Sertoli cell ectoplasmic specializations: a type of actin-associated adhesion junction?

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Summary

In this paper we provide evidence that ectoplasmic specializations are a form of intercellular adhesion junction. Ectoplasmic specializations, found at basal junctions between adjacent Sertoli cells and at sites of adhesion between Sertoli cells and germ cells, consist of actin filament bundles sandwiched between the plasma membrane and a cistern of endoplasmic reticulum. The actin filaments in each bundle are unipolar and are hexagonally packed. The bundles are coupled to the adjacent membranes and to each other. Because ectoplasmic specializations are associated with junctional sites, they may play a role in intercellular adhesion. In this study, we report a procedure for obtaining samples enriched for ectoplasmic specializations and identify polypeptides that may be associated with ectoplasmic specializations. On SDS-polyacrylamide gels, an 83K (K = 10^3 Mr) polypeptide is specific to the ectoplasmic specialization-enriched sample, suggesting that it may be a component of ectoplasmic specializations. Other polypeptides at 38, 53, 56 and 69K also may be associated with ectoplasmic specializations. ImmunobLOTS further indicate that fimbrin and vinculin are present in the ectoplasmic specialization-enriched fraction. In addition, immunofluorescence indicates that vinculin is associated with spermatid-Sertoli cell and Sertoli-Sertoli cell junctions. We suspect that fimbrin, an actin-bundling protein, may be involved in cross-linking the hexagonally packed actin filaments in ectoplasmic specializations while vinculin may be associated with actin-membrane linkages. If so, ectoplasmic specializations may be a new class of actin-associated junctional site. Moreover, the presence of vinculin in testicular fractions enriched for ectoplasmic specializations and at junctional sites supports the view that these structures may play a role in intercellular adhesion, possibly by stabilizing an adhesive membrane domain.

Key words: ectoplasmic specializations, adhesion junction, Sertoli cells.

Introduction

Ectoplasmic specializations (Russell, 1977a) are complex cytoskeletal structures found in Sertoli cells. They occur in submembrane regions adjacent to basally situated tight junctions (known as the blood-testis barrier) and to sites of adhesion with spermatogenic cells (Dym & Fawcett, 1970; Flickinger & Fawcett, 1967; Nicander, 1967; Russell, 1977a,b). Because they are closely associated with junctional sites, ectoplasmic specializations are suspected of playing a role in maintaining or regulating intercellular junctions (Dym & Fawcett, 1970; Masri et al. 1987; Romrell & Ross, 1979; Russell et al. 1988; Vogl & Soucy, 1985; Vogl et al. 1986; Weber et al. 1988).

Ectoplasmic specializations consist of bundles of actin filaments sandwiched between the plasma membrane and a cistern of endoplasmic reticulum (Brokelmann, 1963; Dym & Fawcett, 1970; Flickinger & Fawcett, 1967; Nicander, 1967; Russell, 1977a). The filaments are known to be actin because of their size and their ability to bind actin antibodies (Franke et al. 1978), fragments of the myosin molecule (Masri et al. 1987; Suarez-QuiAn & Dym, 1984; Toyama, 1976; Vogl et al. 1986) and NBD-phallacidin (Suarez-QuiAn & Dym, 1984; Vogl & Soucy, 1985; Vogl et al. 1985). Filaments within each bundle are organized into regular hexagonal arrays (Franke et al. 1978; Russell, 1977a,b) and have a unipolar orientation (Toyama, 1976; Vogl et al. 1986).

Although it was believed initially that ectoplasmic specializations were contractile (Gravis, 1978, 1979; Toyama, 1976), recent studies have ruled out this possibility. The structural organization of the actin filaments is inconsistent with a contractile system. Moreover, myosin is not present at these sites (Vogl & Soucy, 1985), nor do
glycerinated or detergent-extracted models contract when exposed to standard contraction buffers (Grove & Vogl, 1986; Vogl & Soucy, 1985). We have concluded that actin in ectoplasmic specializations is structural without being contractile.

The precise role of actin in ectoplasmic specializations has not been elucidated. However, one of a number of possibilities is that they are involved with maintaining and/or regulating intercellular junctions. Several pieces of evidence indicate that ectoplasmic specializations are associated with junctions and that this association is specific to intercellular adhesion sites. First, ectoplasmic specializations are found at both the base of the cell adjacent to the blood–testis barrier and at the apex of the cell in association with spermatids – the only major junction common to these sites is adhesive. Second, the disappearance of ectoplasmic specializations, either through the normal course of spermatogenesis or by pharmacological perturbation, is correlated with changes in intercellular adhesion and junctional permeability (Russell, 1977a, b; Russell et al. 1988; Weber et al. 1988). Finally, when spermatids are mechanically detached from the seminiferous epithelium, ectoplasmic specializations, together with the Sertoli cell plasma membrane, remain attached to them (Franke et al. 1978; Masri et al. 1987; Romrell & Ross, 1979; Vogl & Soucy, 1985; Vogl et al. 1985, 1986). We have postulated that the actin filament bundles in ectoplasmic specializations may establish and maintain adhesive domains in the plasma membrane (Masri et al. 1987; Vogl et al. 1986).

Actin filaments are associated with several types of adhesive junctions in other cells. These include intermediate junctions (zonula adherens) of most epithelial cells (Hirokawa & Heuser, 1981; Hirokawa et al. 1982; Hull & Staehelin, 1979; Mooseker, 1985), focal contacts in cultured cells (Burridge, 1986; Geiger, 1979; Geiger et al. 1984a, b), and small intercellular punctate attachments in intact tissue and in cultured cells (Geiger et al. 1981). All cell adhesion sites appear to contain a number of ubiquitous elements. Vinculin, a 130K (K = 10^3 M⁻) protein, is believed to be involved with linking actin to the membrane (Geiger, 1979; Geiger et al. 1981, 1984a, b). It is also considered to be one of the major marker proteins for defining sites of adhesion between cells and between cells and the substratum. Alpha-actinin, an actin binding protein, is also generally present at adhesion sites. It links actin filaments to each other and may also participate in linking actin to the membrane (Burridge, 1986). If ectoplasmic specializations are sites of intercellular adhesion, they may contain one or both of these elements. In fact, data from immunofluorescence studies have suggested that alpha-actinin is present (Franke et al. 1978; Russell & Goh, 1988).

Although we predict that ectoplasmic specializations contain elements common to intercellular adhesion sites, we also suspect that they contain novel elements as well. Actin bundles in ectoplasmic specializations differ from those associated with adhesion sites in other cells in two important ways: they are not part of a contractile mechanism and they are organized into paracrystalline, hexagonal arrays. Because the hexagonal organization of the filament bundles in ectoplasmic specializations is similar to that of the actin core of microvilli, we suspect that these two structures also may have similar molecular components.

If ectoplasmic specializations play a role in maintaining and/or regulating adhesive junctions in Sertoli cells, they are likely to be of fundamental importance in controlling spermatogenesis. One approach to investigating the suggested role of ectoplasmic specializations in intercellular adhesion is to examine the protein composition of these structures. A knowledge of the proteins present in ectoplasmic specializations not only provides a basis for comparing adhesive junctions in Sertoli cells with those in other cells, but is the initial step in identifying elements that link the actin bundles to the membrane and determining if these linkages are associated with adhesive elements in the membrane.

To do this we have developed a procedure for isolating testicular fractions enriched for ectoplasmic specializations from rat Sertoli cells. In this study, we report this procedure and provide a tentative assessment of the proteins in this enriched preparation. Our data indicate that vinculin is present in these samples, as is fimbrin, an actin-binding protein found in microvilli that organizes filaments into unipolar arrays (Bretscher, 1981; Glenney et al. 1981; Matsudaira et al. 1983).

Materials and methods

General morphology

Images to illustrate the general morphology of ectoplasmic specializations and the relationship of actin filaments to each other and to adjacent membranes were obtained from testes removed from two adult Sprague-Dawley rats (340 and 314 g) anesthetized with sodium pentobarbitone administered intraperitoneally. A single testis from one animal was perfused with a fixative containing 1·5% paraformaldehyde, 1·5% glutaraldehyde and 0·1 M-sodium cacodylate (pH 7·3). After 30 min, the testis was cut into small pieces and immersed in the same fixative for an additional 2 h. The tissue was washed in buffer, then post-fixed on ice for 1 h, with 1% OsO₄ in 0·1 M-sodium cacodylate (pH 7·3). The material was washed with distilled water, stained en bloc with 1% uranyl acetate for 1 h, and then processed further using standard techniques for electron microscopy.

To accentuate the filaments and their associated linkages, a testis from the second animal was processed as follows. Sheets of seminiferous epithelium were isolated from the testis generally as described (Vogl & Soucy, 1983) and resuspended in PBS (150 mm-NaCl, 5 mm-KCl, 3·2 mm-Na₂HPO₄, 0·8 mm-KH₂PO₄, adjusted to pH 7·3 with 0·1 M-NaOH) containing 5·0 mm-EDTA. To remove soluble proteins, the sheets were mechanically fragmented by aspirating the material with a Pasteur pipette five times every 30 s for 15 min. Previous studies have shown that ectoplasmic specializations remain attached to spermatids mechanically dissociated from the epithelium (Masri et al. 1987; Romrell & Ross, 1979; Vogl et al. 1986). All buffers used throughout the protocol were stored on ice and used cold. The dissociated spermatids, together with their attached ectoplasmic specializations, were collected by centrifugation and then resuspended in PBS containing 5·0 mm-EDTA and 1% glutaraldehyde (pH 7·3) at room temperature. After 30 min, the sample was washed three times with 0·1 M-sodium
phosphate (pH 7.0) and then fixed for an additional 2 h in 0.1 M-sodium phosphate buffer containing 1% glutaraldehyde and 0.2% tannic acid. After washing with buffer, the tissue was post-fixed for 1 h on ice with 1% OsO₄ in 0.1 M-sodium phosphate buffer (pH 6.0), washed with distilled water, stained en bloc with 1% uranyl acetate, and then processed further using standard techniques for electron microscopy.

Sections were stained with uranyl acetate and lead citrate and photographed on a Philips 300 electron microscope operated at 60–80 kV.

Isolation of ectoplasmic specializations

This procedure (summarized in Fig. 1) takes advantage of the fact that ectoplasmic specializations remain attached to spermatids mechanically detached from the seminiferous epithelium (Masri et al. 1987; Romrell & Ross, 1979; Vogl et al. 1986). Fifty male Sprague-Dawley rats (220–350 g) were used per preparation in this study. Testes were surgically removed from animals killed by decapitation, decapsulated and stored in ice-cold PBS (150 mM-NaCl, 5 mM-KCl, 3.2 mM-Na₂HPO₄, 0.8 mM-KH₂PO₄, buffered to pH 7.3 with 0.1 M-NaOH) containing 5 mM-EDTA until all testes were collected. The testes were minced for 20 min in ice-cold PBS containing 5 mM-EDTA and then aspirated for 15 min through 10-ml pipettes. The fragmented tissue was centrifuged for 2.5 min at low speed (setting 4) in a clinical centrifuge (International Equipment Co.) and the supernatant, containing spermatids and fragmented cellular debris, was collected. To maximize the yield of spermatids, the pellet from this centrifugation was aspirated

Sertoli cell ectoplasmic specializations

Fig. 1. Diagram summarizing the procedure for isolating a sample enriched for ectoplasmic specializations. The regions on the sucrose gradients that contain the material of interest are blackened. See text for complete description of method.

Fig. 2. Electron micrographs indicating the general morphology of ectoplasmic specializations. Shown in A is an ectoplasmic specialization associated with an elongate spermatid. The plasma membrane of the spermatid is indicated by small arrowheads. The ectoplasmic specialization, consisting of actin filaments (large arrowheads) and a closely associated cistern of endoplasmic reticulum (er), is evident in the Sertoli cell cytoplasm juxtaposed to the spermatid. Shown in B is a field similar to that shown in A; however, the material has been mechanically extracted and treated with tannic acid to accentuate the filaments and membrane. A, B. Bar, 0.1 μm, ×177 000.
Fig. 3. Electron micrographs illustrating the arrangement of actin filaments and the presence of filament–filament and filament–membrane linkages in ectoplasmic specializations. The images are of ectoplasmic specializations attached to spermatids mechanically dissociated from the seminiferous epithelium and treated with tannic acid. The hexagonal packing of the actin filaments is evident in A and B. In A the filaments are indicated by the white dots, while in B similar filaments are indicated by arrowheads. Linkages between actin filaments are indicated by arrows in C, D and E. Linkages between actin filaments and adjacent membranes are indicated by the arrows and arrowheads in F and G. In F, amorphous clumps (arrowhead) of material occur between some filaments and the membrane, while other filaments appear to be connected to the membrane by linear strands (arrows). In G, the linkages (arrowheads) can be best appreciated by tilting the image and looking down the long axis of the micrograph. Bars: A, 0.05 μm, ×414 000; B, 0.05 μm, ×393 000; C, 0.05 μm, ×386 000; D, 0.05 μm, ×395 000; E, 0.05 μm, ×439 000; F, 0.05 μm, ×324 000; G, 0.1 μm, ×200 000.

and centrifuged again in the same way and the supernatant was collected. Both supernatants were centrifuged for 5 min at 3200 g at 4°C, after which the pellets were washed three times in homogenization buffer (10 mM-imidazole, 75 mM-KCl, 5 mM-MgCl2, 1 mM-EGTA, 0.2 mM-phenylmethylsulfonyl fluoride (PMSF), 0.2 μg ml−1 leupeptin, 0.2 μg ml−1 pepstatin) by repeated resuspension and centrifugation. Approximately 10–20 ml of washed pellet were obtained at this step. The pelleted material was then diluted approximately 1:1 (v/v) with 60% sucrose in homogenization buffer, layered onto a sucrose step gradient (35%/40%/45%/50%/55%/60%) and centrifuged at 26 000 revs min−1 in a Beckman SW27 rotor for 45 min. The sucrose solutions for the step gradient were prepared by diluting a 60% (w/w) stock solution (772 g of sucrose 1~ of homogenization buffer) with an appropriate amount of homogenization buffer. The contents of the fractions were assessed using fluorescence, phase-contrast, and electron microscopy.

Material at the 45%/50% interface down to the 55%/60% interface was collected, diluted with ice-cold homogenization buffer and pelleted by centrifugation (Sorvall SS34 rotor, 13 000 revs min−1, 15 min). This pellet, consisting mostly of elongating and late spermatids with attached ectoplasmic specializations, was homogenized for 30 strokes in a 30 ml Dounce homogenizer and then resuspended in an equal volume of homogenization buffer containing 30% sucrose. The suspension was layered onto a sucrose step gradient (20%/25%/30%/35%/40%/45%/50% /55%/60%) and centrifuged at 26 000 g in a Beckman SW27 rotor for 45 min. On the basis of their contents, determined by fluorescence and phase-contrast microscopy, the following fractions were collected: an upper fraction (top of gradient down to, but not including, the 30%/35% interface), a middle fraction (30%/35% interface down to, but not including, the 40%/45% interface), and a lower fraction (40%/45% interface to the bottom of the gradient). Each fraction was diluted with ice-cold homogenization buffer and centrifuged for 15 min at 26 000 revs min−1 in a Beckman SW-27 rotor. In some cases, pellets from the upper, middle and lower fractions were resuspended for 30 min in ice-cold homogenization buffer containing 1-0% Triton X-100, centrifuged, and rinsed twice with homogenization buffer by repeated resuspension and centrifugation. All pellets were
Fig. 4. Fluorescent and phase-contrast images of the contents of the pooled upper fractions (A and A') and the pooled lower fractions (B and B') of the first sucrose step gradient. The upper fractions, consisting of material from the top of the gradient to the third interface inclusive, contain cellular debris and some spermatids at later stages of spermatogenesis. The lower fractions, taken from the top of the 45% sucrose step down to the last interface, are highly enriched for elongating and late spermatids. The pattern of NBD-phallacidin fluorescence in both sets of fractions indicates that ectoplasmic specializations remain attached to the spermatids. The pooled lower fractions are further processed to separate the ectoplasmic specializations from the spermatids. A–B. Bars, 100 μm, ×160.

Fluorescence microscopy

The presence of actin in each of the fractions taken from the sucrose gradients was determined by fluorescence microscopy using NBD-phallacidin as a probe for filamentous actin. Samples from each of the fractions were diluted with homogenization buffer, centrifuged at 13,000 revs min⁻¹ for 20 min in an SS34 rotor (Sorvall) and then resuspended for 10 min in PBS containing 3.7% paraformaldehyde. The fixed sample was centrifuged again at 13,000 revs min⁻¹ for 20 min, resuspended in PBS, centrifuged, and then resuspended in 100 μl of 1-65 μM-NBD-phallacidin in PBS for 20 min at room temperature. Following this, the sample was diluted with PBS, centrifuged at 13,000 revs min⁻¹ for 20 min, resuspended in a small volume of PBS, and placed on a microscope slide for viewing.

For immunofluorescent localization of vinculin, a testis, fixed by perfusion with 3% paraformaldehyde in PBS for 5 min and washed by perfusion with PBS for 30 min, was frozen and sectioned. Sections were attached to polylysine-coated slides and then immersed in acetone at −20°C for 5 min, air-dried, and processed by standard procedures for indirect immunofluorescence microscopy. An affinity-purified antibody to human platelet vinculin, prepared by us, was used at a concentration of approx. 50 μg ml⁻¹. A goat anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (Sigma Co.) was used at a 1:32 dilution. Control sections received either the secondary antibody alone or no antibodies.

Samples were viewed by epifluorescence, using either a Zeiss Photomicroscope III or a Zeiss Axiophot microscope fitted with filters for detecting fluorescein isothiocyanate.

Electron microscopy of fractions from the sucrose gradients

Samples from each of the fractions were diluted with homogenization buffer and centrifuged at 13,000 revs min⁻¹ for 20 min in an SS34 rotor (Sorvall). The pellets were fixed for 2h with 1.5% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M-cacodylate buffer (pH7.3), postfixed, on ice, for 1h in cacodylate-buffered OsO₄, and then processed further by standard techniques for electron microscopy. Sections were photographed on a Philips 300 electron microscope operated at 60 kV.

SDS–polyacrylamide electrophoresis

Samples were run on 7.5% SDS–polyacrylamide gels according to the method of Laemmli (1970) and stained with Coomassie Blue. Molecular weights were calculated on the basis of the following molecular weight markers obtained from Sigma Chemical Co.: ovalbumin (45,000), bovine plasma albumin (66,000), rabbit muscle phosphorylase b (97,400), beta-galactosidase (116,000), and rabbit muscle myosin (205,000). Actin, which we purified from rabbit skeletal muscle according to the procedure of Pardee & Spudich (1982a,b), was also used as a marker. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Immunoblotting procedures

Immunoblotting procedures were carried out as outlined by Towbin et al. (1979). A polyclonal antibody to chicken skeletal...
Fig. 5. Transmission electron micrographs of the upper (A) and lower (B,C) fractions from the first sucrose step gradient. The pooled upper fractions consist mostly of cellular debris, including mitochondria and other membranous elements. As B illustrates, the pooled lower fractions are enriched for late-stage spermatids. Ectoplasmic specializations remain attached to the spermatid heads (arrowhead). Actin filaments of ectoplasmic specializations are visible at higher magnification (C, arrowheads). A,B. Bar, 5 μm; X6200; C, bar, 1 μm, X27 000.

Vinculin isolation and antibody production

Vinculin was isolated from human platelets following the procedure of Rosenfeld et al. (1985). The isolated protein was confirmed to be vinculin by its cross-reactivity on Western blots to a polyclonal antibody to human platelet vinculin provided by Dr Rosenfeld.

Three virgin female New Zealand White rabbits were initially inoculated with 300 ng of vinculin mixed with complete Freund’s adjuvant. The rabbits were boosted, at 3 weeks and 5 weeks after the first injection, with 300 μg of protein mixed with incomplete Freund’s adjuvant. Two weeks after the final boost, the rabbits were exsanguinated and the IgG fraction was collected from the serum by ammonium sulfate precipitation.

Affinity-purified antibodies were prepared by passing the IgG fraction through a CNBr-activated Sepharose 4B (Pharmacia) column to which was bound human platelet vinculin. The bound IgG was eluted with 0.2 M-glycine (pH 2.3) and the eluted fractions were immediately neutralized with equivalent volumes of 0.2 M-Tris-base. The eluted protein was concentrated using Millipore CX10 ultrafilters and dialyzed against PBS diluted 1:10. Samples (1 ml) were lyophilized and, just before use, reconstituted with 100 μl of water and further concentrated by ultrafiltration.

Results

Ultrastructure of ectoplasmic specializations

Our observations of the ultrastructure of ectoplasmic specializations are consistent with those of several investigators (Dym & Fawcett, 1970; Romrell & Ross, 1979; Russell, 1977a; Russell et al. 1988). Ectoplasmic specializations at sites of adhesion between Sertoli cells and germ cells consist of bundles of actin filaments sandwiched between a cistern of endoplasmic reticulum and the Sertoli cell plasma membrane (Fig. 2A). The bundles consist of four to six rows of actin filaments that display a paracrystalline, hexagonal organization when cut in cross-section. Spermatids lying directly adjacent to ectoplasmic specializations are separated from the Sertoli cell by a space approximately 11 nm wide. A light-staining flocculent material is occasionally visible in this space.

The visibility of structural linkages between filaments within the bundles, between filaments and the endoplasmic reticulum, and between filaments and the Sertoli cell plasma membrane, is enhanced by treatment with tannic acid. It is evident from Fig. 2B that the overall organization of ectoplasmic specializations at sites of adhesion

314  B. D. Grove and A. W. Vogl
between Sertoli cells and spermatids is the same as in the perfusion-fixed material. However, the cytoplasmic ground substance is less visible and the actin filaments appear to be more prominent than in the untreated material.

Fig. 3A–G illustrates, at high magnification, the structural organization of actin bundles in ectoplasmic specializations from mechanically fragmented and tannic acid-treated material. The hexagonal packing of the actin filaments is readily apparent in Fig. 3A,B. Spacing between filaments in micrographs such as these is approximately 10–11 nm. Within the bundles, strands of electron-dense material frequently appear to link filaments to each other (Fig. 3C–E). In addition, there appears to be material linking the filaments to the plasma membrane and to the endoplasmic reticulum membrane. Filament–membrane linkages occur mostly as periodic clumps of amorphous material (Fig. 3F,G), but strands connecting individual filaments to the membrane are also occasionally visible (Fig. 3F).

**Isolation of ectoplasmic specializations**

The initial steps for mechanically disrupting the testicular tissue are adapted from procedures previously developed in our laboratory for obtaining sheets of seminiferous epithelium and for detaching spermatids from the epithelium without dislodging the associated ectoplasmic specializations (Masri et al. 1987; Vogl et al. 1986). In those studies, mincing testicular tissue after treatment with high concentrations of EDTA resulted in detachment of sheets of epithelium from other components of the seminiferous tubule. Aspirating the minced tissue further fragmented the epithelium and detached spermatids, together with their attached ectoplasmic specializations, from the Sertoli cells.

Elongate and late spermatids with attached ectoplasmic

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**Sertoli cell ectoplasmic specializations**

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Fig. 7. Transmission electron microscopy of the upper (A), middle (B,C), and lower (D) fractions of the second sucrose gradient confirms the results from fluorescence and phase-contrast microscopy. The upper fractions contain large amounts of membranous material and other unidentifiable cytoplasmic components. Actin filament bundles are not readily visible. The middle fractions also contain large amounts of membranous material and fragments of spermatid flagella, but ectoplasmic specialization fragments are easily seen (arrowheads). Ectoplasmic specialization fragments consist of bundles of actin filaments attached to membrane (B and C). In some cases, what appears to be spermatid membrane remains attached to ectoplasmic specializations (arrowhead in C). The asterisk in C indicates actin filaments. The lower fractions contain mostly spermatid heads and tails (D). A,B,D. Bar, 2.5 μm, ×6200; C, bar, 0.5 μm, ×21000.

specializations are separated from the unwanted testicular tissue by low-speed centrifugation followed by ultracentrifugation on a sucrose step gradient. As illustrated in Figs 4 and 5, the sample collected from the 45%/50% interface to the last interface, but excluding the pellet, is highly enriched for elongate and late spermatids. Samples collected from the top of the gradient down to and including the third interface consist of cellular debris and some elongate and late spermatids (Figs 4 and 5). Fluorescence microscopy using NBD-phallacidin reveals a fluorescent image associated with most of the spermatids in the spermatid-enriched sample that is typical of ectoplasmic specializations attached to spermatids (Masri et al. 1987; Vogl et al. 1985). The finding that actin bundles from ectoplasmic specializations remain attached to spermatids is confirmed also by transmission electron microscopy (Fig. 5C).

By homogenizing the spermatid-enriched sample and centrifuging the homogenate on a second sucrose step gradient, ectoplasmic specializations are detached and separated from the spermatids. Fluorescence and phase-contrast microscopy reveal that the middle fractions (30%/35% interface down to, but not including, the 40%/45% interface) of the gradient are enriched for actin from ectoplasmic specializations (Fig. 6). Although a considerable amount of cellular debris is also present in this middle fraction, the size and apparent concentration of actin bundle fragments is greatest here. Fluorescence microscopy of the gradient steps above (upper fraction; top of gradient down to, but not including, the 30%/35% interface) and below (lower fraction; 40%/45% interface down to the bottom of the gradient) the middle fraction also reveals actin. However, the actin fragments in the upper fraction are much smaller and relatively less concentrated than in the middle fraction (Fig. 6A,A'). The lower fraction contains primarily spermatid heads and flagella (Fig. 6C,C').

Transmission electron microscopy reveals that the upper fraction consists mostly of small, membranous vesicles and what may be cytoplasmic ground substance (Fig. 7A). Actin filaments and filament bundles are not readily discernible by electron microscopy in this fraction. The middle fraction also contains a large amount of membranous, subcellular debris as well, but in contrast to the upper fraction large bundles of actin filaments with membrane attached are conspicuous (Fig. 7B,C). The other cellular debris in this fraction consists mainly of larger membranous vesicles and, occasionally, fragments of spermatid flagella. The lower fraction is enriched for spermatid heads, flagella and nuclear debris (Fig. 7D). Much less vesicular material is present in the lower fraction than in the upper and middle fractions, but intermediate filaments are visible and ectoplasmic specialization fragments are associated with some spermatid heads.

**SDS–polyacrylamide gel electrophoresis**

SDS–polyacrylamide gel electrophoresis reveals a number of major polypeptides ranging from approximately 30K to 100K in Triton-extracted samples of the ectoplasm-
Fig. 8. SDS–polyacrylamide gel electrophoresis of the upper, middle, and lower fractions from the second sucrose gradient. Samples were extracted with Triton X-100. Lane 1, upper fraction (55 μg protein); lane 2, upper fraction, diluted; lane 3, middle fraction (75 μg protein); lane 4, middle fraction (15 μg protein); lane 5, lower fraction (75 μg protein); lane 6, lower fraction (15 μg protein); lane 7, actin standard (0.25 μg protein). A band at approximately 45K, which co-migrates with actin, is present in all three fractions, but is proportionately most concentrated in the upper and middle fractions. Arrowheads indicate polypeptides at approximately 43, 54 and 65K that are proportionately more concentrated in the lower fraction than in the middle and upper fractions. Asterisks indicate polypeptides at approximately 38, 53, 56, 69 and 83K that are proportionately more concentrated in the middle fraction than in the lower fraction. The 83K protein is restricted to the middle fraction.

mic specialization-enriched fraction (middle fraction) (Figs 8, 9, 10). However, because the middle fraction contains subcellular debris in addition to ectoplasmic specializations, not all of these polypeptides are components of ectoplasmic specializations. Fig. 8 compares the polypeptide composition of Triton-extracted samples of the middle fraction with that of the upper and lower fractions. Polypeptides at approximately 43, 55 and 65K are proportionately more concentrated in the lower fraction than in the middle fraction. Because the lower fraction is enriched for spermatid heads and flagella, these polypeptides are probably spermatid components. On the other hand, a 45K polypeptide, which comigrates with actin purified from rabbit skeletal muscle, and major polypeptides at approximately 38, 53, 56, 69 and 83K are proportionately more concentrated in the middle fraction than in the lower fraction (Fig. 8, asterisks). The 45, 38, 53, 56 and 69K polypeptides are also major components of Triton-extracted samples of the upper fraction, but the 83K polypeptide is present only in the middle fraction. While these polypeptides are possible candidates for ectoplasmic specialization components, we are unable to

Sertoli cell ectoplasmic specializations

317
Fig. 10. SDS–polyacrylamide gel electrophoresis (Coomassie Blue) and immunoblots of the upper, middle and lower fractions using an affinity-purified polyclonal antibody to human platelet vinculin. Lane 1, upper fraction; lane 2, middle fraction, Triton extracted; lane 3, middle fraction, unextracted; lane 4, lower fraction; lane 5, human platelet vinculin (1 μg). The lanes marked blocked were stained with the antibody pre-adsorbed with the antigen. The total protein in each of the testicular fraction samples was matched as well as possible but, owing to difficulty in preparing the lower fraction for SDS–PAGE because of its high DNA content, the lower fraction may not be reliably represented in this figure.

rule out the possibility that those also present in the upper fraction are components of the membranous contamination in the middle fraction.

Western blots
Fig. 9 shows Western blots of the ectoplasmic specialization-enriched (middle) fraction stained with polyclonal antibodies to chicken skeletal muscle actin and chicken intestinal brush-border fimbrin. The actin antibody cross-reacts strongly with the band that comigrates with purified skeletal muscle actin, confirming that this band is actin.

The antibody to fimbrin cross-reacts strongly with a polypeptide of similar molecular weight to that of serum bovine albumin and to a lesser extent with some lower molecular weight bands. The mobility of the major immunoreactive band is similar to that reported for chicken fimbrin (Bretscher & Weber, 1980). Because fimbrin has been reported to be susceptible to proteolysis (Bretscher, 1981), the minor bands may represent proteolytic breakdown fragments.

Blots of the upper, middle and lower fractions also were tested against an affinity-purified antibody to human platelet vinculin. As Fig. 10 illustrates, a single, immunoreactive band comigrating with human platelet vinculin is present in all fractions. The immunoreactive band is most pronounced in the ectoplasmic specialization-enriched (middle) fraction in this figure, although it should be noted that, owing to difficulty in preparing the lower fraction for electrophoresis because of its high DNA content, the relative amount of vinculin in the lower fraction may not be reliably represented here. Antibody pre-adsorbed with the platelet vinculin does not cross-react on parallel blots, confirming the specificity of the antibody. It should also be noted that the apparent mobility of vinculin at 116K on our gels, rather than the generally accepted 130K, is due to a reassessment of the molecular weight of beta-galactosidase, one of the molecular weight standards used (Burridge, 1986).

Immunofluorescence
As shown in Fig. 11A,B, the affinity-purified antibody, which reacts with only one band on immunoblots of testicular fractions enriched for ectoplasmic specializations, specifically labels regions of Sertoli cells known to contain ectoplasmic specializations. We obtained specific staining adjacent to spermatid heads at the apex of the epithelium and at the base of the epithelium in areas that also stain with NBD–phallacidin (see Suarez-Quian & Dym, 1984). As expected, specific staining also occurs in myoid cells and smooth muscle cells associated with small...
blood vessels. We observed no specific staining in the controls.

**Discussion**

In this paper we present data consistent with the argument that ectoplasmic specializations in Sertoli cells are a class of intercellular adhesion complex.

Ectoplasmic specializations, consisting of actin filament bundles sandwiched between the plasma membrane and a cistern of endoplasmic reticulum, are found at basal junctions between adjacent Sertoli cells (blood–testis barrier) and at junctions between Sertoli cells and germ cells. The possibility that the actin filament bundles in ectoplasmic specializations may in some way play a role in establishing and/or regulating these junctions has been recognized by numerous investigators (Dym & Fawcett, 1970; Masri et al. 1987; Romrell & Ross, 1979; Russell, 1977a; Russell et al. 1988; Vogl & Soucy, 1985; Vogl et al. 1986; Weber et al. 1988). Several lines of evidence support this. First, the movement of germ cells across the blood–testis barrier during spermatogenesis and the release of spermatozoa from the seminiferous epithelium during spermiation are associated with a loss of the actin filament bundles (Russell, 1977a,b; Vogl et al. 1983). Second, the fact that intact ectoplasmic specializations remain attached to spermatids mechanically detached from Sertoli cells indicates that the filament bundles are linked to the plasma membrane and that adjacent plasma membranes are adherent to each other (Franke et al. 1978; Masri et al. 1987; Vogl et al. 1986). Visible evidence of linkages comes from ultrastructural studies, reported here and elsewhere (Franke et al. 1978; Russell, 1977a; Russell et al. 1988; Pelletier, 1988), that reveal strands of material between filaments, between the filaments and adjacent membranes and between adjacent plasma membranes. Finally, the injection of cytochalasin D into rat testes results in the disruption of actin filaments in ectoplasmic specializations and a corresponding change in junction adhesiveness (Russell et al. 1988) and permeability (Weber et al. 1988).

The concept that actin filaments may interact with the plasma membrane at junctional sites is not new (Cereijido et al. 1981; Duffey et al. 1981; Geiger et al. 1981, 1984a; Meza et al. 1980, 1982; Volberg et al. 1986). However, ectoplasmic specializations are unusual in that

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**Fig. 11.** Shown here are phase-contrast (A) and fluorescence (B) micrographs of a fixed-frozen section of rat testis treated with an affinity-purified antibody produced against human platelet vinculin as described in the text. Specific staining is visible adjacent to spermatid heads (large arrowheads) and at sites interpreted as being the blood–testis barrier at the base of the epithelium (small arrowheads). Staining also occurs in myoid cells (arrow) of the tubule wall and smooth muscle cells of small blood vessels (bv). A, B. Bar, 50 μm, ×565.

*Sertoli cell ectoplasmic specializations* 319
they are the only known example where actin bundles associated with intercellular junctions display paracrystalline packing and unipolar orientation. In addition, ectoplasmic specializations, unlike other junction-associated actin networks, are not contractile (Vogl & Soucy, 1985).

Although some of the proteins associated with junction-related actin networks in general may be present in ectoplasmic specializations, novel proteins may also be important in the organization and functioning of these structures. The development of a procedure for isolating ectoplasmic specializations is an important prerequisite to studies of the molecular organization of ectoplasmic specializations.

The procedure reported in this study for obtaining samples enriched for ectoplasmic specializations is the first step towards this goal. The procedure takes advantage of the fact that ectoplasmic specializations remain attached to spermatids mechanically detached from the seminiferous epithelium (Masri et al. 1987; Romrell & Ross, 1979; Vogl et al. 1986). Elongate and mature spermatids are separated from other testicular material on sucrose gradients, then the ectoplasmic specializations are mechanically detached from the spermatids and isolated using a second sucrose gradient.

The middle fractions, collected from the second sucrose gradient, are enriched for ectoplasmic specializations. Though fragments of ectoplasmic specializations are present throughout the gradient, the middle fractions not only contain the highest concentration of these structures, but are also reasonably free of recognizable spermatid components, which are most concentrated in the lower fractions. Also, many of the ectoplasmic specialization fragments in the middle fractions consist of actin filament bundles associated with membrane, indicating that most, if not all, of the components linking actin filaments to each other and to the membrane are retained during the isolation protocol.

Polypeptides in SDS–polyacrylamide gels of the ectoplasmic specialization enriched fractions at 38, 53, 56, 69 and 83K may be candidates for ectoplasmic specialization-associated proteins. The 83K polypeptide is a particularly strong candidate because it is restricted almost entirely to the ectoplasmic specialization-enriched fraction. The relationship of the 38, 53, 56 and 69K polypeptides to ectoplasmic specializations is less certain because these polypeptides are also prominent in the upper fractions. The presence of actin as a major polypeptide on gels of Triton-extracted samples of the upper fractions indicates that the small ectoplasmic specialization fragments in this fraction may form a significant part of the Triton-insoluble material. If so, one or more of these polypeptides may be a component of ectoplasmic specializations. Alternatively, some of these polypeptides may be components of the cytoplasmic and membrane debris that is present in the upper fractions and contaminates the middle fractions.

The presence of fimbrin or a fimbrin-like protein in ectoplasmic specializations is possibly indicated by the strong immunoreactivity of a protein component of the ectoplasmic specialization-enriched fraction with an antibody to chicken brush-border fimbrin. This band has a similar mobility to chicken fimbrin. While the presence of this protein in the ectoplasmic specialization-enriched fraction does not demonstrate conclusively that fimbrin is a component of ectoplasmic specialization, ectoplasmic specializations would be the most likely source of this protein. Because the fractions examined in this study are derived from a preparation consisting almost exclusively of late spermatids with attached ectoplasmic specializations, ectoplasmic specializations are the only actin-containing component apart from the spermatid subacrosomal region (Masri et al. 1987) in the ectoplasmic specialization-enriched fraction. Furthermore, on the basis of in vitro studies and its distribution in other cells, fimbrin is generally considered to cross-link actin filaments into paracrystalline bundles with a unipolar orientation (Glenney et al. 1981; Matsudaira et al. 1983; Moosker, 1985; Moosker & Tilney, 1975). The presence of fimbrin or a fimbrin-like protein in ectoplasmic specializations would be consistent with the paracrystalline organization of actin in these structures.

Because Sertoli cell ectoplasmic specializations are associated with sites of adhesion to germ cells, we suspected that vinculin, a protein characteristic of adherens type junctions (Burridge & Feramisco, 1980; Geiger, 1979; Geiger et al. 1980; Singer & Paradiso, 1981), may be present at these sites as well. The role of vinculin in intercellular adhesion is poorly understood, but work on cell–substrate adhesion indicates that vinculin is closely associated with the plasma membrane and is one of group of proteins believed to link the actin cytoskeleton to adhesive components in the plasma membrane (Burridge, 1986; Geiger et al. 1984a,b). Immunofluorescent staining at junctional sites between spermatids and Sertoli cells and between adjacent Sertoli cells in frozen sections stained with an affinity-purified antibody to human platelet vinculin provides strong evidence that vinculin is a component of ectoplasmic specializations. Immunoblots of the testicular fractions confirm that the antibody is monospecific for vinculin and support the immunofluorescence data by revealing an immunoreactive band in the ectoplasmic specialization-enriched fraction. The precise location of vinculin in ectoplasmic specializations is not apparent from these data; however, it is reasonable to speculate that vinculin may be involved in actin–membrane interactions in these structures.

Fig. 12 summarizes our view of the actin–membrane interactions in an ectoplasmic specialization at the junction between the Sertoli cell and the spermatid. Actin filaments, sandwiched between the Sertoli cell plasma membrane and a cistern of endoplasmic reticulum, are cross-linked to each other and to the adjacent membranes. Direct and indirect morphological evidence alone indicates that these linkages are present (this report; Franke et al. 1978; Russell, 1977a; Russell et al. 1988); however, evidence reported here that fimbrin and vinculin are present in ectoplasmic specializations is consistent also with this model. We postulate that fimbrin or a fimbrin-like protein may be one of the interfilament links, while vinculin may be a component of the actin–membrane linkages. Franke et al. (1978) and Russell & Goh
Fig. 12. Diagram of a mammalian Sertoli cell ectoplasmic specialization adjacent to a spermatid. Actin filaments are organized into hexagonal arrays between the Sertoli cell plasma membrane and cisternae of endoplasmic reticulum and are illustrated as being cross-linked to each other and to the plasma membrane and the endoplasmic reticulum. Because this junction is an adhesive junction, we speculate that actin filaments may be linked, directly or indirectly, to adhesive molecules in the membrane.
(1988) reported that the actin cross-linking protein, alpha-actinin, may also be present in ectoplasmic specializations and, possibly, this protein may play a role in cross-linking filaments to each other or to the adjacent membranes. It should be noted that spermatid–Sertoli cell junctions, but not Sertoli–Sertoli cell junctions, are morphologically asymmetrical, with microfilament bundles being present only in the Sertoli cell.

Ectoplasmic specializations combine a number of diverse features found in actin systems of other cells. They are morphologically associated with junctions and, therefore, are a class of junction-related actin complex. However, unlike other junction-related actin systems, ectoplasmic specializations consist of a non-contractile, paracrystalline arrangement of actin filaments, a feature characteristic of microvilli, and evidence presented here strongly indicates that they may contain fimbrin. We suspect that ectoplasmic specializations are primarily involved with intercellular adhesion. Immunological evidence that vinculin is associated with ectoplasmic specializations is consistent with this view. Although ectoplasmic specializations at the basal junction between adjacent Sertoli cells appear morphologically associated with tight junctions, this basal junction, like junctional complexes of other epithelia, probably also contains adhesive components. The finding that altered tight-junction permeability in Sertoli cells in response to cytochalasin D treatment may result from altered intercellular adhesion (Weber et al. 1988) would be consistent with results of studies using other epithelia where disruption of actin filaments predominantly associated with zonula adherens is correlated with increased permeability of neighboring tight junctions (Cereijido et al. 1981; Dufey et al. 1981; Madara et al. 1986; Meza et al. 1980, 1982). In fact, there is increasing evidence that the position of the zonula adherens may secondarily affect the assembly and localization of tight junctions (Gumbiner & Simons, 1987; Gumbiner et al. 1988). Although the precise nature of the relationship between the cell membrane and actin filaments in ectoplasmic specializations is not known, it is tempting to speculate that the actin filaments may stabilize the position of cell-adhesion molecules in the membrane.

We thank Drs A. Bretscher, K. Fujiiwara and G. Rosenfeld, who kindly provided us with some of the polyclonal antibodies used in this study. This work was supported by the Medical Research Council of Canada grant no. MT-8020 (to A.W.V.) and a Medical Research Council of Canada postdoctoral fellowship awarded to B.D.G.

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(Received 17 June 1988 – Accepted, in revised form, 18 February 1989)