Dynamin II interacts with the cadherin- and occludin-based protein complexes at the blood–testis barrier in adult rat testes

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*(P P Y Lie, W Xia, C Q F Wang and D D Mruk contributed equally to the completion of this work)

Abstract

In adult rat testes, blood–testis barrier (BTB) restructuring facilitates the migration of preleptotene spermatocytes from the basal to the adluminal compartment that occurs at stage VIII of the epithelial cycle. Structural proteins at the BTB must utilize an efficient mechanism (e.g. endocytosis) to facilitate its transient ‘opening’. Dynamin II, a large GTPase known to be involved in endocytosis, was shown to be a product of Sertoli and germ cells in the testis. It was also localized to the BTB, as well as the apical ectoplasmic specialization (apical ES), during virtually all stages of the epithelial cycle. By co-immunoprecipitation, dynamin II was shown to associate with occludin, N-cadherin, zona occludens-1 (ZO-1), β-catenin, junctional adhesion molecule-A, and p130Cas, but not nectin-3. An in vivo model in rats previously characterized for studying adherens junction (AJ) dynamics in the testes by adjudin (formerly called AF-2364, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxyhydrate) treatment was used in our studies. At the time of germ cell loss from the seminiferous epithelium as a result of adjudin-induced AJ restructuring without disrupting the BTB integrity, a significant decline in the steady-state dynamin II protein level was detected. This change was associated with a concomitant increase in the levels of two protein complexes at the BTB, namely occludin/ZO-1 and N-cadherin/β-catenin. Interestingly, these changes were also accompanied by a significant increase in the structural interaction of dynamin II with β-catenin and ZO-1. β-Catenin and ZO-1 are adaptors that structurally link the cadherin- and occludin-based protein complexes together at the BTB in an ‘engaged’ state to reinforce the barrier function in normal testes. However, β-catenin and ZO-1 were ‘disengaged’ from each other but bound to dynamin II during adjudin-induced AJ restructuring in the testis. The data reported herein suggest that dynamin II may assist the ‘disengagement’ of β-catenin from ZO-1 during BTB restructuring. Thus, this may permit the occludin/ZO-1 complexes to maintain the BTB integrity when the cadherin/catenin complexes are dissociated to facilitate germ cell movement.


Introduction

In adult rat testes, the blood–testis barrier (BTB) is composed of co-existing tight (TJ) and adherens junctions (AJ; e.g. basal ectoplasmic specialization (basal ES), an actin–based testis-specific AJ type) between adjacent Sertoli cells (Vogl et al. 1991, 2000, Cheng & Mruk 2002, Toyama et al. 2003, Mruk & Cheng 2004). It also physically divides the seminiferous epithelium into the basal and adluminal compartments. While these specialized junctions that constitute the BTB confer one of the tightest barriers in mammals, the BTB is a highly dynamic cellular structure. For instance, during stage VIII of the epithelial cycle in adult rat testes, preleptotene spermatocytes must translocate across the BTB (Russell 1977), entering the apical compartment for further development, and differentiating into pachytene spermatocytes. As such, the BTB must ‘disassemble’ (or open?) to facilitate preleptotene spermatocyte movement. Thus, it is conceivable that extensive turnover of proteins is occurring at the BTB during spermatogenesis. Five different classes of protein complexes have been found at the BTB. These include occludins/zona occludens-1 (ZO-1), junctional adhesion molecules (JAMs)/ZO-1, claudins/ZO-1, nectins/afadin, and cadherins/catenins (Wong & Cheng 2005, Xia et al. 2005a), although some of these are also found in the apical ectoplasmic specialization (apical ES) at the Sertoli cell-elongating spermatid interface (e.g. nectins/afadin and cadherins/catenins; Wine & Chapin 1999, Chapin et al. 2001, Johnson & Boekelheide 2002, Lee et al. 2003, Sluka et al. 2006). The interaction of the integral membrane proteins (such as occludins and cadherins) and
their adaptors (such as ZO-1 for occludins and catenins for N-cadherin) is regulated by protein/lipid kinases and phosphatases (Mruk & Cheng 2004). Recent studies have illustrated that the dynamic nature of the BTB is regulated, at least in part, by the engagement and the disengagement of integral membrane proteins and their peripheral adaptors of adjacent Sertoli cells (Yan & Cheng 2005). For instance, it was shown that during extensive AJ restructuring that facilitated germ cell movement, there was a loss in association between catenins and ZO-1, the corresponding adaptors of cadherins and occludin respectively (Yan & Cheng 2005). Thus, this disengaged the interacting cadherins and occludins at the BTB, facilitating germ cell movement while the occludin–ZO-1 complexes continued to maintain the BTB integrity (Yan & Cheng 2005). However, the molecule(s) that facilitates such changes in protein–protein interactions, likely via transient protein ‘internalization’ or ‘reshuffling’, has yet to be identified. Recent studies have shown that GTPases are the crucial players of protein trafficking (Takai et al. 2001, Lui et al. 2003a, Mruk et al. 2005). As such, we sought to investigate the role of dynamin in such events.

Dynamin is a family of large GTPases implicated in the formation of nascent vesicles during both endocytosis and the secretory process (for reviews, see McNiven et al. 2000, Sever et al. 2000b, Hinshaw 2006, Kruchten & McNiven 2006, Robinet et al. 2006). Dynamin is known to play an important role in the internalization of integral membrane proteins (e.g. occludin and N-cadherin) in multiple epithelia (Orth & McNiven 2003) and serves as pinchase-like mechanoenzyme to facilitate the formation of endocytic vesicles by severing nascent endocytic pits from the plasma membrane (Thompson & McNiven 2001, Cao et al. 2003). The best studied function of the dynamin family is the promotion of vesicle fission during clathrin-mediated endocytosis (Sever et al. 2000a, Hinshaw 2006, Kruchten & McNiven 2006). Other functions, such as membrane tubulation and phagosome formation, have also been ascribed to dynamin (Orth & McNiven 2003). Members of the dynamin family include three classical dynamins, namely dynamins I, II, and III, and also dynamin-like proteins. Dynamin I is a neural specific isoform pertinent to synaptic vesicle recycling. Dynamin II is ubiquitously expressed in all eukaryotic cells, and it has recently been identified in the testis (Iguchi et al. 2002). It is crucial for the endocytosis of integral membrane proteins (McNiven et al. 2000, Sever et al. 2000b). Dynamin III is a testis-specific isoform (Kamitani et al. 2002). A recent study has reported that dynamin I is absent in the testis, while the other two isoforms are highly expressed (Kamitani et al. 2002). Nonetheless, the functions of dynamins in the testis remain to be elucidated. It was postulated that dynamins may play a role in nutrient provision to germ cells via endocytosis (Kamitani et al. 2002). In this report, we have examined the possible role of dynamin II in BTB dynamics via its specific interactions with the occludin/ZO-1 and the N-cadherin/b-catenin protein complexes at the BTB.

### Materials and Methods

#### Animals

Male Sprague–Dawley rats (~270–300 g body weight) were purchased from Charles River Laboratories (Kingston, NY, USA). The use of animals in this study was approved by the Rockefeller University Animal Care and Use Committee with Protocol Numbers 03017 and 06018.

#### Primary Sertoli and germ cell cultures

Sertoli and germ cells were isolated from the testes of 20- and 90-day-old rats respectively, as detailed elsewhere (Mruk et al. 1997, 2003). In short, Sertoli cells were cultured at a density of 5×10⁵ cells/cm² on 100 mm dishes in F12/DMEM (Ham’s F12 Nutrient Mixture: Dulbecco’s Modified Eagle’s Medium, V/V, 1:1) at 35 °C in a humidified atmosphere of 95% air/5% CO₂ with supplements (Mruk et al. 1997). On day 2, these cultures were subjected to a hypotonic treatment (10 mM Tris, pH 7.4 at 22 °C for 2 min; Galdiari et al. 1981) to lyse residual germ cells, and cultures were terminated on day 4 for lysate preparation and RNA extraction as described (Mruk et al. 2003). On the other hand, total germ cells were isolated from adult rat testes and used for lysate preparation and RNA extraction within 6 h as described (Aravindan et al. 1996). Both primary cultures had negligible contamination of other cell types, and this has been vigorously characterized by RT-PCR, immunoblotting, light and electron microscopy as reported earlier from this laboratory (Siu et al. 2005).

#### Isolation of seminiferous tubules

Seminiferous tubules were isolated from adult rat testes (~300–350 g body weight) as described (Zwain & Cheng 1994). Tubules were incubated in F12/DMEM containing insulin (20 µg/ml), human transferrin (20 µg/ml), gentamicin (100 µg/ml), and penicillin (100 IU/ml) at 35 °C for about 6 h before they were harvested for lysate preparation. The tubules used in this report had negligible contamination of Leydig cells since these tubules failed to respond to hCG (10 ng/ml) treatment when the level of testosterone was quantified as described (Zwain & Cheng 1994).

#### Treatment of rats with adjudin to induce junction restructuring

Adult rats (~270–300 g body weight, b.w.; n=3–5 for each time point) were treated with adjudin (1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, formerly called AF-2364, a molecule that induces adherens junction disruption in testis) via gavage at 50 mg/kg b.w. at time 0. The treated rats were killed after 1, 4, 8 h and 1, 4, 7, and 14 days thereafter. Adjudin was suspended in methylcellulose (0-25% (w/v) in sterile water) as a stock solution of 20 mg/ml. This treatment is known to induce extensive AJ restructuring in the seminiferous epithelium at the Sertoli–germ cell interface,

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most notably between Sertoli cells and elongating/elongate spermatids, followed by round spermatids and spermatocytes, but not spermatogonia (Chen et al. 2003, Cheng et al. 2005). However, the BTB integrity has been observed to be unaffected by day 15 following adjudin treatment (Mruk & Cheng 2004, Cheng et al. 2005).

Sample preparation

Lysates were obtained by treating Sertoli cells, germ cells, testes, or seminiferous tubules with a lysis buffer (50 mM Tris–HCl, 0.15 M NaCl, 1% Nonidet P-40 (v/v), 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM sodium orthovanadate), followed by sonication and centrifugation at 13 000 g for 10 min to obtain the clear supernatant. The use of EGTA instead of EDTA in the lysis buffer as a chelating agent to block metalloprotease activity was important. Since EDTA was shown to chelate with vanadate (Crans et al. 1989, Huyer et al. 1997, Siu et al. 2005), it would reduce the level of free vanadate included in the buffer that blocked the activity of protein-tyrosine phosphatases (PTP) in lysates. If PTP activity remained unchecked, this would affect the phosphorylation status of component proteins at the BTB, adversely affecting protein–protein interactions in the samples to be analyzed. Protein concentration was estimated by Coomassie blue dye-binding assay using BSA as a standard (Bradford 1976).

Antibodies

Commercially obtained antibodies listed in Table 1 were used for immunoblot analysis, immunohistochemistry, fluorescent microscopy, and co-immunoprecipitation. All three anti-dynamin antibodies used in our study cross-reacted with dynamins I and II, but since dynamin I is absent in the testis (Kamitani et al. 2002), dynamin signals reported herein were only from dynamin II.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Usage</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Cadherin</td>
<td>Rabbit</td>
<td>IP, IB</td>
<td>1:100</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>Rabbit</td>
<td>IP, IB</td>
<td>1:100</td>
</tr>
<tr>
<td>β-Actin</td>
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</tr>
<tr>
<td>Occludin</td>
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<td>IP, IB</td>
<td>1:100</td>
</tr>
<tr>
<td>ZO-1</td>
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<tr>
<td>ZO-1-FITC</td>
<td>Mouse</td>
<td>IF</td>
<td>1:100</td>
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<tr>
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<td>IF</td>
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<tr>
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<td>Rabbit</td>
<td>IHC, IF</td>
<td>1:300</td>
</tr>
</tbody>
</table>

*All rabbit antibodies were polyclonal and mouse antibodies were monoclonal.

Working dilution

The sources and working dilutions of antibodies used for different studies in this report

Immunoblot analysis

One-hundred micrograms protein from each sample within an experimental group were resolved by SDS-PAGE using 7.5, 10, or 12.5% T SDS-polyacrylamide gels under reducing conditions, depending on the relative molecular mass of the target proteins. Proteins were then transferred onto nitrocellulose membranes for immunoblot analysis as described earlier (Mruk et al. 2003). Commercially obtained antibodies against different target proteins are listed in Table 1. All immunoblots were densitometrically scanned using SPSS SigmaGel software (version 1.05) from SPSS Inc. (Chicago, IL, USA) and normalized against the level of β-actin to confirm equal protein loading.

Immunohistochemistry and fluorescent microscopy

For both immunohistochemistry and fluorescent microscopy experiments, the rabbit anti-dynamin antibodies from Cell Signaling Technology (Beverly, CA, USA) and Abcam, Inc. (Cambridge, MA, USA; see Table 1) were used, whereas the mouse antibody from BD Transduction Laboratories (San Jose CA, USA) failed to work in these studies. While both rabbit antibodies yielded specific staining in the seminiferous epithelium in preliminary studies, the antibody from Abcam produced much better resolution and at a significantly lower working dilution (1:300 vs 1:75, see Table 1). As such, all reported results regarding dynamin were from experiments carried out with the antibody from Abcam. Immunohistochemistry was performed as described earlier (Siu et al. 2005). In short, frozen testes were embedded in optimal cutting temperature (OCT; Sakura Finetek USA Inc., Torrance, CA, USA) compound, and sectioned to ~6–8 μm thickness in a cryostat at −20 °C. Cross-sections from different testes within an experimental group were mounted onto one to two poly-L-lysine-coated slides
using QCapture Suite Imaging Software (version 2.56; an Olympus DP70 Digital Camera. Images were captured microscope equipped with UPlanFl fluorescent optics and micrographs were acquired using an Olympus BX40 FITC-conjugated secondary antibodies (see Table 1). All similar observations. performed by two different investigators, which yielded representative data from four to six different experiments from morphology studies reported in this paper were et al. Journal of Endocrinology Inc. (Silver Spring, MD, USA).

Estimated by Tukey’s honest significant test using GBSTAT for each time point. Data within an experimental group were five times using different batches of cells. For All experiments reported herein were repeated for three to five times to eliminate interexperimental variations. Statistical analysis

All experiments reported herein were repeated for three to five times using different batches of cell lysates. For in vivo experiments, at least six rats, including controls, were used for each time point. Data within an experimental group were analyzed by ANOVA, and statistical significance was estimated by Tukey’s honest significant test using GBSTAT (version 7.0) software package from Dynamic Microsystem Inc. (Silver Spring, MD, USA).

Localization and cellular association of dynamin II in adult rat testes

Figure 1A–F illustrates the representative results of an immunohistochemistry experiment performed on frozen sections of adult rat testes by incubating with the rabbit anti-dynamin antibody. Specific signals from dynamin II were detected, as explained in Materials and Methods. Dynamin II appears as reddish-brown precipitate localized mostly to the apical ES with strongest staining in stages VII–VIII tubules (see Fig. 1A). Figure 1B shows a normal rat testis section incubated with rabbit IgG in place of the primary antibody, illustrating the antibody specificity. Since only very weak background staining was found in the interstitium. The strongest dynamin II staining was detected in an early stage VIII tubule as shown in Fig. 1C. Figure 1D is a magnified view of the boxed area in Fig. 1C, demonstrating the dynamin II staining on the convex surface of the heads of elongating spermatids, consistent with its localization at the apical ES. Weak but still discernible dynamin II staining was found at the apical ES site of elongating spermatids in stages V and X tubules (see Fig. 1E and F vs C and D). Furthermore, dynamin II was detected in the basal compartment of the seminiferous epithelium, localizing at the BTB in virtually all tubules examined (see Fig. 1A and C–F). Dynamin II was also found to be associated with round spermatids, spermatocytes, and spermatogonia in stages VII–VIII tubules, though signals attenuated in stages V and X (see Fig. 1C–F). This was suggestive of its localization at the desmosome-like junctions, since apical ES is absent at the interface between these germ and Sertoli cells in the epithelium. The specificity of this antibody from Abcam (Table 1) was further verified by immunoblotting as shown in Fig. 1G. Only one prominent band with an electrophoretic mobility corresponding to the apparent relative molecular mass of dynamin II (100 kDa) was detected in the lysates of seminiferous tubules and germ cells from adult rats, and Sertoli cells from 20-day-old rats. The relative expression level of dynamin II in different cellular fractions of the testis was also investigated (Fig. 1H and I). It was shown that germ cells expressed approximately three times more dynamin II than Sertoli cells (Fig. 1I). This result is consistent with an earlier report describing the relative abundance of dynamin in germ vs Sertoli cells (Kamitani et al. 2002).

Co-localization of dynamin II with other target proteins at the BTB and their structural association

We next used fluorescent microscopy to examine the co-localization of dynamin II with proteins at the BTB. Figure 2A–D, E–H illustrate the co-localization of occludin and JAM-A (both are putative integral membrane proteins at the BTB) with their adaptor ZO-1 in the seminiferous epithelium, consistent with their localization at the BTB in adult rat testes. It is worthy to note that dynamin II was
found to be localized at the apical ES as well as the BTB (see Fig. 2I and M), which is consistent with the results of the immunohistochemistry as shown in Fig. 1A–D. Dynamin II partially co-localized with ZO-1 at the BTB (Fig. 2I–L). At the same time, its co-localization with N-cadherin was also observed, but the merged signal was much weaker (Fig. 2M–P). To further verify these observations, specific antibodies against different target proteins (see Table 1) were used for co-immunoprecipitation (Co-IP), and precipitable immunocomplexes were

![Image of immunohistochemistry results](image-url)
Changes in dynamin II steady-state protein level and its cellular association in the seminiferous epithelium during adjudin-induced junction restructuring and germ cell depletion

Adjudin is known to induce germ cell depletion from the seminiferous epithelium without compromising the BTB integrity at the time of extensive anchoring junction restructuring (Mruk & Cheng 2004, Cheng et al. 2005). The drug apparently exerts its effects at the Sertoli–germ cell interface, for instance, by disrupting the integrin/laminin protein complex at the apical ES (Siu et al. 2005). In previous mating studies, the anti-fertility effect of adjudin was not visible until the sperm reserve at the epididymides was exhausted, 3–4 weeks after treatment (Cheng et al. 2005). This observation illustrated that adjudin has no apparent toxicity to germ cells. Otherwise, the infertility effect would have been detected within 1–2 weeks since elongate/elongating spermatids were virtually depleted from the epithelium from days 4 to 14 (see Fig. 3). Therefore, this model was used to examine changes in the dynamin II protein level and its cellular association during extensive junction restructuring in the seminiferous epithelium. As shown in Fig. 3A and B, there was a trend of time–dependent decline in dynamin II protein level in testes when germ cells began to detach from the epithelium from day 1 onwards except for a mild and statistically insignificant increase in hours 1–8 post-treatment. This trend of declining dynamin II in the testis was not entirely unexpected since dynamin II is largely expressed by elongating/elongate spermatids (see Fig. 1). The data shown in Fig. 3A and B were validated by immunohistochemistry studies to visualize the distribution of dynamin II in the seminiferous epithelium during adjudin-induced germ cell loss (see Fig. 3C–F). For instance, a decline in dynamin II staining was detected in the epithelium from days 4 to 14 when elongating/elongate spermatids were being depleted from the epithelium. This event was accompanied by a loss of dynamin II at the Sertoli cell–spermatid apical ES site (Fig. 3D–F vs A). It is also worthy to note that dynamin II was still seen to be surrounding the heads of the elongate spermatids remaining in the tubule lumen (see black arrowheads in Fig. 3D–F). Interestingly, while the overall dynamin II staining in the epithelium was weak, its signals at the BTB did not appear to be diminished by day 14 when the tubules were virtually devoid of spermatids and spermatocytes. Instead, the staining intensity at the BTB was considerably higher as compared with normal testes (see Fig. 3F vs C–E).

Changes in the steady-state levels of target proteins at the BTB during junction restructuring in the seminiferous epithelium

In the same experiment during which the level of dynamin II was quantified (see Fig. 3), the steady-state levels of two known protein complexes at the BTB, namely N-cadherin/β-catenin and occludin/ZO-1, were also measured (Fig. 4A and B). It was found that levels of these proteins increased significantly. As some of these proteins are restricted to (e.g. occludin) or predominantly expressed by (e.g. ZO-1) Sertoli cells, their increase in protein levels shown by immunoblots might reflect changes in cellular contribution instead of an increase in de novo synthesis. For instance, as shown in Fig. 3E and F, the lysates obtained from these testes were largely

![Image](https://via.placeholder.com/150)
Figure 2 (A–Q) A study to assess the association of dynamin II with BTB proteins in the seminiferous epithelium by immunofluorescent microscopy (A–P) and co-immunoprecipitation (Co-IP) (Q). (A–P) Fluorescent micrographs that illustrate the co-localization of occludin and ZO-1 at the BTB (A–D), which is similar to JAM-A and ZO-1 (E–H). It is noted that Cy3 is red fluorescence and FITC is green fluorescence. Cell nuclei were stained with DAPI. Dynamin II also co-localized, at least in part, with ZO-1 (I–L); and to a lesser extent with N-cadherin (M–P), at the basal compartment consistent with its localization at the BTB; although most dynamin II fluorescence was detected at the apical ES site at the elongating/elongate spermatid–Sertoli cell interface. The results shown here are the data from a representative experiment. This experiment was repeated at least twice using sections obtained from different animals which yielded similar results. Bar in A is 60 μm, which applies to B–P. (Q) The results shown above were further confirmed by a Co-IP experiment. Lysates of either ST (seminiferous tubules) or GC (germ cells) from testes of adult rats were used for Co-IP as described in Materials and Methods. Negative controls are samples treated with either normal rabbit (Rb) or goat (Gt) IgG instead of the primary precipitating antibodies. It is worthy of note that dynamin II interacted with occludin (in lysates of ST but not GC since germ cells do not express occludin), N-cadherin, ZO-1, β-catenin, JAM-A, p130Cas, and β-actin, but not nectin-3. The data shown herein are the results of a representative experiment which was repeated thrice using different sets of samples that yielded similar observations. IB, immunoblot.
contributed by Sertoli cells, whereas the lysates from testes on day 4 and earlier time points including normal rats (Ctrl) had more proteins contributed by germ cells. As such, the data shown in Fig. 4B were corrected against the declining testicular weight (see Fig. 4C), and re-plotted as the relative target protein level per pair testes (see Fig. 4D). Consistent with the data shown in Fig. 4B on day 1, most of the target proteins (except β-catenin) were induced at the beginning of germ cell depletion (see Fig. 3). However, on days 7 and 14, when most germ cells, in particular elongating/elongate and round spermatids and most spermatocytes, were depleted from the epithelium, only N-cadherin was still induced. This observation is consistent with an earlier report (Chen et al. 2003). Yet the levels of β-catenin, occludin, and ZO-1, did not appear to be significantly different from the controls (Fig. 4D vs B). In fact, a mild inhibition was detected for β-catenin on day 14 (Fig. 4D vs B).

Changes in protein–protein interactions at the BTB during junction restructuring in the seminiferous epithelium

We next used samples from testes of adjudin-treated rats to examine changes in the association of the BTB target proteins with dynamin II by Co-IP as shown in Fig. 5. When these testes lysates were examined for dynamin II protein level, a significant decline in overall dynamin II level was detected

Figure 3 (A–F) Changes in the steady-state protein levels of dynamin II (A–B) and its localization (C–F) in the epithelium during adjudin-induced germ cell loss. (A–B) Lysates (~100 μg protein) obtained from testes of rats treated with a single dose of adjudin (50 mg/kg b.w., by gavage) were resolved by SDS-PAGE for immunoblottings (A). The results shown in A are summarized in the histogram shown in B with n = 4 rats. It is noted that by day 4 when most of the germ cells were depleted from the seminiferous epithelium (see D–F vs C), a significant decline in the steady-state dynamin II protein level was detected (B), which persisted through days 7–14. ns, not significantly different by ANOVA; *P < 0.05; †P < 0.01. (C–F) These micrographs are the results of an immunohistochemistry experiment using an anti-dynamin antibody (see Table 1). It should be noted that by day 4 after adjudin treatment, most of the elongating/elongate spermatids were depleted from the epithelium, and the loss of dynamin II staining was consistent with the results shown in A–B. However, some elongate spermatids remaining in the tubule lumen were still stained positive for dynamin II (see black arrowhead in D), this trend persisted through days 7–14 (see black arrowhead in E and F). While there was an overall loss of immunoreactive dynamin II in the seminiferous epithelium, its level in the basal compartment of the seminiferous epithelium at the BTB persisted or even appeared to be higher on day 14 vs normal rats (see F vs C–E). This experiment was repeated twice using different sections from different experimental groups with similar results. Bar in C = 80 μm, which applies to D–F. H, hour; D, day; Ctrl, control.
Figure 4 (A–D) Changes in the steady-state protein levels of BTB-associated protein complexes during adjudin-induced germ cell depletion from the seminiferous epithelium. (A) About 100 μg protein from testes lysates on 1, 7, and 14 days after adjudin treatment and the control (Ctrl, normal testes at time 0) were resolved by SDS-PAGE. Immunoblotting was carried out using antibodies against different proteins in the N-cadherin/β-catenin and the occludin/ZO-1 protein complexes. β-Actin served as the protein loading control. This is a representative result from 4 separate experiments, which yielded similar results and are shown in B. (B) This is a histogram using data shown in A and normalized against β-actin (n=4). The steady-state protein level in Ctrl was arbitrarily set as 1, against which other data were compared. *Significantly different, P<0.05 by ANOVA; †P<0.01. (C) Changes in testes weight (per pair testes) after adjudin treatment. (D) Data shown in (C) were corrected against the declining testes weight and expressed as relative protein level per pair testes. Statistical analysis shown in C and D was performed using ANOVA; ns, not significantly different; D, days; Ctrl, control. *P<0.05.

Dynamin II and junction restructuring. P P Y LIE, W XIA, C Q F WANG, D D MRUK and others 579

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Figure 5 (A and B) A study by Co-IP to assess changes in protein–protein interactions between dynamin II, integral membrane proteins, and adaptors at the BTB. (A) Specific antibodies against different target proteins were used to immunoprecipitate dynamin II. It was observed that a loss in association between dynamin II and integral membrane proteins at the BTB: occludin and N-cadherin, occurred on day 7 (7D) after adjudin treatment (see also Fig. 3C–F), which was the time of germ cell depletion from the epithelium. This was concomitant with a loss of dynamin II from the BTB (see the lower panel). However, a significant increase in the association between dynamin II and the two adaptors: ZO-1 and β-catenin was detected. Also, by day 14 post-treatment, while the dynamin II level was still low, an increase in protein–protein association of dynamin II with occludin and N-cadherin was detected (see the fifth panel in which testis lysates were stained for dynamin II by...
N-cadherin (Fig. 5A and B). Although the levels of dynamin II in the testis on days 7 and 14 remained low (vs Ctrl and day 1) and were not statistically significantly different from each other (see Fig. 5B), the binding between dynamin II and the two BTB protein complexes rebound by day 14 (Fig. 5A and B). These observations are physiologically important, as they suggest that when the tubules were almost devoid of germ cells on days 7 and 14 following adjudin treatment (see Fig. 3E and F vs C and D), the association between dynamin II and the two BTB junctional complexes, namely occludin/ZO-1 and N-cadherin/β-catenin, become tighter. These biochemical results are also supported by immunohistochemistry studies, showing that by day 14 when the tubules were virtually devoid of spermatids and spermatocytes (see Fig. 3F), dynamin II staining in the basal compartment of the seminiferous tubule and at the BTB did not diminish vs control rats (see Fig. 3F vs C). In short, the changes in protein–protein association between dynamin II and the adaptors ZO-1 and β-catenin on days 7 and 14 vs controls and day 1 (see Fig. 5) could not be explained simply by an alteration of steady-state protein levels. Instead, there was a shift in affinity between dynamin II and the adaptors.

Discussion

What is the physiological role of dynamin II at the BTB?

Dynamin II is most abundantly found in the testis vs other organs (Iguchi et al. 2002). However, its physiological role in the testis remains unknown. Studies in other epithelia have demonstrated that dynamins are crucial for endocytosis of integral membrane proteins at cell junctions, such as occludin, and they are involved in protein–trafficking events between cell membrane and the actin network (Oh et al. 1998, Schmid et al. 1998, Sever et al. 2000a, Orth & McNiven 2003, Shen & Turner 2005). Moreover, recent studies have shown that membrane protein internalization and its recycling are important for regulating junction dynamics in multiple epithelia (for a review, see Maxfield & McGraw 2004). In general, this process of protein internalization is regulated by clathrin- or caveolin-mediated pathways (Le et al. 1999, Ivanov et al. 2004, Shen & Turner 2005), or alternatively by a clathrin- and caveolin-independent mechanism such as pinocytosis (Utech et al. 2005). Dynamins are known to be involved in both clathrin- and caveolin-mediated protein trafficking (Oh et al. 1998, Shen & Turner 2005), facilitating internalization, and recycling of integral membrane proteins. However, it remains to be investigated if dynamin II indeed is involved in promoting internalization and recycling of occludins, cadherins, JAM-A, and Claudins at the BTB; but since dynamin II was found to co-localize with clathrin in Sertoli cells (Kamitani et al. 2002), its role in cellular protein trafficking was implicated. Furthermore, recent studies have provided strong support for the model in which dynamins function as pinchase-like mechanoenzymes, to sever nascent endocytic pits from the plasma membrane to form endocytic vesicles, rather than acting as molecular switches like small GTPases (Thompson & McNiven 2001, Cao et al. 2003). Thus, it is highly likely that dynamin II acts as a ‘pinchase-like mechanoenzyme’ in Sertoli cells, similar to other epithelial cells, to facilitate internalization of occludin and/or N-cadherin.

It is obvious that the best approach to assess the function of dynamins at the BTB is to examine the testes of dynamin knockout mice. Yet, dynamin knockout mice are presently unavailable, thus it is not known if the deletion of any one of the three classical dynamins would affect spermatogenesis and/or BTB function. Nonetheless, in light of the extensive junction restructuring events at the BTB during spermatogenesis at stage VIII of the epithelial cycle to facilitate preleptotene spermatocyte migration across the barrier (Russell 1977), we sought to examine the possible involvement of dynamin II in the protein complexes at the BTB using a different approach. Studies were carried out by a model of junction restructuring in which adult rats were treated with adjudin (Mruk & Cheng 2004). In normal testes, it was shown that dynamin II was associated with the two most extensively studied protein complexes at the BTB, namely occludin/ZO-1 and N-cadherin/β-catenin. It is also worthy of note that the association between dynamin II and the adaptors in these two complexes, namely ZO-1 and β-catenin, significantly increased during germ cell depletion.

In other epithelia, TJ fibrils are restricted to the apical region...
of epithelial cells, beneath these are the AJ plaques and followed by the desmosomes, forming the junction complexes (Alberts et al. 2002). In light of such intimate association between TJ and AJ, a disruption of AJ is known to perturb the TJ barrier in virtually all the epithelia and endothelia examined to date (Man et al. 2000, West et al. 2002, Guo et al. 2003). However, this general cell physiological response is not applicable to the seminiferous epithelium. Otherwise, the BTB cannot maintain its ‘fence’ and ‘immunological barrier’ functions during spermatogenesis when developing preleptotene spermatocytes are traversing the BTB at stage VIII of the epithelial cycle. Additionally, TJ and AJ are even more intimately associated at the BTB vs other epithelia and endothelia. This is because at the BTB, TJs ‘co-exist’ side-by-side with desmosome-like junctions and two types of testis-specific AJ, namely basal ES and basal tubulobulbar complex (basal TBC). Hence, it is conceivable that although the disruption of AJ usually leads to TJ disassembly, a unique mechanism is in place in the testis to ‘disengage’ the events of TJ and AJ disassembly pertinent to spermatogenesis. Indeed, recent studies have shown that the testis apparently utilizes a ‘disengagement’ and ‘engagement’ mechanism to ensure the integrity of TJs during AJ restructuring (Yan & Cheng 2005).

For instance, it was shown that the co-existing occludin/ZO-1 and N-cadherin/β-catenin complexes were structurally ‘engaged’ in normal testes via their adaptors, ZO-1 and β-catenin, to reinforce BTB integrity, creating the tightest barrier in the mammalian body (Yan & Cheng 2005).

However, when the testes undergo restructuring during spermatogenesis or when AJ restructuring is induced by adjudin treatment, ZO-1 is dissociated from β-catenin, i.e. in a ‘disengagement’ state. As such, the occludin/ZO-1 and N-cadherin/β-catenin protein complexes are not structurally ‘engaged’ but rather ‘disengaged’. This permits AJ restructuring to facilitate germ cell movement without compromising the occludin–ZO-1 interactions at the BTB. As reported herein, dynamin II became more significantly ‘engaged’ with ZO-1 and β-catenin during extensive AJ restructuring induced by adjudin, which may explain the lack of damage to the BTB at the time of germ cell loss during adjudin-induced AJ restructuring (Mruk & Cheng 2004). This phenomenon seemingly suggests that dynamin II may play a role in assisting the ‘disengagement’ of ZO-1 and β-catenin by pulling them away from each other during adjudin-induced junction restructuring so that these adaptors can associate primarily with their corresponding integral membrane proteins, occludin and N-cadherin respectively. In this way, the occludin–ZO-1 complex can continue to maintain the TJ barrier. This postulation is also supported by the protein–protein interaction data. By day 14 when virtually all the tubules were devoid of germ cells with only Sertoli cells and spermatogonia found in the basal compartment, there was an increase in the association between dynamin II and the two major junctional protein complexes at the BTB.

Obviously, future investigations should be expanded to examine the changes in the kinetics of internalization and recycling of BTB integral membrane proteins (e.g. occludin, N-cadherin, and/or JAM-A) following adjudin treatment, using seminiferous tubule cultures and the techniques of biotinylation, immunoblotting, and co-immunoprecipitation. In addition, the functional significance of the changes in association between dynamin II and ZO-1/β-catenin, as presented in this report, should be carefully evaluated.

In this context, it is of interest to note that while dynamin II is found in the BTB and may be crucial to BTB dynamics, it is the most abundantly detected at the apical ES in a stage specific manner (i.e. at early stage VIII). This seemingly suggests that dynamin II may also facilitate protein internalization at the apical ES during spermatogenesis, which should be investigated in future studies.

Is the adjudin-induced AJ restructuring and germ cell loss a reliable model to probe the function of dynamin II?

It is obvious that the results reported herein were based on the use of the adjudin model and comparison with testes from normal rats. As such, one would argue if the observed changes in protein–protein interactions reported herein were simply the result of drug toxicity, and they might be irrelevant to normal testicular physiology. We offer several explanations to support our conclusion. First, recently completed acute toxicity studies in mice and rats, as well as pertinent mutagenicity and genotoxicity conducted by licensed toxicologists according to FDA guidelines, have shown that adjudin is not toxic at doses effective to induce transient infertility in these animals (Mruk et al. 2006). However, as illustrated in a 29-day subchronic toxicity study, a narrow margin between adjudin’s safety and efficacy was detected, making it unlikely to become a male contraceptive unless it can be targeted specifically to the testis to improve its efficacy and selectivity (Mruk et al. 2006). These toxicity studies thus illustrate that the data reported herein were not likely to be the manifestation of drug toxicity. Secondly, if adjudin is indeed acutely toxic to Sertoli and/or germ cells, its infertility effects would have been more rapid when it was administered to adult rats at 50 mg/kg b.w. (once a week for 2–4 weeks) by gavage, without requiring a ~20-day wait period for the sperm reserve in the epididymides to be exhausted (Cheng et al. 2001, 2005). Furthermore, the anti-fertility effect of adjudin was highly reversible when rats were given just two to six doses at 50 mg/kg b.w. (once a week for 2–6 weeks), suggesting that at these dosings, not all Sertoli cells and spermatogonia were killed by the adjudin treatment (Cheng et al. 2005). Nonetheless, these findings do not rule out the possibility that adjudin is a Sertoli and/or germ cell toxicant, in particular if it is given chronically. Thirdly, recent studies from our laboratory using the adjudin model have identified the signaling molecules and pathways, such as focal adhesion kinase (FAK), Src, and extracellular signal-regulated kinase (ERK) which is one of the mitogen–activated protein kinases, crucial for regulating cell adhesion in the epithelium particularly between Sertoli cells and elongating/elongate spermatids at the apical ES (Siu et al. 2005, Xia & Cheng 2005). These findings have largely been confirmed using a well-established and thoroughly
characterized in vivo model for studying AJ dynamics in the testis by testosterone/estradiol implants to suppress the intratesticular androgen level (O'Donnell et al. 1996, 2000, Saito et al. 2000, Beardsley & O'Donnell 2003); (for reviews, see O'Donnell et al. 2001, McLachlan et al. 2002). In this model, the suppression of endogenous androgen level resulted in a selective disruption of apical ES at the Sertoli cell–spermatid (step 8 and beyond) interface without apparent toxic effects to testicular cells since germ cells eventually repopulated the epithelium during recovery, and the BTB integrity was also not compromised (O'Donnell et al. 2000, Beardsley & O'Donnell 2003). For instance, a study using this androgen suppression model has shown that cSrc, FAK, and ERK are indeed the crucial kinases in the ERK-signaling pathway that regulates apical ES dynamics (Wong et al. 2005), which is consistent with the results obtained from the adjudin model (Siu et al. 2005, Xia & Cheng 2005). Lastly and perhaps the most important of all, as shown in studies using the androgen suppression model, the observed changes in cadherins–catenins interactions in the epithelium as a result of increasing tyrosine phosphorylation in β-catenin, ultimately led to germ cell loss from the epithelium (Xia et al. 2005b, Zhang et al. 2005); and these results were consistent with data obtained from the adjudin model (Xia & Cheng 2005, Yan & Cheng 2005). Collectively, the above

Figure 6 A schematic drawing illustrating the dynamic interaction between dynamin II and the adaptors ZO-1 and β-catenin that may play a role in regulating tight junction dynamics and cell adhesion at the BTB. The changes in interaction between dynamin II and the adaptor proteins at the occludin/ZO-1 and the N-cadherin/β-catenin complexes during BTB restructuring may have physiological significance in spermatogenesis of adult rat testes. The panel on the left depicts the BTB in a ‘closed’ state, where ZO-1 and β-catenin interact with each other so that the co-existing occludin/ZO-1 and N-cadherin/β-catenin protein complexes reinforce the BTB integrity (Yan & Cheng 2005). When the BTB is in the ‘open state’ as shown in the right panel (either during junction restructuring in spermatogenesis or induced by adjudin treatment), dynamin II becomes associated more extensively with ZO-1 and β-catenin. This may play a role in causing the ‘disengagement’ of the integral membrane proteins, and their interactions with adaptors in the occludin/ZO-1 and N-cadherin/β-catenin protein complexes, facilitating BTB opening and preleptotene spermatid migration during stage VIII of the epithelial cycle. In addition, the change in association between dynamin II and the adaptor proteins may be used to maintain the ‘disengaged’ state of the BTB. On the other hand, the right panel of this diagram also includes another postulated function of dynamin II in the seminiferous epithelium, based on findings in other epithelia, which is the internalization of integral membrane proteins (e.g. occludin and N-cadherin) by endocytic processes. Dynamin II may act as a ‘pinchase’ to facilitate endocytic vesicle formation by severing nascent endocytic pits from the plasma membrane as proposed earlier (Thompson & McNiven 2001, Cao et al. 2003). It is worthy to note that dynamins per se are not proteases, it is likely that adaptors that are associated with dynamins recruit protease(s) to the site to facilitate its ‘pinchase-like’ action. As such, dynamin II may have multiple functions in the seminiferous epithelium, which must be vigorously investigated in future studies. Nevertheless, the proposed ‘pinchase’ function of dynamin II does not negate our findings. The ‘opening’ state of the BTB is likely to be induced by a surge of cytokines released from Sertoli and germ cells (e.g. TGF-β3 and TNFα), with receptors largely restricted to Sertoli cells (Lui et al. 2003b, Siu et al. 2003, Li et al. 2006, Xia et al. 2006). This is a highly simplified hypothetical scheme, since the BTB is composed of at least five different classes of protein complexes, namely occludin/ZO-1, claudins/ZO-1, JAMs/ZO-1, cadherins/catenins, and nectins/afadin. Different classes of protein complexes are probably regulated differently. This model is expected to be revised rapidly. However, it serves as a biochemical scheme upon which functional experiments can be designed to investigate the functional significance of the change in the dynamin II–adapter proteins association as presented in this report, and also the possible role of dynamin II in integral membrane protein internalization.
arguments illustrate that even if adjudin is a Sertoli cell toxicant, it produces changes in the seminiferous epithelium including the BTB and induces junction restructuring at the cell–cell interface, which can be used to learn something about the normal system. Thus, the results presented in this report are physiologically relevant to events that occur in the seminiferous epithelium during spermatogenesis.

What is the physiological significance regarding changes in protein–protein interactions at the BTB pertinent to preleptotene spermatocyte migration across the BTB at stage VIII of the epithelial cycle?

Recent studies have shown that at least five different structural protein complexes (excluding their isoforms) are found at the BTB, conferring barrier and anchoring functions in adult rat testes. These include claudins/ZO-1, JAMs/ZO-1, and nectins/afadin, in addition to the previously mentioned occludin/ZO-1 and N-cadherin/β-catenin (Wong & Cheng 2005, Xia et al. 2005a). Undoubtedly, we will see this list grow in the years to come. At the BTB, these co-existing protein complexes and the different junction types serve to maintain not only the BTB integrity but also the cell polarity. More importantly, they ensure the timely BTB restructuring that facilitates preleptotene spermatocyte migration during stage VIII of the epithelial cycle in adult rat testes. It would be physiologically ‘difficult’, perhaps even ‘uneconomical’, to have de novo synthesis of all these proteins simultaneously at the BTB each time the preleptotene spermatocytes traverse the BTB. For this reason, protein complexes at the BTB may employ an efficient mechanism to allow rapid BTB restructuring. As illustrated by recent studies using different models, the changes in protein–protein interactions at the BTB or apical ES are indeed being used to facilitate AJ and/or TJ restructuring in the seminiferous epithelium, so as to elicit rapid changes in cell adhesion (Xia et al. 2005b, Yan & Cheng 2005). Studies from other epithelia have shown that cadherins can be shuffled rapidly to the cytoplasm via internalization, and re-shuffled back to the plasma membrane with the help of GTPases, kinases, and adaptors (e.g. Rap1, PKC, p120ctn; Le et al. 1999, 2002, Balzac et al. 2005, Mruk et al. 2005, Xiao et al. 2005). In addition, GTPases (e.g. dynamin II) are likely to be involved in these protein-trafficking events, and some of them may act as molecular switches for trafficking between cell membrane and cytoskeletal networks in various epithelia, including the seminiferous epithelium in adult rat testes (Olkkonen & Stenmark 1997, Takai et al. 2001, Deneka et al. 2003, Mruk et al. 2005). Work is now in progress in our laboratory to assess internalization and recycling of integral membrane proteins at the Sertoli–Sertoli and the Sertoli–germ cell interfaces, and the factor(s) and/or mechanism(s) that regulate these events.

In summary, we have demonstrated that dynamin II is a potentially important regulator for the protein–protein interactions between different adaptors (e.g. catenins and ZO-1) and their corresponding integral membrane proteins (e.g. cadherins and occludins) in rat testes. Based on these recent data including the results reported herein, we have provided a hypothetical model (see Fig. 6) illustrating the dynamic interactions between GTPases (e.g. dynamin II) and the corresponding BTB-associated protein complexes (e.g. occludin/ZO-1 and N-cadherin/β-catenin), to facilitate preleptotene spermatocyte migration across the BTB without compromising its integrity.

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Dynamin II and junction restructuring · P Y LEE, W XIA, C Q F WANG, D M RUK and others 585


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