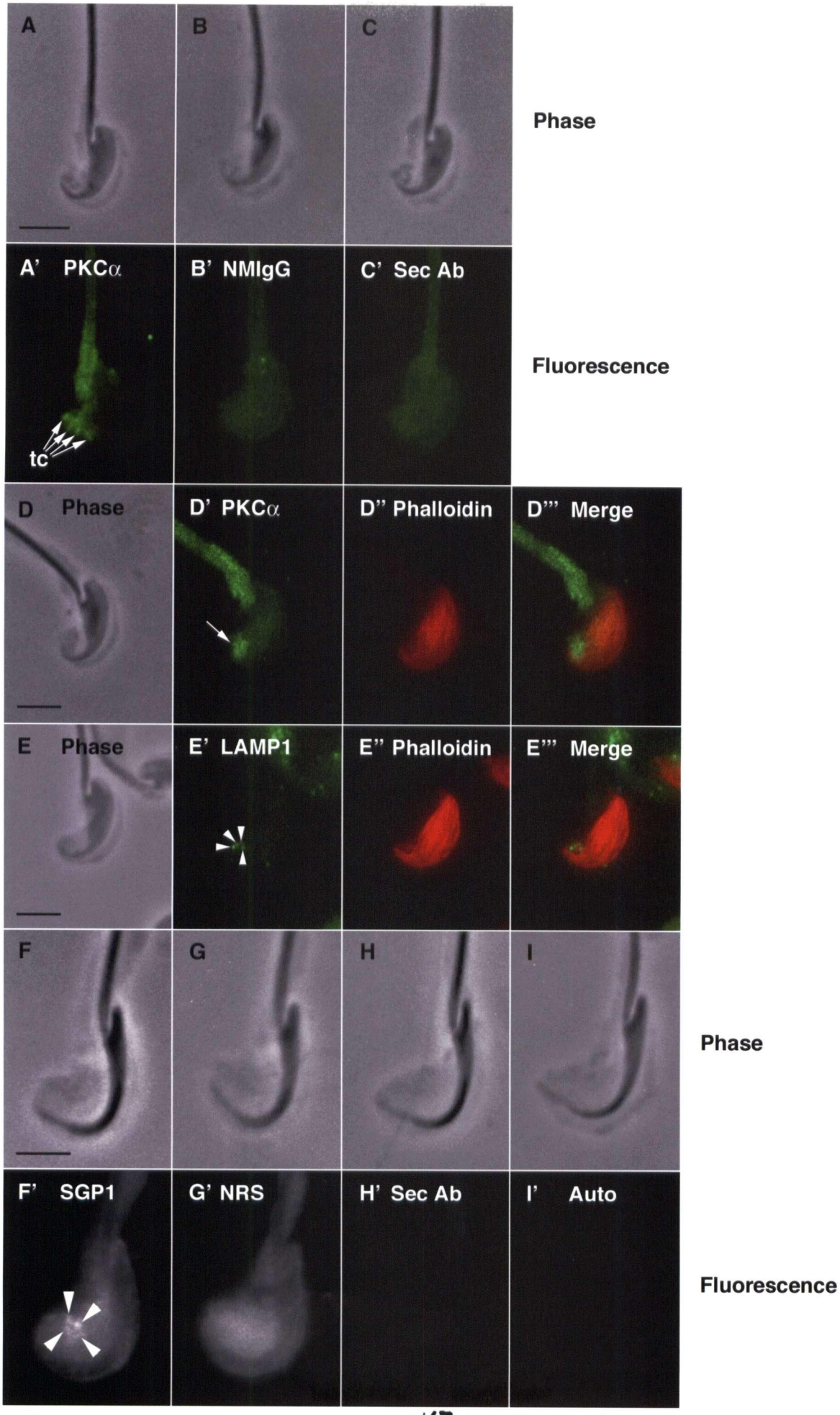
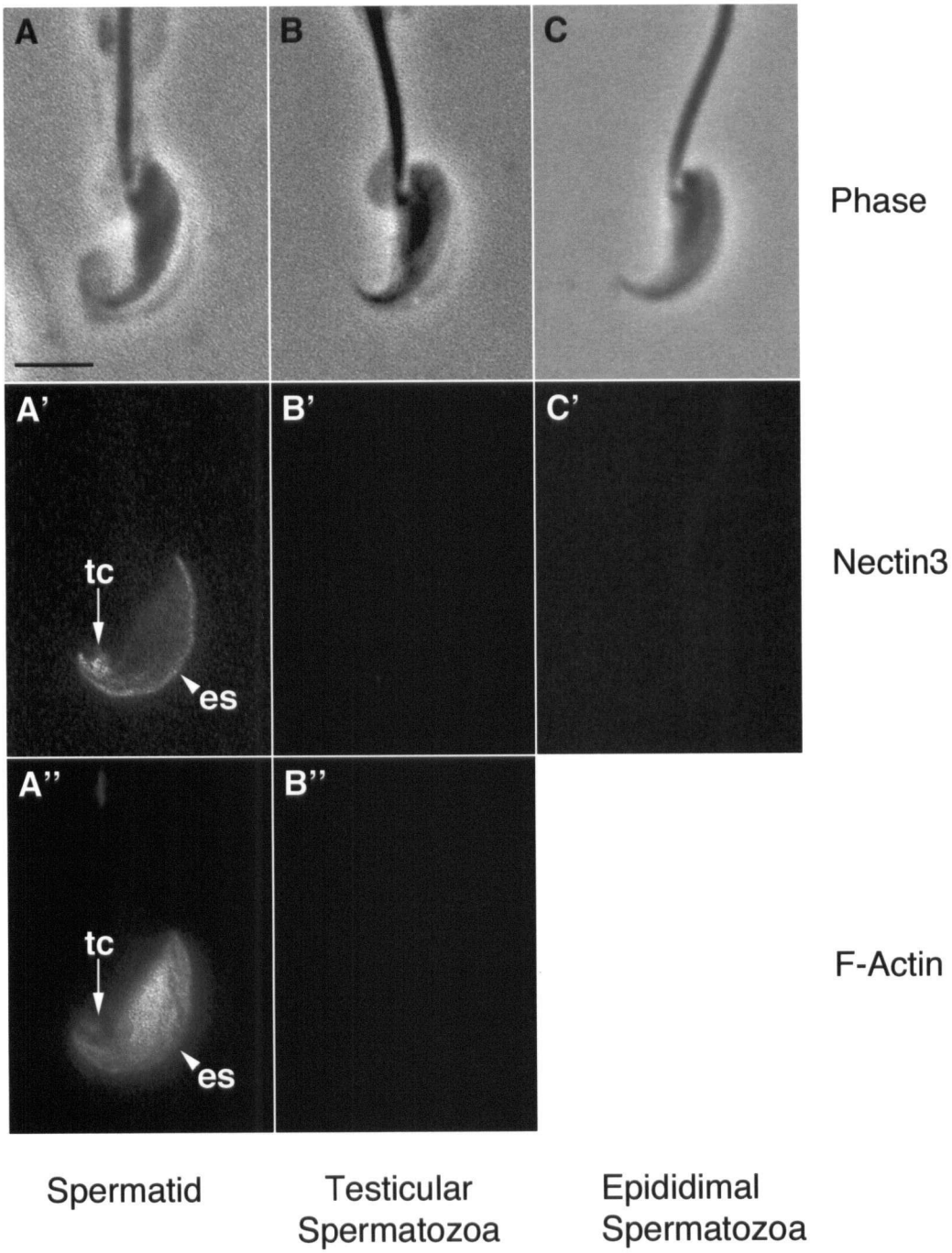

A
B
C
Phase
A' PKCα
B' NMIgG
C' Sec Ab
tc
Fluorescence
D Phase
D' PKCα
D" Phalloidin
D"' Merge
E Phase
E' LAMP1
E" Phalloidin
E"' Merge
F
G
H
I
Phase
F' SGP1
G' NRS
H' Sec Ab
I' Auto
Fluorescence

Figure 5.9. Nectin-3 functional localization in spermatids and spermatozoa. Phase and fluorescent localization of Nectin-3 and filamentous actin on spermatid/tubulobulbar complex containing testis material (A-C'). Filamentous actin labeling (A') confirms the presence of both ectoplasmic specializations (es) and tubulobulbar complexes (tc) associated with the spermatid in (A) and nectin-3 is also present (A''). On both testicular spermatozoa (B) and spermatozoa in the epididymis (C), nectin-3 is absent (B'', C''). Labelling with phalloidin confirms that the spermatozoa do not contain any ectoplasmic specialization or tubulobulbar complex associated actin filaments (B'). Bar in A= 5 μm.


A
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Nectin3
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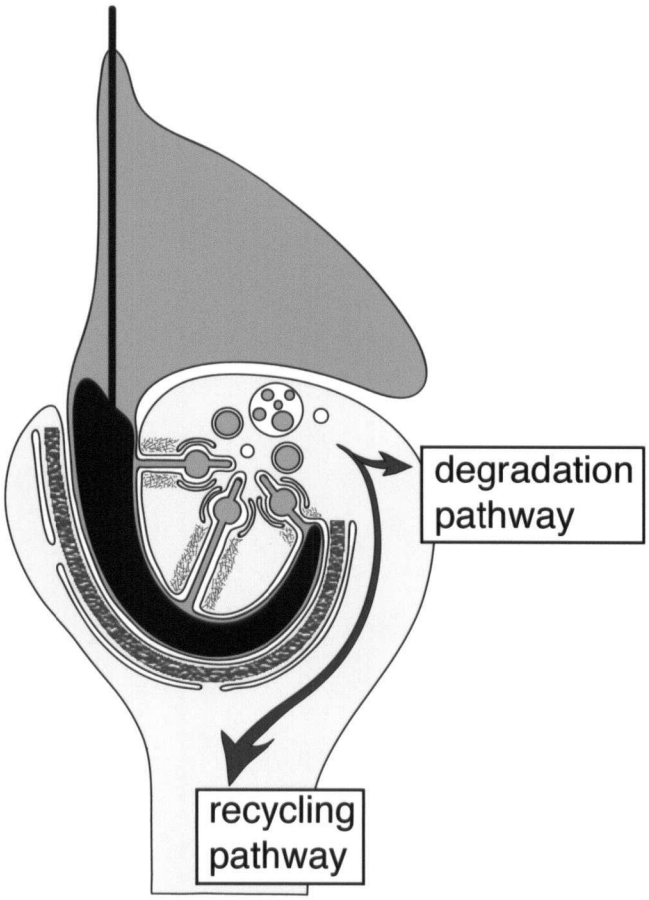
B''
tc
es
F-Actin
Spermatid
Testicular Spermatozoa
Epididimal Spermatozoa

Figure 5.10. Diagram of the paths that the vesicles associated in the tubulobulbar complex region may take, recycling or degradation.


degradation
pathway
recycling
pathway

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CHAPTER 6

Non-muscle cofilin is a component of tubulobulbar complexes in the testis

Introduction

In the mammalian seminiferous epithelium, Sertoli cells interact with one another (basally in the seminiferous epithelium) and with maturing spermatids, (apically in the epithelium), through actin-related intercellular adhesion junction plaques termed ectoplasmic specializations. These junction plaques are tripartite structures composed of the Sertoli cell plasma membrane, a layer of actin filaments packed into paracrystalline arrays and a cisternae of endoplasmic reticulum. Ectoplasmic specializations disassemble at two specific times: 1) prior to when maturing spermatocytes are moved from the basal compartment of the seminiferous epithelium towards the apex and 2) just prior to spermatid release. During ectoplasmic specialization disassembly, finger–like structures called tubulobulbar complexes are formed (Figure 6.1a). These structures continue to develop as the ectoplasmic specializations disassemble (Russell 1979a). At areas previously occupied by apically located ectoplasmic specializations, tubulobulbar complexes are composed of tube-like projections of the elongate spermatid, which protrude into corresponding Sertoli cell invaginations. They are the final structures that link the mature spermatids to the Sertoli cells. Basal tubulobulbar complexes also have been reported (Russell and Clermont, 1976) in which spermatogenic cell components are absent. Both apical and basal tubulobulbar complexes have three segments consisting of a proximal tubular segment (nearest to the spermatid head), a bulbous protrusion distal to

A version of this chapter has been accepted for publication. Guttman, JA., Obinata, T., Shima, J., Griswold, M., and Vogl, AW (2003) Non-Muscle Cofilin is a Component of Tubulobulbar Complexes in the Testis. Biol. Repro.

the proximal tubular segment and smaller distal tubular region, distal to the bulbous region (Figure 6.1b and 6.1c). Interestingly, many of the same structural components present at ectoplasmic specializations (namely the Sertoli cell plasma membrane, actin filaments and endoplasmic reticulum) also are found at tubulobulbar complexes. The cisternae of endoplasmic reticulum are found applied to the Sertoli cell plasma membrane of the bulbous region and the actin filaments form an actin network cuff around the major tubular segment (Figure 6.1d).

Three possible functions have been proposed for tubulobulbar complexes. The first is that tubulobulbar complexes function to attach the mature spermatids to Sertoli cells (Russell, 1979a and b; Russell and Malone 1980), the second is that these structures remove excess spermatid cytoplasm and acrosomal contents prior to spermatid release (Tanii et al., 1999; Russell and Malone 1980`), and the final one is that the structures internalize junction domains in the plasma (Russell 1979b; Vogl 1989).

Because actin is a major component of ectoplasmic specializations, numerous studies have focused on identifying actin-associated components at ectoplasmic specializations (Franke et al., 1978; Jockusch and Isenberg, 1981; Grove and Vogl, 1989; Bartels et al., 1996; Hasson et al., 1995; Guttman et al., 2002b; Ozaki-Kuroda et al., 2002). Recently, the calcium-dependent actin severing and capping protein gelsolin also has been identified at ectoplasmic specializations (Guttman et al.,2002b). This protein is thought to function in the disassembly of the actin layer of the junction plaques. Based

on this finding we were interested to see if a calcium independent actin severing protein also was found at these sites.

Cofilin is a calcium-independent actin depolymerizing protein, which disassembles actin filaments when unphosphorylated. Two isoforms of cofilin (muscle type and non-muscle type) exist and mRNA of both types have been found in the testis (Ono et al., 1994). There are two groups of proteins that have been identified thus far that have the capability of phosphorylating cofilin and rendering it inactive. These proteins are the LIM kinases (LIMKs) and the testicular protein kinases (TESKs) respectively. LIMK2 null male mice exhibit impaired spermatogenesis (Takahashi et al., 2002). Interestingly with all of this data implicating cofilin as an important factor in spermatogenesis, the protein has still not been localized to any specific structures in the testis.

In this study I used a pan-cofilin antibody to immunolocalize cofilin in the seminiferous epithelium. I found that this probe specifically labeled tubulobulbar complexes in the region occupied by filamentous actin. It did not label ectoplasmic specializations, the only other major site of actin filament concentration in Sertoli cells. On 2D western blots of rat seminiferous epithelium, only 2 protein dots appeared, not 4, which would be predicted due to the two cofilin isoforms (muscle type and non-muscle type) and their ability to be phosphorylated and dephosphorylated. Microarray analysis indicated that the message for non-muscle type cofilin is 8.5 fold greater than for the muscle type isoform in Sertoli cells. Antibodies raised specifically against non-muscle

type cofilin labeled tubulobulbar complexes and reacted with the same 2 dots that reacted with the pan-cofilin antibody on 2D rat seminiferous epithelium western blots. The data presented here supports the finding that the non-muscle cofilin isoform is present at tubulobulbar complexes and that neither the muscle or non-muscle cofilin isoforms are concentrated at ectoplasmic specializations.

Materials and Methods

Animals

All animals used in these studies were reproductively active male Sprague Dawley rats. They were obtained from the University of British Columbia animal care colony and were maintained according to the guidelines established by the Canadian Council on Animal Care. Microarray studies were performed using mice from Washington State University. Their use conformed to the protocols approved by the Washington State University Animal Care and Use Committee and the National Institutes of Health standards detailed in the *Guidelines for the Care and Use of Experimental Animals*.

Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were obtained from Sigma-Aldrich Canada (Mississauga, Ontario). The paraformaldehyde and NaCl were obtained from Fisher Scientific (Vancouver, BC). All control immunoglobulins (IgG) as well as all secondary antibodies conjugated to Horseradish Peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pennsylvania). Secondary antibodies conjugated to ALEXA fluorochromes were all purchased from Molecular Probes (Eugene, OR).

Immunofluorescence

Tissue preparation

Testes were removed from male rats under deep anesthesia. Warm (33°C) PBS (150mM NaCl, 5mM KCl, 0.8mM KH_2PO_4, 3.2mM Na_2HPO_4, pH 7.3) was perfused through the spermatic artery using a 26G needle attached to a gravity fed perfusion apparatus for 2 minutes to clear the organ of blood. Following this, warm 3% paraformaldehyde in PBS was perfused through the testis for 30 minutes. PBS was then re-perfused through the organ to wash out any remaining fixative.

Frozen sections

For frozen sections, the fixed testes were frozen (using liquid nitrogen) while at the same time being attached to an aluminum stub by OCT compound (Sakura Finetek USA, Torrance, Ca, USA). Frozen testis sections were cut, attached to poly-l-lysine coated glass slides, immediately plunged into –20°C acetone for 5 minutes allowed to air dry, then processed for immunofluorescence.

Fragmented material

For fragmented material, perfusion fixed testes were de-capsulated, cut into 1mm sized cubes and transferred into a 15ml plastic Falcon tube along with about 5mls of PBS. The material was gently passed through an 18, then 21gauge needle for 2-5 gentle passes. This fragmented material was left to sediment by gravity at room temperature for 10-15 minutes at which point the upper most layer was added to poly-l-lysine coated slides and allowed to incubate in a humidity chamber for 10 minutes. All excess PBS was then

removed and immediately treated with –20°C acetone for 5 minutes and allowed to air dry at which point 5% blocking serum was added.

Antibody labeling

Once the tissue was ready for serum blocking, 5% normal goat serum (NGS) in TPBS-BSA (PBS containing 0.05% Tween-20 and 0.1% Bovine Serum Albumin) from the secondary antibody host was incubated on the testis material for 20 minutes at room temperature. This was used to block the tissue from secondary antibody non-specific binding. The primary antibodies consisting of a mouse monoclonal anti-cofilin Mab-22 used at a 1:200 dilution (Abe et al., 1989)(Gift from Dr. Takashi Obinata), and a rabbit serum anti-non-muscle cofilin used at a 1:250 dilution (Mohri et al., 2000)(Gift from Dr. Takashi Obinata), were added to the experimental slides made up in TPBS-BSA with 1% NGS and incubated overnight at 4°C in a humidity chamber. The material was washed extensively with the TPBS-BSA (wash buffer) then incubated for 60 minutes at 37°C with secondary antibody conjugated to a fluorochrome (goat anti-mouse ALEXA 488 or goat anti-rabbit ALEXA 568). After further washing, coverslips were mounted using Vectashield (Vector Labs, Burlington, Ontario) and visualized by standard fluorescence microscopy using a Zeiss Axiophot microscope.

Filamentous actin labeling was performed using ALEXA 488 or ALEXA 568 phalloidin (Molecular probes, Eugene Oregon).

Controls consisted of replacing the primary antibody with its respective immunoglobulin (IgG) or serum at identical concentrations to the primary antibody,

replacing the primary antibody with buffer alone, or replacing both the primary and secondary antibodies with buffer alone.

1D Western blotting

Material was loaded into wells of 1mm thick 10% SDS-PAGE reducing gels and run according to standard protocols (Laemmlli, 1970). Proteins were transferred onto Immobilon-P transfer membrane (Millipore) then washed for 5 minutes at room temperature with TBST (500mM Tris pH 7.5, 150mM NaCl, 0.1% Tween-20). The blots were then blocked for 8 hours at 4°C using 4% non-fat milk (Blotto, Santa Cruz Biotechnology) in order to decerase non-specific antibody binding. Following blocking, membranes were washed 3 times, 10 minutes each, then incubated with the mouse anti-cofilin Mab-22 antibody used at a 1:2000 dilution or the rabbit anti-non-muscle cofilin antibody, used at a 1:1000 dilution, overnight at 4°C. The following day, blots were washed extensively with TBST followed by a 1 hour incubation with the secondary antibody (goat anti-mouse or goat anti-rabbitt conjugated to horseradish peroxidase; Jackson ImmunoResearch) at room temperature. Upon further washing with TBST followed by TBS, blots were reacted with ECL (Pharmacia) to visualize the reactive bands on X-OMAT film (Kodak).

Controls consisted of replacing the primary antibodies with normal mouse IgG or normal rabbit serum at identical primary antibody concentrations or identical serum dilutions.

2D electrophoresis

Isolated rat seminiferous epithelium, in solution, was first centrifuged at 10,000RPM using an Eppendorf desktop centrifuge for 15 minutes at room temperature. Once completed, the supernatant was collected and treated to acetone precipitation. To do this, three volumes of -20°C acetone was added to the sample and incubated at –20°C for 30 minutes with occasional agitation. The sample proteins were then pelletted and the supernatant was discarded. The resulting protein pellet was allowed to air dry for 10-15 minutes and was re-suspended in 2D-gel sample buffer (8M Urea, 2M Thiourea, 4% w/v CHAPS, 20mM Tris, 0.0025% Bromophenol Blue) at room temperature on a Labquake rotator for 6-8 hours. Once re-suspended, 0.5% of IPG (Immobilized pH Gradient) Buffer (Amersham, Quebec), at the same pH as the IPG focusing strip, was added to the sample. After a 5 minute centrifugation at 10,000RPM in an Eppendorf desktop centrifuge, the remaining supernatant was added to the IPG strip for a 24 hour strip re-hydration at 15°C. Focusing was then performed using the IPGphor isoelectric focusing machine (Amersham). Focused IPG strips were loaded onto large format slab gels (Bio-Rad) and run at 50-85 volts.

Microarray Analysis (Performed by Jim Shima from Dr. Michael Griswold's Laboratory; Washington State University)

Techniques used were identical to those described in chapter 2.

Results

Actin filaments are found concentrated at two locations in Sertoli cells, ectoplasmic specializations and tubulobulbar complexes. Upon staining rat testis sections and fragments with a pan-cofilin antibody, labeling was found localized exclusively to the actin-rich tubulobulbar complex structures (Figure 6.2a-a' and 6.3a-a' arrowheads) and was notably absent from ectoplasmic specializations (Figure 6.2e-e' and 6.3e-e'). Normal mouse IgG (NMIgG), secondary antibody and autofluorescent controls showed no specific staining pattern (Figure 6.2b-d', 6.2f-h', 6.3b-d', 6.3f-h') but both the antibody and NMIgG stained tissue sections showed a similar non-specific staining pattern (Figure 6.2b-b' and 6.2f-f'). The specificity of this antibody in the testis was confirmed using 1D western blots of rat testis and rat seminiferous epithelium which resulted in the appearance of a single band at about 20kD (Figure 6.3a), the appropriate molecular weight for cofilin. Because there are two isoforms of cofilin (muscle and non-muscle type) and mRNA from both isoforms are present in the testis (Ono et al.,1994) I performed 2D western blots of rat seminiferous epithelium expecting to find 4 reactive protein dots to account for both the phosphorylated and unphosphorylated forms of muscle and non-muscle type cofilin. Interestingly, only 2 reactive dots were found at the appropriate molecular weight and isoelectric point (Figure 6.4b).

To identify which isoform of cofilin may be present specifically in Sertoli cells, the region where the filamentous actin surrounds the tubulobulbar complex, GeneChip® microarrays (Affymetrix) were used to quantify the amount of muscle and non-muscle type cofilin transcript found in an enriched murine Sertoli cell population. Transcript

abundances of 1905 and 219 were detected for non-muscle type cofilin and muscle type cofilin respectively, representing an over 8.5 fold increase of non-muscle type cofilin mRNA compared to muscle type.

Although not always reflective of protein abundance, the mRNA data led us to use a previously characterized rabbit serum non-muscle type cofilin antibody (Ono et al.,1994) for immunolocalization on fixed, fragmented testis material. Using this antibody, I found specific labeling at tubulobulbar complexes (Figure 6.5a) as well as non-specific staining along the dorsal curve of the spermatid head (Figure 6.5b). This well-known non-specific staining pattern often is seen when using rabbit antibodies on testis material. 1D western blots clearly labeled a protein at about 20kD (Figure 6.6a) as well as other higher molecular weight non-specific bands that were accounted for by the normal rabbit serum primary antibody control blot (Figure 6.6b). To see if the two reactive protein dots on the pan-cofilin (Mab-22) 2D western blot were in fact the non-muscle isoform of cofilin, I again performed 2D western blots of isolated rat seminiferous epithelium and probed them with the non-muscle type cofilin antibody. As predicted the same 2 reactive protein dots appeared in the appropriate location (molecular weight and isoelectric point) for cofilin (Figure 6.6c).

Discussion

This study demonstrates that, in Sertoli cells, cofilin is concentrated at tubulobulbar complexes and is not a detectable component of ectoplasmic specializations. These results also indicate that the predominant isoform of the protein expressed at tubulobulbar complexes in Sertoli cells is non-muscle cofilin.

Cofilin is a calcium independent actin filament regulatory protein. To date, two isoforms (muscle type and non-muscle type) have been discovered in mammals, and the isoforms are encoded by different genes. mRNA transcripts for muscle type cofilin have been found in skeletal muscle, heart, and testis, where as mRNA transcripts for non-muscle cofilin have been found in brain, lung, spleen, and testis (Ono et al., 1994).

Actin filaments in Sertoli cells are concentrated at tubulobulbar complexes and at ectoplasmic specializations, both of which develop at sites of intercellular attachment. Actin filaments in tubulobulbar complexes occur as a network whereas those in ectoplasmic specializations are unipolar and hexagonally packed into bundles. A cistern of endoplasmic reticulum, which is a potential calcium store, is directly associated with filament bundles in ectoplasmic specializations. A similar cistern of endoplasmic reticulum is not found in association with the filament networks in tubulobulbar complexes. The presence of cofilin, a calcium dependent actin filament severing protein, in tubulobulbar complexes and not at ectoplasmic specializations may be related to differences in local levels of calcium and the way it is regulated at the two sites.

The conclusion that non-muscle cofilin is the isoform of the protein predominantly expressed in the seminiferous epithelium and at tubulobulbar complexes is

supported by three observations. First, there is an 8.5 fold higher abundance of non-muscle type cofilin mRNA over muscle type cofilin mRNA in Sertoli cells. Second, immunoblots probed with the pan-cofilin Mab-22 antibody indicate that mainly one isoform is expressed in the seminiferous epithelium. Third, staining with an antibody specific for non-muscle cofilin labeled tubulobulbar complexes in sections and the same protein as the Mab-22 antibody on immunoblots of seminiferous epithelium. Based on these findings I conclude that the isoform of cofilin at tubulobulbar complexes is likely the non-muscle type.

Because both non-muscle type cofilin and gelsolin are present at tubulobulbar complexes, the two proteins probably act together and in concert with other actin binding proteins to control actin filament dynamics at tubulobulbar complexes. Although non-muscle cofilin is specifically concentrated at tubulobulbar complexes, its precise function at these sites remains to be experimentally verified, although a role in disassembly of actin filaments during cell detachment is likely.

Cofilin previously has been reported in mouse spermatogenic cells (Takahashi et al., 2002) where it is diffusely localized in the cytoplasm. However, I was unable to convincingly demonstrate differences in staining between spermatogenic cells in antibody and control treated normal rat tissue sections in our study. Differences in results between this study and the previous study may be due to differences in tissue handling or to species differences in reactivity. Also, the diffuse nature of cofilin staining in spermatogenic cells may have been below what I could convincingly detect using these

techniques. Perhaps the strongest evidence for the presence of the protein in spermatogenic cells is that testicular fractions (mouse) enriched for spermatocytes react positively on immunoblots treated with cofilin antibodies (Takahashi et al., 2002). In addition, nuclear inclusions of cofilin occur in spermatocytes of LIM-kinase null mice after the testes have been exposed to experimental hyperthermia (Takahashi et al., 2002).

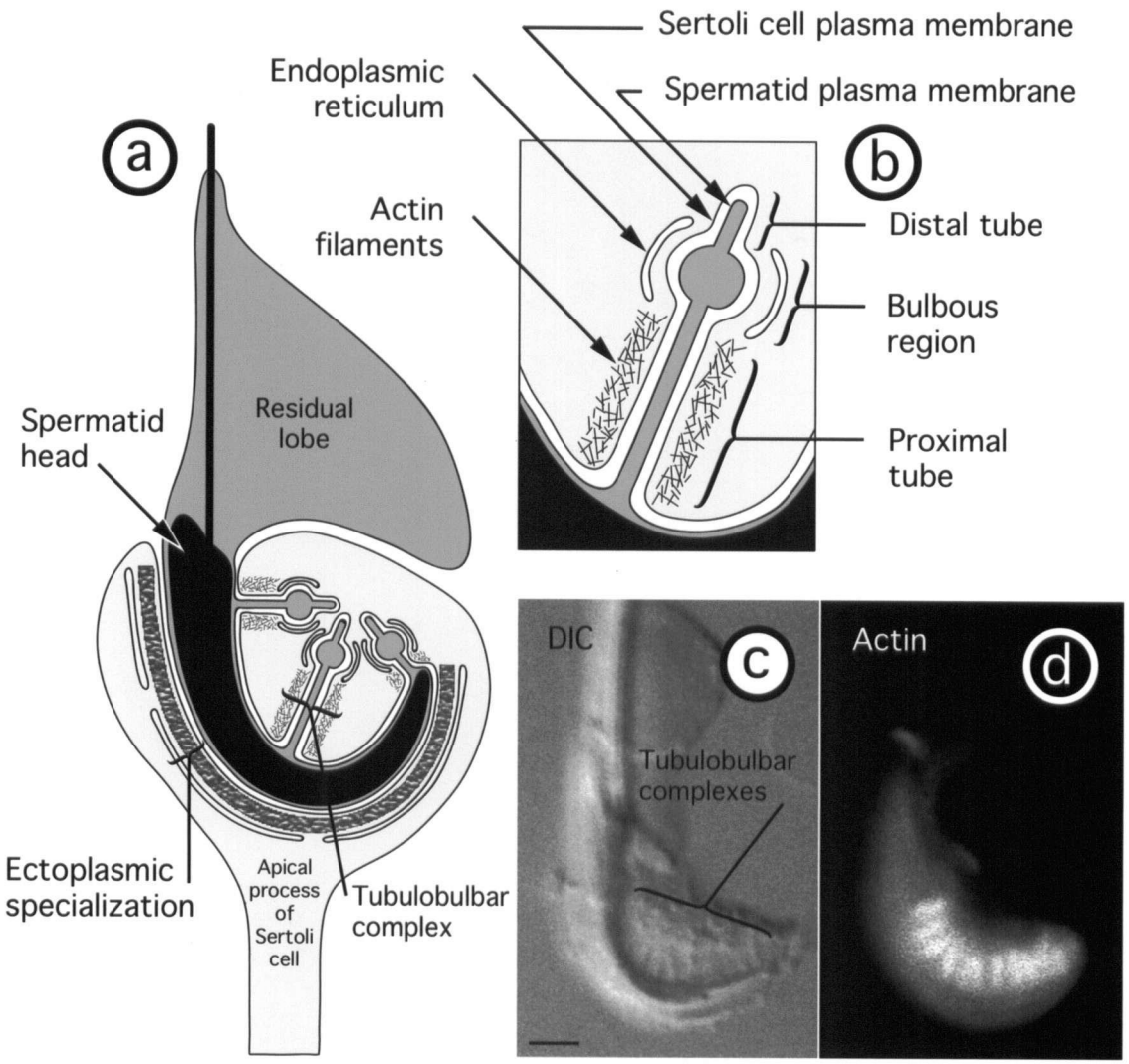
LIM-kinase specifically catalyses the phosphorylation of cofilin (Arber et al., 1998; Yang et al., 1998; Sumi et al., 1999). There are 2 LIM-kinase genes that have been described to date in mouse, rat and human – LIMK1 and 2 (Nunoue et al., 1995; Osada et al., 1996; Koshimizu et al., 1997). LIMK1 is thought to be specifically activated by the small GTPase rac where as LIMK2 is thought to be activated by the rho and cdc42 G-proteins (Sumi et al., 1999; Edwards et al., 1999). The finding of impaired spermatogenesis and increased apoptosis of spermatogenic cells in LIMK2 null mice, is consistent with the conclusion that LIMK2 is important in male reproduction presumably by regulating either the activity of cofilin or its distribution in spermatogenic cells (Takahashi et al., 2002).

Although LIMK2 has been demonstrated in spermatogenic cells (Takahashi et al., 2002), neither isoform of LIM-kinase have been successfully localized in Sertoli cells. It is possible that phosphorylation of cofilin in Sertoli cells is regulated by proteins other than LIM-kinase. Among possible candidates are the TESK proteins.

In contrast to the LIM-kinases, the TESK proteins (LIM-kinase-like proteins) do not appear to be activated by small GTPases, rather they appear to be activated by integrins (Toshima et al., 2001). Interestingly, the β1 integrin is a component both of ectoplasmic specializations and of tubulobulbar complexes (Palombi et al., 1992; Salanova et al., 1995). Thus far two TESK proteins have been described, TESK 1 and 2. TESK 1 is found primarily in testicular germ cells whereas TESK2 primarily is found in Sertoli cells. Although TESK2's function in the testis has not been determined, it has been shown to phosphorylate cofilin *in vitro* (Toshima et al., 2001). This finding together with the observations that TESK2 is primarily expressed in Sertoli cells and is likely activated through an integrin based mechanism make TESK2 a prime candidate for regulating the phosphorylation and therefore activity of cofilin.

Based on my results from immunofluorescence and immunoblots, I conclude that non-muscle cofilin is a component of tubulobulbar complexes and is not a significant component of ectoplasmic specializations in rat Sertoli cells. The presence of this protein at tubulobulbar complexes provides a calcium independent way of controlling actin filament dynamics at these sites.

Figure 6.1. Diagrammatic representation of tubulobulbar complexes attached to an elongate spermatid. (a) Cartoon of a late step spermatid within the seminiferous epithelium. In the rat, tubulobulbar complexes form within the concave region of the scicle shaped spermatid head. These complexes contain both spermatid and Sertoli cell components. (b) diagram of a single tubulobulbar complex. Finger-like projections of the spermatid, the tubulobulbar process, protrude into a corresponding Sertoli cell invagination. The bulb area of the complex is surrounded by endoplasmic reticulum and separates the tubulobulbar complex into three sections. The proximal tube, the bulb and the distal tube. The proximal tube is surrounded by filamentous actin. DIC image and (c) phalloidin stained fragmented rat testis material (d) clearly showing the finger-like staining pattern of filamentous actin at the tubulobulbar complex.


Sertoli cell plasma membrane
Endoplasmic reticulum
Spermatid plasma membrane
a
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Actin filaments
Distal tube
Bulbous region
Proximal tube
Spermatid head
Residual lobe
DIC
c
Actin
d
Tubulobulbar complexes
Ectoplasmic specialization
Apical process of Sertoli cell
Tubulobulbar complex

Figure 6.2. Phase and fluorescent micrographs of rat testis sections stained with a pan-cofilin antibody (Mab-22) and their respective controls. (a-d') Late stage rat testis sections stained when tubulobulbar complexes are present, with either the Mab-22 antibody (a-a'), NMIgG (b-b'), secondary antibody (c-c') or a blank autofluorescent control (d-d'). (e-h') show seminiferous epithelium sections at the stage of elongate spermatid presence, when tubulobulbar complexes are absent and ectoplasmic specializations are found surrounding the spermatid heads, stained with the primary antibody (e-e'), primary antibody control (f-f'), secondary antibody control (g-g') or autofluorescent controls (h-h'). Arrowheads point to specific staining at tubulobulbar complexes. Arrows show non-specific staining appearing in both the Mab-22 micrographs and NMIgG images.

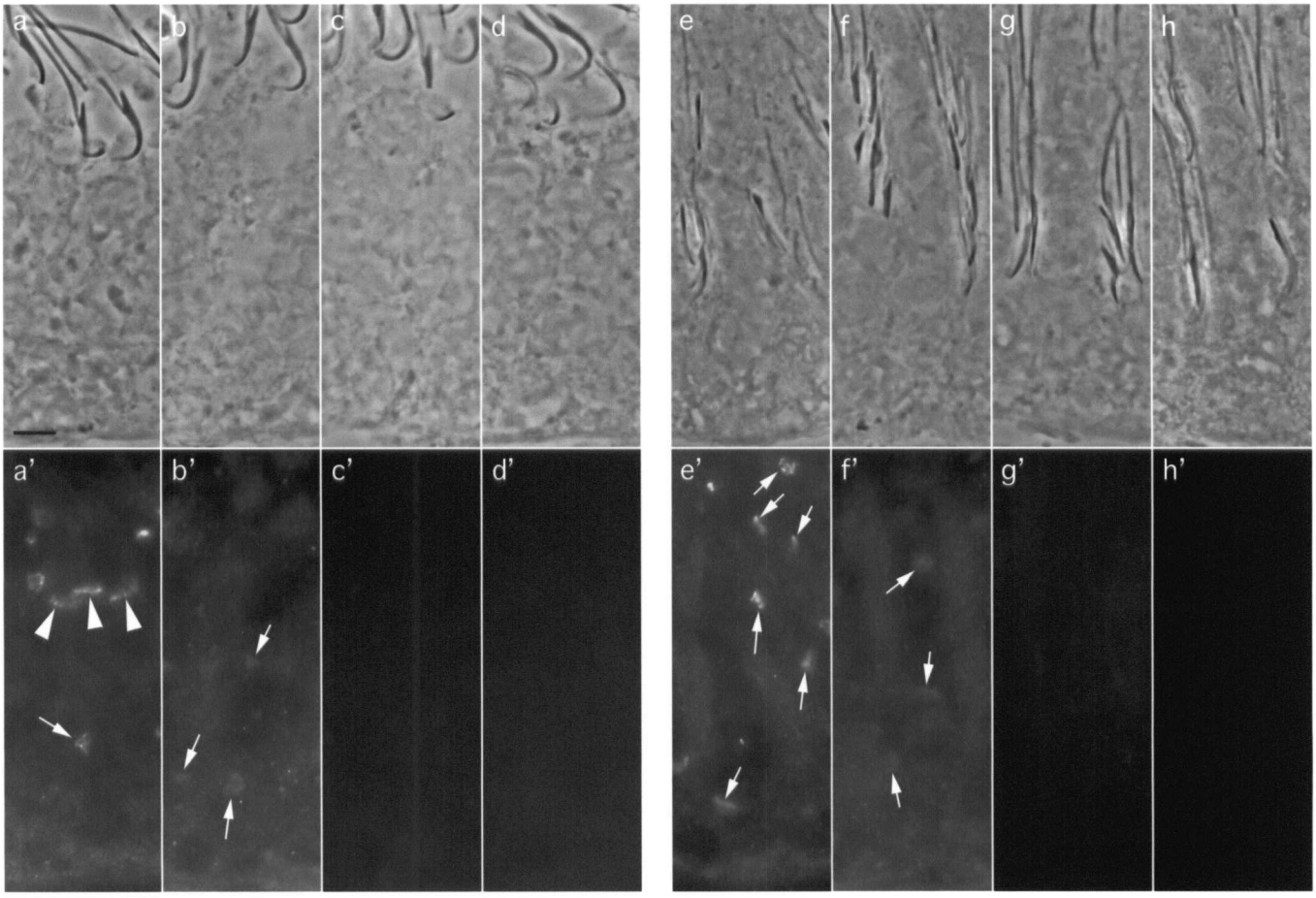
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Figure 6.3. Phase and fluorescent micrographs of rat testis fragments stained with a pan-cofilin antibody (Mab-22) and their respective controls. (a-d') fragmented testis material showing late step spermatids which have tubulobulbar complexes and are stained with either the Mab-22 antibody (a-a'), NMIgG (b-b'), secondary antibody alone (c-c') or carrier buffer alone (d-d'). (e-h') fragmented testis tissue showing earlier step spermatids without tubulobulbar complexes, only with ectoplasmic specializations. (e-e') uses the Mab-22 antibody, (f-f') is the primary antibody control, (g-g') is the secondary antibody control and (h-h') is the autofluorescence control. Arrowheads point to specific staining at tubulobulbar complexes.

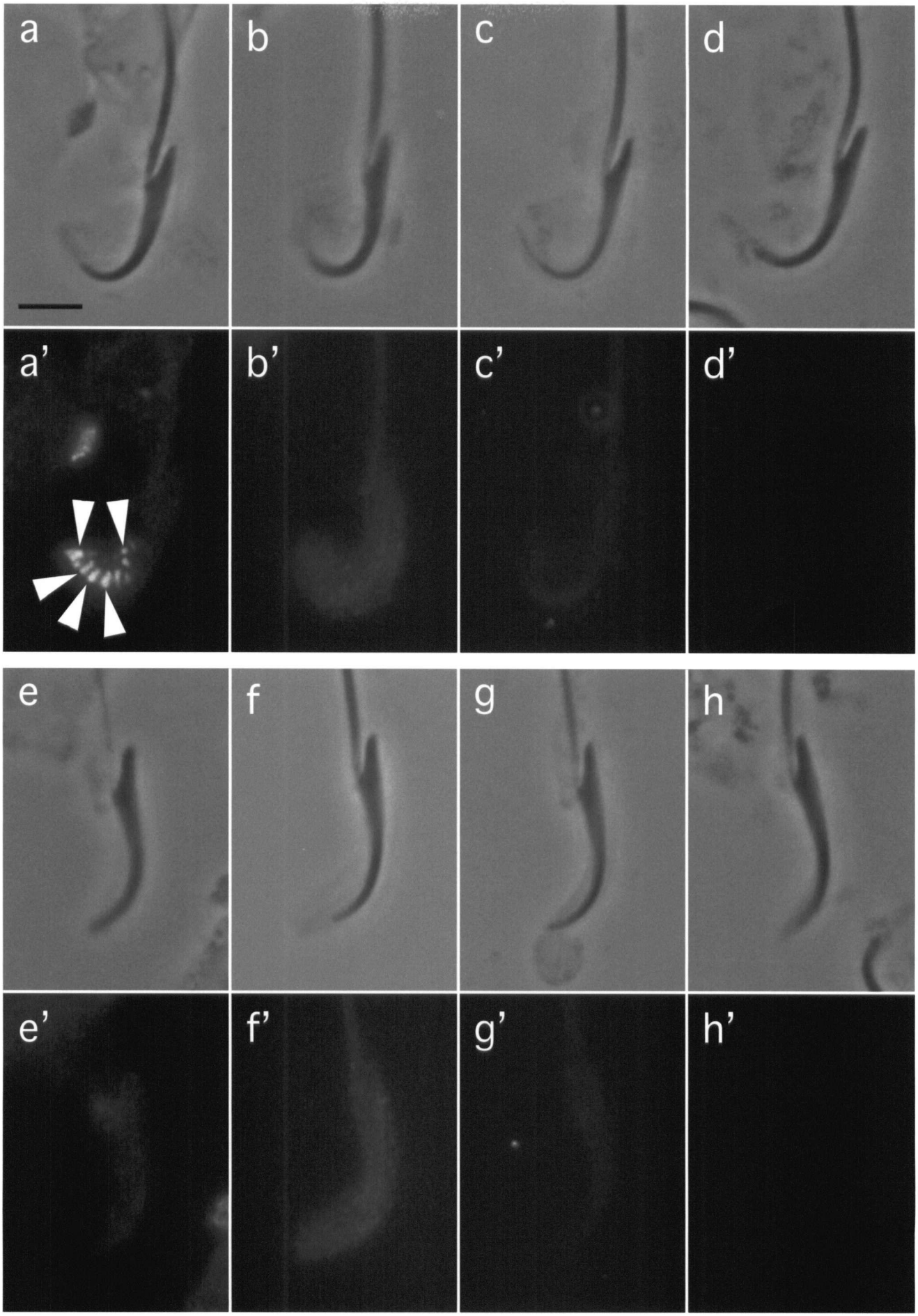
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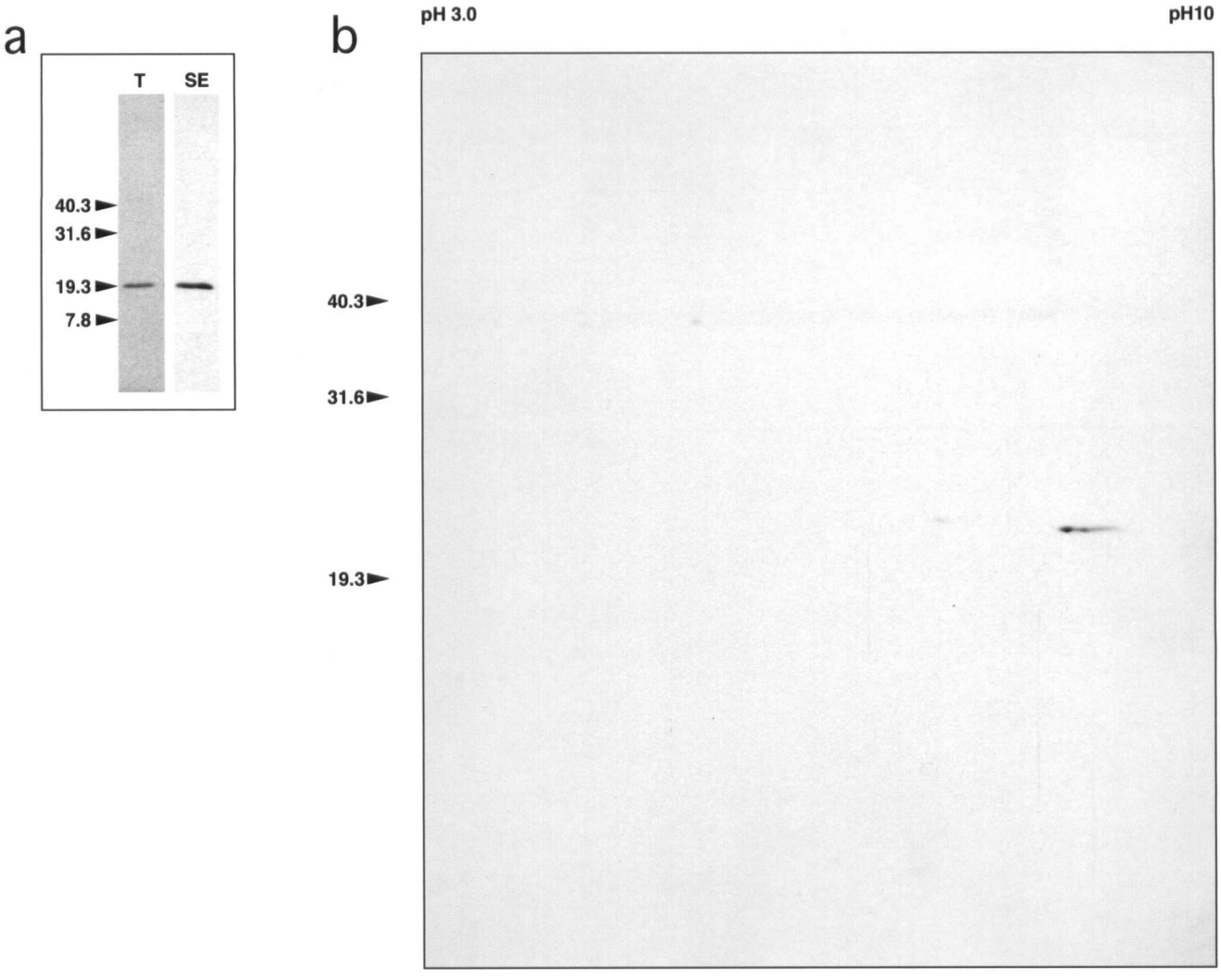
Figure 6.4. 1D and 2D western blots of testicular material probed with the Mab-22 pan-cofilin antibody. (a) 1D blots of rat testis homogenate (T) and isolated rat seminiferous epithelium (SE) probed with the Mab-22 antibody. (b) 2D western blot of isolated rat seminiferous epithelium probed with the MAB-22 antibody.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31.6
19.3
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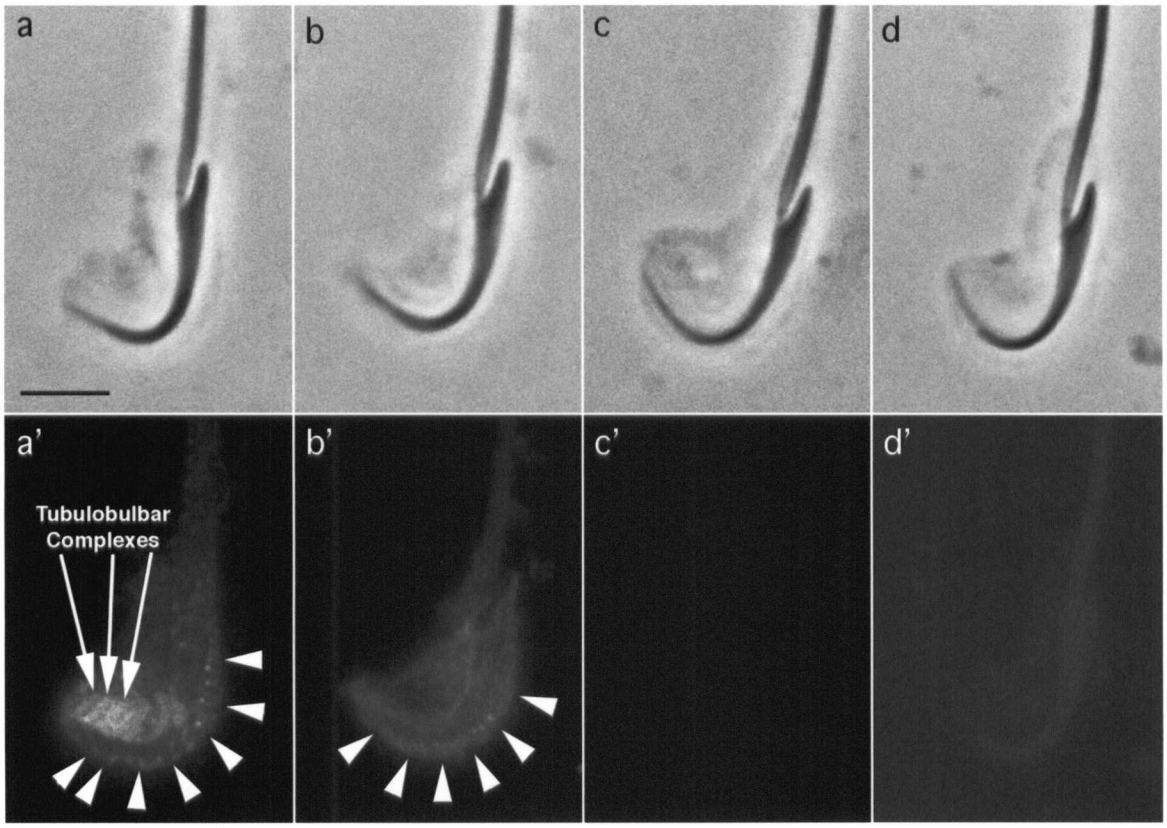
b
pH 3.0
pH10
40.3
31.6
19.3

Figure 6.5. Paired phase and non-muscle cofilin immunofluorescent micrographs of fragmented rat testis late step spermatids with tubulobulbar complexes and their controls. (A-A') phase and fluorescent images using the rabbit serum non-muscle cofilin antibody. Specific staining is seen at tubulobulbar complexes (Arrows) where non-specific staining is also present (arrowheads). (B-B') normal rabbit serum primary antibody control. Non-specific staining is clearly seen associated with the spermatid head (arrowheads) and not at tubulobulbar complexes. (C-C') secondary antibody controls and (D-D') Autofluorescent controls had no fluorescent staining associated with them.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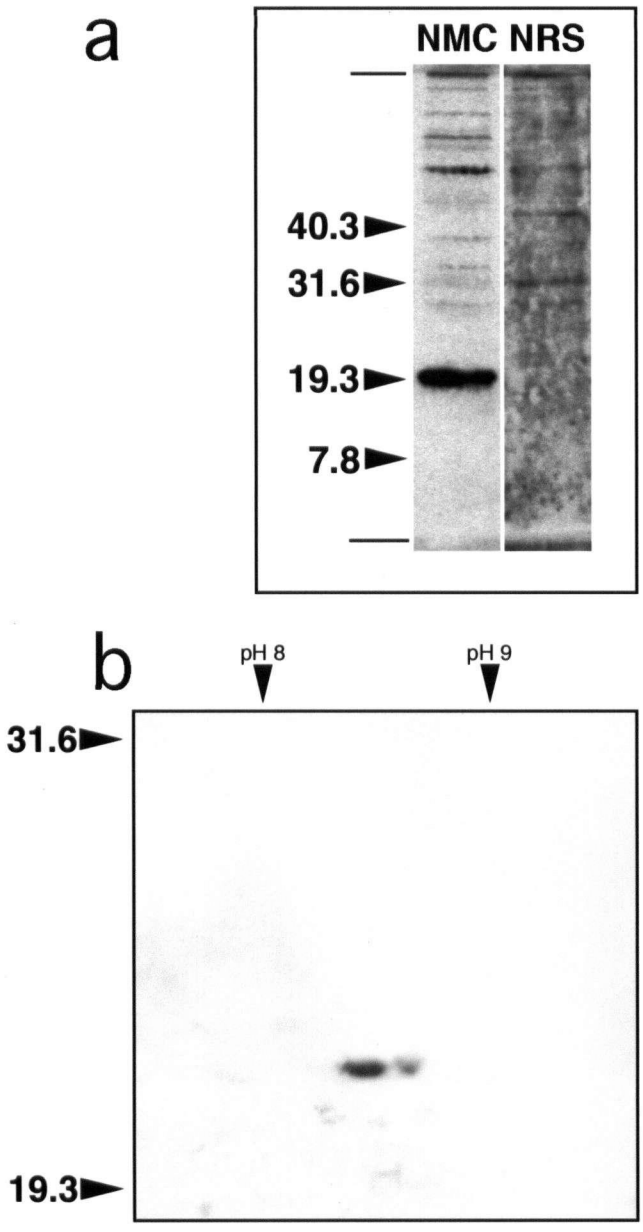
Tubulobulbar
Complexes

Figure 6.6. 1D and 2D rat seminiferous epithelium western blots using the non-muscle type cofilin antibody. (a) 15% acrylamide 1D western blot of rat seminiferous epithelium probed with the non-muscle type cofilin antibody (NMC) and control blot probed with normal rabbit serum (NRS). Top and bottom of the gel are represented by the straight bars. (b) 12.5% acrylamide, 2D western blot of rat seminiferous epithelium probed with the non-muscle cofilin antibody used at identical concentration to that used in the 1D blot.


a
NMC NRS
40.3
31.6
19.3
7.8
b
pH 8
pH 9
31.6
19.3

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CHAPTER 7

Final Discussion and Conclusion

Evidence presented in this thesis provides insight into a number of important aspects of spermiogenesis and cell biology in general. In the following section I will address the advances and impact that the research provided here has on these two fields.

Kinesin project

Spermatogenesis importance

Over the past decade the hypothesis that microtubule-based molecular motors are involved in the entrenchment and return of developing spermatids in the seminiferous epithelium during spermiogenesis has been tested in a number of ways. Based on the morphology of the system, the direction of spermatid translocation, the results of binding and motility assays, and the localization of cytoplasmic dynein at ectoplasmic specializations, this motility event is hypothesized to be due to Sertoli cell motor proteins, which are attached to ectoplasmic specializations.

Because there are no plus-end directed dyneins that have been discovered to date, a likely candidate for the plus-end directed molecular motor(s) at ectoplasmic specializations would be a member (s) of the kinesin superfamily.

In order to screen for a kinesin on ectoplasmic specializations, I initially used an antibody approach in which 3 polyclonal antibodies were generated against peptides to 3 of the most conserved regions of the kinesin molecules. These generated antibodies were

used for immunolocalization on testis tissue and all three reacted at areas in the seminiferous epithelium known to contain kinesins as well as at ectoplasmic specializations, a finding consistent with the microtubule-based spermatid translocation hypothesis and the first evidence placing a kinesin at ectoplasmic specializations. The peptides chosen are identical to those selected by others who have tried to generate anti-pan kinesin antibodies (Sawin et al., 1992). Interestingly, although conserved amongst most kinesins, some kinesins only contain fragments of these peptides and parts of these peptides are also found in other proteins. Due to this, simply the localization of one of these antibodies at structures can not be used to definitely determine if a kinesin is in fact present at the structures, but the localization of all three at the site indicates that the presence of a kinesin or kinesin-like protein should be pursued.

Based on the results of the preliminary antibody screen, an attempt was made to try to identify the kinesin isoform(s) associated with ectoplasmic specializations. To do this both a proteomics-based and an mRNA-based approach were taken. In the proteomics-based approach, concentrated spermatid/junction plaques were treated with the actin severing protein gelsolin and initially loaded onto 2D gels and 2D Blots. The blots were initially probed with antibodies to known ectoplasmic specialization components to determine if those components were released from the underlying spermatids. All antibodies tested showed positive reactivity on the supernatant 2D blots which were then probed with the generated anti-peptide antibodies. This resulted in numerous reactive protein spots. The spots that were able to be visualized under a UV box were excised and sent for mass spectrometry analysis, resulting in proteins other than

kinesins. Reasons for this are 2 fold. First the anti-peptide antibodies appeared to react positively with their peptide of choice and fragments of these peptides were found in abundance in some of the mass spectrometry results. Secondly, due to the abundance of other proteins in the general area as those that were excised by hand, the accuracy of the excision could have been compromised due to overlapping proteins. This is a significant problem and emphasizes the importance of dot purity and of having the proper equipment for accurate dot excision.

In order to circumvent the problems with protein excision and antibody peptide reactivity in proteins other than kinesins, I used a proteomics technique called 2DLCMS. This technique involved loading the entire gelsolin treated supernatant sample (after it has been prepared for mass spectrometry analysis) onto a strong cation exchange column prior to step-wise eluting it and running it through an LCMS. This resulted in some promising results including 2 peptides identified with high (greater than 90%) confidence of a kinesin called kin3 as well as numerous hypothetical proteins. In the kinesin molecule these peptides were found within the head region of the molecule. Based on the location of the 2 identified peptides within the kin3 ptorein there is a possibility that an area within the tail region of the kin3-like protein in the testis may impart a different localization and function on our system. Kin3 is a newly identified kinesin about which little is known. Data available indicate that it may be involved in early endosome sorting.

Using another approach to identify candidate kinesin isoform(s) at ectoplasmic specializations, in collaboration with members of Dr. Griswold's lab (Washington State

University), we screened a testis developmental mRNA database and a Sertoli cell mRNA database for kinesins in the testis and Sertoli cells. In the testis developmental series, an increase in mRNA abundance was seen with a number of kinesins. Interestingly, only 1 had a comparatively large mRNA signal in the Sertoli cell analysis, that was Rab6kinesin (Rab6KIFL). Although there is no guarantee that the mRNA abundance is representative of the protein abundance, this does give us a suggestion of possible protein involvement.

Rab6kineisn (Rab6KIFL) is a kinesin that was initially found interacting with the active form of Rab6 and possibly functioned at the Golgi to regulate membrane traffic in HeLa cells (Echard et al., 1998). In addition to this Hill et al., (2000) found that in HeLa cells the Rab6-kinesinlike was required at the cleavage furrow for proper cytokinesis. Due to the difference in localization and function of this protein in identical cell types and the fact that Sertoli cells do not divide, this provides a possibility that this kinesin-like protein may function differently in Sertoli cells, perhaps in spermatid translocation.

In summary, the kinesin at ectoplasmic specializations has yet to be identified although motility data and antibody work is consistent with a kinesin being present. The potential candidate proteins, Kin3 and/or Rab6KIFL, in Sertoli cells and possibly at ectoplasmic specializations sets the foundation for further investigation into their function in the testis. Future experiments should include generating kin3 and Rab6KIFL antibodies that react in testis and upon positive results, generating Sertoli cell-specific kin3 and Rab6KIFL null mice to investigate deficits in spermiogenesis.

Gelsolin/ PIP_2/PLCγ Project

Spermatogenesis importance

The disassembly of ectoplasmic specializations is an essential event occurring basally when the young spermatocytes move from the basal compartment of the seminiferous epithelium to the apical (or adluminal) compartment as well as occurring apically in the final stages of spermatid maturation. Apically, ectoplasmic specialization disassembly is correlated with spermatid release from Sertoli cells and in some species directly preceeds their release. Ectoplasmic specializations contain an abundance of very stable actin filaments, which must become depolymerized (or severed) in order for the majority of the junction plaque to disassemble.

In the work presented here, I discovered that an actin severing protein, gelsolin, is present at ectoplasmic specializations and that this protein may be involved in one of the major processes of spermatogenesis, junction disassembly. I tested the hypothesis that gelsolin is functionally attached to it's naturally occurring inhibitor PIP_2 and that it can be released from PIP_2 through the hydrolysis of PIP_2 by PLCγ, and activated by endoplasmic reticulum-based calcium (likely through inositol triphosphate receptors) to disassemble ectoplasmic specialization actin filaments. I added to this work by demonstrating for the first time that rac1 a small GTPase is also a component of ectoplasmic specializations. Although not yet demonstrated, I hypothesize that it's function is to recruit PIP_2 synthesis machinery.

This work also gives the first tangible reason for the ectoplasmic specialization's structure. Until this study there was no evidence supporting a reason for a cisternae of endoplasmic reticulum to be present at ectoplasmic specializations. The PLCγ/ PIP_2/gelsolin actin disassembly cascade provides a reason, that is, regulation of local levels of calcium. Future experiments should continue the investigation into rac1's function at ectoplasmic specializations. In addition to this the identification of PI(4)-P5 kinase at ectoplasmic specializations should be pursued.

Tubulobulbar complex project

Spermatogenesis importance

Due to their size, location and transient nature, tubulobulbar complexes have been a largely ignored structure in the testis, in fact there are only 27 papers that mention these structures. The data presented in this thesis offers some of the first molecular based work done on this structures and provides evidence for their possible function in junction component endocytosis.

Tubulobulbar complexes are structures that form at sites previously occupied by ectoplasmic specializations. Based on their location and the junctional components that I have found associated with them, I provided preliminary evidence consistent with the hypothesis that tubulobulbar complexes may be structures involved in ectoplasmic specialization disassembly and detachment of adjacent cells. The hypothesis that tubulobulbar complexes are involved in junction component endocytosis is corroborated by the finding of PKCα, a calcium-dependent protein associated in junction protein

endocytosis in other systems (Ng et al., 1999; Le et al., 2002), in the same general area as tubulobulbar complexes. The PKCα finding suggests a possible role for the structure of the tubulobulbar complexes, with the endoplasmic reticulum distal to the filamentous actin region. This endoplasmic reticulum may provide calcium to the PKCα for distal tubulobulbar complex tip endocytosis. In addition to this both the calcium-dependent and -independent actin-associated proteins gelsolin and non-muscle cofilin are associated with tubulobulbar complexes. Even though the function of these proteins has yet to be determined at tubulobulbar complexes, there exists a number of possibilities. For gelsolin two possibilities exist. It may function to disassemble the filamentous actin encircling the large tubular shaft of the tubulobulbar complex, by using the calcium from the endoplasmic reticulum of the tubulobulbar complex, or it may work to cap the ends of the actin filaments in order to prevent it's premature disassembly. Irrespective of the function of the gelsolin at these structures, with regard to cofilin at these complexes, only one possibility exists, actin disassembly. This protein is only found at tubulobulbar complexes in the seminiferous epithelium, not ectoplasmic specializations suggesting that the tubulobulbar complexes are not only modified forms of ectoplasmic specializations but rather, they are distinct structures which may use cofilin to disassemble their associated filamentous actin, independently of gelsolin. In the future, the use of PKC activating and inhibiting drugs should be used to try to elicit tubulobulbar complex endocytosis. Also the use of non-muscle cofilin transgenic mice should be explored to see if there is a defect in junction turnover and spermatid release.

General ectoplasmic specialization disassembly hypothesis:

By coupling both the gelsolin and tubulobulbar work together, a general hypothesis can be formed for ectoplasmic specialization disassembly. In this hypothesis at both apical and basal ectoplasmic specializations, an unknown signal triggers Rac1 to stop the recruitment and activation of the PIP_2 synthesis enzyme PI4(5)-kinase at the Sertoli cell plasma membrane. At this point PLCγ is triggered through another unknown mechanism to hydrolyze PIP_2 thereby converting it to inositol triphosphate (InsP3) and diacylglycerol (DAG), releasing gelsolin. Upon the InsP3 triggering InsP3 receptors in the endoplasmic reticulum membrane, calcium is released. This calcium activates gelsolin to sever the majority of the actin filaments of the junction plaque. While this is occurring, tubulobulbar complexes form and the actin associated with them is rearranged from hexagonal arrays to a meshwork. This actin is disassembled either by gelsolin or non-muscle type cofilin. Finally, the membrane adhesion proteins eventually make their way towards the tubulobulbar complexes where PKCα elicits their endocytosis.

Cell Biology importance; Kinesin project

In the context of the significance of this study with regard to general cell biology, the evidence presented here is the first, which implicates a kinesin in transporting an entire cell, a spermatid. Also, the stage-specific transport of the ectoplasmic specializations may prove to be a useful tool in studying the regulation of kinesins. In addition to this, this work may provide insight into a new role for Rab6KIFL. Because cytokinesis does not occur in Sertoli cells, and the Rab6KIFL Golgi localization has only been observed by one laboratory, the role for Rab6KIFL in Sertoli cells may prove to be

an exciting new function for this protein. The interest in this protein would be significantly increased if it were found at this intercellular adhesion junction. This could be accomplished by generating antibodies to the entire protein or to specific sequences.

Cell Biology importance; Gelsolin/PIP_2/PLCγ Project

Ectoplasmic specializations in the testis provide easily identifiable, and relatively easily concentrated sites for the general study of gelsolin's functions *in vivo*. The presence of all necessary gelsolin associated components at ectoplasmic specializations (gelsolin, PIP_2, endoplasmic reticulum calcium and actin filaments) will likely prove important since it is the only site identified to date known to contain all of these essential components, conveniently found in a single structure. In addition to this, because the process of spermatogenesis is a cyclical naturally occurring event, and because the exact stages when the junction plaques disassemble are known, the prediction of gelsolin associated events should correlate stage specifically.

Cell Biology importance; Tubulobulbar complex project

Due to their ease of separation (fragmentation of the testis) and their stage specific formation, tubulobulbar complexes may provide researchers with a model system for studying endocytosis of adhesion junction components *in vivo*, possibly recycling and degradation of junction proteins and perhaps actin rearrangement. In addition to this, I show in this study that tubulobulbar complexes can be visualized by DIC microscopy. There is also some evidence (Hadley et al., 1985) that basally located ectoplasmic specializations can be generated in culture. This may provide another tool for junction

protein recycling and actin remodelling research if the formation of tubulobulbar complexes can be elicited.

Summary of Major Contribution of this Study

There are three important contributions that my work has provided. The most significant contribution that this study offers is insight into ectoplasmic specialization disassembly. Until now we have not understood how this incredibly stable junction plaque can disassemble and why it contains a cisternae of endoplasmic reticulum (Vogl and Soucy, 1985). These questions can be explained through my discovery of a PLCγ/PIP_2/gelsolin disassembly mechanism. The second important finding is that tubulobulbar complexes are structures that likely play a role in ectoplasmic specialization turnover. Finally the finding that the kinesin-like proteins Rab6KIFL and Kin3 may be associated with the ectoplasmic specialization provides potential candidates involved with spermatid entrenchment.

Future Directions

Kinesin project:

Future experiments should include raising antibodies to both kin3 and Rab6KIFL. The anti-kin3 antibodies should be raised against the 2 peptide sequences analyzed by mass spectrometry (TVAATNMNETSSR and ANSTGATGARLK) as well as to other unique regions of the protein. These antibodies should be immunolocalized in the testis and, depending on the result, followed by either the generation of site-directed knock-out mice or knocking down the protein(s) using RNAi.

Gelsolin and rac1 project:

The gelsolin/rac1 project should be continued using recombinant forms of both active and inactive rac1 protein. This protein should be added to isolated spermatid/ectoplasmic specialization complexes and assayed under time lapse microscopy in the presence of fluorescently labeled actin monomers to see if it's addition induces actin filament lengthening. In addition to this, the isolated spermatid/ectoplasmic specialization complexes should be incubated with both active and inactive rac1 protein. The spermatids should then be pelletted in a manner similar to how I prepared the gelsolin treated supernatants, then assayed using western blotting for both gelsolin and actin protein levels. This would indicate whether rac1 can in fact trigger plaque disassembly, or gelsolin release from filament ends. In addition to this, using RNAi techniques, a rac1 knock-down in the testis using back perfusion through the rete testes may also provide valuable information.

Tubulobulbar complex/endocytosis project:

The future directions that the tubulobulbar complex project should pursue include experiments in which under time-lapse microscopy, recombinant active PKCα is added to isolated late step spermatid/tubulobulbar complexes to see if vacuole formation can be elicited. Also, using an 18°C temperature block of the material (in our case isolated testis and/or late spermatids with Sertoli cell regions containing tubulobulbar complexes) should be performed to investigate whether endocytosed proteins can be accumulated in early or sorting endosomes (although, here, time course may be a factor). Additionally, the vesicular structures should be studied to investigate whether recycling or degradation pathways are used following internalization. To do this, the pH of the vesicles could be tested and antibodies to EEA1 (early endosome antigen 1), rab5 (another marker for early endosomes) and rab7 (a marker for late endosomes) should be colocalized with the internalized proteins to be able to begin to describe if these proteins are destined for recycling or degradation.

Cofilin project:

The cofilin project should be pursued using Sertoli cell specific knock out mice or RNAi to diminish the function of non-muscle cofilin. This should allow for the investigation of deficits in spermatid release from Sertoli cells. Additionally, the role of muscle-specific cofilin should be pursued, initially using muscle-specific cofilin antibodies that react on testis material.

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