A novel role for SED1 (MFG-E8) in maintaining the integrity of the epididymal epithelium

Adam S. Raymond and Barry D. Shur*
Department of Cell Biology, Biochemistry, Cell and Developmental Biology Graduate Program, Emory University School of Medicine, Atlanta, GA 30322, USA

*Author for correspondence (e-mail: bshur@emory.edu)

Accepted 13 November 2008
Journal of Cell Science 122, 849-858 Published by The Company of Biologists 2009
doi:10.1242/jcs.041731

Summary
The epididymis is a highly convoluted tubule that connects the testis with the vas deferens, and in which mammalian sperm acquire the ability to fertilize eggs. The most proximal portion of the epididymis, or initial segment, secretes numerous factors that are critical for sperm maturation and storage. One such factor is SED1 (also known as MFG-E8) a bi-motif protein composed of two N-terminal EGF domains, the second of which contains an RGD motif, and two C-terminal discoidin domains (also known as F5/8 type C domains). Previous studies have reported that SED1 is secreted into the epididymal lumen, where it coats sperm and later facilitates sperm-egg binding. Herein, we report that SED1-null males also harbor unexpected epididymal pathologies, including detached epithelia and spermatic granulomas. We therefore examined whether SED1 has a tissue-intrinsic role in the epididymis, in addition to its role in sperm-egg adhesion. Improved fixation protocols revealed that SED1 is found in the basolateral domains of epididymal epithelial cells in vivo, and similarly, SED1 is secreted both apically and basally from polarized epididymal cells in vitro. The basolateral distribution of SED1 suggests that it may play a novel role in epididymal cell adhesion. Consistent with this, in vitro assays showed that SED1 supports epididymal cell adhesion via RGD binding to αV integrin receptors on epididymal epithelial cells. Finally, epididymal cells from SED1-null males showed reduced adhesion in vitro, a phenotype that can be rescued with exogenous SED1. These results suggest that SED1 facilitates epididymal cell adhesion, and that its loss leads to breakdown of the epididymal epithelium and consequent development of spermatic granulomas.

Key words: SED1, MFG-E8, Epididymis, Granuloma, αV integrin

Introduction
The epididymis is part of the male excurrent duct and its proper function is critical for male reproductive health. Upon leaving the testis, sperm pass through the efferent ducts and into the epididymis where they are modified by epididymal secretions that are required for the acquisition of motility and fertilizing ability (Ogebim-Crist, 1969). Furthermore, the efferent ducts and proximal segment of the epididymis are responsible for absorbing up to 50% of the testicular fluid and reducing the pH by 0.5 units to compact and inactivate the sperm for storage in the distal segment (Levine and Marsh, 1971). As the molecular nature of these functions has become better understood, the epididymis has received increased attention as a target for postmeiotic male contraceptive technologies, while remaining an excellent model for investigating complex questions in tubule development, differentiation and maintenance.

The epididymis consists of a single highly convoluted tubule of pseudostratified epithelium wrapped in smooth muscle and packaged in a fibrous capsule that resides immediately adjacent to the testis (Setchell et al., 1994). The mouse epididymis can be separated into the initial segment and caput, which collectively are responsible for fluid reabsorption and protein secretion (Abou-Haèila and Fain-Maurel, 1984; Flickinger, 1981). The region proximal to the testis is composed of a cleavable N-terminal signal sequence followed by two domains with homology to Drosophila coagulation factors V and VIII and the animal lectin discoidin (Andersen et al., 1997; Andersen et al., 2000; Ensslin et al., 1998; Andersen et al., 1997; Couto et al., 1996; Ensslin et al., 1998; Andersen et al., 1997; Stubbs et al., 1990). SED1 is composed of a cleavable N-terminal signal sequence followed by two domains with homology to Drosophila coagulation factor (EGF) (Oshima et al., 1999; Stubbs et al., 1990). The second EGF domain contains an arginine-glycine-aspartic acid (RGD) motif that has been identified as a ligand for αVβ3 and αVβ5 integrin heterodimers (Andersen et al., 1997; Andersen et al., 2000; Ensslin and Shur, 2007; Hanayama et al., 2002; Taylor et al., 1997). The C-terminal portion contains two tandem discoidin domains (also known as F5/8 type C domains), similar to those found in blood coagulation factors V and VIII and the animal lectin discoidin
(Ogura et al., 1996; Stubbs et al., 1990). Both discoidin domains are composed of an eight-strand antiparallel β-barrel from which microspikes or hypervariable regions project and which dictate binding specificity to negatively charged matrices and phospholipids (Andersen et al., 1997; Andersen et al., 2000; Fuentes-Prior et al., 2002; Lin et al., 2007; Macedo-Ribeiro et al., 1999; Pratt et al., 1999; Shao et al., 2008; Shi and Gilbert, 2003; Shi et al., 2004; Shur et al., 2004). A long-form splice variant contains a 37-amino acid proline- and threonine-rich O-glycosylation domain that has been suggested to play a role in apical secretion. In support of this, the short isoform, devoid of the O-glycosylation domain, is present in many tissues that do not have polarized secretory epithelia, whereas expression of the long isoform is specifically upregulated during lactation (Oshima et al., 1999).

SED1 serves as an adhesive protein in a number of systems. It was initially discovered as a component of milk fat globules and is expressed by the mammary gland epithelium during branching morphogenesis (Atabai et al., 2005; Ensslin and Shur, 2007). In addition to apical secretion into the milk-filled lumen, basal deposition of SED1 facilitates adhesion of luminal epithelial cells to the adjacent myoepithelium and activates intracellular signaling cascades through αV integrins (Ensslin and Shur, 2007). Similarly, thioglycolate-responsive macrophages secrete SED1 as they approach apoptotic lymphocytes (Hanayama et al., 2002; Hanayama et al., 2004). The C-terminal domains bind to exposed phosphatidyl serine on apoptotic cells, whereas the RGD motif in the second EGF domain serves as a ligand for αVβ3 integrins on the macrophage surface (Hanayama et al., 2002). This SED1 ‘bridge’ leads to macrophage engulfment of the apoptotic lymphocyte (Hanayama et al., 2004). Finally, a porcine homolog of SED1, p47, was isolated from sperm plasma membranes based on its affinity for zona pellucida glycoproteins (Ensslin et al., 1998). Subsequent studies showed that SED1 is secreted from the initial segment of the mouse epididymis, where it coats sperm within the lumen and plays a critical role in sperm adhesion to the zona pellucida (Ensslin and Shur, 2003). In this regard, SED1-null sperm have a reduced capacity to bind eggs in vitro, and SED1-null males have reduced fertility in vivo (Ensslin and Shur, 2003).

Further analysis of the SED1-null male reproductive tract identified an unexpected phenotype in the epididymis: increased incidence of epithelial breakdown and spermatic granulomas. Sperm granulomas contain dense aggregates of immune cells and sperm resulting from an autoimmune response against sperm-associated antigens exposed following damage to the epididymal epithelium. They can result from both biochemical and surgical insults to the epididymis, and can be large enough to totally occlude the epididymal lumen (Hess et al., 2000; Flickinger et al., 1995). The loss of epithelial integrity and the presence of spermatic granulomas prompted us to investigate a tissue-intrinsic role for SED1 in the epididymis. Herein, we report novel localizations of SED1 suggesting that the protein is properly positioned to function in epididymal cell adhesion. Results further suggest that SED1 facilitates epididymal cell adhesion by serving as a ligand for αV integrins expressed on these cells. Loss of SED1 results in reduced adhesion in vitro and may contribute to the described pathologies seen in SED1-null males in vivo.

**Results**

**SED1-null epididymides exhibit a loss in tubule integrity and an increase in spermatic granulomas**

Histological analysis of wild-type and SED1-null epididymides reveals a requirement for SED1 in maintaining the integrity of the epididymal tubule. As discussed above, SED1-null males have an increased incidence of spermatic granulomas, large lesions that occur when sperm-associated antigens breach the blood-epididymal barrier and invoke an autoimmune response (Flickinger et al., 1995). In a representative population of 106 SED1-null epididymides, 42 (40%) showed macroscopic abnormalities visible to the naked eye, occurring as early as 7 weeks of age (Fig. 1A, arrow). Affected SED1-null epididymides often showed complete occlusion of the tubule, evidenced by swelling of tubule cross-sections upstream of the lesion and the absence of sperm in downstream sections (Fig. 1A, arrowhead). Although 40% of the SED1-null males showed macroscopic lesions, the epididymal phenotype was more penetrant than this: histological analysis of macroscopically normal SED1-null epididymides revealed microscopic lesions similar to that seen in Fig. 1B. However, the full penetrance of the epididymal phenotype is unclear, as a comprehensive histological analysis of all epididymides was not undertaken in this study.

Fig. 1. Epididymides from SED1-null males show epithelial breakdown. (A) Photomicrographs of paraffin-embedded epididymal tissue with the major functional regions identified. Sagittal sections from SED1 heterozygous (+/−) and null (−/−) littermates at 8 weeks of age stained with hematoxylin and cosin and imaged at identical magnification. The caudal segment of the SED1-null epididymis (−/−) is characterized by breakdown of the epithelium and resulting in a bolus of sperm, fluid and immune cells. Occlusion of the tubule results in severe tubule swelling upstream of the lesion (compare corpus regions) and an absence of sperm in downstream tubules (arrowhead). (B-E) Other SED1-null pathologies not directly associated with the lesion site include detached, free-floating cells within the lumen suggestive of shed epithelium (arrowheads, B,D,E). (C) is an enlargement of (B). Scale bars: 50 μm (B), 20 μm (C-E).
Although spermatic granulomas are occasionally evident in the caput and corpus segments of SED1-null epididymides, they are most often manifest in the cauda segment. This is similar to the estrogen receptor-null (ERKO) epididymides where spermatic granulomas occur at similar frequency and distribution to that seen in SED1-null males (Flickinger et al., 1995; Hess et al., 2000). Presumably, the preponderance of granulomas in the distal cauda, regardless of where the primary insult occurs, is a consequence of its short, cuboidal epithelium that may be particularly sensitive to disruptions in normal cell and/or fluid dynamics (Flickinger et al., 1995). In addition to spermatic granulomas, SED1-null epididymides displayed absence of an intact epithelium and the presence of shed, free-floating cells morphologically consistent with a detached tubule epithelium (Fig. 1B-E). Other hallmarks of epithelial breakdown were evident, including lymphocyte infiltration, vacuoles in the basal aspects of the epithelium, epithelial metaplasia, and lymphocyte infiltration, similar to that reported by others (Flickinger et al., 1995; Hess et al., 2000). Considering the well-defined role for SED1 in mediating intercellular adhesions in other systems, it is plausible that these pathologies reflect a loss in SED1-dependent adhesion in the epididymis.

**SED1 is expressed in basal and basolateral domains of epididymal epithelia in vivo**

We first characterized SED1 expression throughout the epididymal tubule by immunoblotting and immunofluorescence. Epididymides from wild-type and SED1-null males were isolated, sub-dissected into initial segment, caput and cauda regions, and prepared for western blotting as described in Materials and Methods (Fig. 2A). Probing these preparations with anti-MFG-E8 antibody yielded two predominant bands (Fig. 2B) characteristic of the short and long isoforms of SED1 (Oshima et al., 1999). SED1 was most highly expressed in the initial segment; expression remained high in the caput but was reduced in the cauda. SED1-null tissue showed background immunoreactivity.

Our previous analysis of SED1 expression in the epididymis relied upon traditional submersion-based fixation. When tissue was perfused with 4% paraformaldehyde to preserve histology that is compromised by submersion fixation we identified novel, unexpected localizations for SED1 in epididymal epithelia. Similar results, using two different anti-SED1 antibodies, were seen in all five males prepared by perfusion fixation. The bulk of SED1 expression was found in the principal cells of the initial segment,
where it localized to the perinuclear Golgi region, as well as punctate foci in the apical compartment suggestive of secretory vesicles. However, following perfusion fixation, SED1 was also found in linear plaques that appeared to be between adjacent principal cells in the initial segment (Fig. 2C). Double-label indirect immunofluorescence with E-cadherin illustrated that many of the SED1 plaques were located on the lateral membrane (two examples shown, Fig. 2D, arrowheads). Higher resolution analysis, including immunolabeling at the ultrastructural level, is required for a more complete appreciation of these novel SED1 plaques.

SED1 was also found in vesicles within the apical and basal compartments of clear cells throughout the lower caput, the distal corpus and proximal cauda segments, as previously reported (Fig. 2C). The function of clear cells varies according to their location in the epididymis, with more proximal ones thought to regulate acidification, whereas those in the distal segments function in endocytosis, recycling and/or protein degradation (Robaire and Hinton, 2002). Not surprisingly, the SED1 found in the most distal clear cells (i.e. cauda) appeared in the lysosomal pathway, as judged by colocalization with the lysosomal marker, LAMP-1 (Fig. 2F). However, the majority of SED1-positive clear cells occurred in the distal corpus, and these cells appeared negative for the LAMP-1 lysosomal marker, suggesting that SED1 in these cells is destined for recycling, either apically or basally (Fig. 2E). As, at the current resolution, SED1 was not readily detectable on clear cell borders, its fate in these cells remains to be determined. Nevertheless, SED1 was present in cellular domains that appear to be unrelated to its known apical secretion from principal cells of the initial segment. In particular, the existence of SED1 plaques between adjacent principal cells of the initial segment is suggestive of a function in cell-cell and/or cell-matrix adhesions.

SED1 is deposited basally by polarized epithelial cells in vitro

In light of the known apical secretion of SED1 from a variety of epithelial cells, including principal cells of the epididymal initial segment, we felt it important to confirm the basolateral secretion of SED1 in epididymal cells polarized in vitro. Primary epididymal cells were isolated and cultured using a previously described protocol with modifications (Carballada and Saling, 1997). Mechanical and enzymatic dissociation of the initial segment and caput generated a mixed population of epithelial, smooth muscle and fibroblast-like cells resident in the epididymis. We therefore collected an enriched epithelial cell population by pre-plating this heterogeneous population on fibronectin substrates for 2 hours, followed by aspiration and re-plating of the nonadherent cells. Immunostaining confirmed the removal of desmin-positive smooth muscle cells and the enrichment of cytokeratin-positive epithelial cells (data not shown).

Primary epididymal epithelial cells were isolated as above from wild-type and SED1-null males and cultured on transwell permeable filters coated with a thin layer of Matrigel. Cells of both genotypes proliferated to a confluent monolayer within 4 days. Cell polarization was analyzed by immunolocalization of ZO-1, a zonal occludin protein that localizes to apical tight junctions in confluent epididymal epithelium (Cyr et al., 1999; Levy and Robaire, 1999). In wild-type and SED1-null monolayers alike, ZO-1 was expressed in the traditional cobblestone pattern consistent with tight junction formation in confluent, polarized epithelia (Fig. 3A, A’). (Byers et al., 1992).

SED1 localization in these polarized epithelia was analyzed by z-section confocal microscopy of immunolabeled cultures. SED1 localizes to punctate foci in the apical domain of wild-type cells in agreement with its known apical secretion (Fig. 3B). Importantly, SED1 immunoreactivity was also found in the basal domain and deposited onto the transwell filter beneath the adherent cells (Fig. 3D) confirming that the protein is secreted basally or basolaterally. SED1-null cells exhibited background immunoreactivity (Fig. 3B’-D’).

Recombinant SED1 increases the initial adhesion of primary epididymal epithelial cells

To directly test if SED1 can mediate epididymal epithelial cell adhesion, a quantitative cell adhesion assay was developed.

Fig. 3. Polarized primary epididymal epithelial cells secrete SED1 both apically and basally. (A, A’) Immunofluorescence of the apical zonal occludin protein ZO-1 (green) and basal nuclei (red) in primary epididymal epithelial cells grown on Matrigel-coated transwell filters. X-Y projections and companion z-plane cross-sections (black arrow; white line indicates cross-section location) generated by confocal z-stack imaging show wild-type and SED1-null primary cells polarize and form apical tight junctions in culture. The cell diagram illustrates the approximate depth of confocal scans taken of polarized cultures immunostained with SED1 antisera. (B) In polarized cultures of wild-type cells, SED1 appears as punctate bodies in the apical domain of the cell consistent with its known apical secretion. (C) Little SED1 protein is found at the level of the nuclei; however immunoreactivity returns in the sub-nuclear basal domain (D). Examination reveals this signal is found in the same z-plane as the filter suggesting cells deposit SED1 onto the underlying substrate. (B’-D’) SED1-null cultures assayed in parallel have no immunoreactivity. Scale bars: 20 μm.
Primary epididymal cells were isolated and enriched for epithelial cells as described above from wild-type CD1 males. Cells were cultured on rSED1 (recombinant SED1) for 4 days to increase the population and to maintain expression of any relevant SED1 receptors. Following culture, cells were gently dissociated with a mild enzymatic digestion and allowed to recover for 2 hours in medium containing 5% FBS. Pilot experiments optimized the relevant assay conditions, including substrate concentration, length of assay, number of washes prior to fixation and colorimetric reading of the adherent cells (data not shown). All assays described below were conducted in 96-well microtiter plates at 32°C for 30 minutes and washed twice with PBS prior to fixation with gluteraldehyde. Adherent cells were stained with crystal violet; the dye was solubilized with acid and absorbance was measured at 595 nm.

Epididymal cells exhibited a dose-dependent adhesion to rSED1 substrates, whereas control wells lacking substrate contained no adherent cells (Fig. 4A,B). As expected, the adherent cells were predominately epithelial as judged by cytokeratin immunoreactivity (Fig. 4C). Maximum adhesion was found at 250 ng/well rSED1, and qualitative assays with up to 500 ng of traditional epithelial substrates, including Matrigel and laminin, resulted in similar levels of adhesion (data not shown).

Epithelial cell adhesion to rSED1 is RGD dependent
We first considered whether the RGD motif contained within the second EGF domain is important for epithelial cell adhesion. Isolated primary epithelial cells were treated with either RGD peptide or RAD control peptide, and applied to wells coated with SED1 or laminin substrates. Whereas pre-incubation with 100 μM RGD peptide had no effect on cell adhesion to laminin, adhesion to rSED1 substrates was reduced by 92% compared with cells incubated with control peptide (Fig. 5). At this concentration, the control RAD peptide caused minor nonspecific inhibition of cell adhesion to both rSED1 and laminin substrates, presumably the result of mild changes in the medium pH caused by peptide addition. In any event, the RGD motif of SED1 appeared to be indispensable for epididymal epithelial cell adhesion, which is consistent with findings in other systems, including mammary epithelium (Ensslin and Shur, 2007).

Epididymal epithelial cells express αV integrins
The αVβ3 and αVβ5 integrins have been previously identified as receptors for the RGD motif in SED1 (Andersen et al., 1997; Ensslin and Shur, 2007; Felding-Haberermann and Cheresh, 1993; Hanayama et al., 2002; Ruoslahti, 1996; Taylor et al., 1997); however, it is not known if these receptors are expressed in epididymal cells. Immunoblotting with anti-αV antiserum identified a single band at 125 kDa in all three epididymal regions of both wild-type and SED1-null tissue (Fig. 6A); 125 kDa is the predicted molecular weight for αV under nonreducing conditions (Hirsch et al., 1994; Suzuki et al., 1987). Expression was approximately similar in the initial segment and caput but slightly reduced in the cauda.

In addition to principal cells, these tissue homogenates contained a mixture of other cell types resident in the epididymis, including capillary endothelial cells that are predicted to express αV integrins (Eliceiri and Cheresh, 2000). Therefore, enriched epithelial cells (isolated as above) were probed with αV antiserum, which detected a similar 125 kDa band indicating that αV is expressed in epithelial cells (Fig. 6B). The large reduction in tubulin levels between tissue and single-cell homogenates, without a significant difference in αV levels, reflects the removal of tubulin-rich mouse sperm that are devoid of αV (Ensslin and Shur, 2003).

The presence and distribution of αV integrins in cultures of primary epididymal cells, containing both epithelial and nonepithelial cell types, were analyzed by immunocytochemistry. Cytokeratin-positive cells from both wild-type and SED1-null genotypes expressed αV (Fig. 6C), whereas desmin-positive smooth muscle cells yielded only background immunoreactivity, similar to nonimmune controls (Fig. 6D). Furthermore, confocal analysis revealed that αV integrin was organized into distinct foci or focal plaques when cells were cultured on rSED1 substrates (Fig. 6E). These foci were arranged along the peripheral aspects of the cell lamellipodia and resided along the basal surface as judged by z-
section imaging (arrows). Importantly, these αV-plaques were notably absent from αV-positive epithelial cells cultured on laminin, indicating that this distribution is dependent on the presence of SED1 substrates (Fig. 6F). These data confirm that αV integrins are expressed in epididymal epithelial cells and suggest that SED1 serves as a ligand for these receptors.

αV integrins are required for epididymal epithelial cell adhesion to SED1
To directly test the role of αV integrins in epithelial cell adhesion to SED1, a small molecule inhibitor, L-954, was used. L-954 is similar to other previously characterized inhibitors (Kumar et al., 2001; Murphy et al., 2005) in being specific for both αVβ3 and αVβ5 heterodimers. The addition of 0.1-1000 nM L-954 resulted in a dose-dependent reduction in cell adhesion to rSED1 but had no effect on cell adhesion to laminin (Fig. 7). L-954 inhibited SED1-dependent adhesion at concentrations as low as 1 nM, with nearly 100% inhibition at 1000 nM.

As a further test of αV integrin function in SED1-dependent epithelial cell adhesion, we examined the effects of an αV function blocking antibody (RMV-7) (Takahashi et al., 1990). The addition of 50 μg/ml IgG resulted in a small (18%) reduction in adhesion compared with mock assays, whereas 100 μg/ml reduced initial adhesion by 57% (Fig. 8). Nonimmune IgG had no effect. The degree of inhibition in our studies (i.e. 57%) is similar to that reported by others using the same RMV-7 IgG (Takahashi et al., 1990). Together, these data confirm that SED1 serves as a ligand for αVβ3 and αVβ5 integrins expressed on epididymal epithelial cells.

SED1-null epithelial cells show reduced adhesion in vitro
Although wild-type and SED1-null cells behaved similarly on transwell filters coated with Matrigel, they showed striking differences when cultured on glass substrates lacking exogenous matrix. When similar numbers of wild-type and SED1-null epithelial cells were cultured on glass chamber slides, both wild-type and SED1-null cells grew as epithelial clusters, or islands; however, SED1-null cultures contained 35% fewer islands than wild-type cultures (Fig. 9A,B). Importantly, when cells of either genotype were provided with rSED1 substrates, many small epithelial islands quickly formed with no detectable difference between genotypes. Thus, SED1-null cells have an intrinsic deficiency in their behavior on traditional tissue culture surfaces that is negated when cells are grown on exogenous SED1.

Although these results are consistent with a role for SED1 in epididymal adhesion, we examined the possibility that the reduction in epithelial islands in SED1-null cultures reflects a primary defect in cell proliferation, rather than a defect in initial cell adhesion. This is particularly relevant here, as we have previously described the activation of MAPK-dependent proliferation cascades following SED1 binding to αV integrins on mammary epithelial cells (Ensslin and Shur, 2007), and integrin receptors are known to influence a variety of intracellular signaling pathways (Felding-Habermann and
Cheresh, 1993; Hynes, 1992). To investigate the possibility that differences in epithelial cell growth reflect an SED1-dependent proliferation cascade, the rate of epididymal epithelial cell proliferation in wild-type and SED1-null cultures was determined by BrdU incorporation. Despite differences in the total number of epithelial islands, wild-type and SED1-null cultures showed virtually identical rates of cell proliferation when grown on glass substrates (Fig. 9C). Furthermore, the presence of rSED1 substrates, which results in greatly increased numbers of epithelial islands, did not produce a concomitant increase in cell proliferation (Fig. 9C), and in fact, led to a slightly reduced level of proliferation in wild-type cells, the basis of which remains unexplored. In any event, these data indicate that SED1 can support epididymal cell adhesion, and that the loss of SED1 results in compromised cellular adhesion.

**Discussion**

In this report, we have described an unexpected phenotype in the SED1-null epididymis consisting of an increase in spermatic granulomas and other pathologies characteristic of a breakdown in the integrity of the tubule epithelium (Flickinger et al., 1995; Hess et al., 2000). In light of the role of SED1 in cell adhesion in other systems, we designed a series of experiments to test the possibility that SED1 also facilitates cell adhesion in the epididymis (Andersen et al., 1997; Andersen et al., 2000; Ensslin et al., 1998; Ensslin and Shur, 2007; Hanayama et al., 2002; Taylor et al., 1997).

Using more appropriate fixation conditions, we determined that SED1 is found along lateral cell–cell borders of the initial segment in addition to its previously reported apical localization (Ensslin and Shur, 2003). SED1 also localized to the basal domain of clear cells in the caput and corpus, although it is not readily apparent if these cells synthesized SED1 and/or endocytose luminal SED1 for recycling and/or transcytosis, as reported for the epididymal epithelium (Cooper et al., 1988). Unlike that seen in more proximal regions, SED1 appeared to be targeted for degradation in the distal clear cells of the cauda. In any event, wild-type primary cells grown to confluence and polarized on permeable substrates secreted SED1 in addition to its previously reported apical localization (Cheresh, 1993; Hynes, 1992).

**Fig. 7.** A small molecule inhibitor of αβ3 and αβ5 integrin heterodimers (L-954) selectively blocks epididymal cell adhesion to SED1. Increasing concentrations of L-954 were added to 30-minute adhesion assays, eliciting a dose-dependent decrease in adhesion to SED1 substrates (500 ng/well). The inhibitor has little effect on cell adhesion to laminin-coated substrates (500 ng/well). Data are expressed as a percentage of untreated (mock) cell adhesion to each substrate. As little as 1 nM of inhibitor is sufficient to reduce adhesion to SED1 by 19%, while 1000 nM inhibits adhesion by 93%. Error bars: s.e.m. *P<0.0001.

**Fig. 8.** Function-blocking antibodies against αV integrin block epididymal cell adhesion to SED1. The addition of αV-blocking IgG (RMV-7) (50 μg/ml or 100 μg/ml) results in a dose-dependent reduction in epithelial cell adhesion to SED1 of 18% and 57%, respectively. Control IgG has no effect. Data are expressed as a percentage of untreated (mock) cell adhesion. Error bars: s.e.m. *P<0.0001.

**Fig. 9.** Loss of SED1 leads to deficient cellular adhesion in vitro. Primary epididymal-enriched epididymal cells were isolated and cultured for 4 days on uncoated glass or SED1 substrates. Attached cells were pulsed with BrdU for 4 hours, fixed, and immunostained for BrdU. Epididymal ‘islands’ (arrowheads) containing ≥4 nuclei were counted. (A) Epifluorescent imaging of SYTO24 counterstain illustrates a reduction in the number of SED1-null (−/−) epithelial islands relative to wild type (+/+). This phenotype is rescued when cells are cultured on SED1 substrates; under these conditions cells of both genotypes produce abundant islands. (B) Quantification shows that SED1-null (−/−) cells generate 35% fewer islands than wild-type (+/+) cells when cultured on glass substrates. (C) BrdU-positive nuclei associated with epithelial islands were counted and expressed as a percentage of total nuclei associated with islands. Data from multiple experiments were averaged and the wild-type (+/+) rate of proliferation was normalized to 100%. Although SED1-null (−/−) cells generate fewer islands than wild type, the rate of proliferation for the two genotypes is identical on glass substrates. Furthermore, the presence of SED1 substrates does not lead to increased proliferation relative to cells cultured on glass. Scale bar: 100 μm. Error bars: s.e.m. *P=0.01; **P<0.001.
which does not secrete SED1. The mechanism whereby SED1 is targeted for either apical or basolateral secretion is poorly understood but likely involves vesicle-dependent trafficking, as cleavage of the N-terminal signal sequence produces a vesicle-enclosed soluble protein. Furthermore, a basal localization of SED1 has also been reported for mammary epithelial cells (Ensslin and Shur, 2007), and a SED1 homolog known as Del-1 is a component of the extracellular matrix (Hidai et al., 2007).

The basolateral distribution of SED1 in epididymal epithelial cells is consistent with a role in cell adhesion. This was confirmed by the ability of SED1 to support the adhesion of primary epididymal epithelial cells in a dose-dependent manner. Furthermore, the addition of RGD peptides to primary cell adhesion assays significantly reduced adhesion to rSED1, whereas it had no effect on adhesion to laminin substrates. As integrin heterodimers containing αV subunits have been reported to serve as receptors for the RGD sequence in SED1 (among other adhesive glycoproteins), we determined that epididymal epithelial cells express αV integrin. Interestingly, epididymal cells organized αV receptors into focal plaques when cultured on SED1 but not on laminin substrates suggesting a physiological response to the underlying substrate. Furthermore, αV integrins on epididymal epithelia may only recognize, or bind, the RGD motif in the context of the SED1 polypeptide backbone, as epididymal cells did not adhere to vitronectin, an RGD-containing substrate known to bind αV integrins (data not shown) (Felting-Habermann and Cheres, 1993). In any event, the involvement of αV integrins in mediating SED1-dependent adhesion was confirmed through the use of specific low molecular weight inhibitors as well as by αV function-blocking antibodies.

Consistent with SED1 function as an adhesive component in the epididymis, SED1-null cells were shown to display an intrinsic defect in cell adhesion. When wild-type and SED1-null primary epithelial cells were isolated and applied to uncoated glass substrates, wild-type cultures had characteristically larger numbers of epithelial islands than was seen in the SED1-null cultures. Control experiments eliminated the possibility that these differences reflect genotype-specific differences in cell proliferation. These results were in good agreement with in vivo BrdU assays, which indicated that wild-type and SED1-null initial segment epididymides proliferate at similar rates (data not shown). Perhaps more importantly, cultures of both genotypes grown on rSED1 substrates contained an abundance of epithelial islands while maintaining similar rates of proliferation to wild-type cells grown on glass. Therefore, the reduced number of cells in SED1-null cultures reflects reduced cell adhesion at plating, which we believe is a consequence of their inability to synthesize and secrete SED1 substrates, as the wild-type cells do.

These data allow us to develop a simple working model for SED1-dependent epithelial cell adhesion in the epididymis. It is proposed that SED1 localized to basolateral domains facilitates cell adhesion via binding of its N-terminal RGD motif to cell surface αV integrin receptors, whereas the C-terminal discoidin domains bind to phospholipid bilayers on adjacent epithelial cells, as shown by others (Andersen et al., 1997; Andersen et al., 2000) (Fig. 10A). Alternatively, SED1 may mediate cell adhesion to the underlying basal lamina, as suggested for the SED1 homolog, Del-1 (Hida et al., 2007), by the binding of discoidin domains to negatively charged components of the extracellular matrix (Fig. 10B). Distinguishing between these possibilities would be greatly facilitated by localization of αVβ3 and αVβ5 integrins in vivo; however, multiple attempts using various commercially available αV antibodies and different fixation conditions failed to yield a reliable signal for αV in epididymal sections.

Although SED1 expression is highest in the initial segment, the majority of SED1-dependent phenotypes manifest in the distal corpus and cauda, similar to other systems reporting spermatic granulomas (Flickinger et al., 1995; Hess et al., 2000). Thus, SED1 may impact epithelial integrity either locally and/or further afield. As SED1 is found both within the epididymal lumen and in apical and basal domains of clear cells that are prevalent in the corpus, it is possible that SED1 is synthesized and/or transcytosed by these cells and deposited in the basement membrane where it serves as an adhesive ligand. Alternatively, SED1 may serve an adhesive function in the initial segment that is required, indirectly, for the integrity of the distal epididymis. Such is the case for the estrogen receptor-null (ERKO) epididymis, in which a defect in the expression and localization of sodium exchangers in the efferent ducts is coincident with the appearance of sperm granulomas in the distal epididymis (Hess et al., 2000). Furthermore, binding of SED1 or Del-1 to αVβ3 and αVβ5 integrins is known to activate intracellular signaling cascades in other systems (Ensslin and Shur, 2007; Penta et al., 1999; Wu et al., 2005), such that the loss of this interaction may affect important epithelial cell functions (e.g. protein secretion), ultimately impacting the overall fortitude and function of the epididymis.

The realization that the loss of SED1 leads to an epididymal phenotype raises the possibility that SED1 does not participate in sperm adhesion to the egg coat as previously reported (Ensslin and Shur, 2003), but rather suggests that the defective sperm-egg binding may be a secondary consequence of defective epididymal integrity. A number of observations indicate that this is not a likely possibility. First, a variety of reagents that specifically block SED1 function, such as blocking antibodies, recombinant SED1 and truncated SED1 proteins, are able to competitively inhibit the binding of wild-type sperm to eggs. Second, the penetrance of the fertility phenotype reported previously, as well as the penetrance of the epididymal pathologies reported here, show distinctly different strain-specific expression. In this regard, the original null mutation was generated on the mosaic B6/129 background, which produced a variable, but highly penetrant (~89%) fertility phenotype, whereas epididymal lesions did not occur until late adulthood (data not shown). By contrast, backcrossing the SED1-null mutation onto the B6 congenic background produced a weak fertility phenotype, possibly due to maintaining them by homozygous matings, although B6 SED1-null...
males showed a clearly penetrant (~50%), but variable, epididymal phenotype as early as 7 weeks of age. Thus, the sub-fertility of the B6/129 SED1-null males is not likely the result of defects in epididymal integrity.

Even though many questions remain unanswered, the data presented here clearly show that SED1 plays an important role in the maintenance of the epididymis, in addition to its role in facilitating sperm-egg adhesion. These results further illustrate the complex relationship between seemingly disconnected regions of the epididymis, and may have important implications for the development of new fertility and contraceptive technologies as the epididymis becomes an increasingly important target for reproductive interventions.

Materials and Methods

Mice and recombinant SED1

All experiments were conducted using wild-type and SED1-null congenic C57/B6 males, with the exception of ZO-1 and SED1 immunocytochemistry in vitro, which was conducted with primary cells isolated from the original B6/129 mosaic line. All adhesion assays were conducted with commercially available CD1 males (Charles Rivers). rSED1 refers to purified recombinant protein prepared as described (Ensslin and Shur, 2003) or purchased commercially (R&D System 2005-CF).

Tissue preparation for histology

Tissue was prepared in one of two ways: (1) whole animal perfusion using 4% paraformaldehyde followed by postdissection submersion fixation in the same, or (2) dissection and overnight submersion fixation in Bouin’s fixative (Sigma HT10-1-32). Tissue was dehydrated, infiltrated with paraffin, and 5 or 10 μm sections were prepared using a Microm microtome. Sections of wild-type and SED1-null tissue were stained with hematoxylin and eosin or processed for immunocytochemistry.

Preparation and culture of primary cells

Primary cell isolation and culturing were modified from (Carballada and Saling, 1997). Briefly, initial segment tissue was dissected into RPMI 1640 medium and single cells were generated by mechanical dissociation followed by serial digestions in 0.025% trypsin-EDTA (Invitrogen 15400) at 20 minutes and 2.4 μ/l disperse (Invitrogen 17105-041) supplemented with 51.6 μ/l Type I collagenase (Invitrogen 17100-017) for 30 minutes. The resulting slurry was passed through Nytex mesh (~0.035 mm, ~16 pores/mm2) to remove undigested tissue, washed several times, and resuspended in RPMI 1640 media containing 5% fetal bovine serum (FBS) and supplemented with 100 ng/ml EGF (BD Bioscience 354001) and 200 nM testosterone (Sigma T-5035). Cells were ‘pre-plated’ at approximately 105 cells/60-mm tissue culture dish, 30 minutes at 4°C prior to plating. A total of 15,000 cells in 100 μl of media were added to each assay well and cells were allowed to adhere for 30 minutes. Nonadherent cells were removed by vacuum aspiration and wells were washed twice with 1 ml of media, then collected into a total of 1 ml of media for trypsinization in fresh media for 2 hours at 32°C. For adhesion assays, cells were resuspended in serum-free RPMI 1640 media containing 100 ng/ml EGF, 200 nM testosterone and 10 mg/ml BSA with or without peptide or inhibitors. Peptide inhibitors (GRGDNP, Biomol P-700; GRADNP, BiomolP-701) were found to reduce the pH of the media, and consequently, medium that contained peptides was buffered at 32°C, 5% CO2 for 30 minutes prior to the addition of cells. For antibody blocking experiments featuring RMV-7 (rat anti-mouse Vα monoclonal, Chemicon B1L 1346) and rat IgG control (Abcam RTK2071), cells were pre-incubated with antibody for 30 minutes at 4°C prior to plating. A total of 15,000 cells in 100 μl of media were added to each assay well and cells were allowed to adhere for 30 minutes. Nonadherent cells were removed by vacuum aspiration and wells were washed twice with 100 μl PBS. Adherent cells were fixed and detected with 0.1% crystal violet solution according to Current Protocols in Cell Biology (Humphries, 1998). Unless indicated otherwise, individual data points from multiple experiments were converted to a percentage of mock control and averaged.

BrdU proliferation assays

Single cells were isolated from the initial segment of wild-type and SED1-null epididymides as described above. Enriched epithelial cells were applied to either uncoated or rSED1 (800 ng) coated glass chamber slides at a concentration of 2.5 × 105 cells/chamber in 0.5 ml of serum-free RPMI 1640 media containing 100 ng/ml EGF and 200 nM testosterone. Cells were allowed to adhere and proliferate; adherent and dead cells were removed by washing with RPMI 1640 media. After 96 hours, the medium was spiked with 100 μg/ml BrdU (Sigma 85881). After 5 hours, adherent cells were fixed with acid-alcohol (10% acetic acid, 70% ethanol v/v) and processed for BrdU detection with biotinylated anti-BrdU antibody according to the manufacturer’s protocol (1:50, Genentech GTX29557). BrdU-positive cells were visualized with streptavidin conjugated with Alexa Fluor 594, and all nuclei were counterstained with SYTO24 (1:5000; Invitrogen S7559). Cells were counted using a ×40 objective, and BrdU-positive cells were expressed as a percentage of total cells counted and normalized to wild-type values on glass. To compare the proliferation kinetics between uncoated and rSED1-coated surfaces, only epithelial islands containing greater than four nuclei and less than a single field at ×40 were scored.

This work was supported by NIH grants RO1 HD23479 (B.D.S.) and T32 GM008367 (A.S.R.). The authors wish to thank Victor Faundez for the anti-LAMP-1 antibodies, Michael Ensslin for identifying the epididymal phenotype, and the members of our laboratory for their critical reading of the manuscript. Deposited in PMC for release after 12 months.

References

Abe, K., Takano, H. and Itou, T. (1983). Ultrastructure of the mouse epididymal duct with special reference to the regional differences of the principal cells. Arch. Histol. Jpn. 46, 57-70.
Abou-Hailea, A. and Fain-Mauruel, M. A. (1984). Regional differences of the proximal part of mouse epididymis: morphological and histochemical characterization. Anat. Rec. 209, 197-208.

Sed1 facilitates epididymal integrity 857
