Interactions of laminin β3 fragment with β1-integrin receptor
A revisit of the apical ectoplasmic specialization-blood-testis-barrier-hemidesmosome functional axis in the testis

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Key words: testis, spermatogenesis, seminiferous epithelial cycle, laminin β3 chain, β1-integrin, α6β1-integrin receptor, apical ectoplasmic specialization, blood-testis barrier, hemidesmosome

Recent studies have demonstrated the presence of a functional axis that coordinates the events of spermiation and blood-testis barrier (BTB) restructuring which take place simultaneously at the opposite ends of the seminiferous epithelium at stage VIII of the epithelial cycle of spermatogenesis in the rat testis. In short, the disruption of the apical ectoplasmic specialization (apical ES) at the Sertoli cell-elongated spermatid interface, which facilitates the release of sperm at spermiation near the tubule lumen, is coordinated with restructuring at the BTB to accommodate the transit of preleptotene spermatocytes across the immunological barrier near the basement membrane. These two events are likely coordinated by a functional axis involving hemidesmosome at the Sertoli cell-basement membrane interface, and it was designated the apical ES-BTB-hemidesmosome axis. It was demonstrated that fragments of laminin chains (e.g., laminin β3 or γ3 chains) derived from the α6β1-integrin-laminin333 protein complex at the apical ES, which were likely generated via the action of MMP-2 (matrix metalloprotease-2, MMP2) prior to spermiation, acted as biologically active peptides to perturb the BTB permeability function by accelerating protein endocytosis (e.g., occludin) at the site, thereby destabilizing the BTB integrity to facilitate the transit of preleptotene spermatocytes. These laminin fragments also perturbed hemidesmosome function via their action on β1-integrin, a component of hemidesmosome in the testis, which in turn, sent a signal to further destabilize the BTB function. As such, the events of spermiation and BTB restructuring are coordinated via this functional axis. Recent studies using animal models treated with toxicants, such as mono-(2-ethylhexyl) phthalate (MEHP), or adjudin, a male contraceptive under investigation, have also supported the presence of this functional axis in the mouse. In this short review, we critically evaluate the role of this local functional axis in the seminiferous epithelium in spermatogenesis. We also provide molecular modeling information on the interactions between biologically active laminin fragments and β1-integrin, which will be important to assist in the design of more potent laminin-based peptides to disrupt this axis, thereby perturbing spermatogenesis for male contraception and to understand the underlying biology that coordinates spermiation and BTB restructuring during spermatogenesis.

Introduction

In the mammalian testis, such as rats, developing spermatids (step 8–19) adhere to Sertoli cells in the seminiferous epithelium via a testis-specific anchoring device known as apical ectoplasmic specialization (apical ES).1-3 Apical ES is the strongest anchoring junction type in the seminiferous epithelium,6 significantly stronger than desmosome,7-9 when the force required to disrupt these junctions were quantitatively compared.6 Also, once apical ES develops during spermiogenesis, it is the only anchorage device, surrounding the entire head of elongating spermatids, conferring both anchoring and cell polarity function.1 Structurally, apical ES is constituted by adhesion protein complexes, such as cadherins-catenins and nectins-afadin,4,10-12 which are also found at the adherens junction (AJ) of different epithelia. However, apical ES is unique in that it also contains the integrin-laminin protein complex, which is normally restricted to anchoring junctions at the cell-extracellular matrix (ECM) interface; these include (1) focal contacts, (or focal adhesion complex, FAC; note: focal contact is not found in the mammalian testis13-15 which use actin for attachment and (2) hemidesmosomes, which use intermediate filament for attachment in other epithelia.13,14,16 It is because of the presence of this FAC- and hemidesmosome-based protein complex at the apical ES, but not basal ES at the BTB, the apical ES is being viewed as a hybrid and atypical AJ type.10,13,14,16,17

*Correspondence to: C. Yan Cheng; Email: Y-Cheng@popcbr.rockenfeller.edu Submitted: 05/06/11; Revised: 06/08/11; Accepted: 06/10/11 http://dx.doi.org/10.4161/spmg.1.3.17076
β1-integrin was first identified as a component of the apical ES in the 1990s, and subsequent studies have shown that α6β1-integrin is a functional integrin receptor residing in the Sertoli cell in the seminiferous epithelium; however, the ligand for this α6β1-integrin was not known at the time. Lamnin γ3, which is one of the putative ligands of integrin receptor at FAC and hemidesmosome in other epithelia, was subsequently found to localize to the same site as α6- and β1-integrin at the apical ES in the seminiferous epithelium of mouse testes. Lamnin γ3 was later found to form a bona fide complex with β1-integrin and it is a putative ligand of α6β1-integrin in the rat testis. Other studies have shown that the functional ligand of α6β1-integrin is composed of the laminin α3, β3 and γ3 chains (designated laminin-333), with all three chains residing in elongating spermatids to yield a functional laminin ligand.

This thus creates an α6β1-integrin-laminin-333 adhesion protein complex at the Sertoli cell-elongating spermatid interface, which is a critical adhesion complex at the apical ES.

Interestingly, β1-integrin is not a component of basal ES at the BTB even though basal ES shares similar ultrastructural features with apical ES. Instead, β1-integrin was detected at the hemidesmosome in the testis near the basement membrane. Recent studies in mice have shown that spermatogonial stem cells (SSC) in the stem cell niche also express α6- and β1-integrin, perhaps being used to facilitate the adhesion of SSC to the stem cell niche, which is located adjacent to the basement membrane of the seminiferous tubules that border on the interstitial tissue. More important, β1-integrin was recently shown to be crucial to the homing of SSCs to the germline niche, illustrating the significance of β1-integrin as a crucial molecule for cell adhesion for spermatid and SSC, and it is perhaps intimately involved in signaling function in the stem cell niche in the testis. At present, the ligand for β1-integrin in the stem cell niche remains unknown but it is likely to be either laminins or collagens, which are the putative ligands for integrin receptors in other epithelia.

One of the major functions of the testis, besides producing testosterone that are released by Leydig cells in the interstitium into the systemic circulation for the maintenance of secondary sexual characteristics of the male, is to produce spermatozoa via mitosis, meiosis and spermatogenesis during which type A spermatogonia can divide and differentiate to become spermatozoa via meiosis I and II to take place, followed by spermiation, to be followed by spermiogenesis and spermatid formation, forming a bona fide cell adhesion protein complex. Interestingly, the localization of all three laminin chains in the cross-talks between hemidesmosome and epithelial TJ barrier function, in particular during tumorigenesis, supporting our earlier observations that a disruption of hemidesmosome function can possibly perturb BTB dynamics. Collectively, these observations coupled with recent findings, including studies using animal models treated with environmental toxicants (e.g., MEHP, bisphenol A) and male contraceptives (e.g., adjuvin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxyhydrate, formerly called AF-2364), support the presence of an apical ES-BTB-hemidesmosome functional axis to coordinate and regulate the events of spermatogenesis and BTB restructuring at stage VIII of the epithelial cycle.

In this review, we highlight recent findings in the field, demonstrating adhesion molecules at the apical ES (e.g., laminins, β1-integrin), BTB (e.g., occludin-ZO-1-FAK (focal adhesion kinase) and hemidesmosome (e.g., β1-integrin) are structural molecules which in turn perturbs the BTB integrity.

The Apical ES-BTB-Hemidesmosome Axis in the Seminiferous Epithelium of the Testis

Evidence that supports an apical ES-BTB functional axis in the seminiferous epithelium. Apical ES is a testis-specific and an atypical adherens junction (AJ) because it is constituted by proteins usually restricted to different types of junctions in other epithelia in addition to AJ. These include (1) A (e.g., N-cadherin, E-cadherin, α-catenin, β-catenin), (2) TJ (e.g., JAM-C (junctional adhesion molecule-C)), CAR (coxsackievirus and adenovirus receptor), polarity proteins such as PAR6, partitioning defective protein 6, PAR3, PAR3, and 14-3-3 (also known as PAR5), (3) FAC (e.g., α6-integrin), big integrin, p-Tyr97-FAK, p-Tyr76-FAK, p-Tyr416-c-Src, p130Cas (p130 Crk-associated substrate), vinculin, (4) communicating gap junction (GJ) (e.g., Cx43 (connexin-43), Cx62), (5) hemidesmosome (e.g., laminin-α3, β3, γ3 chains (note: a functional laminin ligand is composed of one each of α, β and γ chains), restricted to the elongating spermatids, forming a bona fide cell adhesion protein complex.
the apical ES of the seminiferous epithelium displays a highly restricted spatial and temporal stage-specific expression. For instance, these laminin chains begin to be detected at the apical ES, surrounding the entire head of step 17 spermatids in the seminiferous epithelium at Stage IV of the epithelial cycle; their localization becomes more intense and peak at the apical ES in step 19 spermatids at Stage VIII of the cycle (but not at step 8 spermatids when the apical ES begins to form at Stage VIII) just prior to spermiation, with their expression diminished considerably to a level almost undetectable by late Stage VIII when the release of sperm takes place. This pattern of restricted expression and stage-specific localization closely mimics that of MMP-2 at the apical ES, which is not unexpected since laminin is a known substrate of MMP-2 and MMP9. Recent findings have shown that MMP-2 that cleaves the apical ES proteins to prepare for spermiation (note: MMP-2 was found to be highly expressed at the apical ES, and laminin chains are putative substrates of MMP-2) is likely to release biologically active fragments of laminin chains (e.g., laminin β3 fragment), which in turn can “potentiate” further breakdown of the apical ES. Additionally, biologically active laminin fragments also perturb BTB disruption directly and indirectly via their action on hemidesmosome. The perturbed hemidesmosome further potentiates the BTB restructuring, perhaps via the activation of nonreceptor protein kinases at the BTB (e.g., FAK, c-Src) to facilitate the transit of preleptotene spermatocytes at the BTB. In short, during the epithelial cycle, apical ES undergoes transition from an “intact” state, to a “degenerating” state in which many of the apical ES proteins can be “recycled” to assist the assembly of “new” apical ES during spermiogenesis, and then a “disrupting” state to prepare for spermiation. And during this “disrupting” state, biologically active laminin fragments released at the apical ES coordinate the event of BTB restructuring, involving hemidesmosome. This apical ES-BTB-hemidesmosome is not only a unique functional axis in the epithelium, it is a prime target for male contraceptives that exert their effects locally in the seminiferous epithelium (e.g., adjudin), but it is also likely a target of environment toxicants (e.g., cadmium, bisphenol A, phthalates) that impede male reproductive function.

Figure 1. A schematic drawing illustrating the apical ectoplasmic specialization-blood-testis barrier-hemidesmosome axis in the seminiferous epithelium of rat testes that coordinates the events of spermiation and BTB restructuring that take place simultaneously at Stage VIII of the seminiferous epithelial cycle of spermatogenesis. In mammalian testes, such as in rats, the BTB divides the seminiferous epithelium into the basal and the apical (adluminal) compartments so that meiosis I and II, spermiogenesis and spermiation all take place in the immune privileged site at the adluminal compartment. However, preleptotene spermatocytes residing in the basal compartment are in transit at the BTB at Stage VIII of the epithelial cycle when spermiation (i.e., the release of sperm) occurs and these two events take place simultaneously at the opposite ends of the seminiferous epithelium. Recent findings have shown that MMP-2 that cleaves the apical ES proteins to prepare for spermiation (note: MMP-2 was found to be highly expressed at the apical ES, and laminin chains are putative substrates of MMP-2) is likely to release biologically active fragments of laminin chains (e.g., laminin β3 fragment), which in turn can “potentiate” further breakdown of the apical ES. Additionally, biologically active laminin fragments also perturb BTB disruption directly and indirectly via their action on hemidesmosome. The perturbed hemidesmosome further potentiates the BTB restructuring, perhaps via the activation of nonreceptor protein kinases at the BTB (e.g., FAK, c-Src) to facilitate the transit of preleptotene spermatocytes at the BTB. In short, during the epithelial cycle, apical ES undergoes transition from an “intact” state, to a “degenerating” state in which many of the apical ES proteins can be “recycled” to assist the assembly of “new” apical ES during spermiogenesis, and then a “disrupting” state to prepare for spermiation. And during this “disrupting” state, biologically active laminin fragments released at the apical ES coordinate the event of BTB restructuring, involving hemidesmosome. This apical ES-BTB-hemidesmosome is not only a unique functional axis in the epithelium, it is a prime target for male contraceptives that exert their effects locally in the seminiferous epithelium (e.g., adjudin), but it is also likely a target of environment toxicants (e.g., cadmium, bisphenol A, phthalates) that impede male reproductive function.

Spermatogenesis volume 1
issue 3

176

Spermatogenesis
Volume 1 Issue 3
coordinate the event of BTB restructuring by perturbing the TJ-barrier function. Indeed, in Sertoli cell cultures that have established a functional TJ-barrier, the addition of recombinant laminin β3 or γ3 fragments was found to perturb the Sertoli cell TJ-barrier by reducing the levels of BTB integral membrane proteins (e.g., occludin, JAM-A) at the Sertoli-Sertoli cell interface. Similar results were obtained by the transient overexpression of laminin fragments in Sertoli cells with an establish Tj-permeability barrier that mimicked the BTB in vivo. These findings thus illustrate the presence of a functional link between apical ES and the BTB.

**Evidence that supports an apical ES-BTB axis based on studies in different animal models.** The presence of this apical ES-BTB-hemidesmosome functional axis is supported by studies in two animal models. First, when mice were treated with MEHP [mono-(2-ethylhexyl) phthalate, the active metabolite of phthalate], this toxicant was found to disrupt the apical ES, inducing germ cell loss from the seminiferous epithelium in vivo and also germ cell detachment from the Sertoli cell epithelium using Sertoli-germ cell cocultures in vitro. The BTB function of these MEHP treated mice was also found to be disrupted, illustrating the likely presence of a functional axis between the apical and the BTB as suggested by these authors. These phenotypes were associated with a significant decline in the steady-state protein levels of laminin-γ3 chain and B1-integrin at the apical ES, as well as claudin-1 and occludin at the BTB which led to a disruption of the apical ES and BTB function. These effects were the result of an initial activation of MMP-2 and the generation of biologically active laminin γ3 fragments from the disrupted apical ES, because both MEHP-induced germ cell loss and BTB disruption were significantly suppressed by the use of MMP inhibitors, including SB-3CT (4-phenoxypyphenylsulfonfonyl)methyliiirane, a specific inhibitor of metalloproteases-2 and -9) and TIMP-2 (tissue inhibitor of metalloproteases-2, a specific inhibitor of MMP-2). In short, these authors concluded that treatment of mice with MEHP induced the activation of MMP-2 at the apical ES, which caused cleavage of laminin-γ3 chain at the apical ES (as reflected by a declining steady-state laminin-γ3 level, and because MMP-2 is restricted to the apical ES59), thereby inducing germ cell loss from the epithelium. The biologically active laminin fragments also perturbed BTB function which was manifested by mis-localization and reduction of claudin-11, occludin and ZO-1 at the BTB. This latter conclusion is supported by the observation that MMP-2 is not a component of the BTB but is restricted to the apical ES, thus activated MMP-2 could not exert its cleavage function at the BTB. While MMP-9 is a component of the BTB which could have been activated by MEHP to induce Sertoli cell BTB disruption, but the use of TIMP-2 (a specific inhibitor of MMP-2 but not MMP-9) was found to significantly block the action of MEHP-induced germ cell loss from the Sertoli cell epithelium only. Thus, the MEHP-induced BTB disruption appears to be mediated by the biologically active laminin fragments released at the apical ES via the action of MMP-2 on the laminin-γ3 chain.

Second, the findings using the MEHP model are analogous to other studies using an animal model in which rats were treated with adjudin (a potential male contraceptive known to induce "premature" spermiation by primarily disrupting apical ES74-76). It was found that the adjudin-induced germ cell loss also associated with an activation of MMP-2 (but not MMP-9, which is associated with the BTB) and a disruption of the apical ES, as well as a significant decline in the steady-state level of laminin-γ3 chain, with these findings being analogous to the MEHP model. These findings thus illustrate that adjudin induces an activation of MMP-2 at the apical ES to cleave the laminin chains locally to generate laminin fragments. The reason that the BTB was not immediately perturbed in this model at the time of apical ES disruption and germ cell loss is that the testis has a unique mechanism to segregate the events of anchoring junction and TJ restructuring. Also, the feedback loop maintained by the hemidesmosome in the apical ES-BTB-hemidesmosome axis as shown in the adjudin model may respond differently to the treatment of adjudin vs. MEHP, which should be vigorously investigated in future studies. Nonetheless, the BTB was found to be transiently disrupted at a later time when most germ cells had already been depleted from the epithelium. These two animal models thus support the concept that there is a physiological link between the apical ES and the BTB in the seminiferous epithelium to coordinate the events of spermatogenesis and BTB restructuring during the epithelial cycle.

**Evidence that supports a BTB-hemidesmosome functional axis in the seminiferous epithelium. Studies in vitro.** Treatment of Sertoli cell epithelium (without detectable elongating/elongated spermatid contamination, and thus apical ES was absent) with a biologically active recombinant laminin fragment or its overexpression not only perturbed the TJ-permeability barrier function, but also resulted in a reduced expression of B1-integrin, implicating a likely disruption of the hemidesmosome. This finding seemingly suggests that there is a physiological link between the apical ES and the hemidesmosome. This conclusion is reached based on several lines of evidence. First, B1-integrin is found at the apical ES18-23,24 hemidesmosome25 and stem cell niche,26 but not at the Sertoli-Sertoli cell interface at the BTB and it did not co-localize with BTB proteins occludin or ZO-1,25 in the seminiferous epithelium of rat testes. Since hemidesmosome were present in Sertoli cell cultures but neither apical ES nor the stem cell niche were found in these cultures, the changes in the steady-state protein level of B1-integrin in the Sertoli cell epithelium in vitro are likely the result of changes at the hemidesmosome. Second, the use of RNAi to knockdown B1-integrin in these cultures, thereby perturbing hemidesmosome function (since apical ES and spermatogonial stem cells are absent in these cultures), was found to perturb Sertoli cell TJ-barrier function by causing mis-localization of occludin and N-cadherin at the Sertoli-Sertoli cell interface. These proteins were found to be internalized, moving from the cell surface to cell cytosol as a result of increased protein endocytosis.25

**Studies in vivo.** The conclusion that there are cross-talks between BTB and hemidesmosome is also supported by the in vivo studies using animal models treated with toxicants. First, MEHP-treated mice displayed a significant decline of B1-integrin steady-state level in the testis (estimated by immunoblotting),
with a loss of β1-integrin at both the apical ES and the Sertoli cell-basement membrane interface at the hemidesmosome when visualized by fluorescence microscopy.90 Thus, the declining β1-integrin protein level in the testis was not only caused by apical ES disruption, it was also contributed in part by the loss of hemidesmosome function. It is noted that germ cell loss does not directly lead to decreased β1-integrin since this protein is restricted exclusively to Sertoli cells at the apical ES and hemidesmosome, while germ cells (e.g., elongating spermatids) do not express any β1-integrin except for spermatogonial stem cells at the stem cell niche.26,27 Thus, the MEHP-induced apical ES disruption caused the generation of biologically active laminin γ3 fragments, which inhibited both the steady-state levels of β1-integrin protein at the apical ES (a direct effect to further “destabilize” the apical ES) and the hemidesmosome (an indirect effect via the action of β1-integrin to perturb the BTB function) in MEHP-treated rats.91 Second, in contrast to the MEHP model, the steady-state β1-integrin level was found to be significantly induced in testes from rats treated with adjudin,60,80 suggesting that the hemidesmosome function was not perturbed (or inhibited), instead it was “activated.” This thus explains the observation that the BTB was not perturbed in the adjudin-treated rats at the time of germ cell loss from the epithelium,60,80 because there is no “negative” feedback signal originated from the hemidesmosome to induce BTB disruption. This is in sharp contrast to MEHP-treated rats in which the declining β1-integrin at the hemidesmosome was associated with a considerable loss of claudin-11, occludin and ZO-1 at the BTB.49 We postulated that the testis has a novel mechanism, such as the engagement and disengagement mechanism,77,82 that “protects” the BTB in the adjudin model during anchoring junction restructing in normal spermatogenesis as discussed in earlier studies.77,78,83 The reason for the differential responses at the hemidesmosome (i.e., expression of β1-integrin) in these two animal models is perhaps due to the fact that MEHP is an estrogenic xenobiotic/toxicant,84,85 whereas adjudin is a nonsteroidal disruptor of germ cell adhesion74–76,86,87 without systemic toxicity.88 The maintained hemidesmosome function in adjudin treated rats may account for the observation that the BTB remained intact, at least through ~day 14, after adjudin treatment,60,81 before it was found to be transiently disrupted but eventually “resealed”.80

**Interaction of Biologically Active Laminin β3 Fragment with β1-integrin**

We have used molecular modeling approach to assess if there are putative interactions between β1-integrin (Fig. 2A) and the biologically active laminin β3 fragment residing in domain I (note: for a description of different functional domains of laminin chain, reviewed in ref. 1). This domain consists of 122 amino acid residues (Fig. 2B) and was shown to regulate Sertoli BTB and hemidesmosome function in the testis.23 While α6-integrin is known to bind β1-integrin at the apical ES to form a functional integrin receptor, the binding partner of β1-integrin at the hemidesmosome remains unknown. Thus, the modeling analysis shown in Figure 2 was limited to β1-integrin and laminin β3 fragment.

The crystal structure of αVβ3-integrin [PDB, Protein Data Bank, ID: 3IJE] was identified as a suitable template for the homology modeling of β1-integrin by BLASTP program. β3-Integrin (template) in αVβ3-integrin receptor was found to share 41.4% of sequence identity and 61.5% of sequence similarity with integrin β1 (target) (Fig. 2C). The 3-D model for target protein sequences was generated by MODELER/Discovery Studio 3.0 software. Furthermore, the quality of the models was improved by loop refinement and energy minimization steps. The Ramachandran plot of the refined models showed that 99.01% of residues in β1-integrin were located in the allowed region of the plot (Fig. S1A). The modeled structure of β1-integrin consists of eight domains (i.e., BA, Hybrid, PSI (where the N-terminus resides), EGF-1 and EGF-2, EGF-3, EGF-4 and BTD (where the C-terminus resides)) (Fig. 2D), which is in a conformation similar to the crystal structure of αVβ3-integrin.89

In the case of laminin β3 fragment (Fig. 2E), the I-TASSER server generated top five structure models as ranked on the confidence score (C-score). The high C-score implies best quality of the modeled structure. Hence, we selected a high scored model from the generated structure for further docking studies. The C-score of the selected model was -1.97. The stereo-chemical quality of laminin β3 model was checked and it showed that 100% residues were located in allowed region of Ramachandran plot (Fig. S1B). Hence, all the generated models are valid for further docking studies to find out binding interface residues of laminin β3 fragment into α6- and β1-integrin.

The active residues found in β1-integrin and laminin β3 fragment used for HADDOCK docking are shown in Table 1. The HADDOCK results of β1-integrin/laminin β3 fragment complex are summarized in Tables 2 and 3. After successful running of docking, HADDOCK displayed top ten clustered complexes. Table 2 shows the various energies that were used for calculation of HADDOCK score to the top three clusters of each of the β1-integrin-laminin-β3 fragment protein complex. The best docked complex was selected with the lowest HADDOCK score from the clustered complexes. Therefore, cluster 1 represents the best lowest energy structures and contains largest number of structures for β1-integrin-laminin β3 fragment complex (Table 2). The protein-protein docking of β1-integrin into laminin β3 fragment resulted in the formation of 14 hydrogen bonds and 16 hydrophobic contacts (Table 3). For instance, residues Glu20, Trp26, Lys30, Glu31, His34, Gln35, Gln39 and Gln42 of laminin β3 fragment were involved in the formation of 14 hydrogen bonds with βA domain residues of β1-integrin (i.e., Tyr131, Ser132, Lys134, Asp136, Lys180, Leu181, Asn222, Leu223, Ser225, Asp257 and Gly340) (Table 3, Fig. 2F). Other residues of laminin β3 fragment involved in hydrophobic contact with β1-integrin were Ser23, Ala27 and Arg28 (Table 3). From these findings, we suggest the possibility that the binding of laminin β3 fragment to β1-integrin subunit could inhibit the formation of biologically active integrin α6β1 interface (i.e., Propeller-βA domain interface). Generally, the N-termini of both α and β subunit form globular head of ligand binding domain for extracellular matrix.89 Hence, the binding of laminin β3 fragment to either β1-integrin or α6-integrin subunit could inhibit the signal
transduction from the extracellular matrix to the cell, which can further “destabilize” the apical ES, facilitating its “degeneration” to prepare for spermiation as depicted in Figure 1.

In short, molecular modeling has identified several putative protein-protein interacting sites between β1-integrin and the biologically active 122-amino acid laminin fragment (Fig. 2F). These findings are useful since they provide the crucial information regarding the stretch of sequence within this 122-amino acid fragment that interacts directly with β1-integrin. Based on this information, shorter synthetic peptides that contain the active domain(s) can be designed for future studies (Fig. 2F).

For instance, based on the findings shown in Figure 2F, a shorter biologically active peptide within the laminin β3 fragment could now be synthesized by deleting residues V1 (Val-1)-M18 (Met-18) and also residues A46 (Ala-46) through G122 (Gly-122) (see Fig. 2B and F) from the N-terminus, keeping only the stretch...
Figure 2D-F. Ribbon representations of modeled structures of (D) β1-integrin based on the primary sequence shown in (A), and of (E) laminin β3 fragment based on primary sequence shown in (B), illustrating different domain architectures. Docking of the β1-integrin and laminin β3 fragment complex was performed using easy interface of HADDOCK (high ambiguity driven protein-protein docking) server.96 The possible binding interface was defined in the form of active and passive residues for protein-protein docking in HADDOCK. Active residues of β1-integrin were identified by performing pairwise sequence alignment of β1-integrin with the corresponding ligand binding residues of β3-integrin in αvβ3-integrin receptor89 (Fig. S1C). This alignments was performed using EMBOSS Pairwise Alignment Tool by global method (http://www.ebi.ac.uk/Tools/emboss/align/index.html). The alignment results show that Asp128, Ser130, Ser132, Asp224 and Glu227 of β1-integrin correspond to Asp119, Ser121, Ser123, Asp217 and Glu220 in β3-integrin (Fig. S1C). Hence, the identified residues were defined as active residues for protein-protein docking of β1-integrin into laminin β3 fragment (see Table 1). For laminin β3 fragment, we defined loop regions, such as Leu32-Glu39 and Asp76-Ser81, as active residues. The passive residues were defined automatically around the active residues. The first complex structure of the cluster with lowest HADDOCK score was selected as the best docked complex to analyze the binding interface residues. The intermolecular contacts of the selected docked complexes were analyzed using DIMPLOT, which is a compiled Windows version of LIGPLOT software.97 The molecular graphics images software UCSF Chimera98,99 was used for interactive visualization and analysis of binding interface residues of our docked complexes. Ribbon drawing of HADDOCK server model of the complex between β1-integrin [shown in green] and laminin β3 fragment [shown in cyan] (F). This complex was rotated around the Y-axis by 30° to properly depict the intermolecular interacting residues. The two networks of hydrogen bonding amino acid residues are tagged by two circles and they are enlarged (see black arrows) in two corresponding stick representations illustrating interacting amino acid residues between β1-integrin and laminin β3 fragment, displaying intermolecular interacting amino acids residues. Hydrogen bond is depicted with a pink dotted line in the large circled area in (F). D, Asp; E, Glu; G, Gly; H, His; K, Lys; L, Leu; N, Asn; Q, Gln; S, Ser; W, Trp; Y, Tyr.
of sequence necessary to interact with β1-integrin. This will significantly reduce the cost of synthesizing this biological peptide for further functional studies, such as exploring the possibility of using this peptide for potential male contraceptive development.

The Interacting Domain of β1-integrin with Adjudin is Similar to that of Laminin β3 Fragment

Based on recent studies, adjudin induces “premature spermatiation” in the mammalian testis including rats, rabbits and dogs,74,75,90,91 causing defoliation of germ cells from the seminiferous epithelium by exerting its effects at the apical ES. It was shown that adjudin disrupted the adhesion protein complexes (e.g., α6β1-integrin/laminin-333) at the apical ES by activating β1-integrin and its downstream signaling molecules58,60 and also other signaling molecules (e.g., testin90) at the site, leading to the release of spermatids, mimicking spermatiation. But since there is no β1-integrin at the BTB, adjudin was shown not to perturb the BTB, at least at the time of germ cell loss from the epithelium.10,81 We thus sought to examine by molecular modeling if adjudin also interacts with similar domain (or interacting pocket) in β1-integrin when compared with the laminin β3 fragment shown in Figure 2F.

As shown in Figure 3, LigPrep (Version 2.3, Schrödinger, LLC, New York) was used to generate the 3-D structure of the adjudin and the adjudin coordinate file was retrieved from NCBI-PubChem database (CID: 9819086). Receptor grid was generated on the centroid of the active site residues such as Asp128, Ser130, Ser132, Glu227 and Asp224 in β1-integrin. Glide standard precision mode was selected for the flexible ligand docking. The results depicted in Figure 3 show that adjudin molecule interacts with the extracellular region of βA domain of β1-integrin, similar to the laminin β3 fragment (Fig. 3 vs. 2F). In the examination of interacting residues, we observed that Ser130 and Ser 132 were involved in hydrogen bond formation and Ser130, Tyr131, Ser132, Met133, Asp135, Asp136, Leu181, Pro184, Cys185, Gly221, Asn222, Leu223, Ser225, Glu227, Asp257, Ala258, Gly340 and Asn341 involved in hydrophobic contacts. It is important to note that the binding conformation of adjudin is similar to laminin β3 fragment by interacting with residues Tyr131, Ser132, Leu181, Asn222 and Ser225 residing in integrin β1 (see Table 4 vs. Table 3).

Based on these molecular modeling data, it seems that the biologically active fragments of laminin chains (e.g., laminin β3 fragment) generated at spermatiation via the action of MMP2 could potentiate disruption of the apical ES to facilitate spermatiation. This is analogous to the disruptive effects of adjudin at the site, since both laminin β3 fragment and adjudin interact with similar “pockets” at the β1-integrin, and β1-integrin is a component of the major adhesion protein complex α6β1-integrin at the apical ES.3,4,10,13 However, unlike laminin β3 fragment, adjudin does not seem to perturb the β1-integrin at the hemidesmosome to induce BTB disruption/restructuring. This is probably because adjudin and laminin β3 fragment interact with different variants of α-integrin at the apical ES vs. the basal ES at the BTB. For instance, α6-integrin is consisted of two molecular variants generating α6Aβ1-integrin receptor and α6Bβ1-integrin receptor,93 and a splice variant of α6-integrin is associated with malignant conversion during tumorigenesis.94 At present, the identity of the α-subunit for β1-integrin at the hemidesmosome is not known; and even if it is α6-integrin, it is not known if it is the same molecular variant as the one found at the apical ES.

Table 1. List of active residues in β1-integrin and laminin β3 fragment used for HADDOCK docking

| Protein                  | Active residues                  |
|--------------------------|----------------------------------|
| β1-Integrin              | Asp-128, Ser-130, Ser-132, Asp-224, Glu-227 |
| Laminin β3 Fragment      | Leu-32, Arg-33, His-34, Gln-35, Ala-36, Gln-37, Glu-38, Glu-39, Asp-76, Arg-77, Leu-78, Gly-79, Gln-80, Ser-81 |

Conclusion and Future Perspectives

Based on recently published data in the field, we have presented strong evidence that supports the presence of a functional apical ES-BTB-hemidesmosome axis to coordinate the events that take place at the opposite ends of the seminiferous epithelium. It is obvious that much work is needed to further define the apical ES-BTB-hemidesmosome functional axis, in particular its regulation during the seminiferous epithelial cycle of spermatogenesis. Several outstanding questions remain to be addressed in future studies. What is the biologically active domain in the laminin β3 domain I which is composed of 122-amino acid residues? Is this the stretch of sequence interacting with the β1-integrin receptor? Based on the molecular interaction data shown in Figure 2 between laminin β3 fragment and β1-integrin, is it possible to synthesize a more potent laminin β3 fragment to perturb this functional axis for male contraception? What is the signaling pathway(s) involved in the activation of MMP-2 for the generation of the biologically active laminin fragments? What is the downstream signaling mechanism(s) following activation of β1-integrin by laminin fragments at the apical ES and hemidesmosome that perturbs apical ES and BTB function, respectively?

Acknowledgments

Studies from the authors’ laboratories were supported by grants from the National Institutes of Health, NICHD R01 HD056034 to C.Y.C., R01 HD056034-02-S1 to C.Y.C., U54 HD029990 Project 5 to C.Y.C.; and Government of India, Department of Biotechnology, BT/BI/03/015/2002 to P.P.M., Department of Information Technology, DIT/R&D/BIO/15(9)/2007 to P.P.M., and Council of Scientific and Industrial Research, No.09/559/(0074)/2011/EMR-I for Senior Research Fellowship to J.M.

Note

Supplemental materials can be found at: www.landesbioscience.com/journals/spmg/article/17076
**Table 2.** Statistical analysis of top three β1-integrin-laminin β3 clusters obtained from HADDOCK

| HADDOCK complex | Cluster | HADDOCK score | Nb | RMSD | $E_{\text{elec}}$ (kcal mol$^{-1}$) | $E_{\text{vdw}}$ (kcal mol$^{-1}$) | $E_{\text{desolv}}$ (kcal mol$^{-1}$) | $E_{\text{strain}}$ (kcal mol$^{-1}$) | BSA (Å$^2$) |
|-----------------|--------|--------------|----|------|---------------------------------|-----------------|-----------------|-----------------|-------|
| **β1-integrin—laminin β3 fragment** | Cluster 1 | -81.4 ± 10.5 | 29 | 0.5 ± 0.3 | -51.3 ± 1.4 | -415.8 ± 49.0 | 33.8 ± 6.2 | 193.0 ± 35.55 | 1869.1 ± 40.3 |
| | Cluster 2 | -71.0 ± 2.5 | 17 | 10.2 ± 0.3 | -38.7 ± 5.8 | -488.8 ± 57.2 | 48.4 ± 10.1 | 169.8 ± 68.44 | 1666.4 ± 65.8 |
| | Cluster 3 | -66.5 ± 8.5 | 15 | 12.9 ± 0.3 | -32.1 ± 5.8 | -425.7 ± 27.1 | 42.1 ± 4.6 | 86.7 ± 45.58 | 1298.8 ± 114.7 |

$^a$HADDOCK score was calculated by weight sum of Van der Waals energy ($E_{\text{vdw}}$), Electrostatic energy ($E_{\text{elec}}$), Desolvation energy ($E_{\text{desolv}}$) and Restraints violation energy ($E_{\text{strain}}$) in concert with buried surface area (BSA). $^b$Number of structures in the given cluster. $^c$RMSD from the overall lowest-energy structure in a cluster.

**Table 3.** Intermolecular interaction between residues of the β1-integrin-laminin β3 fragment complex

| Protein-protein interaction | Hydrogen bond donor | Hydrogen bond acceptor | Distance (Å) | Hydrophobic Contact Residues |
|-----------------------------|---------------------|------------------------|--------------|-----------------------------|
| TYR131(N)                  | Glu31(OE1)          | 2.70                   |              |                             |
| Ser132(N)                  | Glu31(OE2)          | 2.99                   |              |                             |
| Ser132(OG)                 | Glu31(OE2)          | 2.59                   |              |                             |
| Gly35(N2E2L)               | Ser132(O)           | 2.76                   |              |                             |
| Lys134(NZ)                 | Glu39(OE1)          | 2.64                   |              |                             |
| Lys134(NZ)                 | Glu42(OE1)          | 2.90                   |              |                             |
| His34(NE2L)                | Asp316(DO1)         | 2.82                   |              |                             |
| Lys180(N)                  | Glu20(OE1)          | 2.98                   |              |                             |
| Leu180(N)                  | Glu20(OE2)          | 3.11                   |              |                             |
| Asn222(N)                  | Glu31(OE1)          | 2.67                   |              |                             |
| Trp26(NONE1)               | Leu223(O)           | 2.78                   |              |                             |
| Ser225(OG)                 | Lys30(O)            | 3.29                   |              |                             |
| His34(ND1L)                | Asp257(DO1)         | 2.55                   |              |                             |
| His34(NE2L)                | Gly340(O)           | 2.77                   |              |                             |

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Figure 3. The docked complex of β1-integrin with adjudin. Adjudin molecule was found to interact with the extracellular region of βA domain of β1-integrin (see Fig 2D), similar to the laminin β3 fragment (see Fig 2F). It is noted that the β1-integrin shown in Figure 2D was rotated by 30° along the Y-axis to better depict the intermolecular interactions between the interacting amino acid residues of β1-integrin with adjudin. The interacting domain residues are encircled and enlarged as indicated by the black arrow, illustrating the intermolecular interacting residues residing in β1-integrin (shown in green) with adjudin (shown in cyan) by stick representation. A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; L, Leu; M, Met; N, Asn; P, Pro; S, Ser; Y, Tyr.
Table 4. Intermolecular interactions of adjuin into β1-integrin

| Interaction                | Docking score | Glide energy (kcal/mol) |
|---------------------------|---------------|------------------------|
| NH-O(Der310)              | 4.476         | -36.488                |
| NH(Ner312)                | 2.251         | 1.954                  |

*H, Donor; D, Hydrogen; A, Acceptor.

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