β1-INTEGRIN AND CADHERIN MEDIATED SIGNALING MOLECULES IN THE RAT SEMINIFEROUS EPITHELIUM

by

DAVID J. MULHOLLAND

B.Sc. The University of British Columbia, 1996

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Anatomy)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December, 1999

© David J. Mulholland, 1999

ABSTRACT

The aim of this study was to assess whether β1-integrin mediated signaling molecules, known to be present at cell-matrix adhesion sites in other cell types, are present at unique cell/cell junctions in the seminiferous epithelium, called ectoplasmic specializations (ESs). To address this question several immunological techniques were used including Western blotting, indirect immunofluorescence and immunoprecipitation. Western blotting on whole epithelial lysates verified the specificity of each immunoprobe while immunofluorescence studies determined the spatial and temporal expression of each molecule during spermatogenesis and showed whether or not integrin related molecules were detectable at cell/cell junctions. Markers known to be specific for filamentous actin, which is concentrated at ESs, were used to counterstain immunological probes and serve as indicators for the location of the junctional plaques. Based on the specificity and codistribution of molecules observed in tissue sections and isolated cells, lysates of seminiferous epithelia were assessed for stable protein complexes using co-immunoprecipitation. Lysates used for immunoprecipitations contained only a population of seminiferous epithelium and did not contain myoid cells or the basement membrane component of the seminiferous tubule. β1-integrin and Integrin Linked Kinase (ILK) were shown, at the light level, to be spatially and temporally associated, throughout spermatogenesis, with sites known to contain ESs. These molecules co-distributed with the exception of some ILK staining associated with basal germ cells. Integrin staining appeared restricted to junctional areas known to contain ESs. ILK precipitates probed with β1-integrin antibodies showed a specific band at 140 kD. β1-integrin precipitates showed the presence of a specific band at about 55 kD when non-conventional detection techniques were used. Pan-Cadherin antibodies showed a direct protein interaction with β-catenin and showed punctate immunofluorescence staining that was restricted to the Sertoli/Sertoli cell interface.

Ultrastructural data indicates that these molecules are concentrated at desmosome-like plaques and not ESs. Staining for these molecules was not detected at the Sertoli cell/germ cell junction at any point during spermatogenesis. Focal adhesion Kinase (FAK), a known integrin associating molecule, did not codistribute with the integrin at the light level. Rather, it was restricted to the cytoplasm of early germ cells. Additionally, these molecules did not co-immunoprecipitate in whole epithelial lysates. The absence of detectable FAK at junctional sites prompted the question of whether another major tyrosine related protein is present. Polyclonal antibodies against phosphotyrosine containing proteins showed an abundance of expression throughout the epithelium at both ESs and other cell/cell borders. Paxillin was shown to be present abundantly throughout both the cytoplasm of germ cells and Sertoli cells. Additionally, it appeared to be concentrated at tubulobular complexes. FAK and vinculin, proteins both known to have binding domains with paxillin, co-immunoprecipitated with paxillin in whole epithelial lysates. Possible locations of a direct interaction for these molecules were determined by light level co-distribution. A paxillin/vinculin complex would likely be found at the tubulobulbar complexes, the only location where both molecules were observed to be concentrated. A FAK/paxillin complex, however, would likely be located in the basal germ cells, which is the only location where these molecules were shown to be co-distributed. Tyrosine phosphorylation studies suggest the presence of at least five major bands in blots of whole cell lysates. FAK is not phosphorylated at tyrosine while paxillin is highly phosphorylated.

Together, these data provide support for the generation of a model showing the presence of molecules known to be fundamental to cell adhesion and signaling. Possible direct protein complexes occurring at junctional sites, and other locations within the epithelium, are based on the distribution of immunofluorescence, co-immunoprecipitations and known interactions that occur in other epithelial systems. Molecules that are detected at junctional sites likely have direct implications for sperm release and architectural changes in the blood-testes barrier.

TABLE OF CONTENTS

RESULTS

A. Immunofluorescence Localization of Signaling Molecules

B. Western Blots and Immunoprecipitations

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
EDTA	Ethylene Diamene Tetra Acetic Acid
EGTA	Ethylene Glycol-bis (β-Amino Ethyl Ether)-N,N,N',N'-Tetra Acetic Acid
ES	Ectoplasmic Specialization
FAK	Focal Adhesion Kinase
HRP	Horse Radish Peroxidase
IgG	Immunoglobulin
Ij	Intercellular Bridge
ILK	Integrin Linked Kinase
KCl	Potassium Chloride
KH2PO4	Potassium Phosphate
KOH	Potassium Hydroxide
MgCl2	Magnesium Chloride
Na2HPO4	Sodium Phosphate
NaCl	Sodium Chloride
NP-40	Nonidet P-40
PBS	Phosphate Buffered Saline
PIPES	Piperazine-N,N'-bis (2-Ethersulfonic Acid)
PVDF	Polyvinylidene Fluoride (PVDF)
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis
S/Gj	Sertoli Cell/Germ Cell junction
S/Sj	Sertoli Cell/Sertoli Cell junction
TPBS	Tween Phosphate Buffered Saline
Tris-HCl	(Tris[hydoxymethyl]aminomethane)-Hydro Chloride
Tb	Tubulobulbar Process
Tween-20	Polyethylene Sorbitan Monolaurate

ACKNOWLEDGEMENTS

INTRODUCTION

A. Organization of the seminiferous epithelium and spermatogenesis

The seminiferous tubules are lined by a complex stratified epithelium consisting of two main cell types. Sertoli cells are a non-dividing cell population that extend from the basement membrane to the lumen of the seminiferous tubule. The spermatogenic cells are a proliferating population of cells that includes cells at different stages of differentiation (Fig. 1). Spermatogenesis is often divided into three phases including: (1) the spermatogonial phase; (2) the spermatocyte phase (meiosis) and (3) the spermatid phase (spermiogenesis).

The most immature cells of the seminiferous epithelium are located near the basement membrane and move apically as they proliferate and differentiate. These cells form four to five concentric layers which represent different generations of developing spermatogenic cells. Spermatogenic cells are morphologically distinct during their differentiation and are identified as spermatogonia, spermatocytes or spermatids. Spermatogonia are derived from stem cells found at the base of the epithelium and undergo several mitotic divisions while changing slightly in morphology. These cells divide to produce the larger spermatocytes which then divide meiotically into round spermatids. These haploid cells subsequently undergo dramatic morphological differentiation to ultimately produce mature spermatozoa (Fig. 1) (Fawcett, 1975a; Russell, 1977).

B. Cell-Cell junctions in the seminiferous epithelium

During spermatogenesis early round spermatids translocate towards the tubule lumen and then become situated within Sertoli cell crypts. As germ cell development

proceeds the crypts change in shape to accommodate the elongating spermatid which eventually undergoes spermiation, or release into the seminiferous tubule lumen (Russell, 1994). Throughout this process sites of actin based cellular adhesion exist both between Sertoli cells and elongating germ cells, Sertoli cell/germ cell junctions or S/Gj, and between the base of adjacent Sertoli cells, Sertoli cell/Sertoli cell junctions or S/Sj (Russell and Peterson, 1985) (Fig. 2).

Figure 1. Histological organization of the seminiferous epithelium in the rat. Germ cells undergo dramatic morphological change as they translocate from the basal compartment to the adluminal one and become situated in the Sertoli cell crypt. Morphologically mature spermatids undergo spermiation and are released into the tubule lumen (adapted from Vogl, 1989).

ADLUMINAL
COMPARTMENT

Morphologically Mature
Spermatid

Cytoplasmic Lobe
of Germ Cell

Germ Cell
Cytoplasm

Elongate
Spermatid

Sertoli Cell

Sertoli Cell
Crypt

Spermatocyte

Acrosome

Spermatogonia
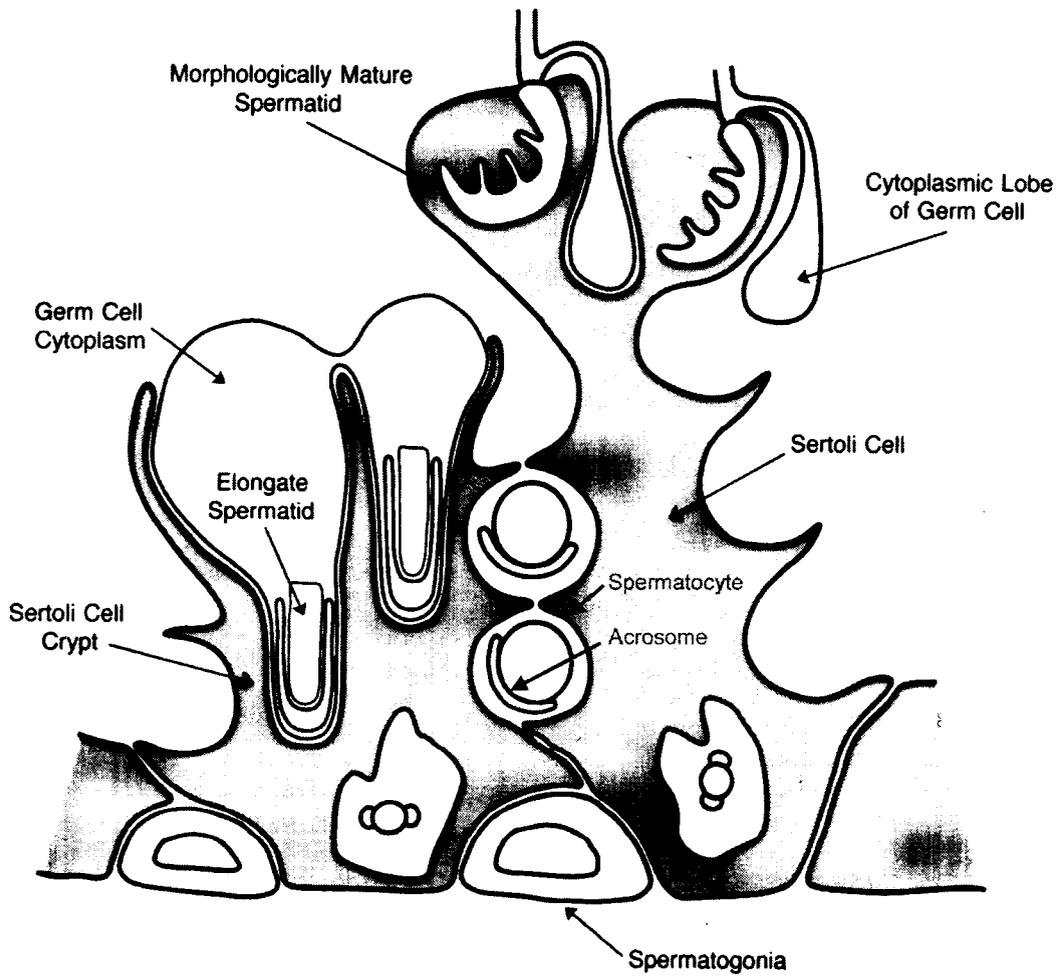
BASAL COMPARTMENT

Figure 2. Major concentrations of filamentous actin in the rat seminiferous epithelium. Ectoplasmic Specializations (ESs) occur in two locations including the Sertoli/Germ cell junction (S/Gj) and the Sertoli/Sertoli (S/Sj) junction. Developing ESs also contain junctional actin in close association with the acrosome as seen in early round spermatids. Non-junctional sites of actin include the tubulobulbar processes, intercellular bridges and subacrosomal actin (adapted from Vogl, 1989).

Tubulobulbar
Process Actin

Sertoli/Germ Cell
Junctional Actin

Intercellular Bridge
Actin

Acrosomal
Actin

Adluminal
Compartment

Developing
Junction

Sertoli/Sertoli
Junctional Actin

Basement
Membrane
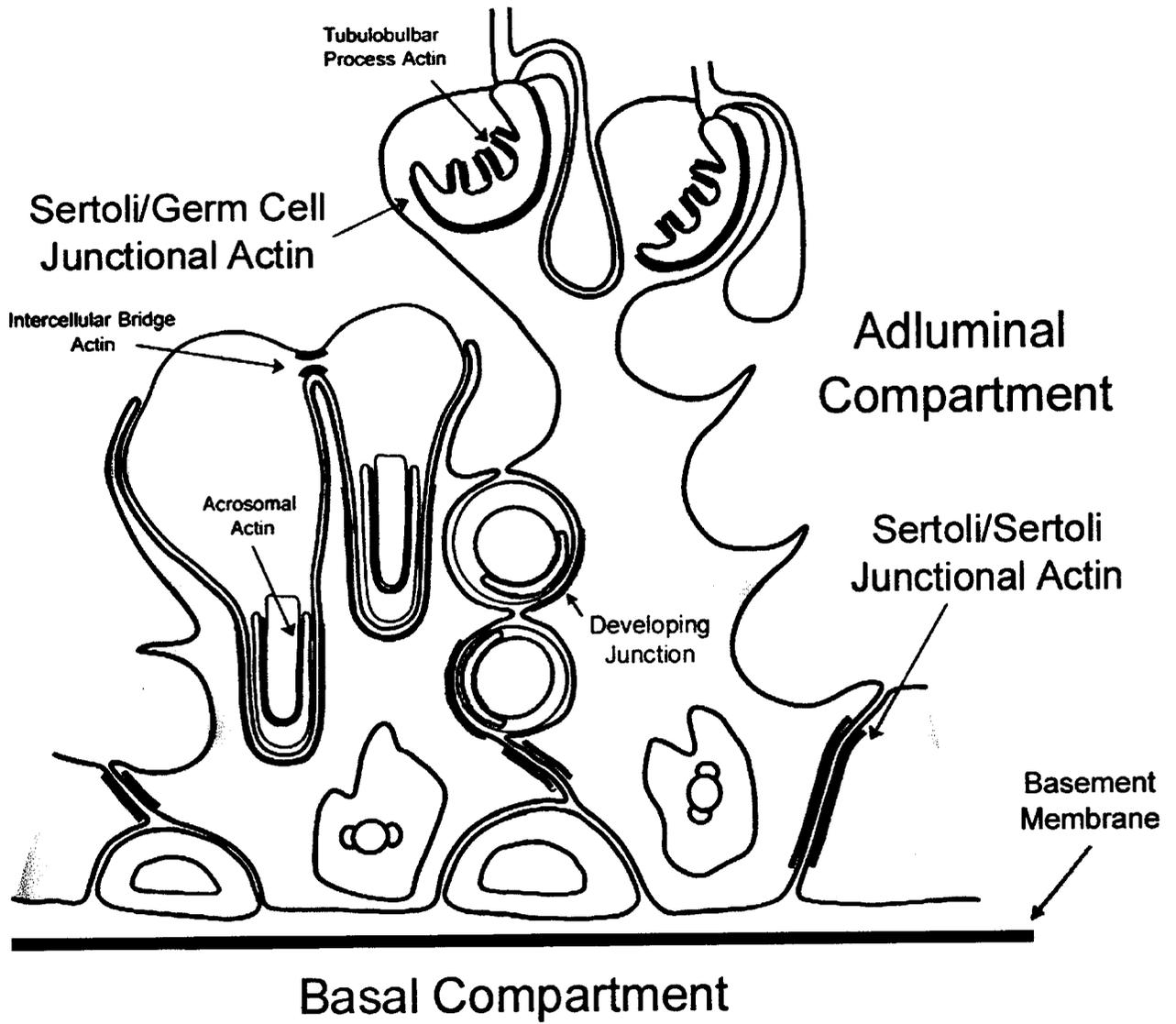
Basal Compartment

The Sertoli cell junctional complex found at the base of the epithelium is morphologically distinct and is not found in any other type of epithelia. This complex functions to divide the epithelium into two compartments including a basal compartment and an adluminal compartment. The basal compartment contains the spermatogonia and younger spermatocytes while the adluminal compartments contains older spermatocytes and all spermatids. During the first meiotic division of spermatocytes, these cells pass through the "blood-testes" barrier and into the adluminal compartment while at the same time maintaining the structurally integrity of the barrier. The Sertoli-Sertoli junctional complex is composed of: (1) tight junctions, (2) gap junctions, (3) desmosome-like junctions, and (4) ectoplasmic specializations or ESs (Russell, 1976; Vogl , 1989). The tight junctions constitute what is called the "blood-testes" barrier, a structure with important immunological functions in the testes. ESs form at the interface between the Sertoli cell and germ cells and is morphologically similar to the ES which occurs at the Sertoli-Sertoli interface. Both the apical (Sertoli/germ cell) and basal (Sertoli/Sertoli) ectoplasmic specializations are thought to function primarily for cellular adhesion (Vogl, 1996).

C. Other organized concentrations of actin filaments: intercellular bridges, tubulobular complexes and subacrosomal actin

In addition to actin associated with ectoplasmic specializations, actin is also concentrated at other major sites in the seminiferous epithelium (Figure 2). Intercellular bridges appear as distinct tubular structures between spermatogenic cells which form as a result of incomplete cytokinesis, ultimately linking the cytoplasm of many spermatids. The function of these structures is not certain but it has been hypothesized that they play a role in maintaining synchrony of spermatogenesis (Fawcett, 1975a).

Tubulobulbar complexes are composed of many finger-like processes which project into the Sertoli cell cytoplasm from the spermatid. Actin filaments occur in the Sertoli cell cytoplasm surrounding the processes and are thought to function as anchoring devices for the spermatids to the Sertoli cells (Russell and Clermont, 1976). They are also thought to be involved in the recycling of junctions at locations of cellular adhesion during spermiation (sperm release). In the rat tubulobulbar processes are particularly prominent in late stage spermatids (Russell and Clermont, 1976.

Actin found in the subacrosomal space changes in distribution during spermatogenesis and tends to spread over the nucleus as the acrosome does during germ cell differentiation (Fig. 2). Interestingly, subacrosomal filamentous actin is more prominent in the rat during early germ cells stages and eventually disappears in later ones. The function of subacrosomal actin has been thought to be involved in the capping process (Russell et al., 1986).

D. Rationale for presence of β1-integrin mediated signaling molecules in rat seminiferous epithelium

As developing spermatids elongate ectoplasmic specializations are formed in Sertoli cells adjacent to spermatid heads. This site of adhesion between the germ cell and Sertoli cell is disassembled as part of the process by which spermatids are released into the tubule lumen (Russell, 1977; Vogl, 1989). Ectoplasmic specializations are also thought to be important in the maintenance of adhesion between neighbouring Sertoli cells (Vogl 1989; Vogl et al., 1991) at the base of the epithelium. Like apical ectoplasmic specializations the structural components of the "blood-testes barrier" are structurally dynamic and undergo cyclic disassembly and reassembly as primary spermatocytes pass into the adluminal compartment (Russell, 1977).

Although much is known about the ultrastructure of ESs in the seminiferous epithelium, comparatively little is known about their molecular composition and their control. It is known, however, that ectoplasmic specializations are dissimilar to other intercellular junctions in that they do not contain the usually composition of adhesion molecules but, nevertheless, contain several known adhesion associated proteins including α-actinin (Franke et al., 1978, Russell and Goh, 1988), and vinculin (Grove et al., 1990; Pfeiffer et al., 1991). While proteins such as these are present, reports of other adhesion molecules have been unclear. For instance, some investigators have suggested the presence of N-CAM at junctional complexes in the seminiferous epithelium grown in culture but their absence in vivo by postnatal day 15 (Li et al., 1998).

Figure 3. (a) Rationale for the presence of integrin related molecules at cell adhesion sites in the seminiferous epithelium. The presence of integrins at cell-cell borders in the seminiferous epithelium, and the absence of other cell-cell adhesion related molecules, suggests that integrin related molecules, such as those found in well-defined cell-matrix attachment sites, may be present at ectoplasmic specializations. Integrin activation, or clustering, at a cell/matrix attachment sites is shown with molecules that associate with the integrin cytoplasmic domain and include Src Kinase, Focal Adhesion Kinase (FAK), talin, paxillin, vinculin and p130^{CAS} (Giancotti and Ruoslahti, 1999). **(b)** A speculative model for the presence of β1-integrin mediated signaling molecules at ectoplasmic specializations in the rat seminiferous epithelium. β1-integrins have been shown to be present in the seminiferous epithelium and concentrated at ESs, but few other integrin related molecules have shown to be present. In other systems protein-protein interactions have been shown between the β1-integrin and ILK. FAK has been shown to associate with many molecules capable of signaling events including paxillin, a vinculin binding protein. These interactions are thought to be mediated by motifs containing phosphotyrosine which are critical to intermolecular interactions.

a)

Matrix

Integrin

Actin Cytoskeleton

b)

NH₂   FAK   COOH

ILK

β1

P-Tyr   Paxillin

Vinculin

Actin Cytoskeleton
(Ectoplasmic Specialization)
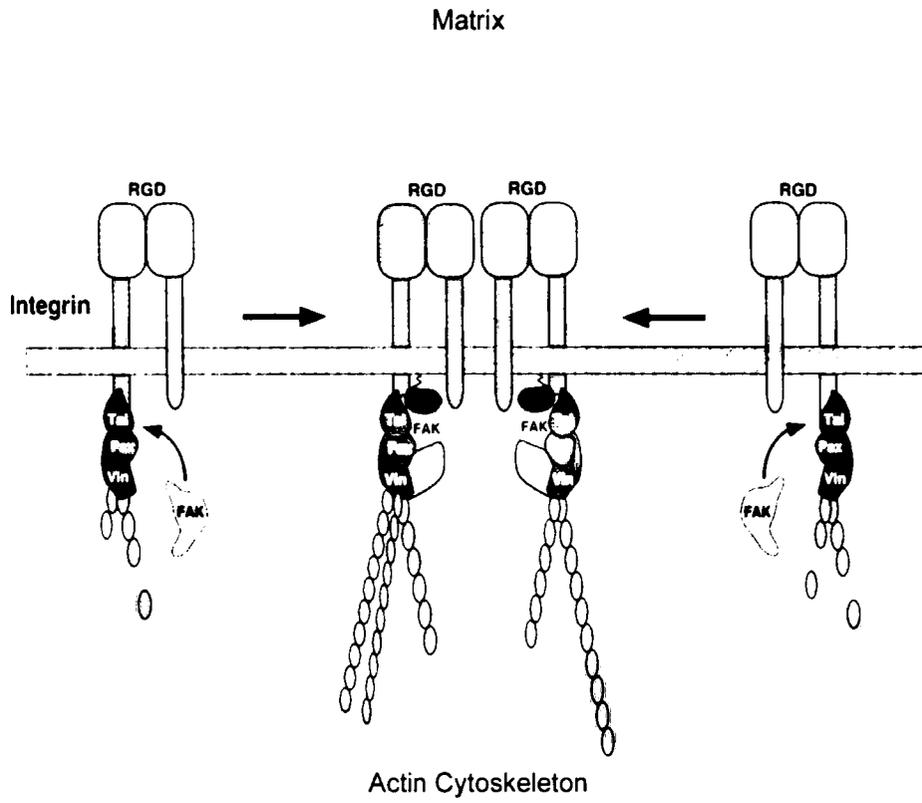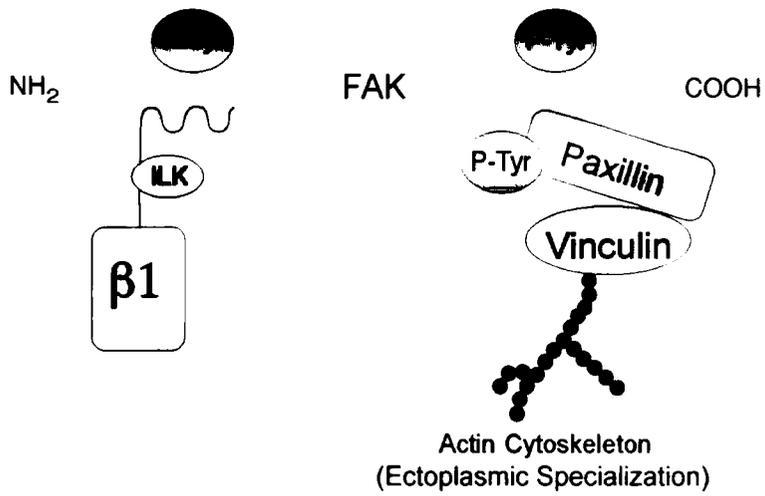
Surprisingly, the only transmembrane adhesion molecule to be identified in vivo both at apical and basal attachment sites is $\beta 1$ integrin (Pfeiffer et al., 1991; Palombi et al., 1992; Salanova et al., 1995 Salanova et al., 1998) and an associated $\alpha 6$ integrin subunit (Salanova et al., 1995, Salanova et al., 1998). Although the $\alpha 6\beta 1$ integrin complex has been previously thought to be mainly a laminin receptor (Sonnenberg et al., 1988), reports have suggested that it may function on the egg surface as a sperm receptor and therefore be involved in cell-cell interactions (Almeida et al., 1995). $\beta 1$-integrins have also been implicated with white blood cells and their migration. Specifically, integrins on the cell surface of eosinophils are thought to interact with ligands on endothelial and epithelial cells (Seminario & Bochner, 1997). Further support for a functional role for integrins in the seminiferous epithelium is based on the disappearance of integrin expression concurrent with the turnover of ESs (Salanova et al., 1995). As spermatocytes translocate from the basal to the adluminal compartment, integrin expression disappears and then subsequently reappears (Salanova et al., 1995). Also, the disappearance of integrin expression has been shown to coincide with disassembly of the apical ESs and spermatid release into the tubule lumen (Palombi et al., 1992). With reports of $\beta 1$ integrin expression coinciding with key spermatogenic events it is possible that integrin-related molecules could also show changing patterns of expression in the seminiferous epithelium. Further motivation for investigating potential signaling pathways associated with ESs comes from preliminary immunoflourescence and biochemistry work, using a pan-antibody for ERK, a serine/threonine extracellular signal regulated kinase. These experiments showed that ERK is localized around the sperm head and is expressed in protein samples that are enriched for ESs.

Integrin mediated signaling molecules (Fig. 3) are often associated with cytoskeletal changes and have been found to be vital for development and maintenance of normal cellular structure and function (Hynes, 1992). Many researchers have shown that integrins are not only able to mediate signals between the extracellular matrix and cells (Clark and Brugge, 1995; Yamada and Miyamoto, 1995; Geiger et al., 1995; Schwartz et al., 1995; Parsons, 1996), but also between adjacent cells (Geiger et al., 1995; Kirkpatrik and Peifer, 1995; Gumbiner, 1996). Although phosphorylation is one of the earliest events associated with integrin activation or clustering (Burridge et al., 1992), there is growing evidence to suggest that the relationship between cell adhesion formation and phosphorylation events may occur independent of each other. Nevertheless there is also increasing evidence that β1-integrin subunits, a structural element found at focal adhesions, can associate, either directly or indirectly via their cytoplasmic domains, with molecules capable of phosphorylation (Burridge et al., 1992; Schoenwaelder SM and Burridge K, 1999). Phosphorylation events have also been shown to be β1-integrin mediated and have been suggested to occur in either an "outside-in" or an "inside-out" manner and are thought to be closely associated with changes in the actin cytoskeleton (Dedhar and Yamada, 1996), This suggests that initiation of signaling events can come from an extracellular ligand or a cytoplasmic protein.

A number of kinases have been shown to associate with integrins in vitro of which include Focal Adhesion Kinase (FAK) (Schaller and Parsons, 1994; Schaller et al., 1995) and Integrin Linked Kinase (ILK) (Hannigan et al., 1996). These submembranous molecules are thought to be closely associated with integrin cytoplasmic tails and to mediate signals to the actin cytoskeleton. FAK, a tyrosine kinase, has been shown to interact, in vitro, with β1. Specifically, it becomes phosphorylated, at tyrosine residues, during integrin activation, and has been shown to induce changes in other signaling

proteins (Schaller and Parsons, 1994, Schaller et al., 1995).   Interestingly, FAK has never been shown to associated with β1-integrin cytoplasmic tails in vivo or in non-cultured cells. This is unlike the situation with ILK, a serine-threonine kinase, which has been shown to have a strong in vivo association with β1, β2 and β3 integrin cytoplasmic domains (Hannigan et al., 1996). Classic signaling molecules downstream of ILK and FAK include vinculin and paxillin. These proteins are thought to have roles in regulation of both adhesion and downstream signaling events and have, therefore, been termed linking or docking proteins to which other components of the actin cytoskeleton can associate (Yamada and Geiger, 1997).

Even though the assembly of many proteins associated with adhesion plaques depend on the ability of FAK to form phosphorylation sites on other proteins, it is very interesting that tyrosine phosphorylation has been shown not to be necessary for the gathering of structural components of focal adhesions (Gilmore and Romer, 1996). When levels of phosphotyrosine levels are enhanced the formation of focal adhesions and adhesion plaques is enhanced while decreased levels of tyrosine phosphorylation can inhibit focal adhesion formation (Burridge and Chrzanowska-Wodnicka, 1996). It appears that despite the importance of tyrosine phosphorylation in the formation of adhesion sites the structural elements of focal adhesions, the phosphorylation of their structural elements, including vinculin and paxillin, is not necessary for adhesion plaque formation. Evidence suggests that molecular regulation by tyrosine phosphorylation is upstream of such focal adhesion elements. Possible protein tyrosine kinases upstream of Rho A, a small GTPase, could have the ability to regulate its activity and the subsequent regulation of Rho guanine nucleotide exchange factor (Nobes et al., 1995). The importance of tyrosine phosphorylation, however, is underscored when considering the number of conditions which can promote dephosphorylation of adhesion sites including many growth factors such

as insulin and platelet derived growth factor. Nevertheless, in either instance of phosphorylation or dephosphorylation it appears that the fundamental phosphorylation event is upstream of Rho A. Both stress fibers and focal adhesions are regulated by Rho-A, a GTP-binding protein (Knight et al., 1995). Further interest in the Rho family stems from the fact that of the molecular composition induced by activation of the small GTPases, focal adhesions, appear to resemble ectoplasmic specializations the closest of any cultured form of cellular adhesion.

Although the tyrosine phosphorylation of the structural elements of adhesion plaques does not appear to be necessary for the formation of these sites, increased levels of tyrosine phosphorylation, especially by non-receptor tyrosine kinases such as Src and FAK, have been implemented with turnover with adhesion plaques (Schoenwaelder and Burridge, 1999). For example, cells which are transformed with v-src show disrupted focal adhesions and elevated tyrosine phosphorylation levels of many structural elements of focal adhesions including integrins, vinculin and also regulatory components including FAK and paxillin (Burridge and Chrzanowska-Wodnicka, 1996). Besides protein interactions which have been shown between FAK and many focal adhesion elements its importance in adhesion turnover is further supported when considering FAK deficient cells. Interestingly, in FAK deficient cells there is increased formation of focal adhesions but reduced motility (Ilic et al., 1995). Similar results have been observed in dominant-negative constructs where FAK expression is repressed (Gilmore and Romer, 1996). Conversely, over expression of FAK results in increased cell migration and decreased formation of adhesion plaques. FAK knock-out mice are lethal during embryonic development but show a phenotype which is similar to that of FAK deficient cells grown in culture (Ilic et al., 1995).

To date, very little is known about the presence and distribution of molecules capable of signaling in the seminiferous epithelium. In this study, using Western blotting, conventional immunofluorescence, immunoprecipitation and immunoelectronmicroscopy we explore the possibility that key members of classically defined β1-integrin signaling pathways are present at cell-cell junctions (ectoplasmic specializations) in the seminiferous epithelium as a prerequisite to determining their role in events such as sperm release and turnover of the basal junctional complex. In addition, we conjecture whether or not cadherins are components of ectoplasmic specializations and therefore a defining feature of their turnover. Together our results have implications for signaling events involved in sperm release and turnover of the "blood-testes" barrier.

E. Statement of hypothesis

The aim of this study was to determine whether classical β1-integrin mediated signaling molecules and complexes found at sites of cellular adhesion in other epithelial systems are present at ectoplasmic specializations in the adult rat seminiferous epithelium. *If ectoplasmic specializations are similar to other forms of cellular adhesion, then ß1-integrins and related signaling molecules should be spatially and temporally co-distributed with markers that are specific for the actin component of these junctions.*

METHODS AND MATERIALS

A. Animals

Tissue for all experiments was obtained from adult Sprague-Dawley rats that ranged in weight from 250 g to 550 g. Animals were anaesthetised using halothane administered via the respiratory tract. Testes were removed and rats were euthanized while under deep anaesthesia.

B. Reagents

Most reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada). Fluorescent phallotoxins were obtained from Molecular Probes (Eugene, OR) and paraformaldehyde was obtained from Fisher Scientific (Vancouver, BC, Canada).

C. Preparation of Tissue for Immunostaining

Testes were perfusion fixed, via the spermatic artery, with 3% paraformaldehyde in phosphate buffered saline (PBS) (150 mM NaCl, 5 mM KCl, 3.2 mM Na_2HPO_4, 0.8 mM KH_2PO_4, pH 7.3, 33 °C) for 30 min. Following fixation, testes were perfusion washed for an additional 30 min. with PBS. Both fixative and wash solutions were filtered using 0.22 μm Millipore filters and warmed to 33 °C prior to perfusion.

After fixing, the testes were frozen in OCT compound (Fisher Scientific) and then equilibrated to chamber temperature in a cryostat (Brights 5010). For immunofluorescence studies, sections were cut to a thickness of 10 μm, collected on polylysine coated slides, and then immediately treated with −20 °C acetone for 10 min. Sections were then air-dried for 10 min. For immunostaining with the probes for β1-integrin

and ILK, testes were immediately frozen after excision from the animal; that is, the tissue was fresh frozen without prior fixation.

D. Immunofluorescence

Prior to immunostaining, tissue was treated for 30 min. with 5% normal goat serum in TPBS (0.1% BSA, 0.1% Tween-20, PBS) to minimize nonspecific binding of secondary antibodies. Incubation times with primary antibodies were either 1-2 hours at 37°C or overnight at 4 °C. Primary antibodies were used at the following dilutions: polyclonal anti-β-catenin (Sigma, cat. C2206) at 1:1000; monoclonal anti-β-catenin (Sigma, cat. C7207) at 1:400; polyclonal anti-Pan Cadherin (Sigma, C3678) at 1:1000; monoclonal anti-Pan Cadherin (Sigma, C1821) at 1:400; serum anti-β1 integrin (Chemicon International, cat. AB1952) at 1:200, polyclonal anti-ILK (gift of Dr. Shoukat Dedhar, University of British Columbia) at 1:100, monoclonal anti-FAK (Transduction Laboratories, cat. F15020) at 1:50, monoclonal anti-Paxillin (Transduction Laboratories, cat. F15020) at 1:400, monoclonal anti-Vinculin (Sigma, cat. V9131) at 1:400 and polyclonal anti-phosphotyrosine (Transduction Laboratories, cat. P11230) at 1:100. After primary antibody staining, tissue sections were washed 3 times (10 min. each wash) with TPBS and then incubated with secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA) for 1 hour at 37°C at a 1:100 dilution. After secondary antibody staining, sections were washed 3 times (10 min each wash) with TPBS and then mounted with Vectashield (Vector Laboratories, Burlinkgamem, CA) and a coverslip. In some cases the second wash included rhodamine or fluorescein Phalloidin to specifically label f-actin structures. Slides were observed and photographed using a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NJ).

E. Tissue preparation for gel electrophoresis and immunoblotting

Testes were decapsulated (see appendix II) and the seminiferous tubules put in a glass petri dish containing cold PEM (80.0 mM PIPES, 1.0 mM EGTA, 0.1 mM $MgCl_2$, 250 mM sucrose, pH 6.8 with KOH). Tissue was minced with two scapel blades decapsulated in cold PEM containing a cocktail of protease inhibitors (0.5 μg/ml Leupeptin, 0.5 μg/ml Pepstatin, 0.5 μg/ml aprotinin, 0.1 mM PMSF), for a maximum of 60 min. at 4 °C. Using fine dissecting tools and a Zeiss Dissecting Microscope fitted with dark-field condenser (Don Mills, Ontario, Canada) epithelia were separated from tubule walls by securing one end of the tubule and using another probe to squeeze the tubule contents out towards the other end. Ejected epithelia were collected with a micropipette and placed in fresh buffer on ice.

Epithelia were lysed both under denaturing (Western blots of whole cell lysates) and native (all co-immunoprecipitations) conditions. Denaturing conditions included 30 min. of NP-40 lysis buffer (30 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxylacholate, 0.1% SDS, 2mM EDTA) followed by boiling and 2 sec. of sonication at 60 MHz. Native conditions was with NP-40 lysis buffer (30 mM Tris, pH 8, 150 mM NaCl; 1% NP-40, 10% glycerol, 2mM EDTA) without sonication or boiling. Lysis buffers included a cocktail of protease inhibitors (0.5 μg/ml Leupeptin, 0.5 μg/ml Pepstatin, 0.5 μg/ml aprotinin, 0.1 mM PMSF and 0.1 mM Na Vanadate). Whole cells lysates, but not immunoprecipitated samples, were assessed for total protein using a bicinchoninic protein assay. Samples were standardized to approximately 1 mg/ml and a total of 15 μg of protein loaded per well during electrophoresis.

F. Gel Electrophoresis & Western Blotting

Tissue samples were then mixed with 2x SDS buffer (0.125 M Tris-HCl, pH6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), boiled at 100 °C for 5 min., put on ice and then loaded into gels. Tissue samples were electrophoresed on 7.5 %, 10% and 12 % SDS-PAGE (Laemmli, 1970) minigels and transferred to PVDF membranes for 4 hours or overnight. After transfer, membranes were washed twice in TBST (100 mM-Tris-HCl, pH 7.5, 0.9% NaCl, 0.1% Tween-20) (5 min each wash) and then blocked with 10% dried skim milk in TBST for either 1 hour at room temperature or overnight at 4 °C. Primary antibodies for Western blotting were used at the following dilutions: monoclonal anti-actin (1:1000), polyclonal anti-actin (1:1000), monoclonal anti-β-catenin (1:1000), polyclonal anti-β-catenin (1:5000); monoclonal anti-Pan-Cadherin (1:1000); polyclonal anti-Pan-Cadherin (1:5000); polyclonal anti-β1-integrin (Chemicon AB 1952) (1:1000), polyclonal anti-β1-integrin (G. Tarone, Italy) (1:10,000); monoclonal anti-ILK (1:500): polyclonal anti-ILK (1:1000), monoclonal anti-FAK (1:500), monoclonal anti-Paxillin (1:10,000), monoclonal anti-Vinculin (1:2000) and polyclonal anti-phosphotyrosine (1:1000). Following primary incubations membranes were washed 2 x 5 min. and 3 x 10 min. with TBST followed by 2 x 5 min. and 3 x 10 min. with TBS. Membranes were incubated either with anti-mouse-HRP (Santa Cruz Biotechnology, cat # sc-2031) or anti-rabbit-HRP (Santa Cruz Biotechnology, cat # sc-2030) at a dilution of 1:5000. Membrane bound antibodies were detected using chemiluminescence with ECL Western blotting detection agents (Amersham Life Science, cat. RPN 2106) for incubation periods of 1 min and then exposed immediately to radiographic film.

G. Electron Microscopy

Testes were perfused with warm (33 °C) fixative (80 mM PIPES, 1.0 mM EGTA, 1.0 mM $MgCl_2$, 0.5% gluteraldehyde, 3.0% paraformaldehyde adjusted to pH 6.8 with KOH) for 30 min. After perfusion, tissue was mixed by inverting the test tube and left at room temperature for 1 h. Cells were sedimented by centrifugation at 11,000 rpm in an Epindorf centrifuge and the supernatant was replaced with 0.1 M sodium cacodylate (pH 6.9). Samples were washed twice with fresh buffer and post-fixed for 1 h on ice in 0.1 M sodium cacodylate containing 1% osmium tetroxide. Samples were washed three times with dH_2O (10 min. each wash) and then dehydrated through a series of increasing concentrations of ethanol. Tissue was then embedded in resin (J.B. EM Services Inc.), sectioned followed by staining with uranyl acetate and lead citrate. Photography was done on a Philips 300 electron microscope.

H. Immunogold Labelling

Testes were perfused for 30 min. by the spermatic artery briefly with PBS followed by fixative (3% paraformaldehyde and 20 mM ethylacetimidate in PBS, pH 7.3). After perfusion the testes was cut into small pieces and immersion fixed (3% paraformaldehyde and 0.1% gluteraldehyde in PBS, pH 7.3) for an additional 60 min. After fixation, the tissue was washed three times with PBS and dehydrated through a series of increasing concentrations of ethanol. Tissue was then embedded in lowicryl KM4.

Tissue sections were collected on carbon/formvar coated nickel grids and treated for 5 min with a pre-block solution (0.01 M glycine, 0.1% BSA, 1:20 NGS and 0.05% Tween-20). Sections were incubated with primary cadherin antibodies (Sigma, cat. 1821) in

antibody solutions (0.01 M glycine and 1% NGS) at a dilution of 1:50 for 2 h at 37 °C. After

staining grids were washed three times (5 min each wash) with wash buffer (0.01 M glycine

and 0.1% BSA) and then incubated for 60 min at 37 °C with the secondary antibody

(Sigma, cat. G7652) diluted 1:20 in secondary antibody solution (0.1% BSA, 0.05% Tween-

20, and 5% fetal bovine serum). Sections were washed with PBS and fixed for 10 min with

2% gluteraldehyde in PBS followed by two washes with dH_2O. Grids were stained with 1%

uranyl acetate, washed and dried.

I. Immunoprecipitation

Whole cells lysates of seminiferous epithelia were pre-cleared for at least 60 min by

adding 30-50 ul of a 50% slurry of protein A-sepharose beads (Sigma, cat. P9424) to 500-

750 ul of total cell lysate. After pre-clearing, the lysates were centrifuged 5-10 sec. at high

speed to pellet beads. Supernatants were then transferred to new pre-chilled tubes.

Primary antibodies were added to cell lysates and incubated overnight without agitation.

During the last 60 min. of incubation 30-50 ul of 50% protein A or protein G-sepharose was

added to the lysates and allowed to incubate while on a platform roller. After tubes were

centrifuged at high speed for 15-20 sec. to sediment immune complexes. The

supernatants were saved for western blotting to assess the extent of specific antigen

precipitation. Immune complexes were then washed 4-5 times by sedimenting the

sepharose beads and then resuspending them in lysis buffer at a volume of 10 times that of

the beads. In the third wash beads were resuspended in 20 mM Tris, pH 7.5 to remove

detergents contained in the wash buffer. After washing liquid was removed with a 23 G

needle on a syringe. Immunoprecipitate complexes were then resuspended in 50-100 ul of

2x SDS-PAGE sample buffer and boiled for 5 minutes to elute bound proteins. Beads were

pelleted and the supernatants were removed and either used immediately for gel electrophoresis or frozen at -20 °C.

J. Controls: Immunofluorescence, Western blotting, Immuno EM and Immunoprecipitation

In immunofluorescence, immuno EM and immunoblotting studies, three types of controls were used. First, controls for nonspecific primary antibody binding included non-immune sera used at an equivalent IgG concentration to that of the primary antibody. Controls for non-specific binding of secondary antibodies included tissue samples treated with buffer instead of primary antibodies and then incubated with secondary antibody. Controls for autofluorescence or autochemiluminescence consisted of incubation with buffer alone without primary or secondary antibodies.

To control for non-specific binding during immunoprecipitation, several controls were used. Controls for non-specific binding of the monoclonal and polyclonal antibodies primary antibody included using non-immune IgG or sera, respectively, at a concentration the same as that of the immune antibody. Specificity of the precipitating antibody was determined by running immunoprecipitates on SDS-PAGE and probing with the immunoprecipitating antibody while also comparing to specific bands seen with non-precipitated whole cell lysates with Western blots. To determine whether there was an antibody or antigen excess non-precipitated supernatants were examined on SDS-PAGE and immunoblotted for the presence of non-precipitated antigen. In addition, the remaining non-soluble pellet from cell lysates was assessed for the presence of specific antigen. To separate cross reaction of denatured IgG molecules from specific detection of ILK two additional methods to conventional immunoprecipitation were used. Rather than using a secondary IgG conjugated to HRP, protein A-HRP (Sigma, P-8651) was used for secondary detection.

Using this method cross-reaction with electrophoresed, and therefore denatured, IgG molecules was avoided and detection only of ILK primary antibodies would occur. Secondly, proteins were electrophoresed under nonreducing conditions achieved by not including β-mercaptoethanol in the treatment buffer. Non-denatured IgG molecules were then separated from faster migrating prospective ILK precipitates.

RESULTS

A. IMMUNOFLUORESCENCE

Distribution of actin

The distribution of ectoplasmic specializations was determined by staining for filamentous actin using FITC-phalloidin in fixed frozen sections of seminiferous tubules. Figure 4 shows paired phase and fluorescence double exposures (FITC-phalloidin, green; DAPI, blue) illustrating the changing distribution of filamentous actin structures. Sertoli-germ cell junctional (S/Gj) actin is apparent at different points of development of spermatogenesis. It is most obvious in regions surrounding spermatid heads during stages I-IV (Fig. 4a, a') and Stages V-VI (Fig. 4b, b'). Subsequently, at stage VII/VIII (Fig. 4c, c'), S/G junctional actin shows greater concentration on the dorsal side of the elongating spermatid. In addition to well established junctions, junctional actin is present in developing ectoplasmic specializations (dj) and is observed to be in close apposition to round spermatids during stages IX (Fig. 4d, d'). Specifically, these developing sites of adhesion are recognized by a circular sheath of actin forming in Sertoli cell regions adjacent to germ cell acrosomes. Sertoli cell-Sertoli cell junctional (S/Sj) actin is identified by a linear array of actin between the bases of adjacent Sertoli cells (Fig. 4a). This junction is present during most spermatogenic stages but undergoes structural change as spermatocytes pass through the blood-testes barrier during stages VII/VIII (Fig. 4c). Tubulobulbar complexes (tb), transient structures found predominately at stages VII/VIII (Fig. 4c), are recognized by thin parallel arrays of actin that interdigitate between the Sertoli cell and late spermatids. These structures can be seen to distribute along the ventral portion of a late spermatid and are particularly evident in a digitally reconstructed serial image obtained using confocal microscopy (Fig. 4c"). Intracellular bridges (ij) between adjacent spermatids are recognized as punctate staining at the apex of the epithelium (Fig. 4b).

Figure 4. Paired fluorescence and phase micrographs of filamentous actin (FITC-phalloidin) counterstained with a blue nuclear stain (DAPI) in cryosections of adult rat seminiferous epithelium during stages I-IV (a, a'), V-VI (b, b'), VII-VIII (c, c'), IX (d, d'), and X-XIV (e, e') of spermatogenesis. Actin filaments in ectoplasmic specializations are clearly seen at apical Sertoli-germ cell junctions (S/Gj) and basal Sertoli-Sertoli junctions (S/Sj). Other major f-actin associated structures are stained including intracellular bridges (ij, b) and tubulobulbar complexes (tb, c). Figure 4c" is a confocal micrograph of a late spermatid stained with FITC-phalloidin showing S/Gj actin and actin filaments associated with tubulobulbar processes (tb) (a'-e' bar = 10 μm; c" bar = 5 μm).
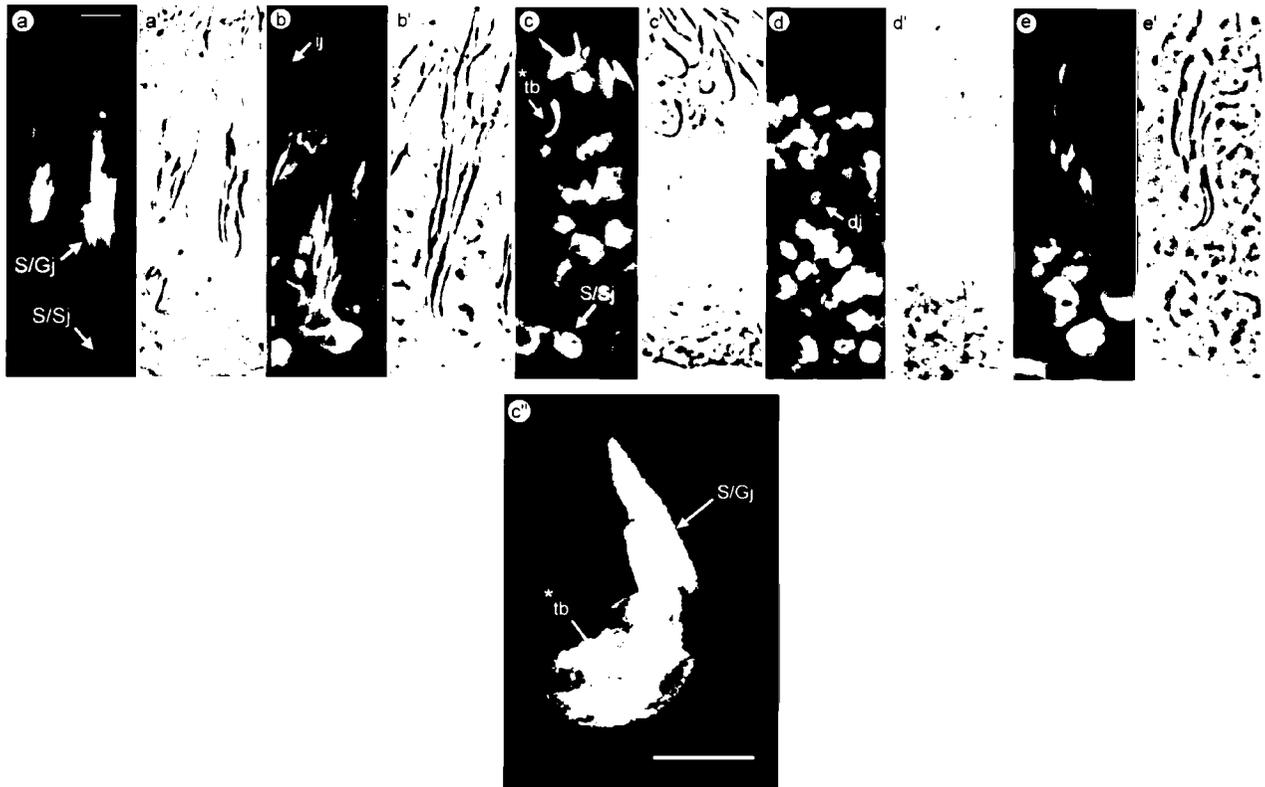
Distribution pan-Cadherin & β-Catenin

β-catenin was detected in both the seminiferous epithelium and in the interstitium of rat testes while Cadherins appeared most highly expressed in the seminiferous epithelium. Epithelial staining for β-catenin (Fig. 5a', b', arrow) and Pan-Cadherin (Fig. 5a", b", arrow) appears restricted to the Sertoli cell-Sertoli cell junctional complexes. Overlay staining shows Cadherin to be codistributed with β-catenin throughout the epithelium with nearly identical spatial and temporal distribution. In both double label experiments and single label experiments neither molecule was detectable at the Sertoli cell/germ cell interface during any stage of spermatogenesis (Fig. 5a-a''', arrow head). Even with high concentrations of primary antibodies (1:10) and extended incubation periods (2-3 hours at 37 °C) junctional staining was not observed at these sites. At all stages the staining pattern for the cadherin and catenin molecules appear punctate and localized rather than linear and continuous. Single labelled tissue sections counterstained with phalloidin (data not shown) revealed that neither molecule co-distributed with junctional actin found at Sertoli/Sertoli junctional sites. In addition to the intense staining observed at basal sites discrete punctate staining for the cadherin and catenin was observed in more apical positions (Fig. 5e'-e''', arrow head). This staining pattern appeared to localize to areas of contact between adjacent cells and was observed in association with most early germ cells including spermatocytes, round spermatids but not elongate spermatids. Specificity of the catenin probe was verified by a convenient internal marker, a blood vessel in the interstitium, which showed intense staining coincident with the borders of cells likely to be smooth muscle cells (Fig. 17a, panel 4a)

Figure 5. Paired immunofluorescence and phase micrographs showing localization of ß1-catenin (a'-e'), pan-Cadherin (a''-e''), and overlay (a'''-e''') in adult rat seminiferous epithelium cryosections during stages I-IV (a-a'''), V-VI (b-b'''), VII-VIII (c-c'''), IX (d-d'''), and X-XIV (e-e''') of spermatogenesis. Staining both for β-catenin and pan-Cadherin appear highly expressed in regions of the Sertoli-Sertoli junctional complex but appear not to co-localize with junctional actin (not shown) nor is staining detectable at apical junctions. The staining pattern both for β-catenin and pan-Cadherin appears punctate and localized rather than linear and continuous (scale bar = 10 μm).
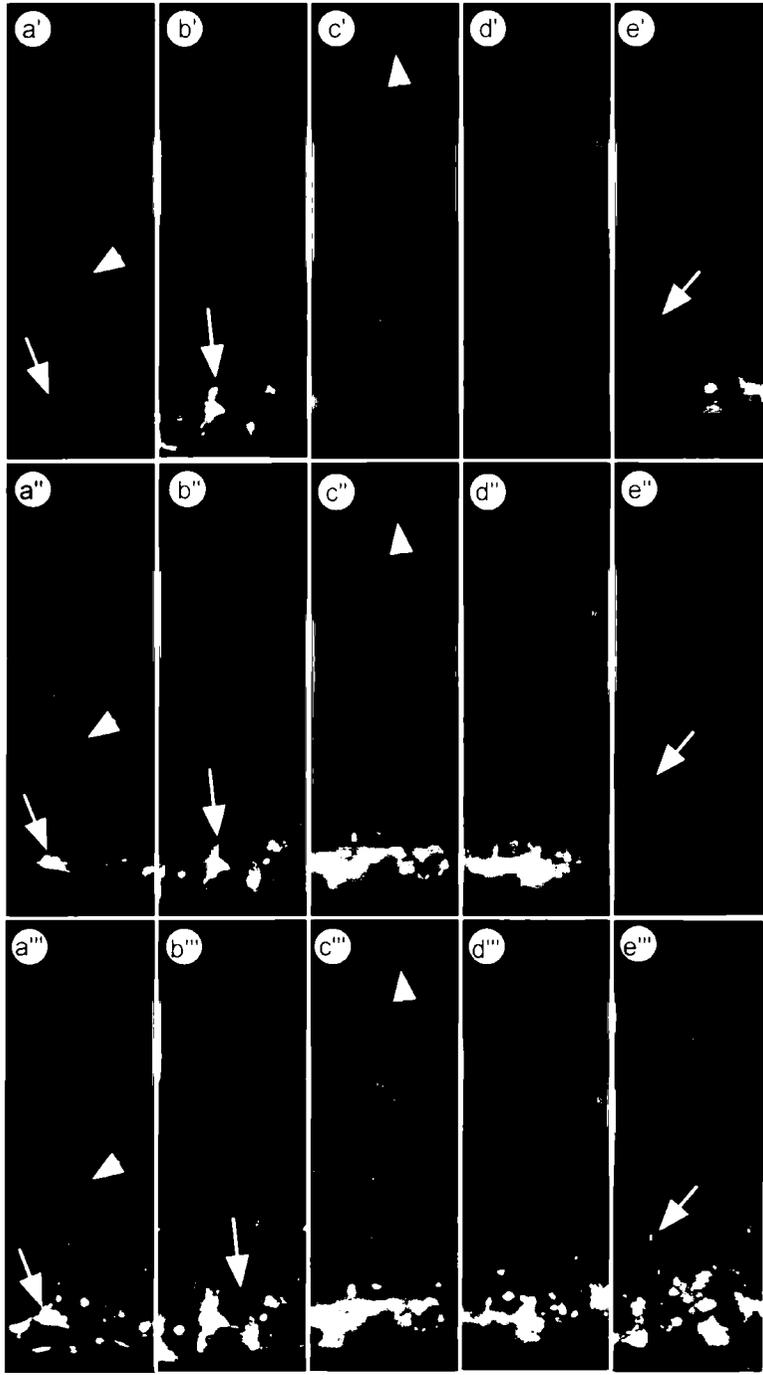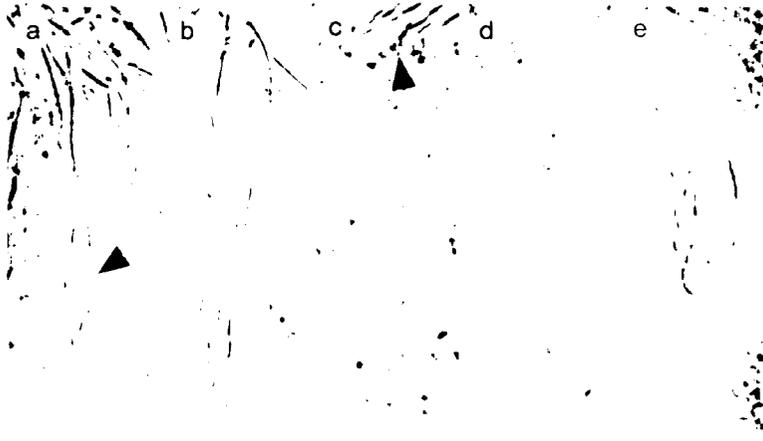
Distribution of β1-integrin

The spatial distribution of the β-1 integrin subunit in unfixed cryosections (Fig. 6) and fixed isolated cells is consistent with actin staining and the presence of junctional actin. Although preservation of morphology is poorer in these tissue sections, integrin expression is clearly coincident with the presence of junctional actin as ectoplasmic specialization remodeling during spermatid developed from round to elongate in shape. Integrin staining was most abundant on dorsal sides of the spermatid (Fig. 6c) throughout development and is consistent with the presence of the greatest amounts of ES actin. Tissue sections also show developing junctions (dj) to express the integrin (Fig. 6a, d) and is even more evident in isolated cells (Fig. 7, a"-c"). Interestingly, immunocytochemistry shows β1-integrin staining in some locations where phalloidin staining (a'-c') is not detected where integrin expression appears including mid- (b") and late- staged (c") spermatids. Integrin expression was not detected at other actin structures throughout the epithelium including intracellular bridges and tubulobulbar complexes. Intense staining at the basal lamina is non-specific and can be accounted for by normal IgG and secondary antibody alone negative controls.

Distribution of ILK

Specific staining for ILK (Fig. 8) is highly expressed at Sertoli-Germ cell junctions as recognized by a linear staining pattern running the length of spermatids heads (Fig. 8; b, c), (Fig 9; b', c'). ILK was detected both at newly forming junctions and in those which have undergone considerable morphologically change (Fig. 8; c, d; Fig 9; a'). As with staining for the integrin and actin, ILK expression was most prominent along the dorsal ridge of developing spermatids. This staining pattern is particularly evident in isolated cells (Fig. 9, c'''). ILK expression, although weaker, can also be observed in the basal compartments of the epithelium outlining spermatogonia and spermatocytes. Although, at these sites, ILK

appeared to co-localize to areas of specific staining for β1-integrin, this molecule was also expressed at non-junctional areas. That is, areas where the β1-integrin subunit or actin is not detectable (Fig. 8, e) including the margins of spermatocytes and spermatogonia (Fig. 8, e). In general, ILK was more difficult to localize at basal sites as it does not appear to form a linear contour that follows the junctional complex as defined by actin staining.

Figure 6. Paired immunofluorescence and phase micrographs of β-1 integrin localization in adult rat seminiferous epithelium cryosections during stages I-IV (a, a'), V-VI (b. b'), VII-VIII (c, c'), IX (d, d') and X-XIV (e, e') of spermatogenesis. Expression of the β-1 integrin complex is consistent with staining for actin both at Sertoli cell-Germ cell junctions (S/Gj, a, c) and Sertoli cell-Sertoli cell junctions (S-Sj, c). Expression of the integrin is also observed in developing junctions (dj, d) (scale bar = 10 μm).

Figure 7. Immunofluorescence showing phase (a-c), f-actin (a'-c', green), β1-integrin (a"-c", red) and overlay (a'''-c''') in isolated germ cells during early (a), mid- (b) and late (c) stages of spermatid development. Integrin staining is expressed at junctional sites and is coincident with the development of the Sertoli cell-Germ cell junction (S/Gj) as verified by the distribution of FITC-phalloidin (scale bar = 10 μm).
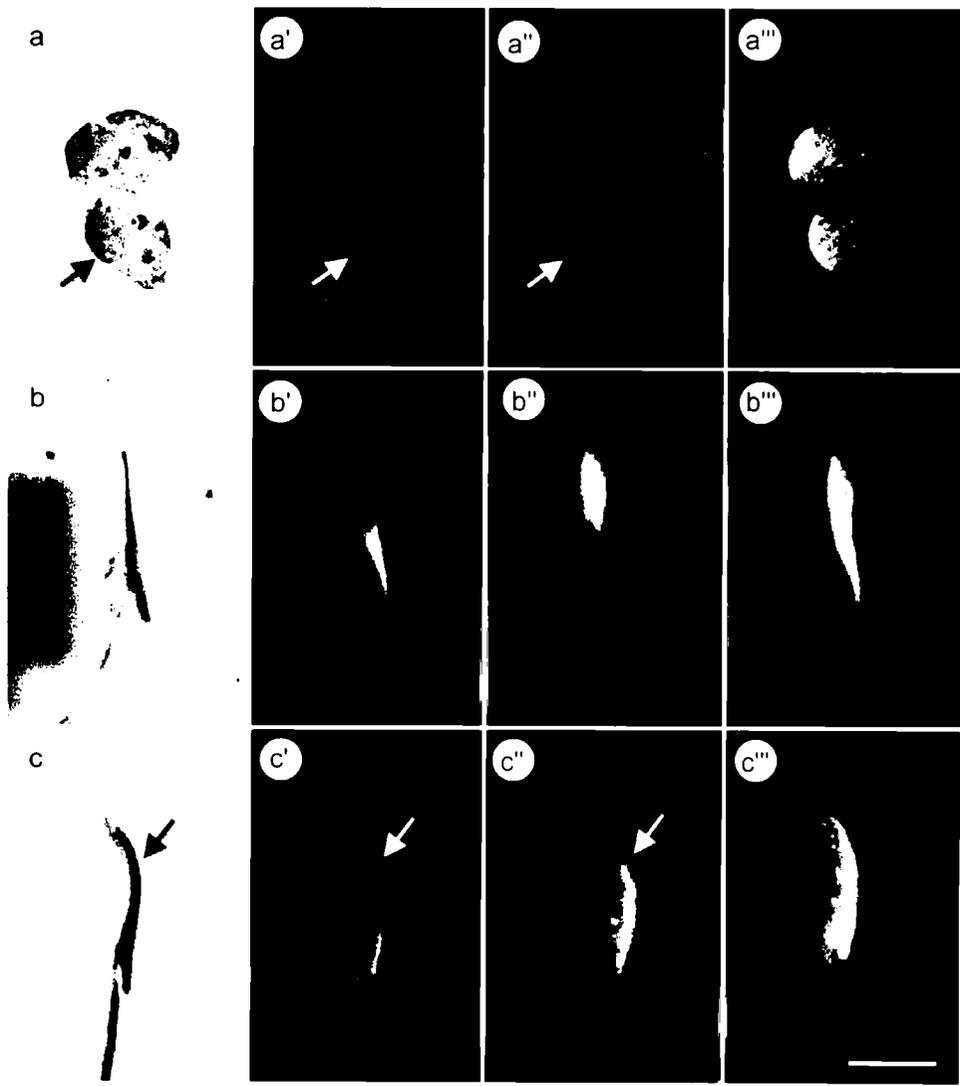
Figure 8. Paired immunofluorescence and phase micrographs of ILK localization in adult rat seminiferous epithelium cryosections during stages I-IV (a, a'), V-VI (b. b'), VII-VIII (c, c'), IX (d, d') and X-XIV (e, e') of spermatogenesis. Specific staining is seen at Sertoli cell-Germ cell junctions (S-Gj), (b, c), at Sertoli cell-Sertoli cell junctions (S-Sj), (a, c) junctions and at developing junctional sites (dj, d). Staining is also observed at non-junctional areas around spermatogonia and spermatocytes found in the most basal aspect of the epithelium (scale bar = 10 μm).
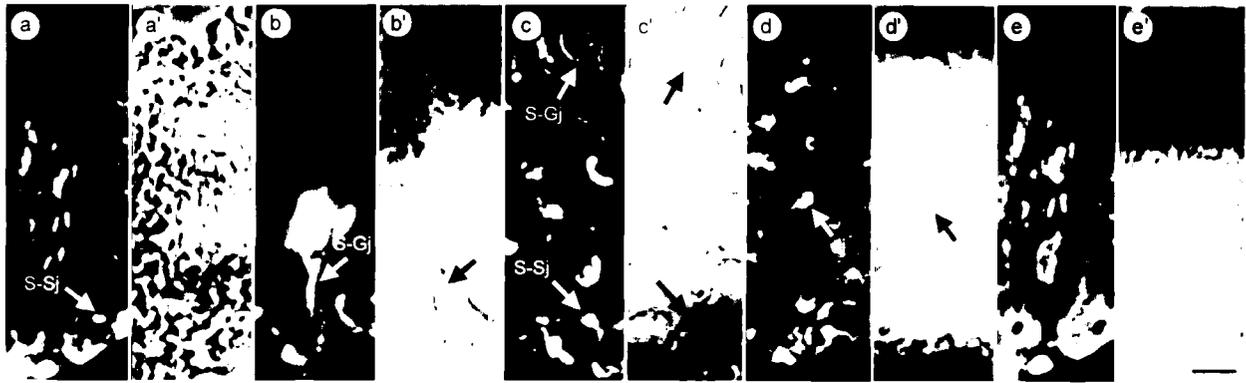
Figure 9. Immunofluorescence showing phase (a-c), f-actin (a'-c', green), ILK (a''-c'', red) and overlay (a'''-c''') in isolated germ cells during early (a), mid- (b) and late (c) stages of development. ILK staining is coincident with development of the Sertoli cell-germ cell junction (S/Gj) as shown by counterstaining with FITC-phalloidin (scale bar = 10 μm).
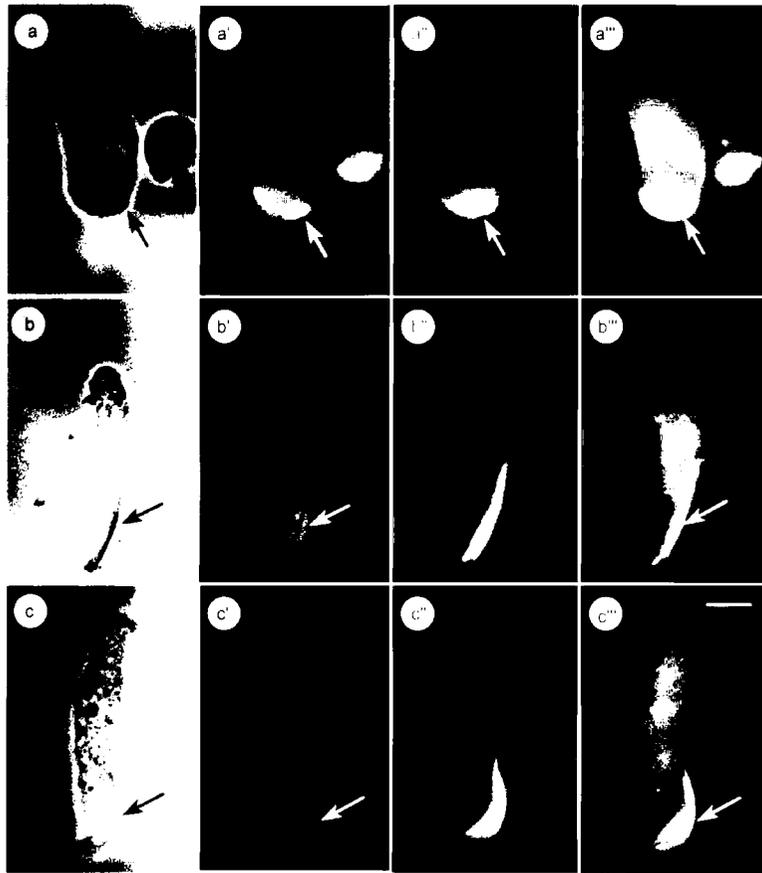
Distribution of FAK

Expression of FAK was not detected at any cell-cell junctional sites including Sertoli-Sertoli junctions and Sertoli-germ cell junctions (Fig. 10, a, c, arrow head). To verify the absence of detectable FAK at basal junction sites tissue sections were counter-stained with phalloidin. In addition, by using components of the testis intersitium (Fig. 17, 5a) as a positive control, a relative measure of FAK expression in the seminiferous epithelium could be determined. The intense cytoplasmic staining observed in basal germ cells was not observed at any point in the cell lineage apically of primary spermatocytes (Fig. 11, b", c"). FAK expression seemed to be the highest in the cytoplasm of germ cells juxtaposing the extracellular matrix, the spermatogonia, where staining intensity was comparable to that of endothelial cells (Fig. 11, d, arrowhead). Figure 11 (a") shows the localization of FAK to the cytoplasm of two adjoining spermatogonia.

Distribution of Vinculin

Immunostaining for vinculin (Fig. 12) localized to Sertoli cell-germ cell junctions (S-Gj) and Sertoli cell-Sertoli cell junctions (S-Sj). Staining at apical junctions is observed around the entire aspect of elongate spermatid heads and becomes restricted to the dorsal aspect of the spermatid head as it becomes more sickle shaped (Fig. 12, b). Vinculin staining is readily observed at developing junctions (dj) (Fig. 12, d'). Vinculin also is expressed at intercellular bridges (ij) (Fig. 12, b) and, just prior to spermiation, in association with tubulobulbar processes (tb) (Fig. 12, c'). Vinculin staining appears to be less abundant along the dorsal aspect of the elongate spermatids just prior to spermiation.

Distribution of Paxillin

Paxillin, has a very high expression throughout the cytoplasm of both Sertoli cells and germ cells in almost all stages of spermatogenesis (Fig. 13, Fig. 14, a"-c"). Paxillin staining

does not concentrate at junctional sites (Fig. 14, a", arrow) but appears diffuse and cytoplasmic. Primary spermatocytes showed considerably less Paxillin staining than any other cell type (Fig. 13, a, arrow) while it appeared highly expressed in the tubulobulbar processes (tb, c') and the apical aspect of Sertoli cells just prior to spermiation (Fig. 13, d). At stage VII/VIII (Fig. 13, d), paxillin staining appears less intense in basal, columnar portions of the Sertoli cell and increased in apical expression. Apical staining of Sertoli cells is considerably less intense by stage X-XIV (Fig. 13, e).

Figure 10. Paired immunofluorescence and phase micrographs of FAK localization in cryosections of rat seminiferous epithelium during stages I-IV (a, a'), V-VI (b, b'), VII-VIII (c, c'), IX (d, d') and X-XIV (e, e') of spermatogenesis. Staining does not appear to localize to either Sertoli-germs cell junctions or Sertoli-Sertoli junctions but is highly diffuse throughout the cytoplasm of spermatogonia and spermatocytes. Specific staining is not observed at any point in the cell lineage past primary spermatocytes (scale bar = 10 μm).

44
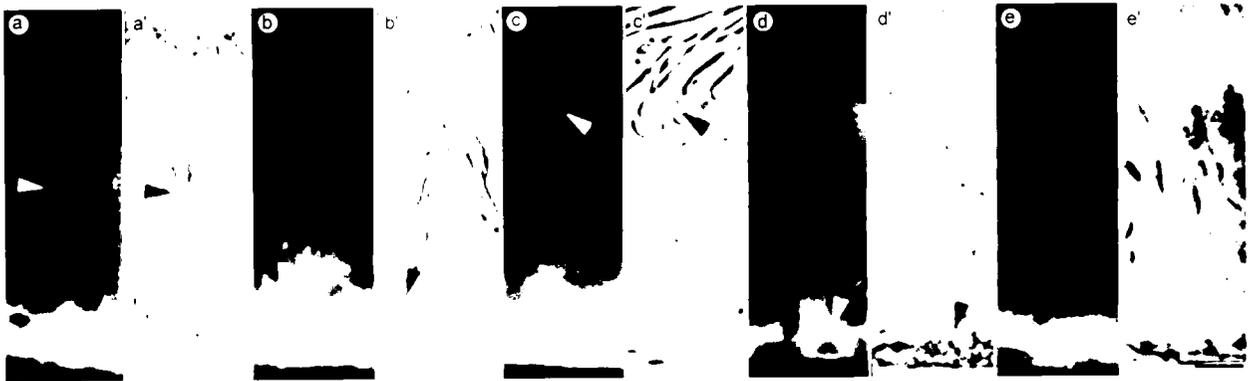
Figure 11. Immunofluorescence showing phase (a-c), f-actin (a'-c', green), FAK (a"-c", red) and overlay (a''') in isolated germ cells during early (a), mid- (b) and late (c) stages of development. FAK staining is not detected at either the Sertoli cell-germ cell junction or Sertoli-Sertoli junction but is restricted to the cytoplasm of spermatogonia and early spermatocytes (scale bar = 10 μm).
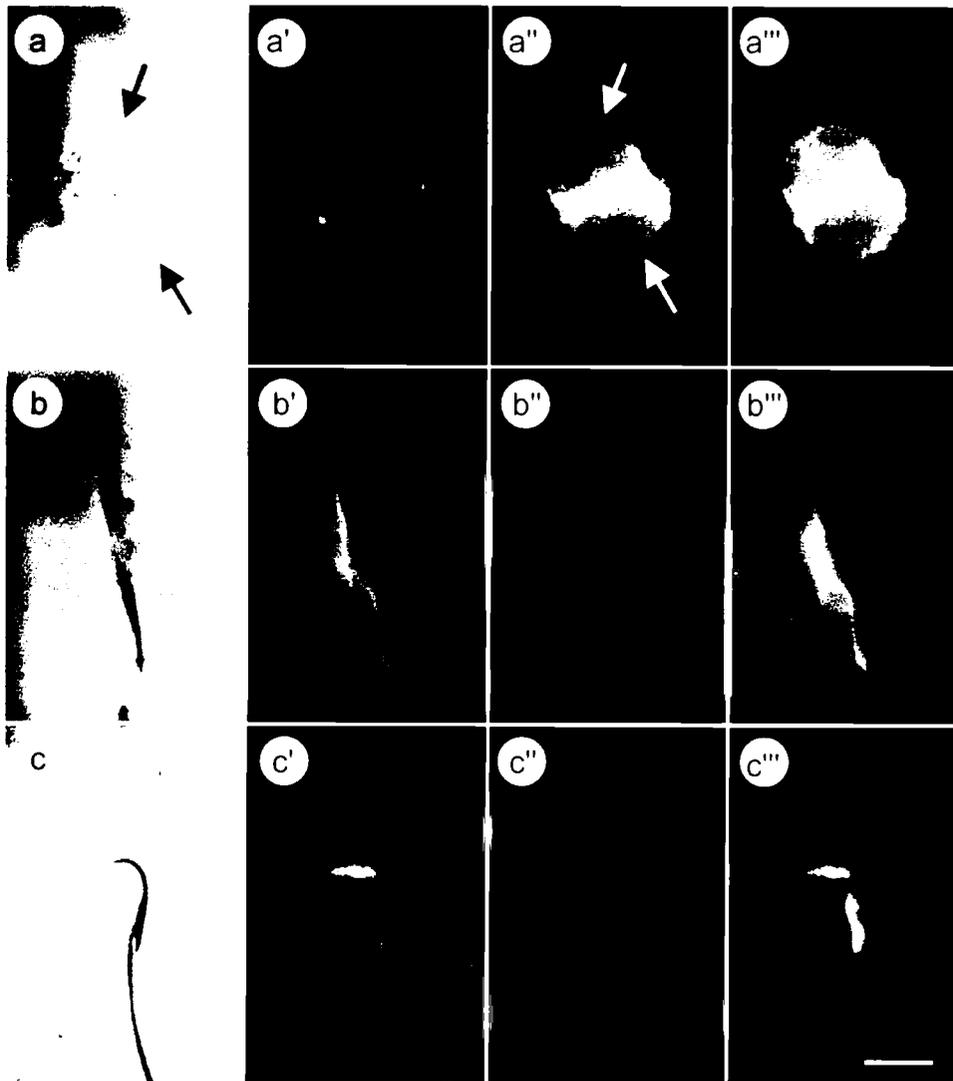
Figure 12. Paired immunofluorescence and phase micrographs of Vinculin localization in cryosections of adult rat seminiferous epithelium during stages I-IV (a, a'), V-VI (b, b'), VII-VIII (c, c'), IX (d, d') and X-XIV (e, e') of spermatogenesis. Vinculin is localized to Sertoli cell-Germ cell junctions (S/Gj) and Sertoli cell-Sertoli cell junctions (S/Sj) in most spermatogenic stages. Vinculin also appears at other actin related structures in the epithelium including tubulobulbar processes (tb, c) and intracellular bridges (ij, b) (scale bar = 10 μm).
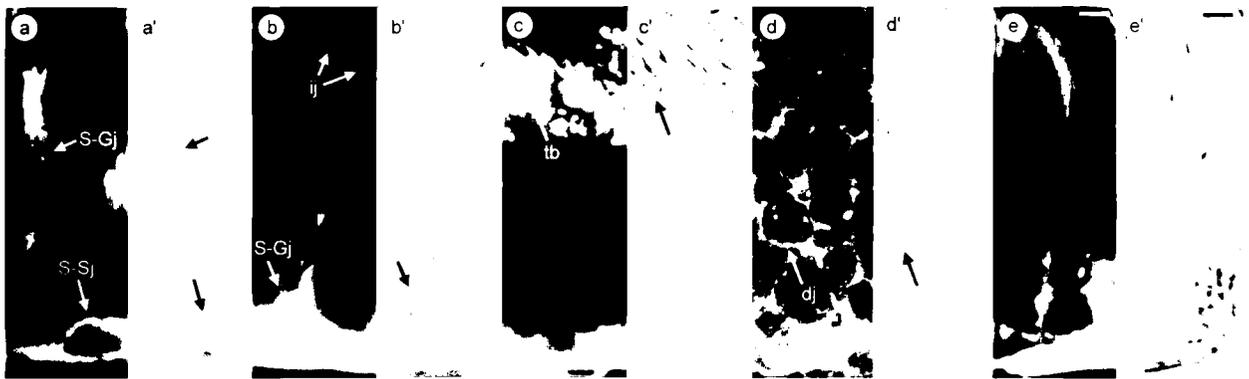
Figure 13. Paired immunofluorescence and phase micrographs of paxillin localization in cryosections of adult rat seminiferous epithelium during stages I-IV (a, a'), V-VI (b, b'), VII-VIII (c, c'), IX (d, d') and X-XIV (e, e') of spermatogenesis. Although staining is not concentrated at junctional sites, staining is abundant throughout both germ cell and Sertoli cell cytoplasm. The exception was that in primary spermatocytes which showed considerably less expression of paxillin throughout their cytoplasm (a, arrow). Tubulobulbar complexes show abundant levels of paxillin (c, arrow) (scale bar = 10 μm).
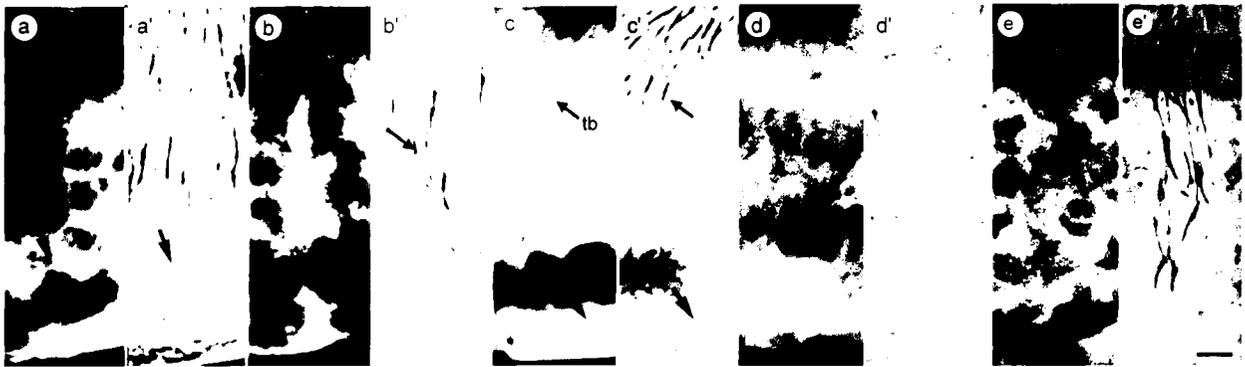
Figure 14. Immunofluorescence showing phase (a-c), f-actin (a'-c', green), paxillin (a''-c'', red) and overlay (a'''-c''') in isolated germ cells during early, mid- and late stages of development. Paxillin staining is not concentrated at either the Sertoli cell-germ cell junction (a'', arrow) or Sertoli-Sertoli junction but is highly expressed throughout the cytoplasm of both germ cells (a'', b'') (scale bar = 10 μm).
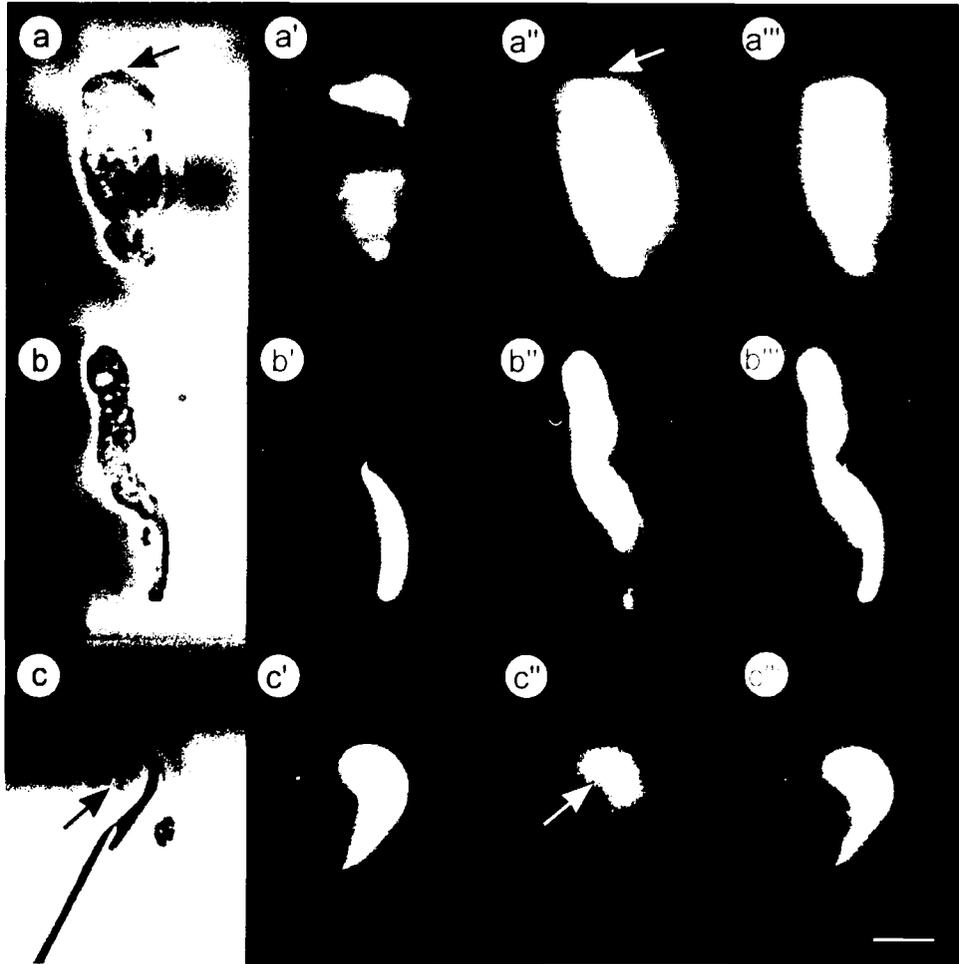
Distribution of Phosphotyrosine

Immunostaining with a rabbit polyclonal antibody revealed phosphotyrosine-containing proteins to be widely expressed throughout the seminiferous epithelium (Fig. 15, 16) including non-junctional components of the epithelium. Expression is concentrated at cell borders of most cells including germ cells (Fig. 15, c, arrow head) and adjacent Sertoli cells (Fig. 15, d, nj). Expression, however, was most intense at S/S junctions (Fig. 15, a) and S/G junctions (Fig. 15, b) at sites where ESs are known to occur. Unlike the other molecules studied, phosphotyrosine showed staining around both the dorsal and ventral components of the spermatid head (Fig. 16, c"). Stage VII/VIII staining at S/Sj appears distributed along only a small portion of the length of the junction (c', arrow) which differs from the more linear staining pattern (Fig. 15, a) that coincides with the majority of the basal actin as observed in stages I-IV. Phosphotyrosine also appears concentrated at tubulobulbar complexes (tb) (Fig15, c; Fig.16, c", arrow) but disappears after sperm release (Fig. 15, d). Phosphotyrosine staining is not obvious in developing junctions (Fig. 15, c, d).

Controls for Immunohistochemistry and Immunocytochemistry

Staining with normal rabbit and mouse IgG, used at a protein concentration equal to that of the most concentrated primary antibody was used to control for non-specific staining of primary antibodies. In fixed tissue sections normal mouse IgG staining (Fig. 17a, 1a) typically showed some diffuse staining throughout the cytoplasm of round germ cells while normal rabbit IgG staining (Fig. 17, 2a) at concentrations equal to the IgG of the β1-integrin primary antibody showed intense staining at the basal lamina that could be accounted for, in part, by staining with the secondary rabbit antibody alone (Fig. 17, 2b). Fresh, or non-fixed, tissue sections treated with normal IgG (Fig. 17, 6a) showed strong reactivity with the basal lamina and also reacted in a diffuse manner throughout the cytoplasm. Rabbit

secondary antibodies alone (Fig. 17, 7a) showed a similar reaction but was not as intense.

Blank controls (Fig. 17, 3a) for tissue sections showed no non-specific staining.   Controls

for isolated cells (Fig. 18) show no non-specific staining with any control treatment.

Figure 15. Paired immunofluorescence and phase micrographs of phosphotyrosine localization in cryosections of adult rat seminiferous epithelium during stages I-IV (a, a'), V-VI b, b'), VII-VIII (c, c'), IX (d, d') and X-XIV (e, e') of spermatogenesis. Expression of phosphotyrosine residues appears at most cell-cell borders but is most highly expressed at apical (b) and basal junctions (a). Sertoli/Sertoli junctions (S/Sj) show prominent phosphotyrosine expression at stages I-IV (a) and VII/VIII (c) (scale bar = 10 μm).
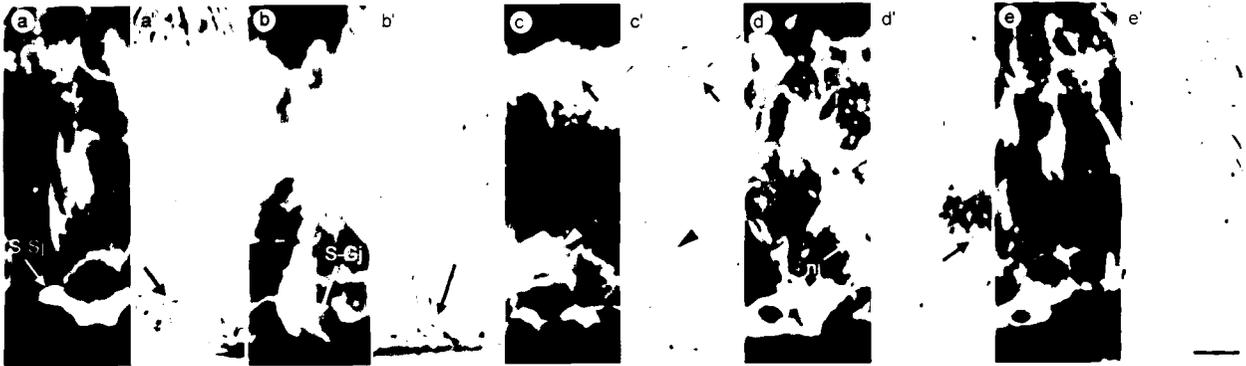
Figure 16. Immunofluorescence showing phase (a-c), f-actin (a'-c', green), phosphotyrosine (a''-c'', red) and overlay (a'''-c''') in isolated germ cells during early (a), mid- (b) and late (c) stages of spermatogenesis. Phosphotyrosine is highly expressed at Sertoli cell-germ cell junctions (c'', arrow) but is also associated with non-junctional areas of germ cells including germ cell borders (a'', arrow). Staining also occurs in locations where tubulobulbar complexes (c'', arrow head) are known to be present. Phosphotyrosine does not appear abundant in the germ cell cytoplasm (scale bar = 10 μm).
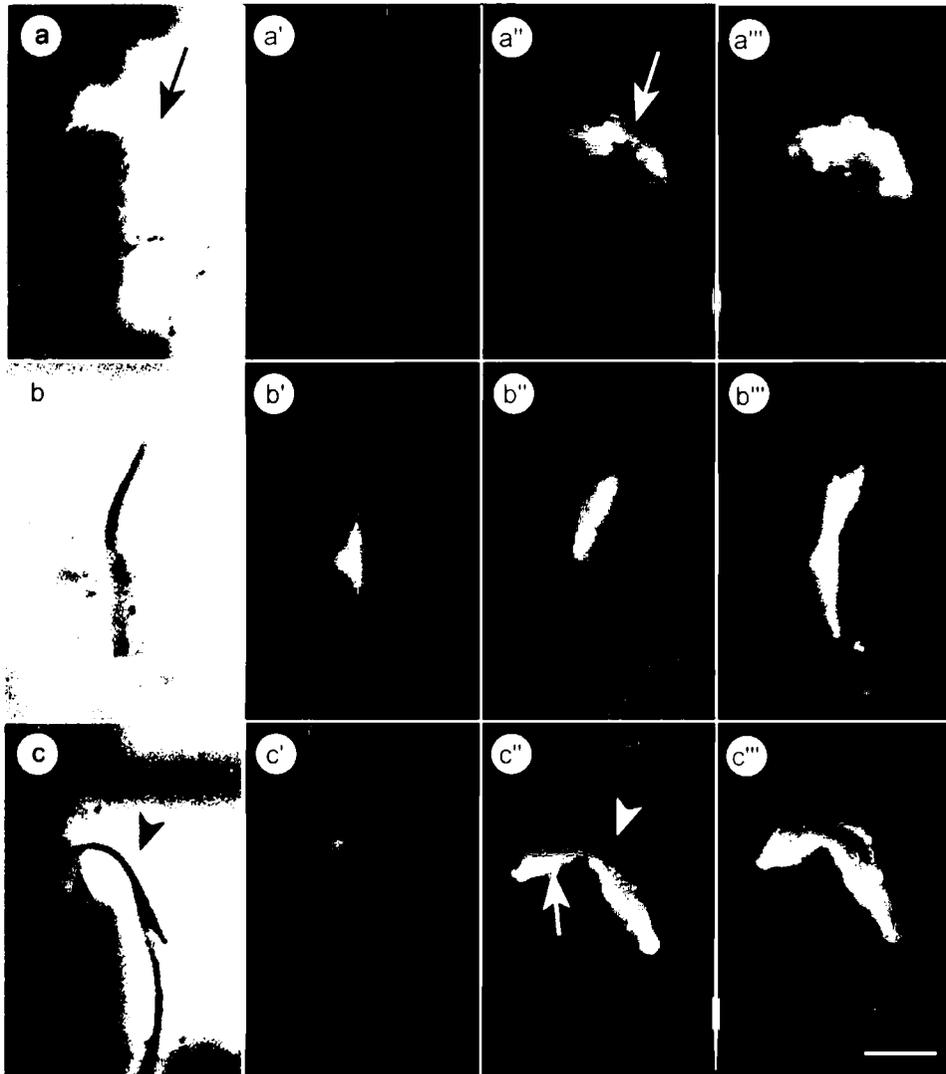
Figure 17. Paired phase and fluorescence controls for immunohistochemistry including staining with normal rabbit IgG (stage VII/VIII) (1a), Texas Red conjugated anti-rabbit IgG (1b) (stage V-VI), normal rabbit IgG (2a) (stage X-XIV) and Texas Red conjugated anti-rabbit IgG (2b) (Stage V-VI). A blank control was also included (3a) (stage VII/VIII). Since many different antibodies at different protein concentrations were used for immunostudies fluorescence controls shown are at a protein concentration equal to that of the most concentrated primary antibody used. Internal positive controls, interstitial blood vessels, are included for β-catenin (4a), and FAK (5a) as a measure of antibody specificity and the relative abundance of antigens. Normal rabbit IgG (at a concentration equal to the working dilution of the β1-integrin) (6a) (stage IX) and secondary antibody alone (7a) (stage IX) controls for non-fixed tissue are also included to account for non-specific staining in experiments with β1-integrin and Integrin Linked Kinase (scale bar = 10 μm).
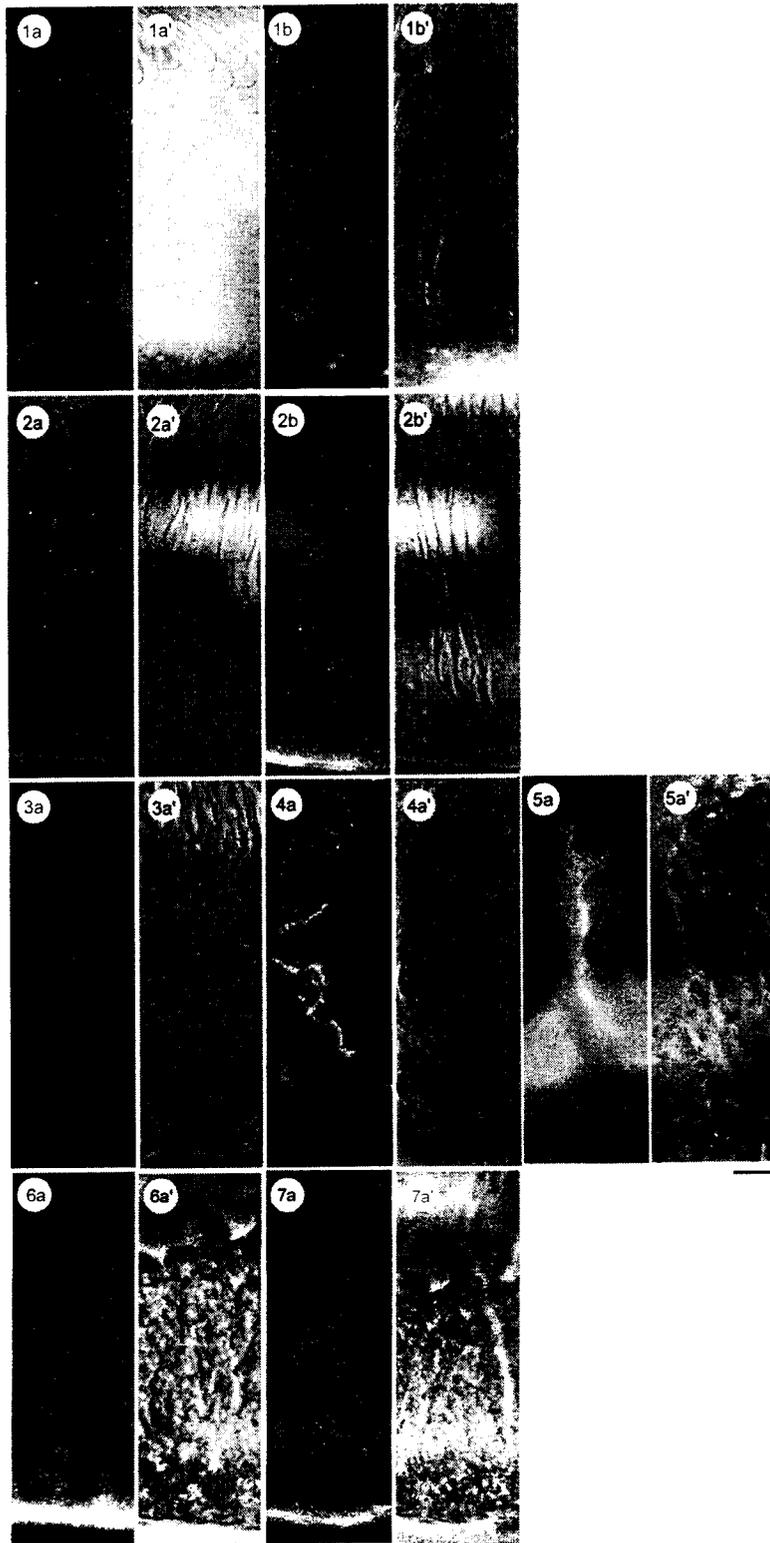
Figure 18. Paired phase and fluorescence controls for immunocytochemistry including staining with normal mouse IgG (1a), Texas Red conjugated anti-mouse IgG (1b), normal rabbit IgG (2a) and Texas Red conjugated anti-rabbit IgG (2b). A blank control (3b) was also included (scale bar = 10 μm).
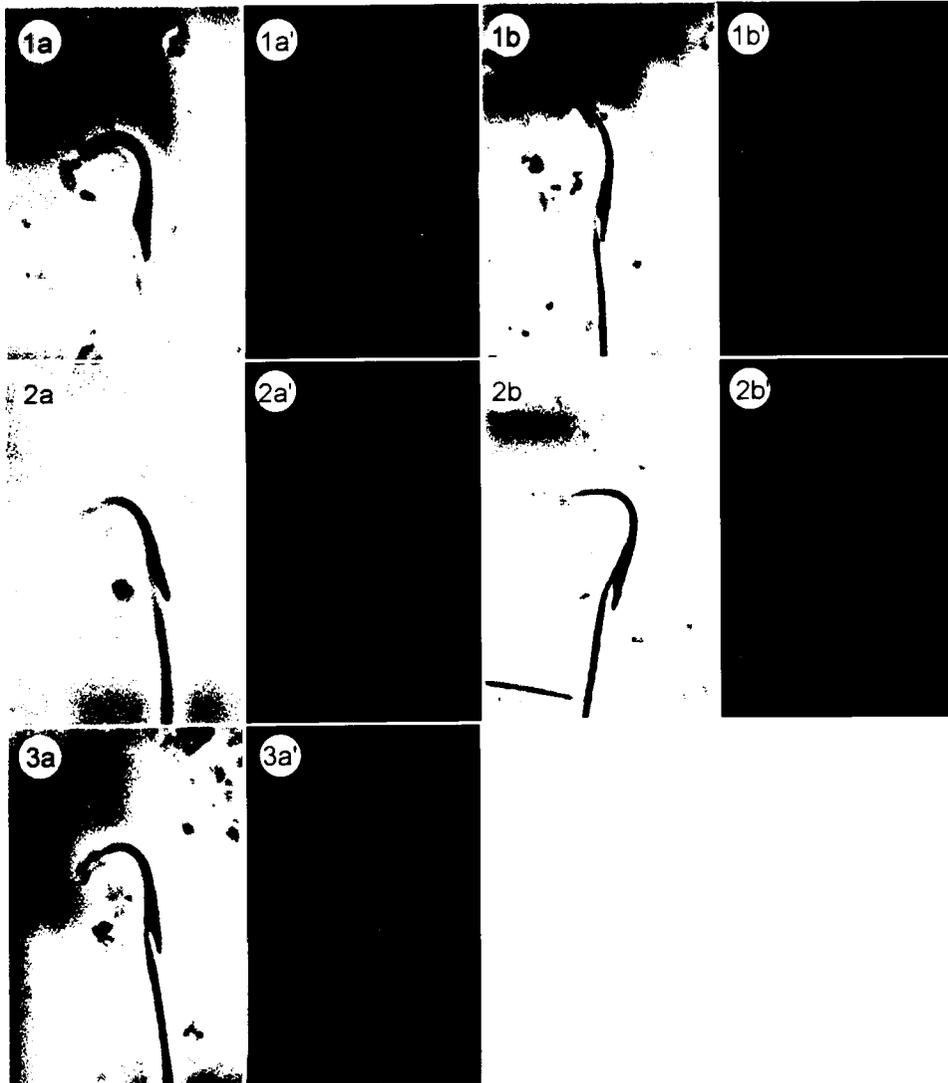
Summary of Spatial & Temporal Distribution of Immunofluorescence During Spermatogenesis

Temporal and spatial staining patterns of molecules used for immunolabelling studies are summarized in the form of a table (Figure 19). Expression of each molecule is seen as a function of the five spermatogenic stages used in immunofluoresence studies. Expression of each molecule is classified as either junctionally related or as being associated with the cytoplasm of either germ cells or Sertoli cells. Because it was not possible to definitively determine whether staining is associated with ESs or with other components of the basal junctional complex, apical junction can be inferred as being ES while basal junction cannot (even though ES staining at basal complexes shows a very particular staining pattern at the light level). β-catenin and Pan-Cadherin are indicated to be highly expressed but restricted to the basal junctional complex. β1-integrin and ILK are shown to be expressed at both apical and basal junction areas throughout all five stages with no detectable staining in the cytoplasm of either germ cells or Sertoli cells. Vinculin is indicated being concentrated at basal and apical stages throughout the stages except for stage VII/VIII spermatids where staining appears to be predominately associated with the tulbulobulbar complexes and not the ES. FAK expression is intense but restricted to spermatogonia and spermatocytes and is not detected at any junctional site. Similarly, we indicate that paxillin is not detected at junctional sites but is highly expressed throughout the cytoplasm both of germ cells and Sertoli cells except during stage IX where staining is less abundant but more apical within the columnar Sertoli cell. Of all the molecules studied phosphotyrosine seemed to show the greatest stage related changes in expression. Although phosphotyrosine was not observed in the cytoplasm of either germ cells or Sertoli cells staining was observed to be intense at apical junctions during most stages except just after spermiation. In addition, during architectural rearrangement of the blood-testes barrier (stage VII/VIII) phosphotyrosine staining is particularly prominent at basal junctions and

appears restricted to only a portion of the Sertoli/Sertoli junction which, at this stage, is co-distributed with the actin component of this interface.

Figure 19. Summary of staged immunofluorescence expression of signaling molecules in adult rat seminiferous epithelium. The relative amount of expression of each molecule at junctional and cytoplasmic locations is indicated. Expression was classified as either not detectable, relatively weak, or relatively high based on the intensity and distribution of immunofluorescence.

a) Junctional Complex Concentration

Stage of Spermatogenesis

Basal Junction

	Cd/Ct#	ß1	ILK	FAK	Pax	Vinc	p-Tyr
I-IV	■	▨	▨	□	□	■	■
V-VI	■	▨	▨	□	□	■	□
VII-VIII	■	▨	▨	□	□	■	■
IX	■	▨	▨	□	□	■	□
X-XIV	■	▨	▨	□	□	■	□

Apical Junction

	Cd/Ct	ß1	ILK	FAK	Pax	Vinc	p-Tyr
I-IV	□	■	■	□	□	■	■
V-VI	□	■	■	□	□	■	■
VII-VIII	□	■	■	□	□	□	■
IX	□	■	■	□	□	■	□
X-XIV	□	■	■	□	□	■	■

b) Cytoplasmic Concentration

Germ Cells

	Cd/Ct	ß1	ILK	FAK*	Pax*	Vinc	p-Tyr
I-IV	□	□	□	▨	■	□	□
V-VI	□	□	□	▨	■	□	□
VII-VIII	□	□	□	▨	■	□	□
IX	□	□	□	▨	□	□	□
X-XIV	□	□	□	▨	■	□	□

Sertoli Cell

	Cd/Ct	ß1	ILK	FAK	Pax	Vinc	p-Tyr
I-IV	□	□	□	□	■	□	□
V-VI	□	□	□	□	■	□	□
VII-VIII	□	□	□	□	■	□	□
IX	□	□	□	□	▨	□	□
X-XIV	□	□	□	□	■	□	□

□ Expression is not detectable

▨ Expression is relatively weak

■ Expression is relatively high

---

*Staining is resticted to immature germ cells or spermatogonia

+Staining is much less intense in spermatocytes

#Puctate staining observed between adjacent round spermatids

B. WESTERN BLOTS & IMMUNOPRECIPITATIONS

Western blotting of non-precipitated whole cell lysates (WCL) show the specificity for probes used throughout immunofluorescence studies while blotting of immunoprecipitates are used in corroboration with light level data to show possible protein complexes that could occur throughout the epithelium in vivo.

Pan-Cadherin/Catenin

Specificity of antibodies for pan-cadherin (130 kD) and catenin (92 kD) in WCL was shown by the presence of a single band at the appropriate molecular weight for each molecule. The presence of stable protein-protein interactions for pan-Cadherin and β-catenin (Fig. 20) are supported by immunoprecipitations with monoclonal pan-Cadherin, polyclonal pan-Cadherin and polyclonal β-catenin primary antibodies. Both monoclonal (Fig. 20i) and polyclonal Cadherin blots (Fig. 20ii) of β-catenin immunoprecipitations show a specific band at approximately 130 kD (arrow). Immunoprecipitations with the monoclonal pan-Caderin probed with antibodies against the C-terminus of the β-catenin molecule (Fig. 20iii) show a specific band at about 95 kD. Expression of both the cadherin and catenin bands appear prominent suggesting that these molecules are binding at roughly an equivalent ratio.

β1-integrin & ILK

Specificity of antibodies for β1-integrin (140 kD) and ILK (58 kD) in WCL was shown by the presence of a single band at the appropriate molecular weight for each molecule (Fig. 21). ILK immunoprecipitates show the presence of a band at approximately 140 kD when probed with polyclonal antibodies for β1-integrin (i). Precipitates of normal rabbit IgG used at an equivalent IgG concentration did not show a specific band at 140 kD. Both the

precipitating ILK antibody and the normal IgG showed an intense band at about 55 kD representing the heavy chain of the precipitating IgG molecule. β1-integrin immunprecipitates that were probed with polyclonal ILK antibodies, using conventional IgG-HRP detection methods, proved to be problematic because of only a 3 kD difference in molecular weights between a ILK band and the heavy chain of the precipitating β1-integrin antibody (Fig. 21ii). Even though β1-integrin precipitates showed a more intense band at 55 kD than the normal IgG precipitates, it could not be confirmed

Figure 20. Whole cell lysate (WCL) and immunoprecipitates (IP) probed with monoclonal Pan-Cadherin (i),polyclonal Pan-cadherin (ii), and polyclonal β-catenin (iii) showing that each primary antibody reacts with a band at the appropriate molecular weight (arrow heads). Western blots of immunoprecipitates suggest that a stable in vivo cadherin/β-catenin complex occurs in whole cell lysates. For each primary antibody used for immunoprecipitation a non-immune IgG of equal protein concentration was used as a control.

i) BLOT: pan-Cadherin (monoclonal)
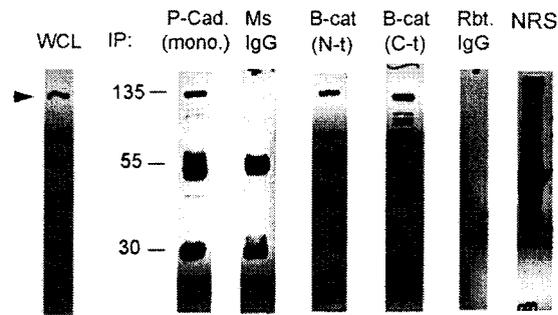
ii) BLOT: pan-Cadherin (serum)
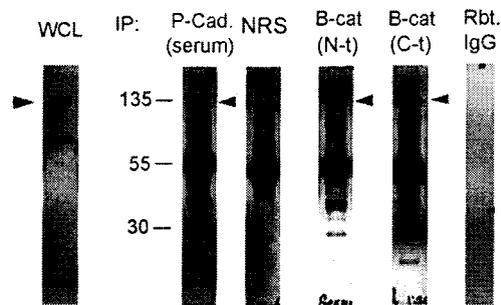
iii) BLOT: B-catenin
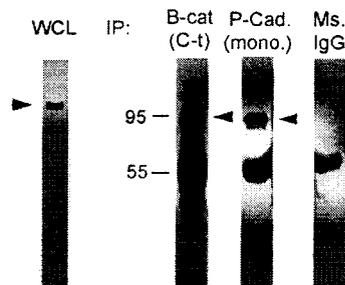
Figure 21. Whole cell lysates (WCL) and immunoprecipitates (IP) probed with polyclonal anti- β1-integrin (i), polyclonal ILK (ii-iv) and monoclonal ILK (v) antibodies. Since the IgG heavy chain of the precipitating antibody cross reacts at a molecular weight close to that of ILK several controls were used to verify the precipitation of ILK with β1-integrin antibodies. These include the use of protein A-peroxidase (iii), a non-reducing gel and a monoclonal ILK antibody (v). For each primary antibody used for immunoprecipitation a non-immune IgG of equal protein concentration was used as a control.

72

i) BLOT: B1-integrin

WCL   IP: β1   Rbt   ILK   Rbt
              IgG   IgG
              (β1)   (ILK)

~140 ►

ii) BLOT: ILK (anti-rbt IgG-HRP)

WCL   IP: β1   Rbt
                  IgG

iii) BLOT: ILK (protein A-peroxidase)

WCL   IP: β1   ILK   Rbt   Pure
                        IgG   IgG

~ 55 ►

iv) BLOT: ILK (non-reducing gel)

WCL   IP: ILK IP   Rbt IgG   Rbt IgG   Gel
            NR   R   NR   R   NR

~ 150 ►
~ 58 ►

v) BLOT: ILK (monoclonal)

WCL   IP: β1   Rbt
                  IgG

~ 60 ►   ~ 60 ►
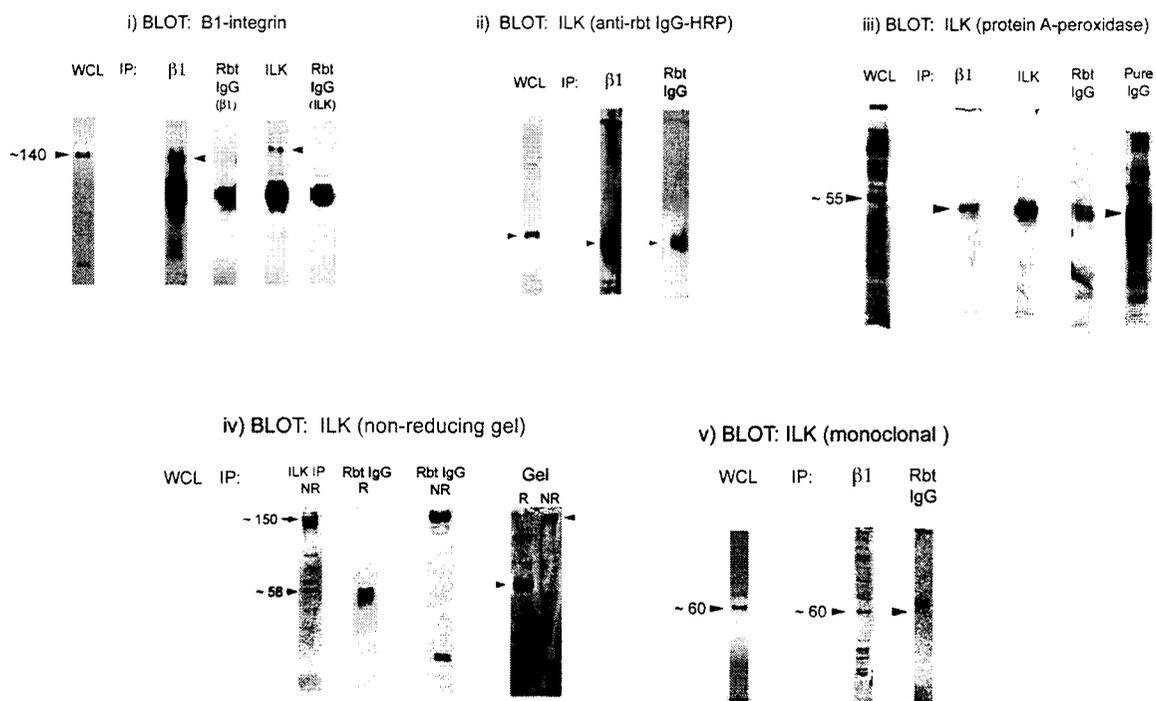
that ILK was present in the integrin precipitates. As a result, several additional controls were employed to determine whether the β1-integrin was actually precipitating the ILK molecule.

The first control was the use of protein A-peroxidase (Fig. 21iii) rather than a conventional IgG-HRP secondary antibody as a means of secondary detection. Although reactivity occurred with control rabbit IgG the intensity of the band for the ILK precipitates was much greater. Similarly β1-integrin preciptites showed a more intense ILK band than the IgG control. Since the precipitating control was done at the same IgG concentration as the experimental antibody this difference in band intensity is likely due to the presence of specific ILK. The second control was the use of a non-reducing gel which was intended to separate the much larger heavy chain, which in a non-reduced state is about 155 kD, from ILK which should would still migrate to about 58 kD (Fig. 21iv). A gel stained with coomassie blue shows the presence of the heavy chains in both in a non-reducing (NR) and reducing gel (R) state. The presence of a band at about 58 kD in the non-reducing blot, and its absence in non-reducing normal IgG, suggests a specific reaction of ILK with β1-integrin precipitates. A reducing gel with normal IgG shows that the heavy chain IgG band migrates close to the specific ILK band. With the recent availability of a monoclonal ILK antibody (Fig. 21v), raised in a different species than the precipitating with β1-integrin antibody, problems of cross reactivity with heavy chain of the IgG molecule could be avoided. Despite the presence of several lower molecular weight bands, the monoclonal ILK antibody showed a relatively intense band at the appropriate molecular weight that did not appear in the IgG precipitating control.

Paxillin/Vinculin & Paxillin/FAK

Figure 22 shows that paxillin and vinculin are able to form stable complexes in whole cell lysates. Pooled monoclonal vinculin antibodies show a strong signal at 70 kD when probed with Paxillin (Fig. 22i). However, paxillin precipitates (Fig. 22ii) appear to contain relatively less vinculin. Also, mouse IgG precipitates, used at an equal protein concentration to that of the paxillin antibody, showed a faint band at a similar molecular weight. Paxillin was also shown to associate with polyclonal FAK precipitates (Fig. 22iii) as recognized by a band at 70 kD. When paxillin precipitates were assessed for specific FAK polyclonal FAK antibodies detected a band at the expected 120 kD eventhough FAK monoclonal antibodies did not.

Figure 22. Whole cell lysate (WCL) and immunoprecipitates (IP) probed with monoclonal anti-paxillin (i, iii), "pooled" monoclonal anti-vinculin (IP only) (ii) and monoclonal FAK (iv) showing that each primary antibody reacts as a single band at the appropriate molecular weight.

i) BLOT: Paxillin
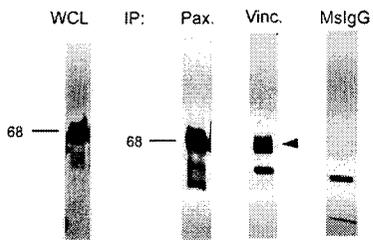
iii) BLOT: Paxillin
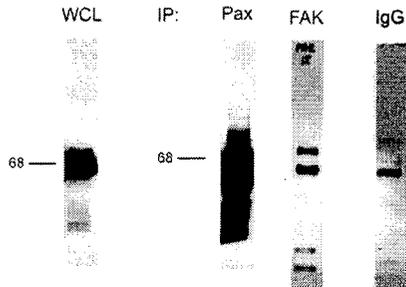
ii) BLOT: Vinculin
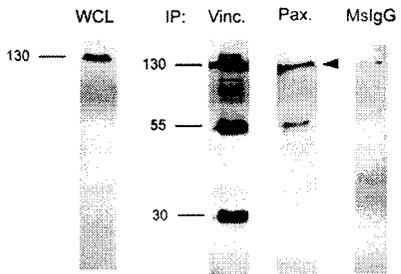
iv) BLOT: FAK
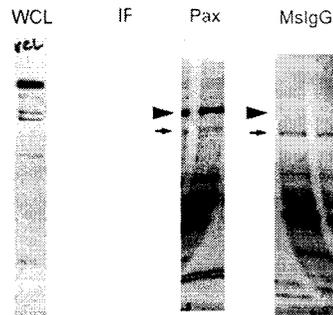
Phosphotyrosine

Polyclonal antibodies against phosphotyrosine residues showed a broad array of bands in WCL. At lower WCL protein concentrations several prominent bands could be discerned and appeared at 55, 70, 120, 130 and 140 kD (Fig. 23i). With major phosphotyrosine bands in WCL being detected at 70 and 120 kD, the possibility that these bands were phosphophorylated forms of FAK and paxillin was considered. To investigate this paxillin and FAK immunoprecipitates, under denaturing conditions, were blotted for phosphotyrosine containing residues. Interestingly, immunoprecipitates for paxillin (Fig. 23ii) showed high level of reactivity while polyclonal FAK precipitates appear to have no detectable tyrosine phosphorylation (Fig. 23iii). When increasing amounts of paxillin precipitates was loaded into the gel increasing band intensity was observed when blotted with phosphotyrosine antibodies.

β1-integrin/FAK

Immunoprecipitates failed to show a direct interaction between β1-integrin and FAK (Fig. 24). Monoclonal and polyclonal integrin antibodies were blotted with monoclonal antibodies to FAK and failed to show any band at 120 kD (Fig. 24i). Similarly, polyclonal FAK precipitates did not show the presence of β1-integrin even after long periods of film exposure (Fig. 24ii).

Vinculin/Actin

With the close spatial and temporal distribution of actin and vinculin their direct in vivo binding was assessed. Pooled monoclonal vinculin precipitates (Fig. 25i) that were probed with a monoclonal actin antibody showed the presence of a high amount of actin indicated by the presence of a prominent band at 43 kD (arrow). Non-pooled vinculin antibodies failed to precipitate actin (data not shown). Polyclonal anti-actin antibodies

precipitated actin but in much smaller amounts than might be expected. Explanations for this include that the polyclonal actin antibody does not co-immunoprecipitate well (no data was available from the supplier) or that the amount of vinculin linked to actin relative to the total amount of actin precipitated is extremely small.

Figure 23. Whole cell lysates (WCL), FAK and paxillin immunoprecipitates (IP) probed for phosphosphotyrosine. In whole cell lysates 4 or 5 major bands appear with ones at 70 kD and 120 kD being candidates for phosphorylated paxillin and FAK respectively. (i). FAK (ii) and paxillin (iii) immunoprecipitates were probed with phosphotyrosine to assess this possibility.

i) BLOT: pTyr

WCL   IP: pTyr   Rbt. IgG
                   (native)   (native)

15 ul   3 ul

~140 —
~120 —   - ~130
~70 —
~55 —

ii) BLOT: Pax   pTyr

IP: Pax   Rbt
                                        IgG
               3 ul   10 ul 15 ul   15 ul
                       Pax

68 —

iii) BLOT: FAK   pTyr

IP: FAK   FAK   Ms
                            IgG

120 —

◄ IgG

Figure 24. Whole cell lysates (WCL) and immunoprecipitates (IP) probed with monoclonal FAK (i) and β1-integrin (ii) showing the specificity of each immunoprobe but the absence of their direct interaction in whole cell lysates.

i) BLOT: FAK

WCL IP: FAK β1 mab β1 pab

120 —
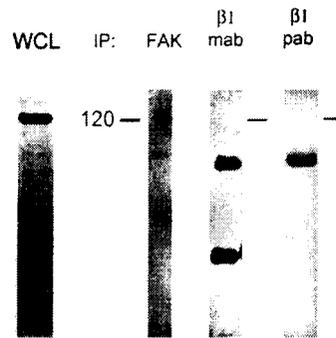
ii) BLOT: B1-integrin

WCL IP: β1 FAK

140—
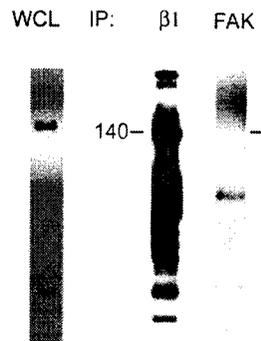
**Figure 25.** Whole cell lysates (WCL) and immunoprecipitates (IP) probed with monoclonal vinculin (i) and monoclonal actin. Although both antibodies reacted specifically in whole cell lysates (WCL), actin immunoprecipitations probed for vinculin (i) failed to detect any specific antigen while actin was readily detected in vinculin immunoprecipitations (ii).

i) BLOT: Vinculin
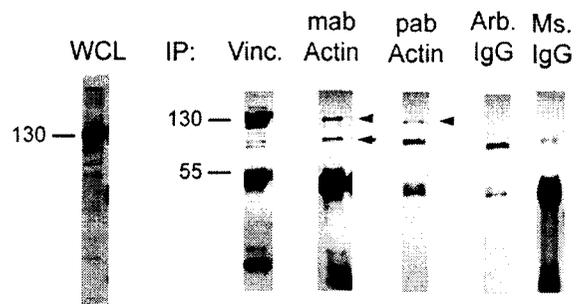
ii) BLOT: mab Actin
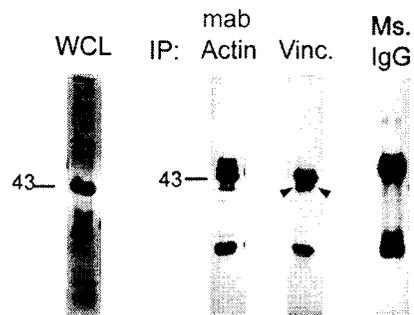
C. IMMUNOELECTRONMICROSCOPY

Immunoelectronmicroscopy using monoclonal pan-Cadherin antibodies showed specific staining at desmosome-like junctions. These junctions which, in part, compose the junctional complex found at the base of adjacent Sertoli cells are found in close apposition to the ectoplasmic specialization component of the junctional complex. They are also recognized by direct attachment to the Sertoli cell intermediate filaments.

Immunostaining, at the ultrastructural level, supports light level observations that Cadherins do not localize to the Sertoli cell/germ cell junction. Fig. 26 (ai & aii) show an elongate spermatid in cross section with no gold particles. Arrows show the location of the ectoplasmic specialization which includes a junctional actin. Fig. 26 (b-f) show lower magnification electronmicrographs of Cadherin staining indicating that Cadherin antibodies are specific to junctional sites and do not stain at other locations within the tissue section. Higher magnifications (b'-f') show localization of gold particles to Sertoli/Sertoli cell junctions and the radiating intermediate filaments. Gold particles appear to localize near to areas that are electron dense, which is likely, the submembranous plaque of the desmosome. Fig. 26g shows specific staining along an electron dense circular structure which when observed at higher magnification shows a cluster of intermediate filaments (Fig. 26g') leading to a group of gold particles. This structure is likely a desmosome that has been cut in an oblique section. In general, gold particles were observed in groups possibly as a result of discontinuous exposure of the antigen within the tissue section. Fig. 26 (g) shows a portion of a basal junctional complex with a desmosome-like junction on the left (arrow) and part of the ectoplasmic specialization on the right (arrowhead). The desmosome-like portion of the complex shows a linear array of gold particles while the actin portion shows almost no gold (Fig. 26g). Some gold appears to be present at the actin component of the junctional complex (Fig. 26g') but the presence of intermediate filaments

suggests that they could be associated with desmosome-like junctions in a different plane of section. Normal mouse IgG controls (Fig. 26h, h'), used at an equivalent protein concentration as the primary antibody, showed no gold particles at desmosome-like junctions.

Figure 26. Immunoelectronmicroscopy localization of pan-Cadherin viewed at low and high magnification. Panel ai/aii shows a spermatid in cross section including the Sertoli cell membrane and the accompanying actin layer. The absence of gold is consistent with light level data and suggests that cadherin molecules are not concentrated at apical ESs. Panels b-g show the presence of specific p-Cadherin staining at Sertoli/Sertoli junctions. Gold particles (arrow heads) appear not to be concentrated at ectoplasmic specializations but, rather, to desmosome-like junctions which can be identified by the presence of radiating intermediate filaments (arrows). Panels b-e show the presence of clusters of gold localizing to desmosome-like junctions. Panel d' (big arrow head) shows a tight junction next to a desmosome-like junction. Panel f shows a desmosome-like junction cut in an oblique section with gold particles following the length of the plaque. Panel g shows that gold is concentrated at portions the Sertoli/Sertoli interface and is associated with intermediate filaments but absent from the ectoplasmic specialization. Normal mouse IgG controls (Panel h) show no gold particles at desmosome-like junctions.
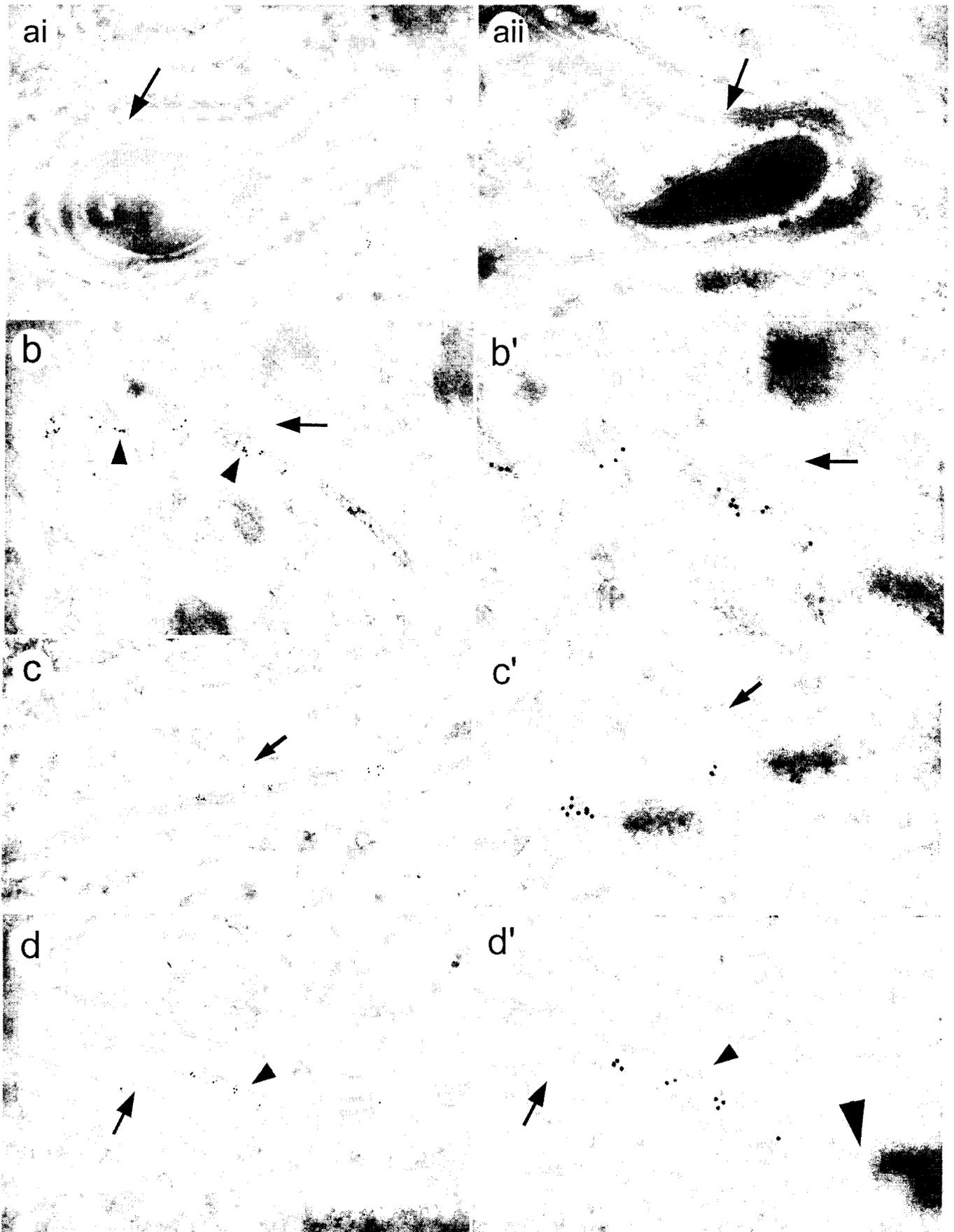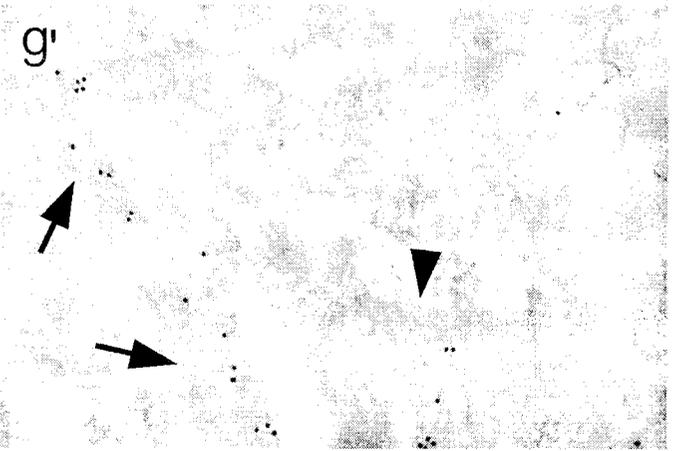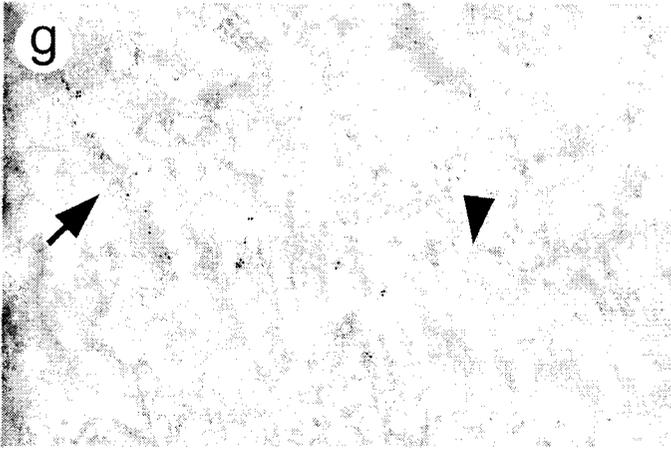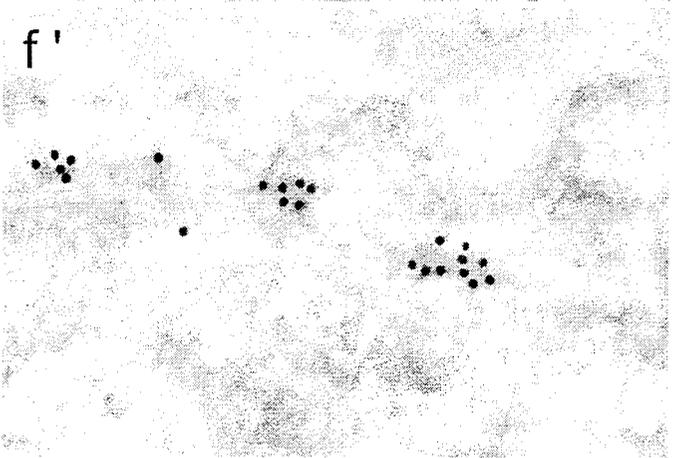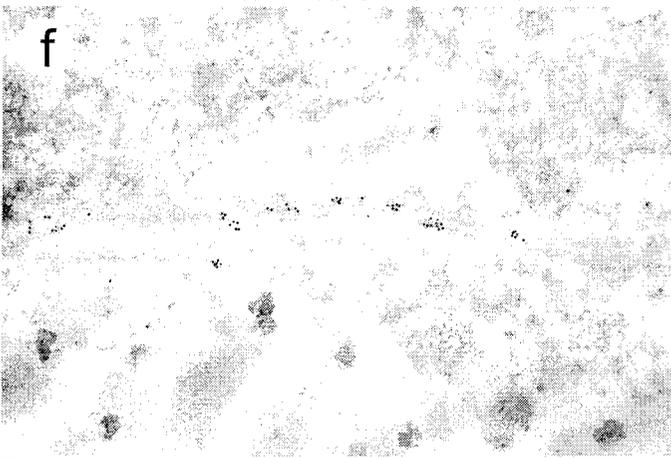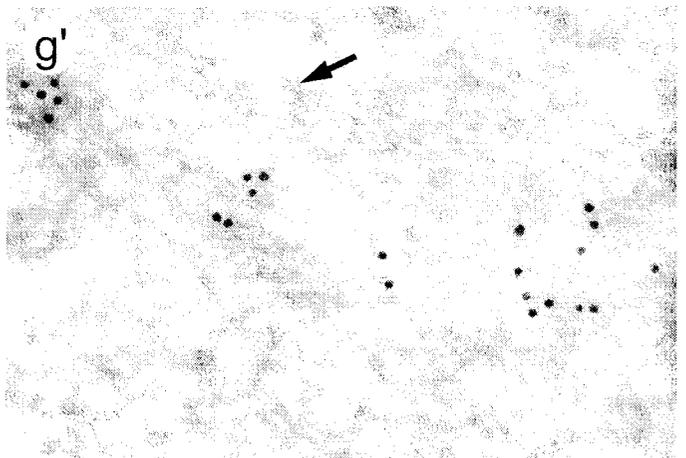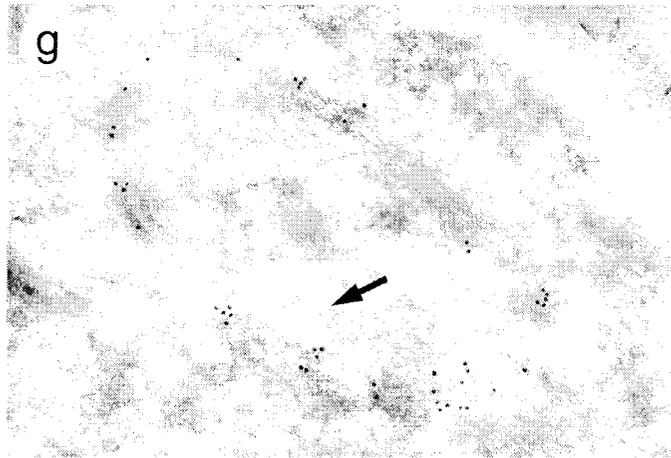
DISCUSSION

Overview

By using the distribution of filamentous actin as a marker for sites of cellular adhesion between adjacent Sertoli cells (S/S junctions) and between Sertoli cells and germ cells (S/G junctions) I have confirmed the presence of β1-integrin and vinculin at these sites. I have further determined that Integrin Linked Kinase is associated with these sites while FAK and paxillin are not detectable. Pan-Cadherin and β-catenin were not observed at the Sertoli cell-germ cell ES but, rather, were highly expressed in areas associated with basal junctional complexes. Ultrastructural data support the absence of a cadherin molecule from the Sertoli/germ cell interface and indicates that that the cadherin complex does not appear to have an ES pattern of distribution at basal sites but rather is concentrated at desmosome-like junctions. Antibodies against phosphotyrosine containing proteins appear distributed abundantly throughout the epithelium but stain most intensely at junctional sites. Paxillin, but not FAK, appears to be present in a phosphorylated state of lysates of whole seminiferous epithelium. To corroborate immunofluorescence co-localization we have used co-immunoprecipitation to assess the possibility of direct protein-protein interactions in the epithelium.

Previous experiments have shown that in the adult rat seminiferous epithelium there is a curious absence of several key adhesion molecules that have been implicated with signaling events associated with cellular adhesion. Some members of the cadherin family, such as E-cadherin, have been detected at cell-cell junctions in almost all types of epithelia (Hirano et al., 1987, Geiger et al., 1990) and have been shown to promote cellular adhesion, but appear not to be expressed in the rat seminiferous epithelium (Byers et al., 1994). Some investigators have suggested that N-cadherin may be present at adhesion

sites between Sertoli cells and early spermatids when grown in vitro (Newton et al., 1992, Byers et al., 1994). A recent study even reported the possibility of N-cadherin expression at sites of spermatid/Sertoli cell adhesion in whole seminiferous epithelium. The same study, however, was not able to detect the β1-integrin at any site within the seminiferous epithelium (Wine and Chapmin, 1999), possibly because they used fixed tissue and harsh processing techniques. Further discrepancy arises when considering the presence of the α- and β-catenins, two cadherin- associated molecules, at basal S/S junctional complexes but their absence from apical S/G junctions (Byers et al., 1994). The only adhesion molecule found to be present both at apical and basal ESs is β1-integrin (Pfeiffer et al., 1991; Palombi et al., 1992; Salanova et al., 1995 Salanova et al., 1998).

The absence of a representative in vitro model of seminiferous epithelium has yielded a significant void in the literature pertaining to the control processes of cellular adhesion. As a prerequisite to understanding such events I have, for the first time, shown the spatial and temporal distribution of molecules likely to be involved in the regulation of turnover of cell-cell junctions in the seminiferous epithelium. In addition, this study is the first to indicate direct protein interactions which may exist in distinct locations in the epithelium as based on immunofluorescence localization. I have also shown the ultrastructural localization of p-Cadherin to a component of the blood-testes barrier, the desmosome-like junction.

The changing morphology of actin in ectoplasmic specializations throughout spermatogenesis provides a basis for the notion that these adhesion sites are not static structures but ones that are constantly being remodeled as the germ cell changes from round, to elongate, to sickle-shaped. At basal sites, junction turnover allows the new

generation of spermatids to pass through the blood-testes barrier into the adluminal compartment. Fluorescent phallotoxins have been routinely used as markers for ectoplasmic specializations, which contain filamentous actin as a main structural components, at both S/G and S/S junctional sites. The staining pattern of phalloidin provides a sensitive and reliable marker for the presence and spatial distribution of these junctions throughout the spermatogenic stages. In addition to ectoplasmic specializations, other filamentous actin containing structures are readily labeled including tubulobulbar processes and intracellular bridges.

β1-integrin & Integrin Linked Kinase are Present at ESs

My immunofluorescence and immunoblotting results have confirmed earlier reports (Pfeiffer et al., 1991; Palombi et al., 1992; Salanova, et al., 1995, Salanova et al., 1998) that the β1-integrin subunit is expressed and concentrated at both S/S junctions and S/G junctions. My immunofluorescence has shown a high expression of the integrin at S/G junctions and moderate levels at S/G junctions that, at both sites, is co-distributed with actin filaments within ESs as indicated by phalloidin staining.

ILK has a spatial and temporal distribution similar to that of β1-integrin. Maximum immunofluorescence staining of both molecules is observed adjacent to spermatids deep within the Sertoli cell crypts. Just prior to sperm release, staining occurs along the dorsal side of the spermatid head in the same location as actin staining in ESs. Staining for β1 and ILK also appear co-distributed with newly forming ESs, which develop in close association with early spermatids during stage IX of spermatogenesis. The close spatial and temporal association of β1-integrin, ILK and ES actin filaments at ES sites indicates that the β1-integrin/ILK complex may participate in junction turnover during spermiation and

during turnover of the basal Sertoli cell junctional complex. Co-immunoprecipitation of these two molecules provides biochemical support for a direct β1-integrin/ILK interaction in vivo. This interaction is likely to occur at junctional sites because the only instance where these two molecules are expressed together is at ES sites as defined by phalloidin staining. Because the expressions of the β1-integrin and ILK are not restricted to ESs only during events such as spermiation and turnover of the blood-testes barrier, it is possible that this complex is involved with continuous structural changes of the junction particularly at apical sites where the adhesion complex must conform to changes in shape of the associated spermatid head. As β1-integrin and ILK appear to be co-expressed throughout the spermatogenic stages it possible that the β1-integrin/ILK complex is a constitutive one rather than an induced one. The only location in the epithelium where β1-integrin and ILK is not co-distributed is in the early germ cells. Here, some ILK staining occurs at the periphery of primary spermatocytes. This staining could be attributed to ILK associating with other known substrates such as the β2- and β3- integrin cytoplasmic tails, two molecules which ILK has shown to have an in vivo association with (Hannigan et al., 1996) but have not been identified as being present in the adult rat seminiferous epithelium. Despite the fact that the tubule wall was removed during tissue collection and epithelium cells were washed prior to lysis the possibility remains that the detected β1-integrin/ILK interaction is occurring at sites between the base of epithelial cells and remaining portions of the limiting membrane even after dissection. Nevertheless, it is this investigators opinion that with the removal of the tubule wall, tissue sections controls accounting for intense staining at the basement membrane, absence of detectable ILK staining at the limiting membrane, and the intense spatial co-distribution of these two molecules at ESs throughout the spermatogenic stages that the detected β1-integrin/ILK interactions is occurring at ES sites. Additionally, during immunofluorescence studies of isolated cells integrin and ILK

expression was not detected at basal portions of Sertoli cells. If the $\beta1$-integrin is not expressed at these sites, then the question arises as to what is the adhesion molecule which is maintaining adherence between the base of the Sertoli cell and the limiting membrane. The known presence of hemidesmosome-like junctions at the base of Sertoli cells indicates that desmosome related molecules such as $\alpha6\beta4$-integrin could be present at these sites, although this has not been investigated. Even thought previous studies have not detected desmosome related proteins, such as Plectin (Guttman et al., 1999) at these sites, intermediate filaments show contacts to these sites both at the light level and the ultrastuctural level.

Focal Adhesion Kinase is not associated with ESs

FAK is not detectable at junctional sites in the adult rat seminiferous epithelium as verified by the absence of staining at sites labelled with fluorescent phalloidin. FAK staining is restricted to basal germ cells and appears diffusely, but intensely, expressed throughout the cytoplasm of spermatogonia and spermatocytes. Expression of FAK in spermatogonia cytoplasm is more intense than that in spermatocytes and is comparable to FAK staining in endothelial cells, a convenient positive control found in the interstitial blood vessels. To further corroborate that FAK does not associate with the $\beta1$-integrin in vivo co-immunoprecipitation failed to show a direct interaction in whole cell lysates of these proteins. It is interesting that a direct in vivo association, in tissue or primary epithelia, between $\beta1$-integrin and FAK has never been shown despite their strong interaction in cultured cells (Schaller et al., 1995; Guan, 1997).

By reason of the fact that FAK has been shown to be present at cell adhesion complexes in many other systems, the absence of this molecule at ectoplasmic

specializations (an actin related intracellular adhesion junction) is a significant finding. The spatial distribution of FAK staining is intriguing when considering that FAK is known to be one of the major mediators of integrin activated signaling events and is also a protein that has been shown to co-distribute with integrins, paxillin and vinculin at sites of cellular adhesion in cultured cells. Also, the apparent lack of phosphorylated FAK, as suggested by the lack of a FAK/phosphotyrosine interaction and co-distribution of FAK and phosphotyrosine immunofluorescence, is intriguing. A possible explanation for the absence of FAK at adhesion sites in the testes is that it may not be required for junction turnover. For instance, FAK has been shown to have many functions which are not all directly related to cell adhesion and has been implicated for roles in cell mobility, migration and shape change (Ilic et al., 1997, Zachary, 1997). Studies have shown FAK expression to be more prominent in embryonic epithelia and developing systems than adult tissue (Tani et al., 1996). In fact, some studies have shown that in developing tissues FAK is highly expressed, but in the same adult tissue expression is faint, if not undetectable (Tani et al.,1996). The seminiferous epithelium is developmentally one of the most dynamic tissues known and undergoes complex morphological changes as diploid spermatogonia proliferate, divide meiotically and differentiate into spermatozoa. Our data indicates strong FAK expression in spermatogonia, the stem cells of the seminiferous epithelia. Perhaps, FAK functions in the initial developmental changes of the spermatogenic cell lineage or is associated with signals from the extracellular matrix. If this is true, then perhaps FAK also shows greater levels of phosphorylation in embryonic tissue than in adult ones. Some authors have suggested that expression of FAK in vitro is an adaptation to culture conditions providing an explanation for the differences in staining patterns often observed between in vivo and in vitro conditions (Tani et al., 1996).

Phosphotyrosine Proteins are Concentrated at ESs

The absence of FAK at ectoplasmic specializations suggests that another major tyrosine kinase may be downstream of the β1-integrin. The observation that a probe for phosphotyrosine intensely labels both S/S junctions and S/G junctions is consistent with this possibility. Significantly, phosphotyrosine staining dramatically highlights basal junctions coincident with movement of spermatocytes through the blood-testes barrier, an event known to occur during stage VII/VIII of spermatogenesis in the rat. This observation indicates that a major phosphotyrosine containing protein may be involved with the rearrangement of the S/S junctional complex. In addition, since phosphotyrosine was prominent at S/G junctions just prior to spermiation, the same or a similar phosphotyrosine containing protein could be functionally important for breakdown of the junction. When whole cell lysates were probed with polyclonal phosphotyrosine antibodies several prominent bands appeared at about 55, 70, 120, 130 and 140 kD. Although the presence of a band at about 120 kD seems to suggest the presence of the tyrosine kinase, FAK, this does not appear to be the case. FAK immunoprecipitates appear not to contain an detectable phosphotyrosine. Differently, is the level of phosphorylated paxillin which appears to be relatively high in cell lysates With the detection of a major phosphotyrosine band at about 70 kD and the abundance of paxillin detected at the light level, it is possible that paxillin is one of the major phosphotyrosine containing proteins in the adult rat seminiferous epithelium. It would be interesting to address which proteins could possibly account, completely or in part, for the bands observed at 55, 120, 130 and 140 kD. For instance, with the absence of junctional FAK it is possible that Src, a 60 kD protein, could be another major phosphotyrosine containing peptide with important roles at ESs. Additionally, with the apparent high expression of phosphotyrosine protein(s) at the ESs it would be interesting to evaluate whether or not tyrosine phosphorylation is a necessary

element for their formation. Primary seminiferous epithelia could be potentially incubated with a variety of growth factors or chemical inhibitors to evaluate this. Subsequent to incubation with phosphotyrosine junctions could be evaluated for the relative amounts of adhesion elements including actin and vinculin.

Paxillin is Widely Distributed in the Seminiferous Epithelium

Paxillin, a vinculin-binding protein, has a nearly ubiquitous spatial distribution throughout the seminiferous epithelium cytoplasm but is not concentrated at ectoplasmic specializations. The most outstanding observation regarding paxillin expression throughout spermatogenesis is its increase in abundance during stages VII/VIII in round spermatids and in the columnar portion of Sertoli cells. Curiously, paxillin does not appear to be present in the spermatocytes. The recruitment of paxillin to the tubulobulbar complexes, structures thought to have an anchoring function between the Sertoli and germ cell, just prior to spermiation suggests that Paxillin could be functionally involved with the turnover of the actin network and therefore a possible candidate for involvement with sperm release. The co-immunoprecipitation of Paxillin and FAK, but the absence of immunostaining for FAK in apical regions of the epithelia, suggests that a direct FAK/Paxillin interaction occurs in the basal portions of the epithelium.

The localization of vinculin, a known paxillin binding protein, to both S/S junctions and S/G junctions occurs throughout the spermatogenic stages. Vinculin also occurs at other actin related structures such as the tubulobular complexes. These structures are the only site during spermatogenesis, where the codistribution of vinculin and paxillin is detectable by immunofluorescence, thus suggesting a location in the epithelium that accounts for their direct interaction. However, even though paxillin is not detectable at

junctional sites the abundance of this molecule throughout the epithelium indicates that a direct interactions may be occurring elsewhere in the epithelium. Since FAK and Paxillin are not concentrated at ESs, it is likely that a FAK/Paxillin protein complex is not directly associated with control of junctional actin architecture but may be associated with other developmental roles elsewhere in the epithelium. Vinculin, an actin linking protein, showed a close spatial and temporal distribution with actin throughout spermatogenesis. Expression of vinculin appeared to be particularly high at ectoplasmic specializations. Co-immunoprecipitations verified that vinculin binds to actin although the relative amount of vinculin detected in actin precipitates was much less than the amount of actin detected in vinculin precipitates. This could be explained by the fact that most vinculin precipitates contain actin while a much smaller portion of actin precipitates contain vinculin. Furthermore, total precipitated actin includes both globular and filamentous types while fluorescent phalloidin staining detects only the later form.

Cadherin vs. Integrins at Junctional sites

The apparent absence of a cadherin/catenin complex at apical ESs and its localization to desmosome-like junctions at S/S cell junctional complexes suggests that an integrin could be a key molecule involved in the control of ectoplasmic specialization turnover. Nevertheless, with the co-distribution of the Cadherin and ß-catenin immunofluorescence, their co-immunoprecipitation and the ultrastructural localization of the pan-Cadherin to desmosome-like junctions it is likely that such a complex plays an turnover of the Sertoli/Sertoli junctional complex. The fact that the pan-Cadherin/catenin complex does not appear to be associated with ESs between adjacent Sertoli cells nor between Sertoli cells and germ cells indicates that a Cadherin mediated signaling pathway may not be a component of ES control. The absence of a Cadherin or catenin molecule between the

Sertoli cell and the germ cell at ESs is unusual, but the known association of catenins with desmosomes (Cowin et al., 1986) provides justification for their presence at desmosome-like junctions in the seminiferous epithelium. It is possible, however, that both integrin (directly) and cadherin (indirectly) related signaling pathways are required for control of the Sertoli cell/Sertoli ectoplasmic specialization. This could be explained by the cross-talk in signaling pathways that may occur between integrin and Cadherin associated molecules. With the presence of both molecules at the Sertoli cell junctional complex it seems almost certain that the coordinated involvement of both molecules are involved in its architectural change. The presence of β1-integrin, Integrin Linked Kinase and vinculin at both apical and basal ES sites suggests that integrin mediated signaling events have a prominent role in the architectural rearrangement of ES-related intracellular adhesion junctions.

Possible Ligands for β1-integrin

With the presence of a β1-integrin mediated signaling pathway at major sites of intercellular adhesion in the testes, it is interesting to contemplate what might initiate or activate such a sequence of signaling events in either an "outside-in" or an "inside-out" manner. This question seems particularly important since these sites are intimately related with sperm release and turnover of the basal junctional complex.

Although integrins have been shown to be associated with a variety of ligands, one ligand stands out as being a good candidate in the seminiferous epithelium. Previous investigations have explored the relationship between an ADAM protein (fertilin) on the surface of the mammalian sperm acrosome, and the α6β1 receptor on the surface of mouse eggs (Chen et al., 1999). Fertilin has a strong affinity for the α6β1 receptor, and when blocked, disrupts sperm to egg adhesion suggesting that α6β1-fertilin binding is

important for adhesion and initiation signaling events (Almeida et al., 1995). Since it is known that the $\alpha6\beta1$ dimer is found at ectoplasmic specializations, it is possible that a member of the ADAM family is responsible for activation and or clustering of the integrins during ES structural modification.

Activation of integrin-mediated signaling pathways may be initiated by submembranous molecules capable of changes in phosphorylation in an inside-out manner. Obvious candidates include ILK, which has been shown to affect the structural integrity of cell-cell junctions when up-regulated (Hannigan et al., 1996, Dedhar and Hannigan, 1996). It is possible that a submembranous event or internal signal could prompt ILK to phosphorylate the cytoplasmic tail of the $\beta1$-integrin and thereby cause integrin activation. Since it is likely that an alternative tyrosine kinase, to FAK, exists in close association with the integrin cytoplasmic domain, it would be interesting to evaluate what the effects of inhibiting or up-regulation of such a kinase would be on sites of adhesion in the seminiferous epithelium. Some authors have speculated that elements that would contribute to inside-out cascades leading to junctional turnover may be hormones such as follicle-stimulating hormone. (Salanova et al., 1998). Other studies have linked testosterone with the fundamental spermatogenic events such as spermatid detachment (O'Donnell et al., 1996) and progression of round spermatids to elongate spermatids (McLachlan et al., 1994).

Hypothetical Model for Junctional Adhesion and Control Molecules

Based on the distribution of $\beta1$-integrin signaling molecules in the seminiferous epithelium and the hypothesis that ESs are structurally maintained and controlled by an integrin mediated pathway a hypothetical model of construction can be proposed. With the

consideration that the S/S and S/G interfaces are different in design I suggest independent models to represent adhesion molecules and junctional components likely to be involved in signaling events. Paired transmission electronmicographs with each model depict the ultrastructure of both S/G junctions (Fig. 27a) and S/S junctions (Fig. 27b). At both sites of adhesion ectoplasmic specializations consists of an endoplasmic reticulum (ER), a hexagonal array of actin and a submembranous space. The junctional complex found between adjacent Sertoli cells also contain desmosome-like junctions and tight junctions in close association with the ES actin.

In both the S/G and S/S junctional model I have included the transmembrane $\alpha6\beta1$ dimer with integrin cytoplasmic tail extending into the Sertoli cytoplasm. I have also included the presence of a cadherin/β-catenin complex molecule at S/S junctional complexes but not at S/G junctional sites or in association with the actin component of the basal ectoplasmic specialization. I have shown the cytoplasmic tail of the integrin subunit to interact, directly or indirectly, with a putative tyrosine kinase which,

Figure 27. Proposed model for the presence of integrin and Cadherin related molecules at both apical (a) and basal (b) junctional complexes. The presence of the β1-integrin, but absence of a detectable Cadherin associated molecules, suggests that a potential downstream signaling cascade associated with changes in junctional actin could be integrin related. Upon interaction with spermatid surface proteins integrin activation or clustering could occur and a subsequent phosphorylation of its cytoplasmic domain by downstream kinases such as ILK. Alternatively, an initial signal could originate from a submembranous member of the cascade. The absence of detectable FAK, but prominent distribution of phosphotyrosine residues, suggests that other tyrosine kinases could be present and function as mediators to downstream molecules such as vinculin, which could directly interact with the actin cytoskeleton.

a

Sertoli Cell Germ Cell
ES
Endoplasmic Reticulum
Junctional Actin
Spermatid

a'

pTyr
Paxillin
Vinculin
Tubulobulbar Process
Actin Array
Endoplasmic Reticulum
Junctional Actin
α6 β1
α6 β1
ILK
Integrin Clustering?
Vinculin
Tyrosine Kinase?
Vinculin
Small GTPases (Rho ?)
Vinculin

Sertoli Cell-Germ Cell Junction

b

Cell-Sertoli Cell ES
Endoplasmic Reticulum
Junctional Actin

b'

Paxillin? pTyr
Vinculin
Desmosome-like Junction
β-Cat
Cad.
Cad.
β-Cat
Tyrosine Kinase?
Paxillin? pTyr
α6 β1
ILK
Ectoplasmic Specialization
α6 β1
Paxillin? pTyr
ILK
Tyrosine Kinase?
Tight Junction
Vinculin
Junctional Actin
Sertoli Cell
Paxillin pTyr
Spermatogonia
Hemi-desmosome like junctions
pTyr
? ? ? ?

Sertoli Cell-Sertoli Cell Junction

based on my data, is probably not FAK. Since vinculin typically does not directly interact with the β1-integrin, but is known to link directly to the actin cytoskeleton, I have diagrammed it downstream of a possible tyrosine kinase and make the speculation that it signals to a member of the small GTPases, possibly rho or rac. Molecules such as these could cause direct rearrangement of the actin cytoskeleton.

At both the S/S junctions and S/G junctions, I have removed FAK from the ectoplasmic specialization to indicate that it does not concentrate at junctional sites but, rather, is found to be highly expressed throughout spermatogonia cytoplasm. Paxillin, but not FAK, appears with phosphotyrosine residues. It is also shown to be present in the cytoplasm of both the Sertoli cell and the germ cell but with question at S/S junctions. At S/G junctions paxillin and vinculin can be seen in close association with the actin network surrounding the tubulobulbar processes and are diagrammed to have a direct interaction. An unidentified transmembrane molecule is indicated opposite to the integrin dimer as a possible ligand of the β1- integrin. With the known association of the $\alpha6\beta4$ integrin with desmosomes I have included the integrin dimer as a molecule that may be localized to the hemidesmosome-like junctions found at the base of the Sertoli cells. This molecule along with other unidentified transmembrane molecules may function in maintaining adhesion between the seminiferous epithelium and the limiting membrane.

CONCLUSION

The presence and spatial co-distribution of $\beta 1$-integrin, ILK, vinculin, phosphotyrosine and actin-related cell-cell junctions in the rat seminiferous epithelium suggests the importance of integrin mediated molecules during the structural modification of basal and apical intracellular adhesion junctions. The absence of FAK, and concentration of paxillin, from junctional sites is intriguing and suggests that these molecules may not be directly associated with the control of junction turnover but could assume a different function during spermatogenesis. My data has provided the first evidence showing that ILK is present at an in vivo actin-related site of adhesion and the absence of a cadherin molecule from an in vivo epithelial cell/cell interface. The reported absence of E-cadherin and N-cadherin, but the positive reactivity of pan-Cadherin in the seminiferous epithelia indicates that another Cadherin molecule remains to be described at the basal junctional complex in the seminiferous epithelium. Based on the molecules detected at ectoplasmic specializations it appears that these junctions have properties both of focal adhesions and epithelial cell-cell junctions. The question of whether junction formation and control is integrin related, cadherin-related or both, during spermatogenesis is a complex issue and needs to be further addressed.

FUTURE DIRECTIONS

The seminiferous epithelium provides a good system to test fundamental questions about signaling events associated with cellular adhesion and the actin cytoskeleton. The present study has addressed many issues but also raises many others requiring further study. The following are some of the possibilities that seem worthy of trial:

Determining the Ligand of the β1-integrin in the seminiferous epithelium

An important question to answer about a potential β1-integrin signaling pathway is to identify the ligand, or molecule with the potential to activate the integrin pathway. A good candidate is a member of the ADAM family. However, the distribution and expression of such a molecule has not been investigated in the seminiferous epithelium. If identified as being present in the seminiferous epithelium and co-distributed with the β1-integrin, blocking this molecule could provide further answers as to the function of the integrins and closely associated molecules.

Cytoskeletal clustering of integrins and the small GTP-ases

Rho family proteins functions as molecular switches that have the ability to cycle between a GDP-bound state and an active GTP-bound state. These molecules are also known to be involved in integrin clustering (Schoenwaelder and Burridge, 1999). It would be interesting to evaluate whether there is differential GTP-ase expression as integrin expression changes during development of the ectoplasmic specialization. Perhaps there is repetitive integrin clustering and cycling of Rho family members including Cdc42 and Rac.

Is PINCH present at Ectoplasmic Specializations?

The N-terminal domain of ILK has been shown to interact with the recently described adapter protein, PINCH (Tu et al., 1999). Further studies have shown that PINCH may be necessary for localization of the PINCH/ILK complex to focal adhesions (Li et al., 1999, J. Cell Science, in press). Interestingly, this study has also provided evidence that ILK does not localize to E-cadherin mediated junctions. Since ectoplasmic specializations share features of both classical adherens junctions and focal adhesions, the question of whether PINCH is expressed at these sites and whether it directly interacts with ILK, and not β1-integrin, is an interesting one.

FAK and pTyr: Levels of Expression in Embryonic vs. Adult Tissue

In some tissues FAK has been shown to be expressed at higher levels in embryonic tissues than adult ones (Tani et al., 1996). It would be interesting to determine whether this holds true in the seminiferous epithelium. Although FAK does not appear to be directly associated with any type of adhesion structure in the adult seminiferous epithelium, it may in early developing tissues. The question of how the expression of FAK changes in development from embryonic to adult tissue needs to be addressed. It has also been reported that levels of phosphotyrosine are frequently greater in embryonic tissue than in adult (Maher and Pasquale, 1988). Since phosphotyrosine levels are already substantial in the adult seminiferous epithelium it would be interesting to evaluate them in embryonic and pre-pubescent tissue.

Chemical Inhibitors

Phosphorylation, and in particular phosphorylation of tyrosine residues, clearly has important functions in many tissues. With the availability of several protein-tyrosine kinase inhibitors, such as herbimycin, specific events such as spermiation could be evaluated for their dependence upon tyrosine phosphorylation events.

Blocking Peptides

With the shown presence, distribution and possible interaction of several key signaling and adhesion molecules in the seminiferous epithelium associated functions of each molecule need to be explored. Despite the fact that a good representative in vitro model of the seminiferous epithelium does not exist, several methods could be used to ascertain what the functions of individual signaling molecules are. One approach could include the use of blocking peptides such as RGD-containing peptides. Primary epithelia maintained in culture for several days could be incubated with blocking peptides and then assessed for structural changes or changes in spermatogenic processes. Since a cycle in the rat takes about 12.9 days (about 53 days for total duration of spermatogenesis) it may be possible to maintain primary epithelia in culture long enough to allow a spermatogonia to go through many of the stages of spermatogenesis.

Knock Outs

Animals that are deficient for a certain molecule can be used to provide evidence for an in vivo function. Although knock-out animals for FAK are lethal early in embryonic development (Ilic et al., 1995), a comparison of histology between normal and mutant animals could provide clues as to whether FAK has a role in embryonic adhesion.

BIBLIOGRAPHY

Beach S and Vogl AW (1999): Spermatid Translocation in the rat seminiferous epithelium: coupling membrane trafficking machinery to a junction plaque. Biology of Reproduction (60): 1036-1046.

Burridge K , Turner CE and Romer LH (1992): J. Cell Biology (119): 905-912.

Burridge K, Chrzanowska-Wodnicka M (1996): Focal adhesions, contractility and signaling. Ann. Rev Cell Dev Biol. (12): 463-519.

Byers SW, S Sujarit, B Jegou, S Butz, H Hoschutzky, K Herrenknecht, C MacCalman and O Blaschuk (1994): Cadherins and cadherin-associated molecules in the developing and maturing rat testis. Endocrinology (134): 630-639.

Chen MS, EAC Almeida, A Huovila, Y Takahashi, LM Shaw, AM Mercurio and JM White (1995): Evidence that distinct states of the integrin $\alpha 6\beta 1$ interact with laminin and an ADAM (144): 549-561.

Clark EA and Brugge JS (1995): Integrins and signal transduction pathways: the road taken. Science (268): 233-239.

Cowin P, HP Kapprell, WW Franke, J Tamkun and RO Hynes (1986): Plakoglobin: a protein common to different kinds of intercellular adhering junctions. Cell (46): 1063-1073

Dedhar S and G Hannigan (1996): Integrin cytoplasmic interactions and birectional transmembrane signaling. Current Opinion in Cell Biology (8): 657-669.

Fawcett DW (1975a): In "Handbook of Physiology" (RO Greep, ed.) Sect. 7, pp 21-55. Williams & Wilkins, Baltimore. Maryland.

Franke WW., C. Grund, A. Fink, K. Weber, BM Jockusch, H. Zentgraf and M. Osborn (1978): Location of alpha-actinin in the microfilament bundles associated with the junctional specializations between Sertoli cells and spermatids. Biol. Cell (31): 7-14.

Geiger B, T Volberg, D Ginsberg, S Bitzur, I Sabanay and R Hynes (1990): Broad spectrum pan-cadherin antibodies, reactive with the C-terminal 24 amino acid residues of N-cadherin. Journal of Cell Science (97): 607-614.

Geiger B, Yehuda-Levenberg S and Bershadsky AD (1995): Molecular interactions in the submembrane plaque of cell-cell and cell-martix adhesions. Acta. Anat.

Giancotti F and Ruoslahti E (1999): Integrin Signaling. Science (285): 1028-1032.

Gilmore AP, Romer LH (1996): Inhibition of FAK signalling in focal adhesions decreases cell motility and proliferation. Mol. Biol Cell (7): 1209-1224.

Grove BD, DC Pfeiffer, S Allen and AW Vogl (1990): Immunofluorescence localization of vinculin in ectoplasmic ("junctional") specializations of rat Sertoli cell. The American Journal of Anatomy (188): 44-56.

Guan JL (1997): The role of focal adhesion kinase in integrin signaling. Int. J. Biochem. Cell. Biol. (29): 1085-1096.

Gumbiner BM (1996): Cell Adhesion: The molecular basis of tissue architecture and morphogenesis. Cell (84): 384-357.

Guttman J, D. Mulholland, A. Vogl (1999): Plectin is Concentrated at Intercellular Junctions and at the Nuclear Surface in Morphologically Differentiated Rat Sertoli Cells. The Anatomical Record (254): 418-428.

Hannigan GE, C Leung-Hagesteijn, L Fitz Gibbon, M Coppolino, G Radeva, J Filmus, J Bell and S. Dedhar (1996): Regulation of cell adhesion and anchorage-dependent growth by a new β1-integrin-linked protein kinase. Nature (379): 91-96.

Hynes RO (1992): Integrins: versatility, modulation and signaling in cell adhesion. Cell (69): 11-25.

Ilic D, C Damsky and T Yamamoto (1997): Focal adhesion kinase: at the crossroads of signal transduction. Journal of Cell Science (110): 401-407.

Ilic D, Y Furuta, S Kanazawa, N Takeda, K Sabue, N Nakasuji, S Nomura, J Fujimoto, M Okada, T Yamamoto and S Aizawa (1995): Reduced cell motility and enhanced focal contact formation in cells from FAK-deficient mice. Nature (377): 539-544.

Knight JB, Yamauchi K and Pessin JE (1995): Divergent insulin and platelet-derived growth factor regulation of focal adhesion kinase tyrosine phosphorylation, and rearrangement of actin stress fibers. J Biol Chem. (270): 10199-10203.

Kirkpatrick C and Pfeifer M (1995): Not just glue: cell-cell junctions as cellular signaling centers. Curr. Opin. Genet. Dev. (5): 56-65.

Li F, Y Zhang and C Wu (1999): Integrin-linked kinase is localized to cell-matrix focal adhesion but not cell-cell adhesion sites and the focal adhesion localization of integrin-linked is regulated by the PINCH-binding ANK repeats. Journal of Cell Science (In Press).

Ling-Hong Li, WF Jester, and J Orth (1998): Expression of 140-kDa Neural Cell Adhesion Molecule in Developing Testes In Vivo and in Long-term Sertoli Cell-gonocyte Cocultures. Journal of Andrology (19): 365-373.

Maher P and E Pasquale (1988): Tyrosine phosphorylated proteins in different tissues during chick embryo development. Journal of Cell Biology (106): 1747-1755.

McLachlan RI, Wreford NG, Mechem SJ, de Kretser DM, Robertson DM (1994): Effects of testosterone on spermatogenic cell populations in the adult rat. Biol. Reprod. (51): 945-955.

Newton S, Blaschuk OW, Millette CF (1992): N-Cadherin mediates Sertoli-cell spermatogenic cell adhesion. Dev. Dynamics (19): 1-13.

Nobes CD, Hawkins P, Stephens L, Hall A (1995): Activation of the small GTP-binding proteins Rho and Rac by growth factor receptors . J Cell Science (108): 225-233.

O'Donnell L, RI McLachlan, NG Wreford, DM de Kretser and DM Robertson (1996): Testosterone withdrawl promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium (55): 895-901.

Palombi F, Salanova M, Taron G, Garini D, Stefanini M (1992): Distribution of β1-integrin subunit in rat seminiferous epithelium. Biol. Reprod. (47): 1173-1182.

Parson JT (1996): Integrin-mediated signaling-regulation by protein tyrosine kinases and small GTP-binding proteins. Current. Opin. Cell Biol. (8): 146-152.

Pfeiffer and Vogl (1991): Evidence that vinculin is co-distributed with actin bundles in ectoplasmic ("junctional") specializations of mammalian Sertoli cells. The Anatomical Record (231): 89-100.

Pfeiffer DC, S. Dedhar, SW Byers and AW Vogl (1991): Evidence that an integrin may be present at specialized sites ("ectoplasmic specializations") of intercellular adhesion in the seminiferous epithelium. J. Cell Biol. 115 (3): 482a abst. 2803.

Russell L and Clermont Y (1976): Anatomical Record (185): 259-278.

Russell L and J Goh (1988): Localization of alpha-actinin in the rat testis: Preliminary observations. In: Development and function of the reproductive organs. VII Serono Symposia Review, No. 14. Parvinen, I. Huhtaniemi, and LJ Pelliniemi, eds. Ares Serono Symposia, Rome, Italy, pp. 237-244.

Russell L, J Weber and A Vogl (1986): Characterization of filaments within the subacrosomal space of rat spermatids during spermiogenesis. Tissue and Cell (18): 887-898.

Russell and Peterson (1985): Sertoli cell junction: Morphological and functional correlates. Int. Rev. Cytol. (94): 177-211.

Russell, L (1994): Spermiation-the sperm release process: Ultrastructural observations and unresolved problems. In: Ultrastructure of Reproduction. J. Van Blerkom and PM Motta, eds. Martinus Nijhoff, Boston, pp. 46-66.

Russell, L (1977): Observations on rat Sertoli ectoplasmic ("junctional") specializations in their association with germ cells of the rat testes. Tissue and Cell (9): 475-498.

Russell, L (1976): Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. Am. J. Anat. (148): 313-328.

Salanova M, G Ricci, C Boitani, M Stefanini, S De Grossi and F Palombi (1998): Junctional contacts between Sertoli cell in normal and aspermatogenic rat seminiferous epithelium

contain α6β1 integrins, and their formation in controlled by follicle-stimulating hormone. Biol. Reprod. (58): 371-378.

Salanova M, Stefanini M, De Curtis I, Palombi F (1995): Integrin receptor α6β1 is localized at specific sites of cell-to-cell contact in rat seminiferous epithelium. Biology of Reproduction (52): 79-87.

Schaller and Parson (1994): Focal adhesion kinase and associated proteins. Current Opinion in Cell Biology (6): 705-710.

Schaller MD, CA Otey, JD Hildebrand and JT Parson (1995): Focal adhesion kinase and paxillin bind to peptide mimicking β1-integrin cytoplasmic domains. J. Cell Biology (130): 1181-87.

Schwartz MA, Schaller MD and Ginsberg MH (1995): Integrins-emerging paradigms of signal-transduction. Ann. Rev. Cell Dev. Biol. (110): 549-599.

Seminario MC, Bochner BS (1997): Expression and function of beta 1 integrins on human eosinophils. Mem Inst Oswaldo Cruz; 92 Suppl 2:157-64.

Schoenwaelder SM and Burridge K (1999): Bidirectional signaling between the cytoskeleton and integrins. Current Opinion in Cell Biology (11): 274-286.

Sonnenberg A, Modderman PW and Hogervost F(1988): Laminin receptor on platelets is the integrin VLA-6. Nature (336): 487-489.

Tani T, H. Koskull, I. Virtanen (1996): Focal adhesion kinase pp125 FAK is associated with both intercellular junctions and matrix adhesion sites in vivo. Histochem. Cell Biol. (105): 17-25.

Tu Y, Li F, Goicoechea S and Wu C (1999): The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. Mol. Cell Biol. (19): 2425-34.

Turner CE, M Schaller and J Parsons (1994): Tyrosine phosphorylation of the Focal Adhesion Kinase pp125 FAK during development: relation to paxillin (105): 637-645.

Vogl A, Pfeiffer D and D Redenbach (1991): Ectoplasmic ("Junctional") Specializations in Mammalian Sertoli Cells: Influence on Spermatogenic Cells. Annals New York Academy of Sciences (637): 175-202.

Vogl A (1989): Distribution and function of organized concentrations of actin filaments in mammalian spermatogenic cells and Sertoli cells. International Review of Cytology (119).

Wine R and Chapman R (1999): Adhesion and signaling proteins spatiotemporally associated with spermiation in the rat. Journal of Andrology (20): 198-213

Yamada KM and Miyamoto S (1995): Integrin transmembrane signaling and cytoskeletal control. Curr. Opin. Cell Biol. (7): 681-689.

Yamada KM and B Geiger (1997): Molecular interactions in cell adhesion complexes. Current Opinion Cell Biology (9): 76-85.

Zachary I (1997): Molecules in Focus: Focal Adhesion Kinase. Int. J. Biochem. Cell Biol. (29): 939-934.

APPENDIX A

Diagrammatic representation of how seminiferous epithelium is collected for Western Blotting and immunoprecipitations studies (adapted from Beach and Vogl, 1999). When the tubule wall is separated from epithelium components such as the basal lamina, basement membrane and surrounding myoid cells are removed. This dissection was done to decrease the possibility that tubule wall components could be accounting for false positive results when assessing direct protein interactions.

Tubulue Wall Components
- Basal Lamina
- Basement Membrane
- Myoid Cells
- Extracellular Matrix

Mechanical Separation of Epithelium
from Tubule Wall

Mechanical Fragmentation
of Epithelium

Cells Lysed and Used for Western Blotting
and Immunoprecipitation
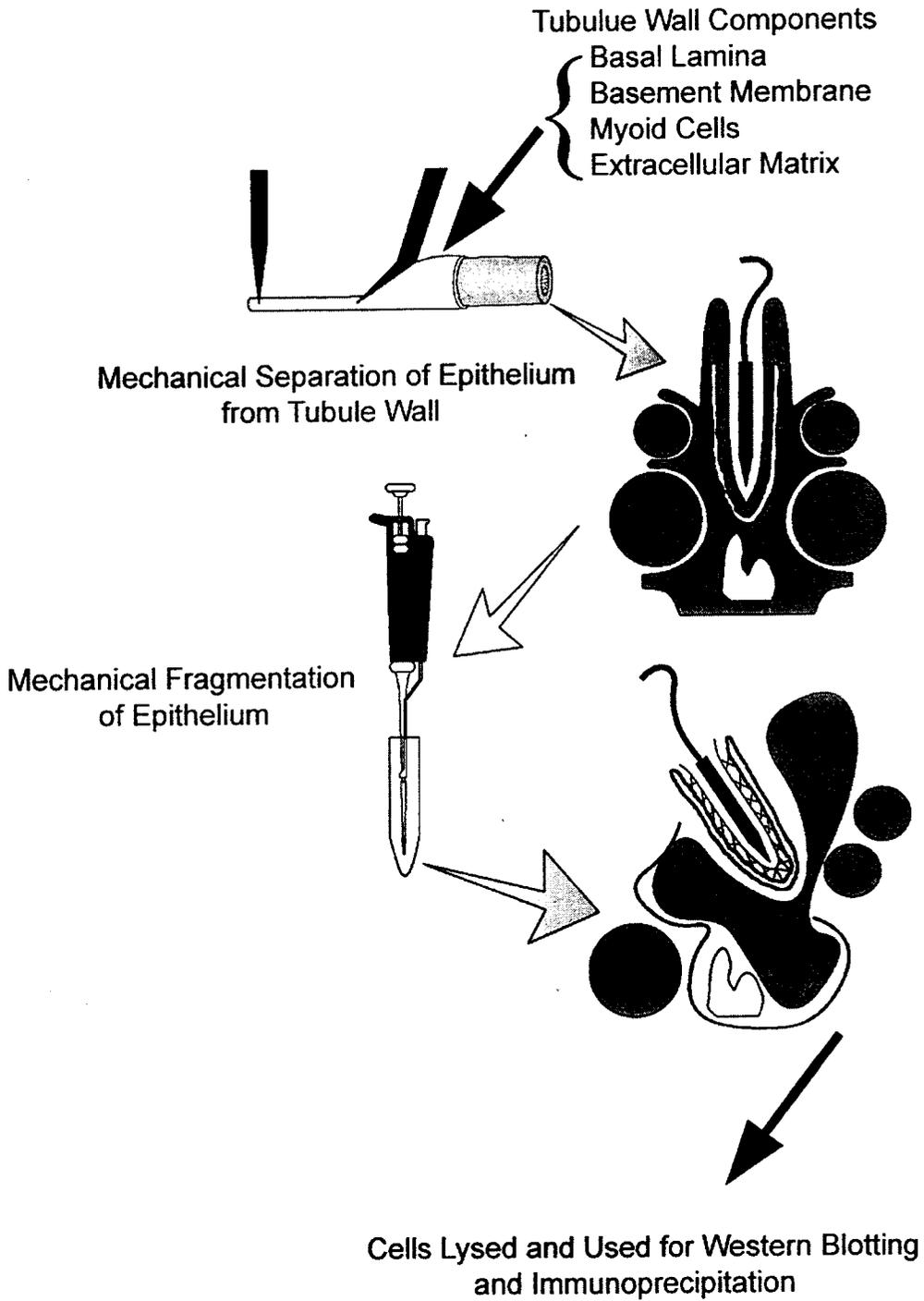
APPENDIX B

Table 1. Descriptive table of primary antibodies

Antiserum	M.W.	Mono./Poly.	Dilution (IF/IB)	Stock (mg/ml)	Source
β1-integrin	140	polyclonal	1:200/1:1000	15	Chemicon (AB1952)
β1-integrin	140	polyclonal	1:1000/1:10,000	15	G.Tarone
ILK	58	polyclonal	1:100/1:1000	0.33	S. Dedhar
ILK	58	monoclonal	1:500 (IB)	1.0	C. Wu, U.S.
pan-Cad	135	monoclonal	1:400/1:1000	10	Sigma (C1821)
pan-Cad	135	polyclonal	1:1000/1:5000	serum	Sigma (C3678)
β-catenin	92	monoclonal	1:400/1:1000	10	Sigma (C7207)
β-catenin	92	polyclonal	1:1000/1:5000	serum	Sigma (C2206)
Actin	43	monoclonal	1:1000 (IB)	1.0	Chemicon (MAB1501)
Actin	43	polyclonal	1:1000 (IB)	0.2	Santa Cruz (sc-1615)
Vinculin	116	monoclonal	1:400/1:2000	8.4	Sigma (V9131)
Paxillin Labor.	68	monoclonal	1:400/1:10,000	0.25	Transduction (P13520)
FAK	120	monoclonal	1:50/1:500	0.25	Transduction (F15020)
FAK	120	polyclonal	1:50/1:500	0.2	Santa Cruz (sc-557-G)
pTyr		polyclonal	1:100/1:1000	0.2	Transduction (P11230)

# APPENDIX C

(continued from page 51-52)

Complete panel of controls used for immunohistochemistry. Paired fluorescence (a, b) and phase micrographs (a', b') include normal IgG (a) and secondary antibody alone (b). Normal IgG concentrations were used an equivalent IgG concentration to that of the working dilution of the primary antibody while secondary antibodies were used at a 1:100 dilution. Primary antibody incubations were either at 1-2 hours for 37 °C or for overnight at 4 °C.

Figure label	Control for primary antibody
8	monoclonal pan-Cadherin
9	polyclonal β-catenin
10	polyclonal pTyr
11	monoclonal FAK
12	monoclonal Vinculin
13	monoclonal Paxillin
14	polyclonal ILK
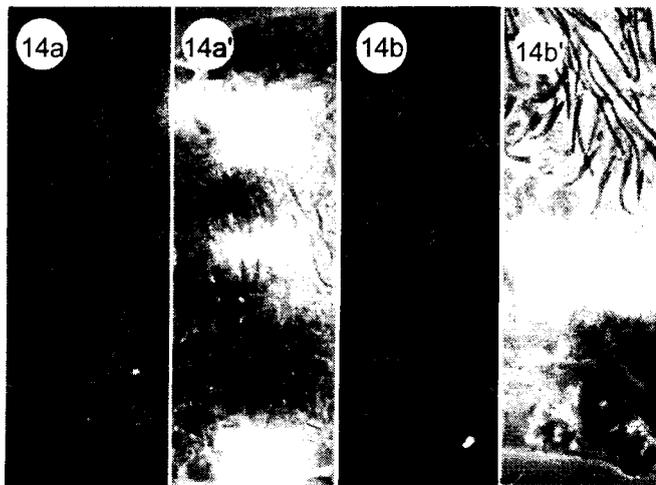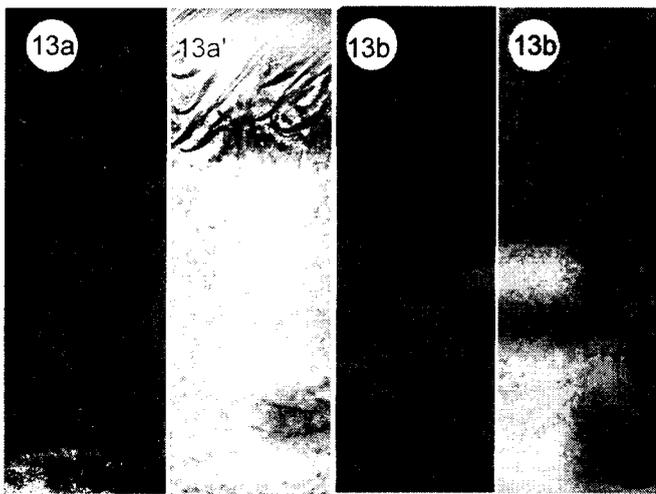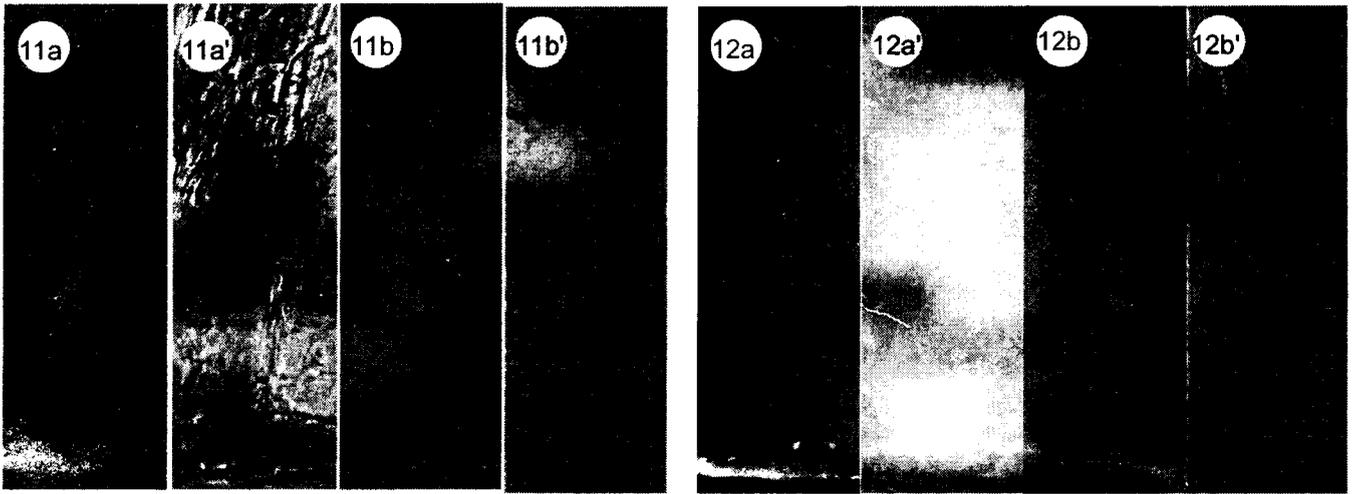
APPENDIX D

Structural features of integrin receptors (adpated from Hynes, 1992) and Integrin Linked Kinase (adapted from Dedhar, 1999). Integrin space filling model (a) and schematic (b) show the locations of cysteine-rich repeats (crosshatched) and metal-binding sites in the α subunit (M⁺⁺). Shaded areas represent the ligand binding domain. Dilsulfide loopes are seen in the schematic representation. Integrin Linked Kinase contain 4 ankyrin repeats at the N-terminus and the β1 cytoplasmic domain binding region at the carboxy terminus. The middle domains contain the PH-like domain and the kinase catalytic domain.

B1-Integrin

Integrin Linked Kinase