Laminin $\alpha 3$ Forms a Complex with $\beta 3$ and $\gamma 3$ Chains That Serves as the Ligand for $\alpha 6\beta 1$-Integrin at the Apical Ectoplasmic Specialization in Adult Rat Testes $^*$

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Apical ectoplasmic specialization (ES) is a testis-specific hybrid cell/cell actin-based adherens junction and cell/matrix focal contact anchoring junction type restricted to the interface between Sertoli cells and developing spermatids. Recent studies have shown that laminin $\gamma 3$, restricted to elongating spermatids, is a putative binding partner of $\alpha 6\beta 1$-integrin localized in Sertoli cells at the apical ES. However, the identity of the $\alpha$ and $\beta$ chains, which constitute a functional laminin ligand with the $\gamma 3$ chain at the apical ES, is not known. Using reverse transcription-PCR and immunoblotting to survey all laminin chains in cells of the seminiferous epithelium, it was noted that $\alpha 2$, $\alpha 3$, $\beta 1$, $\beta 2$, $\beta 3$, and $\gamma 3$ chains were found in germ cells, whereas $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, and $\gamma 3$ chains were found in Sertoli cells, implying that $\alpha 3$ and $\beta 3$ are the plausible laminin chains restricted to germ cells that may be the bona fide partners of $\gamma 3$. To verify this postulate, recombinant proteins based on domain G of $\alpha 3$ and domain I of $\beta 3$ and $\gamma 3$ chains were produced and used to obtain the corresponding specific polyclonal antibodies. Additional studies have demonstrated that the laminin $\alpha 3$, $\beta 3$, and $\gamma 3$ chains indeed are restricted to germ cells at the apical ES, co-localizing with each other and with $\beta 1$-integrin. Furthermore, co-immunoprecipitation studies have confirmed the interactions among laminin $\alpha 3$, $\beta 3$, and $\gamma 3$, as well as $\beta 1$-integrin. When the functional laminin ligand at the apical ES was disrupted via blocking antibodies, such as using anti-laminin $\alpha 3$ or $\gamma 3$ IgG, this treatment perturbed adhesion between Sertoli and germ cells (mostly spermatids), leading to germ cell loss from the epithelium. More important, a transient disruption of the blood-testis barrier was also detected.

During spermatogenesis, preleptotene spermatocytes residing at the basal compartment of the seminiferous tubules must traverse the BTB$^3$ to the apical compartment at late stage VII through early stage VIII of the epithelial cycle in adult rat testes (1). Once in the adluminal compartment, spermatocytes differentiate into round spermatids, and the elongating/elongate spermatids orientate themselves with heads and tails pointing toward the basal lamina and the tubule lumen, respectively. The primary anchoring device that facilitates this process of orientation, while maintaining adhesion at the Sertoli cell/spermatid interface, is the apical ectoplasmic specialization (ES), a cell/cell actin-based adherens junction (AJs) (2–4). In recent years, numerous reports have been published identifying the adhesion molecules at the apical ES. These include the cadherin-catenin, nectin-afadin, and integrin-laminin protein complexes (5–10), which are the primary adhesion protein complexes at the Sertoli cell/desplasming epithelial interface. Interestingly, in virtually all other epithelia, the integrin/laminin complex is usually restricted to the basement membrane at the cell/matrix interface (11, 12). Yet studies have shown that integrin is a crucial adhesion molecule at the apical ES (7, 13) and together with laminin is likely the most important functional protein complex at the Sertoli cell/spermatic interface at the apical ES, suggesting that apical ES is a hybrid cell/cell and cell/matrix anchoring junction type (4, 14).

Laminins are glycoproteins and heterotrimers composed of $\alpha$, $\beta$, and $\gamma$ chains. To date, there are five known $\alpha$-subunits, four $\beta$-subunits, and three $\gamma$-subunits that can give rise to at least 16 different functional laminin ligands that bind to integrins restricted to the cell/matrix anchoring junctions, also known as focal contacts or focal adhesion complexes (15, 16). Laminins are crucial scaffolding proteins that provide structural stability to epithelia/endothelia in the basement membrane (12). More important, laminins and integrins at focal contacts provide adhesion between epithelial cells and basal lamina. This laminin/integrin protein complex also serves as an efficient structure to facilitate cell migration during normal and in pathological conditions, such as tumor invasion. The roles of laminins have been expanded from cell/matrix site to cell/cell interface following the identification of a non-basement membrane-associated laminin $\gamma 3$ chain in mouse testes (5); laminin-423 and laminin-523 (previously known as laminins 14 and 6) are the plausible laminin ligands that bind to integrins restricted to the cell/matrix anchoring junctions, also known as focal contacts or focal adhesion complexes (15, 16). Laminins are crucial scaffolding proteins that provide structural stability to epithelia/endothelia in the basement membrane (12). More important, laminins and integrins at focal contacts provide adhesion between epithelial cells and basal lamina. This laminin/integrin protein complex also serves as an efficient structure to facilitate cell migration during normal and in pathological conditions, such as tumor invasion. The roles of laminins have been expanded from cell/matrix site to cell/cell interface following the identification of a non-basement membrane-associated laminin $\gamma 3$ chain in mouse testes (5); laminin-423 and laminin-523 (previously known as laminins 14 and 15, respectively) were also found to reside outside the retinal basement membrane (17). Although their functions at the apical cell surface remain to be defined, it has been speculated that laminins expressed at the apical retinal epithelium may play a role in photoreceptor morphogenesis (17).

In adult rat testes, laminin $\gamma 3$ chain has recently been shown to be a putative binding partner of $\alpha 6\beta 1$-integrin at the Sertoli cell/spermatic interface (18); however, the $\alpha$ and $\beta$ chains that constitute a functional laminin remain to be identified. In this report, we sought to identify the laminin $\alpha$ and $\beta$ chains that form a functional ligand together with $\gamma 3$ for $\alpha 6\beta 1$-integrin at the apical ES. Using specific blocking antibodies, we also sought to examine the changes in the status of spermatogenesis, the integrity of the Sertoli-germ cell adhesion, and the BTB when functional laminin chains at the apical ES were perturbed. This is an initial attempt to study cross-talk between apical ES and BTB.

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$^4$ The abbreviations used are: BTB, blood-testis barrier; ES, ectoplasmic specialization; EM, electron microscopy; DAPI, 4′,6′-diamino-2-phenylindole; FITC, fluorescein isothiocyanate; IP, immunoprecipitation or immunoprecipitate; TJ, tight junction; AJ, adherens junction; ANOVA, analysis of variance; RT, reverse transcription; PBS, phosphate-buffered saline; FAK, focal adhesion kinase; % T, total gel concentration (g/100 ml) = (acrylamide + N,N,N,N-tetramethylethylenediamine)/100 ml.
EXPERIMENTAL PROCEDURES

Animals—The use of rats and rabbits was approved by The Rockefeller University Laboratory Animal Use and Care Committee with protocol numbers 00111, 03017, 03040, and 06018.

RNA Isolation, RT-PCR, Isolation of Sertoli and Germ Cells, and Primary Cultures and Co-cultures of Testicular Cells—Total RNA from rat testes, testicular cells, Sertoli cell cultures, and Sertoli-germ cell co-cultures were extracted using RNA STAT-60™ (Tel-Test “B” Inc., Friendswood, TX) according to the manufacturer’s instructions. RT-PCR was performed as described (19) using primer pairs specific to different target genes (in particular to all the known laminin chains (20)) and co-amplified with ribosomal S16 (see supplemental Table S1). Sertoli and germ cells were isolated from testes of 20- and 90-day-old Sprague-Dawley rats, respectively, as described previously (19, 21, 22). The purity of Sertoli and germ cells used in studies reported herein was monitored and characterized by primers specific to markers of Sertoli cells (e.g. testin), spermatogonia (e.g. c-Kit receptor), Leydig cells (e.g. 3β-hydroxysteroid dehydrogenase), and myoid cells (e.g. fibronectin), as well as electron microscopy as detailed elsewhere (10, 23, 24). These analyses have shown that the cell preparations (e.g. Sertoli and germ cells) used in our studies had negligible contamination from other cell types. For co-culture, Sertoli cells were plated on Matrigel-coated dishes (Matrigel diluted 1:7 with F12/Dulbecco’s modified Eagle’s medium) and cultured alone in serum-free F12/Dulbecco’s modified Eagle’s medium supplemented with epidermal growth factor, insulin, transferrin, and bacitracin as described (19, 25) for 5 days at 35 °C in a humidified atmosphere of 95% air, 5% CO2 with a hypotonic treatment on day 3 to remove residual germ cells. On day 6, total germ cells isolated from adult rat testes were plated onto the Sertoli cell epithelium using a Sertoli:germ cell ratio of 1:1 or 1:2 to initiate Sertoli-germ cell adherens junction assembly, and co-cultures were terminated at 2, 4, 8, 24, and 48 h thereafter. Cultures (in triplicates) were used for each time point, and each experiment was repeated at least three times using different batches of Sertoli and germ cells. The formation of functional tight junctions between Sertoli cells and adherens junctions (e.g. apical ectoplasmic specialization) at the Sertoli/germ cell interface in these co-

FIGURE 1. A study by RT-PCR to assess the distribution of different laminin mRNAs in rat testes. A, testin, a Sertoli cell-specific marker, was amplified only in testes and Sertoli cells but not in germ cells (left panel), whereas c-Kit receptor, a spermatagonium-specific marker, was amplified only in testes and germ cells but not in Sertoli cells (right panel), illustrating that each cell type (used in studies shown in B–D) was contaminated with negligible numbers of other cell types. B, laminins α1–3 were detected in testes. Laminin α2 was expressed in both Sertoli and germ cells, and laminin α3 was expressed predominantly in germ cells (right panel). C, laminin β-subunits were also amplified by PCR using primers specific to the different β chains (see supplemental Table S1). In testes, all three laminin β-chains were detected. Laminin β2 was expressed in both Sertoli and germ cells, whereas the expression of laminin β3 was restricted to germ cells. D, laminin γ1 was detected in testes and Sertoli cells but not in germ cells, whereas the expression level of laminin γ3 was higher in germ cells than in Sertoli cells. The asterisk illustrates the absence of a specific laminin chain. SC, Sertoli cells; GC, germ cells; D, day, indicating the age of animals from which cells or testes were isolated or obtained.
Functional Laminin Chains at the Apical ES

Production of Recombinant Proteins—Recombinant proteins of laminin α3, β3, and γ3 chains were expressed and produced using Escherichia coli BL21 Star™ (DE3) cells with the Champion™PET Directional TOPO® expression kit (Invitrogen). First, cDNAs corresponding to domain G of laminin α3 and domain I of laminins β3 and γ3 were amplified by PCR with AccPrime™ Pfx SuperMix (catalog no. 12344-040, Invitrogen) using total cDNAs from 90-day-old germ cells that served as the template (note that total RNA isolated from these germ cells were reverse-transcribed to cDNAs with oligo(dT)-15-mer and reverse transcriptase) and specific primers shown in supplemental Table S2 and then subcloned into pET101/D-TOPO® expression vectors. The inserted cDNA was in-frame with the expression vector and was confirmed by direct nucleotide sequencing. The expression vectors were transformed into E. coli BL21 Star™ (DE3) cells by the heat shock method at 42 °C. Protein expression in bacterial culture was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside when the A_{600 nm} level reached between 0.5 and 0.8. Bacteria were incubated at 37 °C for an additional 6 h. Cells were harvested by centrifugation (3000 × g for 10 min). To extract lysates, bacterial cell pellets were suspended in a lysis buffer (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol (v/v), 0.5% Triton X-100 (v/v), 100 mM imidazole, pH 7.8), frozen in liquid nitrogen, and thawed at 42 °C (repeated three times) followed by sonication using a Cole-Parmer model CP 130PB-1 ultrasonic processor, Chicago) and centrifugation. The recombinant proteins were produced with V5 and His6 epitope tags at the C terminus, and the authenticity was verified by a specific mouse anti-V5 epitope antibody (catalog no. R960-25, Invitrogen). Recombinant proteins were purified using a nickel column (Amersham Biosciences). The purity was confirmed by SDS-PAGE and silver-stained gels. The identity of the corresponding laminin recombinant proteins was subsequently confirmed using proteins sliced out of the Coomassie Blue-stained polyacrylamide gels by mass spectrometry, performed at The Rockefeller University Proteomics Resource Center. In some experiments (e.g. Fig. 2A), proteins embedded in Coomassie Blue-stained gel slices were micropurified using electroelution by placing ~10–20 gel slices (containing 200–400 μg of laminin chain protein) in a small dialysis bag (Spectrapor tubing with M_r cut-off at 3500; Spectrum Laboratories Inc., Rancho Dominguez, CA) and suspended in a final volume of ~500–800 μl of gel running buffer. The dialysis bag was then placed in a Hoefer horizontal gel unit, and electroelution was performed at 80 V for 4–8 h at room temperature using SDS-polyacrylamide gel running buffer without SDS as the electroelution buffer.

Preparation of Antiserum and Purification of IgG—Anti-laminins α3, β3, and γ3 were prepared in female New Zealand White rabbits with three immunizations using affinity column and gel-purified recombinant proteins emulsified with Freund’s complete and incomplete adjuvants essentially as earlier described (26). In short, to prepare highly purified recombinant proteins for immunization, recombinant laminin α3, β3, and γ3 proteins isolated from the nickel columns were resolved by SDS-PAGE on 15% T SDS-polyacrylamide gels, stained with Coomassie Blue, and the bands corresponded to the different laminin chains were sliced out. To remove acetic acid and methanol that were used in the Coomassie Blue staining and de-staining steps, gel slices (~10–15 μg protein/slice; about 15 gel slices were used for each immunization per rabbit) were immediately suspended in 5 ml of PBS (10 mM sodium phosphate, 0.15 m NaCl, pH 7.4 at 22 °C) and placed in a rotator at 30 rpm. Gel slices containing ~200 μg of recombinant protein were washed with PBS five times and homogenized in ~400 μl of PBS using a glass homogenizer. Homogenized gel slices were then emulsified with an equal volume of Freund's complete adjuvant with a sonicator and administered subcutaneously to the back of the rabbit at ~4 sites (200 μl/site). Prior to the first immunization, about 30 ml of blood was collected from the ear vein of each rabbit to obtain preimmune serum, which was used for corresponding control experiments described herein. Two booster injections were administered 6 and 8 weeks later using similar amounts of gel-purified recombinant proteins but emulsified with Freund’s incomplete adjuvant. Rabbits were bled, 10 days after the final booster injection, from the marginal vein in the ear, and at least four more bleedings were collected weekly thereafter. Blood allowed to clot overnight at 4 °C was centrifuged at 2000 × g for 15 min to obtain serum. Anti-laminin α3, β3, and γ3 IgG and the IgG from corresponding preimmune sera were isolated from (~5 ml) sera by sequential ammonium sulfate precipitation and DEAE (Bio-Rad) affinity chromatography as described (27). The purity of the IgG was confirmed by SDS-PAGE.
Characterization of Anti-laminin α3, β3, and γ3 Antibodies—To confirm that the antibodies raised against laminin α3 chain in rabbits did not cross-react with β3 and/or γ3 and vice versa, such that the results obtained from co-immunoprecipitation experiments were not artifacts of antibody cross-reactivity among these three laminin chains (namely α3, β3, and γ3), the antigens and these antibodies were characterized as follows. First, the identities of the nickel column-purified recombinant laminin α3, β3, and γ3 proteins were confirmed by mass spectrometry. Protein sequence analyses and their alignments were examined by using the EMBOSS software (European Bioinformatics Institute). The purity of the recombinant proteins used for immunization was also confirmed by SDS-PAGE and Coomassie Blue staining prior to their use for immunization. Second, the three antibodies were characterized by immunoblotting as follows.

**Characterization of Anti-laminin α3, β3, and γ3 Antibodies**

![Figures A, B, and C](image)

**Figure 3.** A study to access the changes in the three laminin chains and their likely binding partners in Sertoli-germ cell co-cultures during functional anchoring junctions assembly. A, antibody specificity was analyzed by immunoblotting using ~100 μg of protein from lysates of testes, seminiferous tubules (ST), Sertoli cells (SC), and germ cells (GC) and the corresponding antisera. All three laminins were expressed almost exclusively by germ cells, whereas β1-integrin was restricted to Sertoli cells (see left panel). When Sertoli and germ cells were co-cultured to initiate anchoring junction assembly, the protein levels of both laminins and β1-integrin, but not FAK, were induced; actin served as a protein loading control. B and C, protein levels of each target protein shown in A were scanned densitometrically and compared. Each bar is a mean ± S.D. of three samples. The protein level at time 0 was arbitrarily set at 1, against which one-way ANOVA was performed. nd, not detectable; ns, not significantly different; *, p < 0.05; **, p < 0.01.
or γ3 construct, were subjected to immunoblotting. The protein blots were immunostained sequentially with mouse anti-V5 epitope antibody followed by anti-laminin α3, β3, and γ3 antibodies to assess cross-reactivity.

Electron Microscopy (EM) and Immunogold EM—Electron microscopy and immunogold EM studies were performed at The Rockefeller University Bio-imaging Resource Center essentially as described previously (28, 29) using adult male Sprague-Dawley rats (~250 g body weight). In short, rat testes obtained from controls (normal rats and testes treated with preimmune IgG) and rats treated with either anti-laminin α3 or β3 IgG at specified time points were cut into small pieces (~1–2 mm) and fixed immediately in ice-cold 2.5% glutaraldehyde in 0.1 M cacodylate, pH 7.4, overnight. Thereafter, samples were embedded in 3% agar to keep the tubules together. After solidification, excessive agar was removed, and the specimens were post-fixed in 1% OsO4 in 0.1 M cacodylate, pH 7.4, on ice for 1–2 h. The tubules in agar blocks were treated en bloc with aqueous uranyl acetate for 1 h at room temperature. Specimens were then dehydrated with ascending graded alcohol, propylene oxide and then embedded in Epon 812. They were incubated with the antiserum (or anti-laminin α3 or β3 IgG, or preimmune serum for control experiments) at room temperature overnight followed by incubation with 10-nm gold-labeled secondary antibody.

**FIGURE 4.** Localization of laminin α3 at the apical ES of adult rat testes and kidneys. A–D, an immunohistochemistry localization study of laminin α3. A, cross-section of an adult rat testis showing the localization of immunoreactive laminin α3 (reddish-brown precipitates) in the epithelium at different stages of the epithelial cycle. Tubules at stages VI–VII (a), stage VIII (b), stages VII–VIII (c), and stages IV–V (d) are boxed, and the magnified views are shown in corresponding panels a–d. B, negative control (Ctrl) in which preimmune serum was used to substitute the primary antibody. C, cross-section of an adult rat kidney showing the localization of laminin α3. D, negative control using preimmune serum. E, laminin α3 (red, Cy3) was detected at the Sertoli cell/elongating spermatid interface (blue, DAPI staining) by fluorescent microscopy. F, an immunoblot using lysates of ~100 µg of protein of testicular cells and stained with an anti-laminin α3 antibody in a 6% T SDS-polyacrylamide gel. D, dye front. Laminin α3 with an apparent Mr of 165,000 was detected. G, apparent Mr analyses of the laminin chains. The Mr of laminins α3, β3, and γ3 was estimated by interpolation of protein standards versus relative mobility (Rf) in multiple SDS-polyacrylamide gels (n = 3). Scale bars: A, 120 µm, applies also to B; A, panel a, 60 µm, applies also to A, panels b–d, C, and D; A, panel b, inset, 20 µm, applies also to insets in A, panel c, and in E.
**Conjugation of Anti-laminin α3 and γ3 IgG with Alexa Fluor 488 Dye and Immunofluorescent Microscopy**—Conjugation of anti-laminin α3 and γ3 IgG with Alexa Fluor 488 dye was performed using the Alexa Fluor® 488 Microscale protein labeling kit from Molecular Probes (Eugene, OR). In short, about 11.3 nmol/μl reactive Alexa Fluor 488 dye was used to label ~60 μg of either anti-laminin α3 or γ3 IgG suspended in PBS. Excessive dye was removed using a spin column. To demonstrate the co-localization of different laminin chains in the seminiferous tubules, immunofluorescent microscopy was performed as described (30). In brief, frozen sections of testes were first incubated with a 1:300 dilution of anti-laminin α3 containing 1% normal goat serum at 35 °C overnight. The sections were then incubated with a 1:100 dilution of Alexa Fluor 488 dye-conjugated anti-laminin γ3 IgG at 4 °C for 7 h. Secondary antibodies conjugated with Cy3 (Zymed Laboratories Inc.), diluted in PBS to 1:50, were incubated with the sections for ~30 min. Negative controls were preformed using corresponding normal rabbit IgG, and these sections were incubated with Alexa Fluor 488 dye-conjugated anti-laminin γ3 IgG followed by secondary antibodies conjugated with Cy3. Sections were then washed and mounted with Vectashield Hardset with 4’,6’-diamino-2-phenylindole (DAPI) (a nucleus stain, Vector Laboratories, Burlingame, CA). Fluorescent micrographs were obtained using a BX40 microscope (Olympus Corp., Melville, NY) equipped with Olympus UPlanF1 fluorescent optics and an Olympus DP70 12.5 MPa digital camera.

**Immunohistochemistry and Immunocytochemistry**—Immunohistochemistry was performed as described previously (30). Control experiments were preformed using corresponding preimmune rabbit serum or IgG instead of the primary antibodies. The sources of antibodies used for all the studies described herein are listed in supplemental Table S3. Immunocytochemistry was performed to study the co-localization of laminin α3/γ3 and laminin γ3/β1 integrin in vitro. Sertoli cells were isolated and cultured for 5 days on a Lab-Tek® Chamber Slide™ system (Nalgene Nunc International) coated with Matrigel™ (Collaborative Biochemical Products, Bedford, MA) at a cell density of 5 × 10^4 cells/well (~1.8 cm²). Thereafter, total germ cells were added to Sertoli cells at a Sertoli:germ cell ratio of 1:2 and cultured for an additional 2 days to allow adherens junction formation. Cells were fixed in ice-cold absolute methanol, blocked with 10% goat serum, and incubated with either an

**Figure 5. Localization of laminin γ3 at the seminiferous epithelium of rat testes and kidneys by immunohistochemistry and immunofluorescent microscopy.** A, cross-section of an adult rat testis showing the localization of immunoreactive laminin γ3 (reddish-brown precipitates) at different stages of the epithelial cycle. Tubules at stages XII–XIII (a), stage VIII (b), stages V–VI (c), and stages VI–VII (d) are boxed, and the magnified views are shown in corresponding panels a–d. B, negative control (Ctrl) using preimmune serum. C, cross-section of an adult kidney showing the localization of laminin γ3. A collecting tubule in the boxed area is magnified in panel e below. Arrowheads in panel e represent immunoreactive laminin γ3 that was found at or near the basement membrane. D, negative control using preimmune serum. E, immunoblot using lysates of seminiferous tubules (ST), Sertoli cells (SC), and germ cells (GC) for SDS-PAGE was probed with the anti-laminin γ3 antibody. Only one prominent band of 146 kDa was detected in lysates of seminiferous tubules and germ cells but not in Sertoli cells. The same gel was also probed with an actin antibody to assess equal protein loading. F, laminin γ3 (green, FITC) was detected at the Sertoli cell/spermatid interface by fluorescent microscopy. Scale bars: A, 120 μm, applies also to B; A, panel a, 60 μm, applies also to A, panels b–d, C, and D; C, panel e, 20 μm, applies also to F.
FIGURE 6. Changes in the localization pattern of laminin γ3 at the seminiferous epithelium during testicular maturation. Cross-sections of testes from rats at 16, 30, and 60 days illustrate changes in the localization of immunoreactive laminin γ3 in the seminiferous epithelium. Laminin γ3 was restricted to the basal compartment near the basement membrane at the time of BTB formation at day 16 (A–C) when apical ES was absent. Laminin γ3 was detected at both the basal and apical compartments when apical ES began to be established at day 30 with elongating spermatids (steps 8 and 9) (D–F), laminin γ3 was expressed predominantly at apical ES when rats were sexually mature at day 60 (G–I). Scale bars: A, 80 μm, applies also to D–I; B, 40 μm, applies also to C and insets in A and D.

Co-immunoprecipitation (Co-IP) and Immunoblotting—To study the binding partners of laminin γ3 as well as other interacting proteins in rat testes, co-IP was performed using lysates of either seminiferous tubules or germ cells as described previously (31). Seminiferous tubules used for studies reported herein were isolated from adult rat testes and were shown to be contaminated with negligible Leydig cells as reported previously (31). Seminiferous tubule and germ cell lysates were prepared in the same lysis buffer as described above. About 50 mg of proteins from each sample was resolved by SDS-PAGE using 7.5, 10, or 12% T SDS-polyacrylamide gels under reducing conditions, depending on the apparent M of the target proteins to be investigated. Immunoblottings were performed as described (30).

Blocking of Laminin-333 Function by Intratesticular Injection of Anti-laminin α3 or γ3 IgG—To assess the physiological function of laminin-333 as a crucial cell adhesion protein complex at the apical ES, rats (n = 3 or 4/time point) received 75 μg of either anti-laminin α3 or γ3 IgG suspended in 200 μl of PBS at three sites per testis (i.e., ~70 μl/site) as described (23). Rats were terminated at day 2, day 4, and day 7 (received one injection), day 10 and day 15 (received two injections at day 0 and day 7), and day 20 and day 25 (received three injections at day 0, day 7, and day 15). There were two groups of control animals in this study. The first group of control rats received 75 μg of preimmune rabbit IgG (suspended in 200 μl of PBS) and were terminated on day 7 (received one injection), day 10 and day 15 (received a total of two injections), and day 20 (received a total of three injections). The second group of control rats received 200 μl of PBS and were terminated on day 7 (received one injection) or day 15 (received a total of two injections) and served as vehicle control. Because of space constraints, results obtained from the first control group in which rats received preimmune IgG were shown.
Yet it must be noted that the results obtained from both control groups were similar, illustrating that changes in the status of spermatogenesis in the seminiferous epithelium that were detected following anti-laminin α3 or γ3 IgG treatment were not artifacts of IgG administration. At the time rats were sacrificed, one testis from each rat was frozen immediately in liquid nitrogen and stored in −80 °C until used. Another testis was fixed in Bouin’s fixative and processed for paraffin sections and staining with hematoxylin and eosin. To assess damaged tubules, a total of ~450 tubules from at least three testes of different rats were photographed and printed with an Epson RX300 printer, using a 10× objective in an Olympus BX40 microscope with a built-in Olympus DP70 digital camera. This thus permitted random scoring of ~450 tubules with ~150 tubules/tissue. Two parameters were used to determine a damaged tubule. First, a tubule was considered damaged when >10 germ cells (e.g. round spermatids and spermatocytes) were found in the tubule lumen, because in control (i.e. normal rats and rats treated with vehicle control) testes, only elongated spermatids were found in the tubule lumen at stage VIII of the epithelial cycle, and fewer than 2% of the tubules had round spermatids/spermatocytes in the tubule lumen. Second, the thickness of the germ cell layer (i.e. elongating/round spermatids and spermatocytes) in the seminiferous epithelium of a tubule in a treatment group was measured and compared against normal tubules, and a >30% loss in the germ cell layer was considered significant and scored as a damaged tubule. Furthermore, the diameter of the tubules was also scored and compared with control testes to assess tubule damage following blocking antibody treatment.

**Statistical Analysis**—Statistical analyses were performed by ANOVA with Tukey’s honestly significant difference (HSD) tests or Student’s t test using the GB-STAT statistical analysis software package (version 7.0, Dynamic Microsystems, Silver Spring, MD).

**RESULTS**

**Identification of the Bona Fide Partners of Laminin γ3 in Adult Rat Testes**—As an initial step to identify the likely laminin α and β chains that interact with laminin γ3 at the apical ES, RT-PCR was used to survey different laminin chains in Sertoli and germ cells (see Fig. 1 and supplemental Table S1). Virtually all laminin chains were found in adult rat testes (Fig. 1), consistent with several earlier reports (33–36). We next examined Sertoli and germ cells in the seminiferous epithelium that express different laminins. Fig. 1 illustrates the representative results of this survey using Sertoli and germ cells with negligible contamination from other cell types, using markers specific for Sertoli (e.g. testin) and germ (e.g. c-kit receptor) cells (Fig. 1A). It is interesting to note that laminin γ3 was expressed predominantly in germ cells isolated from adult rat testes (containing spermatogonia, spermatocytes elongating/elongate spermatids at a ratio of 16.7:18.65% as determined by DNA flow cytometry as reported previously (37)), whereas laminins α3 and β3 were restricted to germ cells (see Fig. 1, B–D). These data thus provide the first clue to the likely combination of laminin chains at the Sertoli cell/elongating spermatid interface.

**Expression of Recombinant Laminin Proteins and Characterization of These Recombinant Proteins and Their Corresponding Specific Antibodies**—To verify the speculation that laminins α3, β3, and γ3 indeed form a functional protein complex based on the RT-PCR results, several cDNA constructs based on domain G of laminin α3 and domain I of β3 and γ3 were expressed in E. coli (supplemental Table S2 and Fig. S1A), thereby obtaining the corresponding recombinant proteins.
These laminin recombinant proteins were isolated by affinity chromatography using nickel columns and then further gel-purified for antibody production (see supplemental Fig. S1B). Polyclonal antibodies were raised by immunizing rabbits with gel slices excised from the Coomassie Blue-stained SDS-polyacrylamide gels (see “Experimental Procedures”). Fig. 2A shows the purity of the recombinant proteins, laminin α3, β3, and γ3, on a Coomassie Blue-stained gel (Fig. 2A, left panel), which were also verified by immunoblotting using an anti-V5 epitope antibody (Fig. 2A, right panel). Monospecific polyclonal antibodies against laminin α3, β3, and γ3 chains were raised in rabbits, and each antibody was shown to react with the corresponding antigen specifically without cross-reactivity to the other antigens (Fig. 2B). The homology of these recombinant proteins at the levels of nucleotide and amino acid sequences is also shown in Fig. 2C, and protein sequence alignment among laminin α3, β3, and γ3 chains using the EMBOSS software program is shown in supplemental Fig. S1C. The analyses shown in Fig. 2C and supplemental Fig. S1C illustrate that cross-reactivity between these antibodies and the corresponding laminin chains is highly unlikely, particularly as gel-purified recombinant proteins (see Fig. 2A) were used for antibody production, consistent with the data shown in Fig. 2B. Using immunoblot analyses, laminin α3, β3, and γ3 chains were indeed detected in lysates of testes, seminiferous tubules, and germ cells but not in Sertoli cells (Fig. 3A, left panel, and B).

**Induction of Laminin α3, β3, and γ3 Chains during Sertoli-Germ Cell Anchoring Junction Assembly**—If these laminin chains are indeed the constituent proteins of the apical ES, it is anticipated that their produc-
tion will be induced during adherens junction assembly. Indeed, the protein levels of these three laminin chains were induced in Sertoli-germ cell co-cultures during anchoring junction assembly as manifested by the formation of functional apical ES and desmosome-like junctions when examined by electron microscopy (data not shown, see Refs. 23 and 38) (Fig. 6, right panel, and C). The protein levels of β1-integrin, the binding partner of laminin residing on Sertoli cells (25), was also induced during A) assembly in this co-culture experiment, whereas the level of FAK remained relatively constant (Fig. 3, A and C).

Localization of Laminin α3 Chain at the Apical ES in Adult Rat Testes—Localization of laminin α3 chain in the seminiferous epithelium of normal rat testes at different stages of the epithelial cycle versus normal rat kidneys is shown in Fig. 4. A–E. In adult rat testes, laminin α3 was associated mostly with elongating and elongated spermatids (Fig. 4, A, panels a–d, versus control (Ctrl) in B). The strongest signal was detected at stage VIII, predominantly surrounding the heads of elongated spermatids (Fig. 4A, panels b and c); this was consistent with its localization at the apical ES, similar to the results of immunofluorescent microscopy shown in Fig. 4E. In kidneys, laminin α3 was detected at or near the basement membrane in the collecting tubules (Fig. 4C); however, only very weak staining was found in the basement membrane in adult testes (Fig. 4, A versus C). Control experiments (Fig. 4, B and D) were performed in which the primary antibody was replaced by preimmune serum. Specificity of this anti-laminin α3 antibody was shown by immunoblotting using lysates of either germ cells (Fig. 4F) or testes (data not shown) in which an immunoreactive band at 165 kDa was detected. Fig. 4G is the result of an analysis that estimated the apparent molecular weight of the three laminin chains by SDS-PAGE using different protein markers and laminin chains.

Localization of Laminin γ3 Chain at the Apical ES in Adult Rat Testes—Similar to laminin α3, the γ3 chain was associated predominantly with elongating and elongated spermatids at the apical ES (Fig. 5A, panels b–d). The strongest signal was also detected at stage VIII, largely surrounding the heads of elongated spermatids at the apical ES site (Fig. 5A, panel b). The immunohistochemical results are consistent with immunofluorescent microscopy (Fig. 5, F versus A), illustrating that the laminin γ3 chain was restricted almost exclusively to the apical ES in adult rat testes. Fig. 5B is a control experiment in which the primary antibody was replaced with preimmune serum. A weak signal was also detected at the apical ES in spermatids at steps 8, 9, and later (Fig. 5A, panels a and c). Similar to laminin α3, the γ3 chain was also detected mostly at the basement membrane in the collecting tubules of kidney (Fig. 5C; Fig. 5D is the corresponding control using preimmune serum in place of the anti-laminin γ3 antibody in sections of kidney). The specificity of this anti-laminin γ3 antibody was illustrated by immunoblotting, as shown in Fig. 5E in which a prominent immunoreactive band of 146 kDa was detected in lysates of seminiferous tubules and germ cells but not Sertoli cells. The distribution of laminin γ3 in the seminiferous epithelium of rat testes during maturation was examined next. Interestingly, it was noted that in 16-day-old rats at the time the BTB was being established, laminin γ3 was largely confined to the basal compartment of the seminiferous epithelium, consistent with its localization at the BTB (Fig. 6, A–C). Also, at this age, the apical ES was not found because no elongating/elongate spermatids were present at this age (Fig. 6, A–C). However, the expression of laminin γ3 was detected at both the basal and apical compartments in 30-day-old rat testes when step 8 and 9 spermatids were found in the epithelium with functional apical ES (Fig. 6, D–F). In adult rats at 60 days of age, when well developed apical ES was found in the epithelium, laminin γ3 became mostly restricted to the apical ES (Fig. 6, G–J). These results illustrate a shift in the localization of laminin γ3 chain in the seminiferous epithelium during testicular maturation.

Ultrastructural Localization of Laminin α3 and γ3 Chains to the Apical ES by Immunogold EM—To further validate results of the immunohistochemistry and immunofluorescent microscopy studies showing that α3 and γ3 chains were confined to the non-basement membrane site in adult rat testes, immunogold EM was used. Consistent with the above findings (see Figs. 4–6), immunogold EM has laminin γ3 (see black dots in Fig. 7, B, C, and E versus A and D) and α3 (Fig. 7, F versus A and D) chains localized almost exclusively to the apical ES in adult rat testes. Fig. 7A is a cross-section of a seminiferous tubule showing a step 8 spermatid in which the entire sperm head (see the developing acrosome (Ac) above the condensed nucleus (Nad)) was invaginated into a Sertoli cell and attached to the seminiferous epithelium via the apical ES. Apical ES was typified by the presence of actin filament bundles (Fig. 7A, white arrowheads) sandwiched between the cisternae of the endoplasmic reticulum (ER) and the Sertoli cell plasma membrane (apposing arrowheads represent the apposing Sertoli and germ cell plasma membranes). Fig. 7D is another tubule of a normal rat testis showing a step 19 spermatid in which the entire sperm head is attached to the epithelium via apical ES having the same ultrastructure features as shown in Fig. 7A. Laminin γ3, which appeared as black grains in immunogold EM, was localized almost exclusively to the apical ES site of a step 8 spermatid (Fig. 7B; ~120 grains were found at the apical ES surrounding the head

FIGURE 9. A study by co-IP to access the structural relationship among laminin-333, β1-integrin, and other regulatory proteins at the apical ES. A, co-IP was performed using germ cell lysates and antibodies against laminins α3, β3, and γ3, c-Src, and paxillin (see supplemental Table S3). Laminin γ3 was shown to associate with laminins α3, β3, and γ3 as well as c-Src but not with paxillin (upper panel). c-Src was shown to associate with laminins α3, β3, and γ3 in this reverse co-IP experiment (lower panel). B, co-IP was also performed using seminiferous tubule lysates and antibodies against pFAK, c-Src, and β1-integrin, and the resultant blot was probed with an anti-laminin α3 antibody, illustrating that pFAK, c-Src, and β1-integrin were indeed structurally associated with laminin γ3. These data were validated in a reverse co-IP experiment using an antibody against c-Src (middle panel). c-Src was associated with laminins α3, β3, and γ3 individually or with the laminin-333 complex using a mixture of the three antibodies (lower panel). –ve, negative controls using either rabbit or mouse IgG as the precipitating antibody. IB, immunoblot; Lam, laminin.
of a step 8 spermatid) and more developed spermatids (Fig. 7, see arrowheads in C and E). Fig. 7F is the result of an immunogold EM study that also illustrates the localization of laminin α3 chain at the apical ES in an elongated spermatid in the rat testis. More important, both laminin α3 and γ3 chains were detected near or adjacent to the germ cell membrane (see Fig. 7, B, C, E, and F), confirming the results of the immunoblotting that they are exclusive germ cell products (see also Fig. 3, A and B).

Co-localization of Laminin α3-γ3-Integrin and Laminin γ3-β1-Integrin-c-Src in the Seminiferous Epithelium in Vivo and Sertoli-Germ Cell Co-cultures in Vitro—Based on the results of immunogold EM, laminin α3 was localized to the similar site as of γ3 chain, near the germ cell membrane in the epithelium. Immunofluorescent microscopy was used to confirm the co-localization of laminin γ3 and β1-integrin (Fig. 8, A and B, panels a–d). Furthermore, laminin α3 and laminin γ3 were also co-localized to the same site at the apical ES, consistent with their presence in germ cells in the seminiferous epithelium as well as in the co-culture system (Fig. 8, A, panels e–h, and B, panels e–h). We next sought to examine whether the laminin-333/β1-integrin-c-Src is a possible regulatory protein unit at the Sertoli/germ cell interface. It was shown that laminin γ3 co-localized with phospho-Src-Tyr116 in vivo...
In Sertoli-germ cell co-cultures, laminin γ3 was detected exclusively in the germ cells, whereas N-cadherin was found mostly at the Sertoli/Sertoli interface. However, cadherin was also a component of Sertoli-spermatid apical ES, and Fig. 8B, panels m–p, thus illustrating the co-localization of laminin γ3 and N-cadherin to the Sertoli/germ cell interface in the co-cultures with functional apical ES.

Structural Interactions of Laminin α3, β3, and γ3 Chains, Which Form a Functional “Laminin-333” Complex at the Apical ES and Its Association with β1-Integrin-pFAK-c-Src—To elucidate whether laminin-333 and integrin are a putative protein complex at the apical ES, a biochemical study was performed using lysates of either germ cells (Fig. 9A) or seminiferous tubules (Fig. 9B) from 90-day-old rats. Using antibodies against laminin α3, β3, and γ3 chains, with c-Src or paxillin for co-IP (see supplemental Table S3), laminin γ3 was found to associate with α3 and β3 chains as well as c-Src, but not with paxillin, in germ cells (Fig. 9A, upper panel). Interestingly, c-Src was also found to interact structurally with laminin α3, β3, and γ3 chains in both lysates of germ cells (Fig. 9A, lower panel) and seminiferous tubules (Fig. 9B, bottom panel; note that c-Src was pulled out by anti-laminin α3, β3, or γ3 antibody alone as well as a combination of these three antibodies). Additionally, c-Src was shown to associate with pFAK and β1-integrin (Fig. 9B, middle panel). This implies that c-Src may play a crucial role in signal transduction between Sertoli cells and elongate/elongating spermatids pertinent to spermatogenesis, consistent with two recent reports (30, 32). pFAK, c-Src, and β1-integrin were also shown to associate with laminin γ3 (Fig. 9B). Negative controls were performed using either rabbit or mouse IgG instead of the precipitating antibodies. Taking these results collectively, it is seen that the laminin α3, β3, and γ3 chains indeed form a functional laminin-333 protein complex that may be the ligand of β1-integrin at the apical ES. This, in turn, forms a functional adhesion complex with pFAK and c-Src.

A Disruption of Laminin-333 at the Apical ES by Blocking Antibodies Perturbs Both AJ and TJ Functions in the Seminiferous Epithelium of Rat Testes—To assess the physiological function of the laminin-333 complex at the Sertoli cell/elongating spermatid interface, laminin-333 was blocked by intratesticular injections of either an anti-laminin α3 or γ3 antibody. Following administration of either anti-laminin α3 or γ3 IgG versus preimmune rabbit IgG, sloughing of spermatids from the epithelium was observed beginning on days 4–15 (Fig. 10C–H versus A, normal rat testis; or see Fig. 10B, where the testis received preimmune IgG). ~40–60% of the tubules were damaged when a total of 450 tubules from different testes were scored (Fig. 10I), whereas the blood vessel remained intact (see Fig. 10Fv). Multinucleated giant germ cells (see Fig. 10, E:iii and iv, G:vi, and H:viii) were found in some tubules of rat testes treated with anti-laminin IgG from day 7 onward, an indication of germ cells undergoing necrosis. A drastic reduction was seen in the tubular diameter by up to 40% versus control rats treated with preimmune

*FIGURE 10—continued*
IgG for 20 days (Fig. 10J). There was no statistical difference in testes weight throughout the whole treatment (Fig. 10K). To further study the changes in cell adhesion function in both the basal and apical compartments of the seminiferous epithelium when the laminin-333 function was perturbed, immunoblot analyses were performed using testes lysates (Fig. 11A). The protein levels of both occludin (a TJ-integral membrane protein in the testis) and ZO-1 (a TJ adaptor) were reduced by ~3-fold at day 15 after anti-laminin γ3 IgG administration.

**FIGURE 11.** A study by immunoblotting to assess changes in protein levels of TJ, AJ, and signaling molecules in testes after administration of blocking laminin γ3 antibody. A, 50 μg of protein lysates from testes of rats after anti-laminin γ3 IgG administration (75 μg/testis) were resolved by SDS-PAGE using either 7.5 or 10% T SDS-polyacrylamide gels. Immunoblotting was done using different primary antibodies. All blots shown here were stripped and reprobed with an anti-actin antibody to assess equal protein loading. B–D, results of immunoblots from A were scanned densitometrically. Each bar represents the mean ± S.D. of three experiments. The protein level of normal testes (Ctrl) was arbitrarily set at 1, against which one-way ANOVA was performed. ns, not significantly different; *, p < 0.05; **, p < 0.01.

**FIGURE 12.** A study using immunofluorescent microscopy to co-localize occludin and ZO-1 in the seminiferous epithelium of rat testes at the BTB during anti-laminin antibody-induced BTB damage. Shown are immunofluorescent micrographs co-localizing occludin and ZO-1 to the BTB site in the seminiferous epithelium of normal rats (A–D), and after treatment with vehicle control on day 10 (E–H) versus testes after anti-laminin γ3 IgG treatment on day 4 (E–H), day 10 (I–L), and day 20 (M–P). Occludin (red, Cy3 in A, E, and M) was found to co-localize with ZO-1 (green, FITC in B, F, and H) in merged images (orange in C, G, and O). The fluorescence of occludin and ZO-1 at the BTB site was mildly weakened by day 4 (E–H versus A–D). However, the fluorescence of occludin and ZO-1 was virtually nondetectable on day 10 after treatment (I–K) when germ cells were depleted from the epithelium (see L, a voided tubule), but the fluorescence of occludin and ZO-1 bounced back by day 20 (M–O) and was significantly higher than the signals at day 10 (M–O versus I–K). DAPI staining is shown in D, H, L, and P. Scale bar: 100 μm (applies to A–P). V.Ctrl, vehicle control.
were metabolized and new laminin-333 was being synthesized. However, JAM-1 (another TJ-integral membrane protein also known as JAM-A) seems to be less susceptible to the blocking antibody. For AJ proteins that are also found at the BTB, such as N-cadherin and β-catenin, their protein levels remained relatively steady up to day 4 but were reduced gradually; unlike the TJ proteins, this protein complex failed to bounce back, as shown by immunoblot results (Fig. 11, A and C). Although the protein levels of several TJ and AJ protein markers were found to decrease during germ cell loss induced by the blocking antibodies of the laminin-333 complex, the expression of laminin α3 and β3 were stimulated after treatment, and the steady-state laminin γ3 protein level was mildly induced (Fig. 11, A and D). For some downstream signal proteins known to be activated by the laminin-integrin complex, the protein level of FAK remained relatively unchanged, whereas c-Src level displayed a significant reduction by days 15–20 after anti-laminin α3 IgG treatment (Fig. 11, A and C). To further confirm that the disruption of BTB by the blocking antibodies is transient and reversible, immunofluorescent microscopy was performed to monitor the BTB integrity. In testes with or without (normal testes) rabbit IgG (isolated from preimmune serum) administered, an almost continuous belt of fluorescent staining of occludin and ZO-1 was detected near the basement membrane of the seminiferous epithelium consistent with their localization at the BTB (Fig. 12, A–D). Earlier studies have shown that this is a reliable indication that the BTB was intact when the study was performed in conjunction with a micropuncture technique, where rats were administered 125I-labeled bovine serum albumin via the jugular vein prior to fluid collection at the rete testis and seminiferous tubule compartment (22). A weaker signal was detected by day 4 after administration of anti-laminin γ3 IgG (Fig. 12, E–H). Staining of both proteins became hardly visible by day 10 after the treatment (Fig. 12, I–L). The BTB damage caused by these blocking antibodies seems to be transient, consistent with results of immunoblot analysis (Fig. 11A), because the fluorescent signals of occludin and ZO-1 bounced back by day 20 (Fig. 12, M–P). This pattern is similar to the pattern obtained by fluorescent microscopy in which the co-localization of N-cadherin/β-catenin at the BTB site was examined (data not shown), except that this protein complex failed to bounce back by day 20, which is also consistent with the immunoblot data shown in Fig. 11, A and C.

An Electron Microscopy Study to Assess BTB Integrity following a Blockade of the Laminin Function at the Apical ES—To further demonstrate the disruption of the BTB when the function of an apical ES protein such as laminin was perturbed, electron microscopy was used to examine any ultrastructural changes in the BTB after intratesticular injection of the anti-laminin γ3 IgG (Fig. 13). Fig. 13A is the seminiferous epithelium of a normal tubule in which the BTB was shown to comprise co-existing TJ and basal ES. The BTB damage caused by these blocking antibodies was transient, consistent with results of immunoblot analysis (Fig. 11A), because the fluorescent signals of occludin and ZO-1 were detected by day 10 after the treatment (Fig. 12, I–L). The BTB damage caused by these blocking antibodies seems to be transient, consistent with results of immunoblot analysis (Fig. 11A), because the fluorescent signals of occludin and ZO-1 bounced back by day 20 (Fig. 12, M–P). This pattern is similar to the pattern obtained by fluorescent microscopy in which the co-localization of N-cadherin/β-catenin at the BTB site was examined (data not shown), except that this protein complex failed to bounce back by day 20, which is also consistent with the immunoblot data shown in Fig. 11, A and C.

An Electron Microscopy Study to Assess BTB Integrity following a Blockade of the Laminin Function at the Apical ES—To further demonstrate the disruption of the BTB when the function of an apical ES protein such as laminin was perturbed, electron microscopy was used to examine any ultrastructural changes in the BTB after intratesticular injection of the anti-laminin γ3 IgG (Fig. 13). Fig. 13A is the seminiferous epithelium of a normal tubule in which the BTB was shown to comprise co-existing TJ and basal ES. The BTB damage caused by these blocking antibodies was transient, consistent with results of immunoblot analysis (Fig. 11A), because the fluorescent signals of occludin and ZO-1 were detected by day 10 after the treatment (Fig. 12, I–L). The BTB damage caused by these blocking antibodies seems to be transient, consistent with results of immunoblot analysis (Fig. 11A), because the fluorescent signals of occludin and ZO-1 bounced back by day 20 (Fig. 12, M–P). This pattern is similar to the pattern obtained by fluorescent microscopy in which the co-localization of N-cadherin/β-catenin at the BTB site was examined (data not shown), except that this protein complex failed to bounce back by day 20, which is also consistent with the immunoblot data shown in Fig. 11, A and C.
FIGURE 14. Schematic diagram showing the molecular architecture of integrin-laminin adhesion complex at the apical ES of seminiferous epithelium and its dynamic functional relationship with the BTB. Integrin-laminin, nectin-afadin, and cadherin-catenin are the primary adhesion protein complexes at the Sertoli cell/developing spermatid interface (right top panel). Laminin-333 is restricted to elongating spermatids that serve as the ligand for αβ1-integrin receptor limited to Sertoli cells at the apical ES. Given that laminin-333 cannot anchor onto the spermatid cell surface without a scaffolding protein, because it lacks a transmembrane domain (based on its primary amino acid sequence), it is...
the anti-laminin γ3 IgG treatment that blocked the laminin function at the apical ES was consistent with the results of studies by immunoblotting and fluorescent microscopy (shown in Figs. 11 and 12).

DISCUSSION

Laminin-333-a6β1-Integrin Is a Putative Adhesion Protein Complex at the Apical ES in the Seminiferous Epithelium of Adult Rat Testes—For the past three decades, laminins, collagen (mostly type IV), and fibronectin have been known as the major structural and biologically active components of basement membrane in the seminiferous epithelium of mammalian testes (39–43); basement membrane is a modified form of extracellular matrix (for reviews, see Refs. 14 and 44). The first report appeared in the literature in 1999, demonstrating that laminin γ3 is a likely non-basement membrane, extracellular matrix protein in mouse testes (5). Subsequent study has confirmed this earlier report that laminin γ3 is a product of spermatids and a potential binding partner of β1-integrin at the apical ES in adult rat testes (18). However, the other two laminin chains that can form a functional heterotrimer with laminin γ3 were not known. Interestingly, laminin γ3 chain was recently shown to localize at the basal compartment of seminiferous epithelium in mice at 26–30 days of age (45). These apparently conflicting data were likely the results of animals at different ages that were used in different reports. Using a monospecific polyclonal anti-laminin γ3 antibody to study the localization of laminin γ3 chain during testicular maturation, we have shown herein that there is a “shifting” of γ3 chain from the basal compartment in 16-day-old rats when BTB was being assembled to the apical ES of the seminiferous epithelium in adult rats at 60 days of age. At age 30 days when apical ES began to form in the epithelium, laminin γ3 was detected in both the basal and the adluminal compartments. It is noted that laminin γ3 chain was detected at or near the basal lamina in the collecting tubules of adult rat kidney in the same experiment, which is consistent with an earlier report (46). This spatial and temporal change in the expression pattern of laminin chain in the tests during development is reminiscent of that of the retina (47). For instance, during retina development at the embryonic stage in rats, laminin β2 chain was detected at both the basal and apical regions of the retinal pigmented epithelium by immunohistochemistry. However, at later stages, laminin β2 was concentrated and restricted to the apical area of the retinal pigmented epithelium, which persisted until adulthood. This shifting in the localization of laminin β2 in the retina was found to assist in the maturation of photoreceptors (47). Similarly, laminin γ2 chain was also detected at the non-basement membrane site of cartilage and smooth muscle fibers during development (48). The precise role of laminin γ3 at the basal compartment in rats remains to be determined, but it is likely associated with BTB development.

In this report, laminin α3 and β3 were shown to be the putative binding partners of laminin γ3, residing at the apical ES. Additionally, they interact with each other and with β1-integrin, and they also link structurally to pFAK and c-Src. Because the interaction with c-Src was detected using lysates of germ cells with negligible contamination of Sertoli, Leydig, or myoid cells in addition to seminiferous tubules and/or testes, it is logical to conclude that the laminin function in germ cells may be regulated by the c-Src protein, which was recently shown to be a crucial regulator of Sertoli-germ cell adhesion in the rat testis (30, 49). Collectively, the data reported herein illustrate that laminin α3 chain forms a complex with β3 and γ3 chains. This, in turn, serves as the ligand for α6β1-integrin receptor at the apical ES in adult rat testes. Although it seems to be a provocative concept that laminin-333 is produced by spermatids and serves as the ligand of the α6β1-integrin receptor residing in Sertoli cells at the cell/cell interface instead of at the focal contact (or focal adhesion complex) anchoring junction site, an earlier report has demonstrated the expression of laminin β2 chain in male germ cells and at the apical compartment of Drosophila testes (50). For instance, laminin β2 chain not only was localized at the basement membrane but also was detected in the nuclei of primary spermatocytes, spermatids, and most importantly surrounding the heads of elongated spermatids in Drosophila. This earlier report illustrates the existence of a non-basement membrane-associated laminin chain in insects, even though the presence of a functional laminin, namely laminin-333, in germ cells in mammalian testes was not known until now.

Can Laminin-333 at the Apical ES Play a Role in the Maintenance of BTB Integrity, i.e. Is There Evidence of Cross-talk between Alf and TJ in the Seminiferous Epithelium?—Herein we present an important observation that a blockade of laminin-333 function at the apical ES, with the use of blocking antibodies against either laminin α3 or γ3 chain, led to a loss in Sertoli/spematid adhesion with a concomitant decline in AJ proteins such as N-cadherin and β-catenin. Additionally, a significant decline in the levels of TJ proteins, e.g. occludin and ZO-1, at the BTB was also detected beginning on day 7 after administration of the blocking IgG. A disruption of the BTB was also confirmed at the ultrastructural level by electron microscopy, which further demonstrates the physiological significance of the laminin/integrin protein complex at the apical ES, i.e. a primary disruption of the laminin can lead to a secondary but transient damage of BTB integrity. This seemingly suggests that there is cross-talk between apical ES and BTB (possibly via the basal ES) in the seminiferous epithelium and that laminin-333 may be a crucial player in this intriguing but yet to be elucidated pathway. However, the damage to BTB appears to be transient, because studies by fluorescent microscopy coupled with immunoblottings have illustrated a transient disruption of the BTB integrity. This observation is physiologically significant in the context of spermatogenesis. For instance, spermatids that take place at stage VIII of the epithelial cycle in the adluminal compartment coincides with the time frame in which preleptotene spermatocytes traverse the BTB in the basal compartment (38, 51). Yet stage VIII is also the stage in which laminin-333 displays the most intense staining at the apical ES in the seminiferous epithelium just prior to spermatiation. The results reported in the present study thus support the postulation that one of the laminin chains in laminin-333 (e.g. the α3 or γ3 chain) that is cleaved at spermatiation may serve as a biologically active fragment and may be one of the crucial signaling complex components that facilitates (or assists?) a transient “disruption” of the BTB to facilitate the migration of preleptotene spermatocytes across the barrier. This is achieved by reducing the levels of occludin and ZO-1 at the BTB at stage VIII of the epithelial cycle. This speculation is not entirely unexpected, because fragments of laminins and collagens can act as biological peptides to regulate junction dynam-
ics as reported in other epithelia (14). Apparently, the immunological barrier of the BTB was not compromised. This conclusion is supported, at least in part, by the lack of infiltrating lymphocytes in the seminiferous epithelium, as noted in the histological data shown in Fig. 10. It is also plausible that pFAK and c-Src also take part in this process, because these two kinases were found to tightly associate with laminin-333 as reported herein, and recent studies have illustrated that these are crucial molecules in regulating junction dynamics in adult rat testes (30, 32, 49).

Is Laminin-333 the Only Non-basement Membrane Ligand for α6β1-Integrin at the Apical ES?—It is interesting to note that the protein level of JAM-1 (also a TJ-integrin membrane protein in the testis) was less affected by the blocking anti-laminin α3 or γ3 IgG treatment that impaired both the apical ES and BTB function. There was also a recovery of occludin and ZO-1 protein levels around day 20 after treatment with blocking antibodies, which could not be detected for the cadherin-catenin protein complex. Moreover, only ~40–60% of the tubules displayed signs of damage by day 15. Based on these results, it is logical to speculate that another yet to be identified non-basement membrane laminin isoform(s) may be present at the apical ES (e.g. laminin-223 or laminin-323), which interacts with α6β1-integrin receptor on Sertoli cells and can supersede the function of laminin-333. This thus accounts for the ~40–60% of damaged tubules detected when the laminin-333 function was blocked. This also suggests that different isoforms of laminins may be expressed during the epithelial cycle to facilitate Sertoli-germ cell interaction during spermatogenesis. This hypothesis is in agreement with the complex nature of spermatogenesis, because it is conceivable that different adhesion protein complexes (e.g. different laminin isoforms) are associated with different steps of developing spermatids to facilitate the rapid turnover of junctions during spermatid movement (e.g. orientation, migration). This is also consistent with the recent findings that apical ES is a hybrid cell/cell and cell/matrix anchoring junction type that utilizes the most efficient cellular structures namely focal contacts found in other epithelia to regulate germ cell movement (4, 14). From the results of RT-PCR reported herein and those of microarray studies, laminin α2, β1, β2 subunits were detected in germ cells, and the mRNA level of laminin β2 chain was also induced during anchoring junction assembly in Sertoli-germ cell co-cultures, implying that another non-basement membrane laminin isoform(s), e.g. laminin-223, may be present in germ cells.

Is Laminin-333 a Crucial Signaling Molecule between Sertoli Cells and Developing Spermatids?—In the present study, we have unequivocally demonstrated the existence of a novel laminin-333 isoform restricted to developing spermatids at the apical ES in adult rat testes that binds to the α6β1-integrin receptor residing on Sertoli cells. This complex, in turn, may regulate AJ junction dynamics through c-Src protein complex can indeed perturb germ cell adhesion in the seminiferous epithelium. Even though we have shown that laminin-333 is associated with c-Src and that a blockade to either one of these proteins leads to a loss of spermatid adhesion in the epithelium, it is not likely that c-Src, a peripheral non-receptor protein-tyrosine kinase, can interact directly with laminin chains on the spermatid cell surface, because laminin per se does not possess a transmembrane nor an intracellular domain. As such, a transmembrane protein that can anchor laminin-333 to the spermatid cell surface to structurally link c-Src in spermatid cytosol to mediate the signal transduction function, as well as to facilitate interactions with integrin residing on Sertoli cells, remains to be identified (see Fig. 14). During tumor invasion and metastasis, cancer cells are known to interact with laminin. For instance, a nonintegrin receptor known as M, 67-kDa laminin receptor, which can interact with α6-integrin, has been shown to induce laminin signaling in tumor cells through mitogen-activated protein kinases cascades (52, 53). This 67-kDa laminin receptor has high affinity toward laminin through the binding site of peptide G (54). Both α6β1-integrin and the 67-kDa laminin receptor are expressed in a lung carcinoma cell line, and this α6β1-integrin/67-kDa laminin receptor complex is known to facilitate cell/matrix adhesion and cell movement in which the α6β1-integrin can bind to laminin at different sites of the 67-kDa laminin receptor. This scenario may be applicable to the testis because of rapid junction restructuring pertinent to germ cell movement during spermatogenesis. In fact, the presence of the 67-kDa laminin receptor in spermatogenic cells has been reported in mouse testes (55). Furthermore, the 67-kDa laminin receptor is predominant in round spermatids (55). It is plausible that laminin-333 anchors on germ cells via this 67-kDa laminin receptor as the transmembrane partner and/or scaffolding protein. An earlier study has shown that this 67-kDa laminin receptor is an integral membrane protein (56). Another study has suggested that this receptor is a cell surface protein that can bind to elastin, an integral membrane protein that is linked to actin (57). Nonetheless, the precise structural relationship between laminin-333 and 67-kDa laminin receptor in spermatids at the apical ES remains to be determined. Based on the results reported herein, a hypothetic model is shown in Fig. 14, which illustrates the significance of the α6β1-integrin-laminin-333 complex that mediates cross-talk between apical ES and BTB during the epithelial cycle in adult rat testes.

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