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Loss of SED1/MFG-E8 results in altered luminal physiology in the epididymis

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SUMMARY

SED1/MFG-E8, herein referred to as SED1, is a bimeric adhesive protein with ascribed functions in a range of cell-cell interactions, including sperm-egg binding. In the male reproductive tract, SED1 is secreted by the initial segment of the epididymis, where it coats sperm and subsequently facilitates binding to the egg zona pellucida. We have recently reported that SED1-null epididymides show an unexpected incidence of spermatogenic granulomas, reflecting breakdown of the epithelium and a consequent autoimmune response against sperm antigens. However, spermatogenic granulomas are most often manifest in the distal segments of the epididymis, whereas the bulk of SED1 is expressed in the proximal epididymis. In some models, the presence of granulomas in the distal epididymis is associated with an underlying defect in the maintenance of luminal fluid homeostasis. Herein, we report that SED1-null epididymal fluid is both hypo-osmotic and alkaline, relative to wildtype epididymal fluid. Furthermore, the SED1-null epididymal epithelium exhibits various hallmarks of disrupted fluid reabsorption and pH regulation, including altered morphology of clear cells, increased intracellular vesicles, and apical distribution of VAMPs. Results indicate that the SED1-null epididymal pathologies are not the secondary consequences of defective testes or efferent ducts or of improper epididymal differentiation, unlike that seen in other epididymal models. The expression and distribution of various ion exchangers, channels, and enzymes that mediate fluid transport and pH regulation are examined in wildtype and SED1-null epididymides, and models to account for how SED1 functions in luminal fluid dynamics are discussed.

Keywords

SED1; MFG-E8; epididymis; granuloma; luminal fluid

INTRODUCTION

Following spermatogenesis in the testis, sperm pass through the efferent ducts and into the epididymis where they acquire the ability to become motile and fertilize the egg (Orgebin-Crist 1969). The epididymis is a single, highly convoluted tubule composed of three primary anatomical regions: the caput, the first portion of which is termed the “initial segment”, the corpus, and the cauda (Setchell et al. 1994). The tall columnar principal cells of the initial segment secrete factors into the luminal fluid that bathe the sperm, while adding various membrane-associated proteins to the sperm surface (Hinton and Palladino 1995). Protein secretion occurs through traditional secretory pathways as well as apocrine shedding, a process that involves the detachment of membrane-bound apical protrusions. Once free

within the lumen, the membrane vesicles fragment, thereby releasing their cache of soluble and membrane-associated proteins (Hermo and Jacks 2002). The membrane-associated components are packaged into small (50–800 nm) vesicles called epididymosomes, which encounter and/or exchange lipid and protein components with sperm (Hermo and Jacks 2002; Rejraji et al. 2006).

The cells of the initial segment and caput also express a host of acid/base transporters and enzymes that facilitate water absorption and acidification of the luminal fluid, and which contribute to sperm compaction and low metabolic activity, respectively (Pastor-Soler et al. 2005). Sperm maturation continues in the corpus, and is completed and sustained in the cauda epididymis where sperm are stored until ejaculation (Setchell et al. 1994). The acidic pH is maintained in the cauda by clear cells that secrete protons through membrane-associated VATPase complexes localized at the luminal plasma membrane (Breton et al. 1996; Brown et al. 1992). Localization of VATPase is tightly tethered to luminal pH, cycling between the apical membrane and late endosomes under acidic conditions, but remaining static at the membrane when bicarbonate levels are high (Pastor-Soler et al. 2003). High bicarbonate levels and alkaline pH are known activators of sperm, and therefore the acidic, low bicarbonate environment of the cauda is thought to maintain the concentrated sperm in a quiescent state during storage (Acott and Carr 1984; Carr et al. 1985; Hinton and Palladino 1995; Pastor-Soler et al. 2005).

A number of epididymal proteins have been identified as important factors in luminal fluid modification and sperm maturation. One such protein is SED1, also known as MFG-E8, lactadherin, among other names (Andersen et al. 1997; Couto et al. 1996; Ensslin et al. 1998; Larocca et al. 1991; Stubbs et al. 1990). SED1 is a secreted bimotif cell adhesion molecule composed of a cleavable signal sequence followed by two N-terminal EGF-like domains with homology to *Drosophila* Notch, and two C-terminal F5/8C domains similar to those found in blood coagulation factors V/VIII and the animal lectin discoidin. The second EGF domain contains an arginine-glycine-aspartic acid (RGD) sequence that binds α_v -containing integrins, and each F5/8C discoidin-like domain is composed of an eight-strand antiparallel beta-barrel that presents microspike hypervariable regions capable of binding to negatively charged surfaces, including anionic phospholipids (Andersen et al. 1997; Andersen et al. 2000; Ensslin and Shur 2007; Fuentes-Prior et al. 2002; Lin et al. 2007; Macedo-Ribeiro et al. 1999; Pratt et al. 1999; Shao et al. 2008; Shur et al. 2004; Taylor et al. 1997).

SED1 was initially identified as an epididymally-secreted protein that coats sperm and subsequently facilitates sperm binding to the egg zona pellucida (Ensslin and Shur 2003). However, we have recently reported that the loss of SED1 leads to breakdown of the epididymal epithelium, with the consequent development of spermatc granulomas (Raymond and Shur 2009). Using perfusion-based fixation protocols, SED1 was found to be localized to lateral “plaques” of principal cells in the initial segment, in addition to its previously described intracellular distribution as revealed by traditional postmortem fixation (Raymond and Shur 2009; Ensslin and Shur 2003). Results suggest that SED1 present on epididymal cell borders may facilitate intercellular adhesion by binding to $\alpha_v\beta_{3/5}$ integrin receptors on adjacent epididymal cells (Raymond and Shur 2009). In this scenario, loss of SED1 leads to epithelial breakdown, exposure of sperm-associated antigens, and development of spermatc granulomas.

Surprisingly, the majority of SED1-null spermatc granulomas occur in the distal segments of the epididymis, whereas SED1 is primarily expressed in the proximal epididymis. In some epididymal models, the presence of granulomas in the distal epididymis is associated with an underlying defect in the ability to properly regulate the composition of the luminal

fluid (Zhou et al. 2001; Joseph et al. 2010a,b), which is known to be absolutely critical for sperm maturation and for their ability to fertilize eggs. Therefore, in this study, we undertook a more detailed analysis of the SED1-null epididymis and show here that the loss of SED1 leads to an inability of the epididymal epithelium to properly regulate the luminal fluid. Results show that SED1-null epididymal fluid is both hypo-osmotic and more alkaline than is wildtype fluid, and does not appear to be a secondary consequence of abnormal epididymal development or differentiation, nor is it the result of defects in the upstream tissues of testis or efferent ducts, as is the case in other systems (Hess et al. 2000; Zhou et al. 2001). Rather, the failure to properly regulate the luminal fluid is accompanied by alterations in cell morphology, the frequency of intracellular vesicles, and the subcellular distribution of VAPase, a transporter involved in luminal acidification, as well as other abnormalities that collectively contribute to the defective regulation of the luminal fluid.

RESULTS AND DISCUSSION

Experimental rationale

We have previously reported that SED1-null epididymides exhibit an increased occurrence of spermatoc granulomas (Raymond and Shur 2009), which are characterized by loss of epithelial integrity, breakdown of the blood-epididymal barrier, and a consequent immune response against sperm antigens (Flickinger et al. 1995). Approximately 40% of SED1-null males show macroscopic lesions, although the penetrance is likely much greater since microscopic lesions are evident in macroscopically normal SED1-null epididymides (Raymond and Shur 2009). Lymphocytes can be seen infiltrating the interstitial spaces, and the resulting granuloma can, in extreme cases, completely occlude the tubule. Other granuloma-associated pathologies are also seen in SED1-null males, including metaplasia of the epididymal epithelium (Fig. 1). In this study, we undertook a more detailed analysis of SED1 localization throughout the epididymal epithelium to appreciate more fully how its loss contributes to the observed pathologies. Since in other epididymal models spermatoc granulomas are associated with, and possibly result from, alterations in epididymal fluid homeostasis, we determined that the loss of SED1 is also associated with abnormal regulation of the luminal fluid, which is both hypo-osmotic and more alkaline than wildtype fluids. To address the underlying molecular defects, we examined the expression of ion transporters, channels and enzymes known to be involved in fluid regulation, as well as the possibility that these pathologies result from abnormal upstream tissues and/or aberrant epididymal development. Finally, we considered the possibility that SED1 may function in luminal regulation via secretion in association with epididymosomes.

SED1 localizes to numerous domains in principal, clear, and basal cells of the epididymis

The three major anatomical segments of the epididymis are subdivided by fibrous septa into ten regions that exhibit subtle differences in cell morphology (Johnston et al. 2005), and consequently, this analysis required precisely oriented sections to appreciate each of five caput, two corpus, and three cauda sub-regions that were not carefully considered in previous studies. As reported earlier, SED1 is highly expressed in the tall columnar principal cells of the initial segment, where it is concentrated in the Golgi region and punctate apical vesicles characteristic of the classical secretory pathway (Fig. 2) (Ensslin and Shur 2003; Raymond and Shur 2009). When tissue is prepared using perfusion-based fixation procedures, rather than traditional postmortem submersion fixation as done here, SED1 is also expressed in principal cells as linear plaques that align with basolateral cell-cell borders (Raymond and Shur 2009).

Caput II is characterized by a shortened epithelial cell height and an increase in the number of “prominent” clear cells (Abou-Haïla and Fain-Maurel 1984). SED1 localization is

somewhat reduced in the principal cells of this region, however the protein is concentrated at the apical membrane and stereocilia remain strongly positive (Fig. 2). Clear cells are also immunoreactive in this region (data not shown). In caput III, the tubule narrows and numerous multivesicular bodies fill the apical domain of the principal cells (Abou-Haëila and Fain-Maurel 1984). Here, SED1 is absent from the Golgi region and the shortened stereocilia, however a subset of multivesicular bodies are strongly positive (Fig. 2). Some punctate vesicular staining returns in caput IV, but fades again in caput V, which is devoid of SED1 immunoreactivity at this resolution. SED1 expression remains low or absent from the proximal corpus epididymis, but returns strongly in a subset of clear cells and basal cells of the distal corpus and proximal cauda before disappearing in the most distal cauda region adjacent to the vas deferens (Fig 2).

Clear cells are highly endocytic and these cells are not labeled when probed for SED1 by in situ hybridization, which suggests the protein is endocytosed by these cells (data not shown) (Robaire and Hinton 2002). However, in gene array experiments by others low levels of SED1 mRNA are detected in all three segments of the cauda, leaving unresolved the possibility that the protein is also synthesized in this region (Johnston et al. 2005). In any event, we have previously reported that the majority of clear cells in these areas do not express LAMP-1, a hallmark of lysosomes, which suggests the protein is not destined for degradation in these cells, but rather plays some unknown function (Raymond and Shur, 2009) Furthermore, although the subcellular distribution of SED1 in basal cells is not evident at this resolution, the protein may contribute to the ability of these cells to regulate the adjacent luminal epithelial cells (Cheung et al. 2005). Additional investigation is required to determine the function of SED1 in these non-principal cell types.

Loss of SED1 results in increased luminal pH and decreased osmolality

In some epididymal models, such as the estrogen receptor (ER) alpha knockout, spermatogenic granulomas are associated with an underlying defect in the maintenance of luminal fluid homeostasis (Hess et al. 2000; Zhou et al. 2001; Joseph et al. 2010a,b). Similarly, infertile *c-ros* knockout males are also characterized by elevated luminal pH, although they do not exhibit epididymal spermatogenic lesions (Yeung et al. 2004). We therefore determined if the loss of SED1 leads to a similar alteration in luminal fluid homeostasis by assessing luminal pH and osmolality. Wildtype and SED1-null animals were sacrificed in pairs, and each epididymis was sub-dissected into the caput and cauda. Luminal contents from the distal cut surface were gently massaged onto litmus paper and compared to a series of buffered standards to determine the pH. Luminal fluid harvested from both the initial segment and cauda regions of SED1-null epididymides are ~0.3 pH units higher than wildtype ($p \leq 0.006$) (Fig. 3). This increase in pH is similar to the difference detected when comparing wildtype and *c-ros* knockout animals (Yeung et al. 2004), and indicates that the SED1-null epididymis fails to properly acidify the epididymal lumen. Importantly, the difference can be measured as early as the caput indicating that the defect most likely occurs in the proximal region of the epididymis.

As an independent means to assess luminal fluid homeostasis, osmolalities were compared between SED1-null and wildtype epididymal fluids. The osmolality of wildtype caput epididymal fluid is 430 Osm/kg, consistent with that recently reported for mouse (Joseph et al. 2010b). In contrast, the SED1-null caput epididymis is 380 Osm/kg, a decrease of 50 Osm/kg relative to wildtype, and similar to that reported for the ER alpha-null epididymis (Joseph et al. 2010b). In contrast to that seen in the caput epididymis, the osmolalities of testicular fluids are similar between wildtype and SED1-null animals, illustrating that the hypotonic milieu is not a characteristic of the entire male reproductive tract (Fig. 3B). Collectively, the elevated pH and reduced osmolality of SED1-null epididymal fluid indicate that the lack of SED1 leads to a failure to properly regulate the epididymal fluid.

SED1-null epididymides exhibit morphological correlates of defective fluid regulation

Consistent with a failure to properly regulate the epididymal fluid, the SED1-null epithelium is characterized by a number of subtle morphological features that have been associated with defective regulation of luminal fluid in other systems. Clear cells throughout the epididymis express VAPase, a protein complex that transports protons across cell membranes to acidify the luminal fluid. The importance of VAPase in luminal fluid acidification is illustrated by the Foxil knockout mouse, in which the absence of VAPase leads to alkaline epididymal fluid (Blomqvist et al. 2006). Under normal conditions, VAPase recycles between endosomes and the apical membrane where it delivers protons to the luminal fluid; however, VAPase remains at the apical membrane under alkaline pH and high bicarbonate levels (Brown et al. 1992). It is therefore interesting that the distribution of VAPase in clear cells of the SED1-null initial segment, where they are called narrow cells, and in the cauda is more apically localized than in wildtype (Fig. 4A,B). These observations are consistent with the elevated pH of SED1-null epididymal fluids, and suggest a potential compensatory mechanism.

Additionally, transmission electron microscopy reveals that some aspects of the subcellular architecture are affected in the absence of SED1. Within the principal cells of the SED1-null initial segment, the mitochondria are often swollen and damaged, a phenotype that can reflect hypo-osmotic conditions (Fig. 4C) (McGill et al. 1973). Although this phenotype may be, in part, accentuated by submersion fixation of the tissue, wildtype and knockout material were prepared in parallel and clearly show that the SED1-null is more sensitive to hypotonic conditions than is normal tissue.

These observations drew our attention to other hallmarks of defective osmolarity and fluid regulation. Semi-thin sections were prepared, stained with toluidine blue, and used to quantify vesicles present in different regions of the epididymis. In some SED1-null males, presumed secretory vesicles in the initial segment were decreased relative to wildtype, but this phenotype was not observed in all animals. However, there was a clear, reproducible increase in intracellular vesicles in caput III of SED1-null males, irrelevant of the presence of a macroscopic granuloma (SED1-null devoid of granuloma: 133.1 ± 8.7 vesicles/100 μm tubule circumference; SED1-null with distal granuloma: 88.9 ± 6.5 vesicles/100 μm ; wildtype: 28.1 ± 3.1 vesicles/100 μm ; $p < 0.0001$) (Fig. 5A). Since caput III is believed to reflect a highly endocytic region of the epididymis (Abou-Haïla and Fain-Maurel 1984), the increase in intracellular vesicles is suggestive of increased, compensatory endocytosis in the SED1-null. Furthermore, the SED1-null epididymal epithelium is often highly vacuolated, a characteristic of abnormal luminal fluid adsorption (Fig. 5B) (Itoh et al., 1999).

Finally in this regard, the narrow cells of the SED1-null initial segment show an unusual pathology not previously seen in other epididymal models. Under traditional submersion-based fixation protocols, wildtype narrow cells extend cap-like protrusions into the lumen containing large membrane-bound vacuoles that presumably facilitate endocytosis and/or acidification, as they are VAPase immunoreactive (see Fig. 4A) (Abou-Haïla and Fain-Maurel 1984; Hinton and Palladino 1995). However, when prepared identically to wildtype, the apical domains of virtually all SED1-null narrow cells have a rough, shredded morphology, devoid of vesicles (Fig. 5C). Similar results are seen when these cells are immunostained for intracellular markers, such as chloride channels. The basis of this defective apical morphology remains unknown, but suggests that the cell's endocytic, and/or VAPase recycling machinery is unable to be regenerated as efficiently as in wildtype.

Due to the high frequency of spermatic granulomas, it is difficult to know for certain if these characteristics are the result of the underlying SED1-deficiency or excess fluid created by a

lesion-obstructed tubule. It is important to note, however, that *ex vivo* pH measurements were consistently greater than wildtype regardless of the presence or absence of a lesion. Similarly, the increased incidence of intracellular vesicles in caput III occurred independent of granulomas, and it has been reported that the vacuolated epithelium, similar to that seen in Fig. 5B, precedes granuloma formation (Itoh et al. 1999). In any event, similar observations to these have been reported by others in the context of dysfunctional fluid homeostasis, and collectively support our assertion that the SED1-null epididymis is defective in regulating the luminal fluid.

Many proteins that regulate the composition of the luminal fluid are expressed and localized normally in the SED1-null epididymis

We therefore considered possible mechanisms whereby loss of SED1 could contribute to this aberrant physiology. Analogous to the proximal convoluted tubule of the kidney, fluid reabsorption and pH regulation in the proximal epididymis require proper expression, localization, and function of a broad spectrum of ion transporters, membrane pumps and channels, and enzymes that facilitate acid-base chemistry. Some epididymal models, including the ER alpha knockout and the *c-ros* knockout, show defects in the expression and localization of sodium/hydrogen exchangers at the apical plasma membrane of epithelial cells in the efferent ducts and initial segment, respectively (Yeung et al. 2004; Zhou et al. 2001). Since SED1 is thought to influence intracellular vesicle formation and/or secretion (Oshima et al. 2002), we considered the possibility that loss of SED1 may affect the proper targeting and/or turnover of fluid regulatory proteins at the apical and/or basolateral membranes.

We identified a subset of these proteins with known expression in the mouse or rat epididymis and characterized their expression and localization throughout wildtype and SED1-null epididymides. Proteins examined by western blot analysis and immunocytochemistry include: NHE2, NHE3 sodium/hydrogen exchangers; NBC1 sodium/bicarbonate co-transporter; CIC3 and CIC5 chloride channels; AE2 anion exchanger; EAAC1 glutamate transporter; Aquaporin-9 (AQP-9) water channel; VATPase proton pump; CAII, CAXIV, CAIV carbonic anhydrases; Cx43 gap junction component; and ZO-1 tight junction component. The level of protein expression as judged by western blot analysis was found to be similar between wildtype and SED1-null tissue in all epididymal segments (initial segment shown in Fig. 6), although some proteins, such as CAXIV, are occasionally reduced in SED1-null samples. However, these differences are not consistently seen. Furthermore, despite their utility for Western blot analysis, not all antibodies proved useful for immunocytochemical studies in all segments, such as AE2, CAXIV, NHE2 and NHE3 (Fig. 6). In all instances, the antibody-specific reactivities were lost using irrelevant primary antibodies.

SED1-null epididymal pathologies are not the secondary consequence of defective upstream tissues or abnormal epididymal development

Given the lack of an obvious and reproducible molecular defect that could account for the SED1-null pathologies reported here, we considered the possibility that these pathologies result from abnormalities in the upstream tissues of the testis and/or the efferent ducts, as is the case for other epididymal models displaying similar pathology (Hess et al., 2000; Zhou et al., 2001). Although SED1 is expressed in the Golgi region of developing spermatocytes (Ensslin and Shur, 2003), SED1-null testes are intact and both the seminiferous tubules and spermatozoa appear morphologically normal (Fig. 7A). Both wildtype and SED1-null testes contain the expected cell types, including Leydig cells, Sertoli cells, spermatogonia, spermatocytes at various stages of differentiation, and sperm-filled lumens. Other structures, such as the rete testes, are also easily identified in the SED1-null, and appear normal.

Similarly, the efferent ducts of SED1-null males are morphologically normal (Fig. 7B), unlike that seen in the ER alpha knockout in which efferent duct morphology and function are compromised due to a lack of estrogen-dependent synthesis and localization of NHE3 sodium/hydrogen exchangers to the apical surface of efferent duct cells (Zhou et al. 2001). Together, these data suggest that the cause of epididymal spermatic granulomas detected in SED1-null males is intrinsic to the epididymis.

We considered the possibility that the SED1-null epididymal pathologies may be the secondary consequence of defective epididymal development and/or differentiation, as in the *c-ros* knockout (Sonnenberg-Riethmacher et al. 1996). All ten above-described epididymal regions are present in SED1-null epididymides (data not shown). Of particular interest is the initial segment, which expresses SED1 in numerous prominent localizations.

Morphologically, the epithelium of the initial segment consists of tall columnar principal cells featuring lengthy stereocilia that project into the lumen and provide an expanded membrane surface to which channels and transporters are localized (Badran and Hermo 2002). In the ER alpha knockout, both epithelial cell height and microvilli length are reduced in the dysfunctional epithelium of the efferent duct (Hess et al. 2000). Therefore, we quantified the epithelial cell height and stereocilia length for tubules in initial segments of six different wildtype and knockout epididymides. The average epithelial height of 38.1 ± 4.4 μm in wildtype is statistically similar to that in SED1-null, 38.6 ± 5.2 μm (Fig. 7C). Stereocilia are also similar: 14.2 ± 3.8 μm and 13.3 ± 2.9 μm in wildtype and SED1-null, respectively; however, the small (7%) decrease in stereocilia height seen in SED1-null cells borders on statistical significance ($p=0.049$). Nevertheless, these data suggest the SED1-null initial segment and caput regions are properly patterned and morphologically differentiated.

Finally, we examined the functional differentiation of the tissue. Endogenous beta-galactosidase activity has been used previously to identify epididymal regions that are improperly differentiated (Avram and Cooper 2004). Both wildtype and SED1-null epididymides illustrate the appropriate region-specific expression of beta-galactosidase, indicating that the knockout epithelium is functionally differentiated (Fig. 7D). This is supported by the proper apical localization of tight junction components (ZO-1), adherens junctions (E-cadherin, not shown), and the t-snare syntaxin 3 (Fig. 7E) (Cyr et al. 1999; Levy and Robaire 1999). Together, these data indicate that the SED1-null epididymis is properly patterned and differentiated, and that the appearance of normal junctional components suggests the emergence of spermatic granulomas is not the de facto result of a non-existent blood-epididymal barrier.

SED1 is associated with mouse epididymosomes

Given our inability to identify a clear underlying mechanism to account for the SED1-null pathologies, we considered the possibility that SED1 may influence luminal fluid homeostasis by virtue of its known secretion into the epididymal lumen. In many cell types, SED1 displays an unusual mode of secretion that involves the release of small 30–60 nm vesicles, or exosomes, resulting from the fusion of multivesicular bodies with the apical membrane, and/or the fragmentation of exosome-filled apical protrusions released into the epididymal lumen. Exosomes function, in part, as paracrine factors transducing long-range signals to distant cells, and are thought to play a critical role in the transfer of membrane-associated proteins and lipids on and/or off of sperm (Gatti et al. 2005; Hermo and Jacks 2002; Rejraji et al. 2006; Thimon et al. 2008). Epididymally-derived exosomes, or epididymosomes, have been reported to contain carbonic anhydrase and VATPase that may contribute to modification of the luminal fluid (Gatti et al. 2005; Thimon et al. 2008). We therefore determined if SED1 is associated with epididymosomes in the mouse, and whether the loss of SED1 leads to a consequent loss of epididymosomes, which may account for the alteration in luminal fluid homeostasis in the SED1-null.

Luminal fluid was isolated from the caput and cauda regions and resolved by ultracentrifugation, which results in an insoluble, transparent-yellow membrane fraction similar to that described by others (Gatti et al. 2005). Proteins associated with the vesicle fraction were separated by SDS-PAGE and immunoblotted for SED1. Consistent with a proteomic analysis reporting SED1 is a component of ram epididymosomes, the vesicle fractions of both caput and cauda regions are immunoreactive for SED1 suggesting it is also associated with mouse epididymosomes (data not shown) (Gatti et al. 2005).

To confirm that SED1 is associated with vesicles and not simply fragmented membranes created during the isolation protocol, fluid was collected from wildtype and SED1-null caput regions and applied to a continuous 15–45% sucrose gradient. Fractions were collected, and migration of unknown proteins and SED1 were monitored by silver stain and western blot, respectively. The sucrose gradient resolves a broad size spectrum of vesicles, ranging from small secretory vesicles to organelles such as endosomes. Silver staining reveals that the overall protein profile changes along the gradient (data not shown), and immunoblotting indicates SED1 is concentrated in gradient fractions known to contain small vesicles, and to a lesser extent with larger membranes, including plasma membrane fragments and endosomes (Fig. 8) (Clift-O'Grady et al. 1998). Vesicle fractionation was repeated using a continuous 5–25% glycerol gradient, which offers higher resolution of the small vesicle population. Immunoblotting reveals a peak of SED1 activity across several fractions in the center of the gradient (Fig. 8), which are of similar size to 30–60 nm synaptic vesicles isolated from PC12 cells (Clift-O'Grady et al. 1998; Clift-O'Grady et al. 1990). In agreement with these data, mouse epididymosomes have been estimated to range in size from 20–200 nm (Rejraji et al. 2006). The distribution of SED1 across both sucrose and glycerol gradients is highly reproducible, and only background immunoreactivity (non-specific reactivity against input material that remains at the top of the gradient) is detected in SED1-null preparations.

We next investigated the presence of 30–60 nm epididymosomes in SED1-deficient fluid. Epididymosomes contain a wide range of membrane-associated components derived from epididymal cells by apocrine secretion, some of which that may serve to identify epididymosomes in SED1-null fluid (Gatti et al. 2005; Thimon et al. 2008). We made the fortuitous observation that the membrane protein Cx43 migrated coincident with SED1-containing epididymosomes, making it a useful reagent for tracking 30–60 nm vesicles in SED1-deficient fluid. Luminal fluid was collected from wildtype and SED1-null epididymides, and resolved in parallel on glycerol gradients. Immunoblotting for Cx43 reveals that both genotypes feature similar 30–60 nm vesicle populations (Fig 8). It is not clear if small variations in the profile of Cx43 between wildtype and SED1-null blots are the result of minor variation between preparations, or physiologically relevant. Nonetheless, the loss of SED1 does not prevent the production or secretion of all 30–60 nm vesicles.

CONCLUSIONS

The data presented here and in previous reports illustrate that SED1 is secreted both apically and basolaterally by principal cells of the initial segment (Raymond and Shur 2009; Ensslin and Shur 2003). The apically secreted SED1 interacts with sperm and facilitates sperm binding to the egg coat, whereas the basolaterally deposited SED1 may facilitate principal cell adhesion by serving as a ligand for α_v -containing integrins. The loss of SED1 leads not only to an inability of sperm to bind and fertilize eggs, but also to a failure to properly regulate the epididymal fluid as well as breakdown of the epididymal epithelium, ultimately leading to spermatoc granulomas. Although provocative in many respects, the data reported here do not indicate an obvious SED1-dependent pathway that would lead to spermatoc granulomas or defective luminal fluid homeostasis in SED1-deficient animals. One possible

model is that SED1 binding to α_v integrins during epididymal cell adhesion elicits integrin-dependent regulatory pathways. In a similar manner, the SED1 homolog, Del1, binds α_v integrins and activates transcription factors and subsequent protein synthesis in a model for vascular recovery following ischemia (Ho et al. 2004). Alternatively, the membrane-binding C-terminal domains of SED1 may affect the transport and/or targeting of intracellular vesicles to various intracellular domains, or facilitate the secretion of epididymosomes that are thought to be critical for luminal fluid homeostasis and sperm maturation. At this time, however, it is not clear if SED1 is found on a specific subset of epididymosomes that are absent in the SED1-null, and/or whether the loss of SED1 leads to a concomitant loss of critical enzymes required for regulation of the epididymal fluid milieu. These possibilities must await a comparative proteomic analysis of epididymosomes isolated from wildtype and SED1-null males.

MATERIALS AND METHODS

Mice

All experiments were conducted using wildtype and SED1-null congenic C57/B6 males, with the exception of syntaxin 3 staining and endogenous beta-galactosidase detection that were conducted with tissue isolated from the original B6/129 mosaic line.

Tissue preparation for histology and electron microscopy

Tissue for hematoxylin and eosin staining and immunohistochemistry was prepared using the following methods: 1) fresh, frozen cryosections fixed with 2% paraformaldehyde (PFA), 2) paraffin sections of tissue fixed by submersion in Bouin's solution, or 3) whole-animal cardiac perfusion with 4% PFA. Tissue for electron microscopy was freshly dissected and submersion-fixed in 2% glutaraldehyde buffered with cacodylate. Fixed tissue was stained with osmium and processed for semi-thin (1 μ m) and TEM sections by the Emory University School of Medicine Electron Microscopy Core.

Immunohistochemistry and antibodies

Sections were processed for staining and immunolocalization as described previously (Raymond and Shur 2009). Briefly, slides were hydrated, treated for endogenous peroxidase with 0.3% hydrogen peroxide, subjected to antigen retrieval in 10 mM sodium citrate, and processed for light-level or fluorescent immunolocalization. Analysis included use of commercially available antibodies against AE2 (sc-46710, Santa Cruz); AQP-9 (AQP91-A, Alpha Diagnostic); CAII (AB1828, Chemicon); CAIV (sc-25598, Santa Cruz); CAXIV (612140, BD Transduction); Cx43 (C6219, Sigma-Aldrich); EAAC1 (AB1520, Chemicon); NBC1 (AB3208, Chemicon); NHE2 (AB3083, Chemicon); NHE3 (MAB3134 & AB3085, Chemicon); syntaxin 3 (ab4113, Abcam); VATPase (sc-20943 & sc-31464, Santa Cruz); and ZO-1 (61-7300, Zymed). CIC-3/5 antibodies were described previously (Isnard-Bagnis et al. 2003). Signal was detected by one of two methods: 1) fluorescence using appropriate secondary antibodies conjugated with a fluorescent tag excitable at 595 nm, or 2) light level using an appropriate HRP-conjugated secondary antibody developed with DAB substrate.

Immunoblotting

Both epididymides from a single animal were subdissected and pooled by anatomical region to constitute one sample. Tissue was resolved in 20 mM Tris, RIPA buffer, or Cooper Buffer (Yeung et al., 2004) as indicated. Protein concentrations were determined by Bradford assay, and equal protein loads were diluted in reducing or non-reducing sample buffer and resolved by 10% SDS-PAGE. Following transfer to PDVF, blots were blocked, probed, stripped, and reprobed as described previously using antibodies listed above.

Determination of luminal pH ex vivo

A protocol for measuring luminal fluid pH was modified from a previous report (Yeung et al. 2004). Single pairs of age-matched wildtype and SED1-null mice were sacrificed by cervical dislocation, and epididymides were quickly removed and subdissected by anatomical region. Luminal contents were gently expressed onto Hydrion pH paper (Microfine, range 6.0–7.4; MF-1615). pH was determined by immediately comparing expressed luminal fluid to a series of buffered standards ranging from 6.8–7.4. Each epididymis was analyzed separately by two independent observers and data points from all measurements were averaged to generate a pH value for each experiment. Graphed data represents two independent experiments featuring a minimum of eight epididymides from each genotype.

Osmometry

Osmolality was measured as described (Joseph et al., 2010b) using a Wescor 5500 Vapro Vapor Pressure Osmometer (Wescor) outfitted with a 2- μ l sample holder to provide more accurate measurements of small sample volumes. Both caput epididymides (including initial segments) from a single animal were sub-dissected and pooled. Each testis was individually measured. To avoid contamination from blood, prominent vessels were cannulated with a needle and drained. The external capsule was removed and internal tubules were penetrated in multiple locations with a 27½ gauge needle, and luminal fluid contents were gently released through the holes and collected using Wiretrol II microcapillary tubes (Drummond Scientific). The collected fluid was centrifuged at $16,000 \times g$ for 5 minutes. The supernatant (2 μ l) was used to saturate a ¼-inch round Kimwipe sample disc (Kimberly Clark). Prior to each experimental run, the osmometer was calibrated using 2 μ l of both a 290 Osm/kg and 1000 Osm/kg standard. To increase sensitivity the low range switch was actuated for every sample measured. The results represent the average of seven wildtype caput segments, eight SED1-null caput segments, four wildtype and four SED1-null testicular samples. Comparisons of sample means were examined by unpaired Student's *t*-test.

Detection of endogenous beta-galactosidase activity

Fresh frozen sections (7 μ m) were prepared and fixed in 50/50 acetone/chloroform for 10 min. Sections were incubated with 1mg/ml x-gal diluted in staining buffer [5 mM $K_3Fe(CN)_6$, 5 mM $K_3Fe(CN)_6 \cdot 3H_2O$, 2 mM $MgCl_2 \cdot 6H_2O$ in PBS] at 37°C for 24 hours (in the dark). Slides were washed with PBS, counterstained with hematoxylin, and embedded with Aquatex for viewing at the light level.

Epithelial morphometrics

Paraffin-embedded sections were immunostained with anti-AQP-9 and counter-stained with hematoxylin to define basic cell structure and AQP-9-reactive stereocilia. The initial segment region was identified by morphology and proximity to the efferent ducts, and measurements were conducted using IP lab image analysis software calibrated with an optical micrometer. Cell heights of principal cells were measured parallel to cell-cell borders from the basement membrane to the apical membrane, and stereocilia were measured along the same trajectory from the apical membrane to the distal end of the cilia. Only tubules perpendicular to the plane of section (seen as circular, doughnut-like tubule cross-sections) were measured. All data points from each single epididymis were averaged and constitute one experiment. Graphed data represents six distinct experiments from each genotype.

Intracellular vesicle quantification

Tissue was fixed by whole animal cardiac perfusion using 2% glutaraldehyde buffered with cacodylate. Semi-thin sections were prepared by the Emory University School of Medicine

Electron Microscopy Core, and stained with toluidine blue. Images were taken of circular, caput III tubules identified in stained-sections by morphology and presence of multivesicular body-like structures, and vesicles were identified and counted based on the absence of stain. Average vesicle content was expressed as a function of total adluminal membrane perimeter for all counted tubules, also measured using IP lab.

Preparation of epididymosomes

Epididymosomes were collected as previously reported with some modifications (Rejzaji et al. 2006). Briefly, epididymides were removed, subdissected and pooled by anatomical region, and bathed in 250 ml of PBS with 1 mM EDTA, or previously defined “bud buffer” designed to mimic intracellular conditions (Clift-O’Grady et al. 1998). The external capsule and internal tubules were penetrated in multiple locations with a 27G ½ inch needle, and fluid contents were released through the poked holes and into the buffer by gentle massaging of the tissue. Sperm were removed by two serial centrifugations at $1000 \times g$ for 5 min. Total protein of the remaining sperm-free luminal fluid was determined by Bradford assay. The whole membrane fraction was collected from total luminal fluid by ultracentrifugation at $200,000 \times g$ for 1 hr; or 1 mg of total protein in 250 μ l of “bud buffer” was resolved through 10–45% sucrose or 5–25% glycerol gradients prepared as described previously (Clift-O’Grady et al. 1998; Salazar et al. 2004). Fractions were collected by gravity, and proteins were resolved by 10% SDS-PAGE, transferred to PDVF, and probed by western blotting.

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Figure 1.

The SED1-null epididymis exhibits cysts and spermatic granulomas, which occlude the exit of sperm and fluid resulting in dilation of upstream tubules. (A,B) Sections through pairs of cauda epididymides from wildtype (A) and SED1-null (B) 8.5 week-old males. Both cauda epididymides from the SED1-null male possess granulomas (arrowheads). SED1-null epididymides also exhibit other pathologies characteristic of sperm granulomas, including (C) epithelial hyperplasia, (D) infiltration of lymphocytes into the intertubular space. bars = 0.5 mm (A,B); = 50 μ m (C,D)

Figure 2.

SED1 localizes to numerous cellular domains in epididymal epithelial cells in vivo. (A) Diagram of the mouse epididymis illustrates ten sub-regions: caput I (initial segment), caput II–V, corpus VI–VII, cauda VIII–X (adapted from Johnston et al., 2005). (B,C) SED1 is highly expressed in the initial segment of the mouse epididymis, where it localizes to the para-Golgi region of principal epithelial cells. (C) Higher magnification views denote SED1 localization to the Golgi complex (arrow) and stereocilia (arrowhead) in Region I. SED1 is also expressed throughout the proximal caput: in the apical membrane and microvilli in region II, multivesicular bodies in region III (C, arrowheads), and punctate apical granules in region IV (C, arrowheads). SED1 is also found in a subset of clear cells (C, Regions VII & VIII, arrowheads) and basal cells (C, Region VII, arrow). (D) All SED1-null tissue

produces background immunoreactivity (initial segment shown). bars = 50 μm (B,D); = 20 μm (C)

Figure 3.

The SED1-null epididymis fails to acidify the luminal fluid and is hypo-osmotic. (A) Epididymides were isolated, subdissected, and luminal contents were extruded onto litmus paper in ex vivo pH assays. Fluid isolated at the distal end of the initial segment measured 7.03 ± 0.03 for wildtype and 7.27 ± 0.02 for SED1-null ($*p = 0.006$), a difference that persists in the cauda: 7.01 ± 0.02 and 7.32 ± 0.01 for wildtype and SED1-null ($**p < 0.001$), respectively. (B) The osmolality of 2 μ l of epididymal fluid was determined using a Wescor Vapor Pressure Osmometer. The data are presented using Box-and-Whisker plots, with the bars reflecting the range of readings, the shaded boxes representing 50% of the data, and the average osmolality represented by the horizontal bar. SED1-null initial segment fluid was 50 Osm/kg less than wildtype fluid (380 Osm/kg vs. 430 Osm/kg; $*p < 0.004$). In contrast, the osmolality of SED1-null testicular fluid was similar to wildtype levels (338 Osm/kg vs. 344 Osm/kg; $p = 0.7$). The results represent the average of 7 wildtype caput segments, 8 SED1-null caput segments, 4 wildtype and 4 SED1-null testicular samples.

Figure 4.

The SED1-null epididymis exhibits characteristics consistent with an elevated pH and hypo-osmotic luminal fluid. A) PFA-perfused, and B) Bouin's-fixed tissue were immunostained for VATPase, labeling narrow cells in the initial segment (A) and clear cells in the cauda (B). VATPase is consistently distributed throughout the apical domain of wildtype cells (A, bracket; B, arrowheads), while being concentrated at the adluminal surface of SED1-null cells (A, arrowheads), a localization that is consistent with alkaline luminal fluid. C) Transmission electron micrographs of wildtype and SED1-null principal epithelial cells. Mutant tissue contains a high frequency of swollen and damaged mitochondria (arrowheads)

that is indicative of increased sensitivity to hypo-osmotic milieu when processed by submersion fixation. bars = 10 μm (A); 50 μm (B) and 1.7 μm (C)

Figure 5.

The SED1-null epididymis possesses characteristics consistent with compensatory endocytosis. A) Semi-thin sections containing caput region III were prepared and stained with toluidine blue. Vesicles were quantified in wildtype (+/+, light gray: 276 vesicles in 15 tubule sections), SED1-null without apparent spermatogenic granulomas (-/-, dark gray: 932 vesicles in 11 tubules), and SED1-null tissue containing a caudal granuloma (-/-, black: 666 vesicles in 15 tubules). Total vesicle counts were normalized to 100 μm of adluminal membrane length. SED1-null tissue exhibits a greatly increased frequency of intracellular vesicles irrelevant of the presence of spermatogenic granulomas ($*p < 0.0001$). Results are representative of three parallel studies using paired wildtype and SED1-null animals. B) Sections of SED1-null epididymis stained with hematoxylin and eosin show an increased frequency of large vacuoles (arrowheads), a phenotype characteristic of abnormal fluid regulation and which is thought to precede granuloma formation in other systems. C) SED1-null narrow cells exhibit altered morphology. Toluidine blue stained semi-thin sections and paraffin-embedded sections immunolabeled for CIC3/5 illustrate abnormalities in SED1-null cells. Narrow cells of the initial segment typically feature a smooth apical cap that protrudes into the lumen (+/+); however, in the SED1-null (-/-) these cells have a rough, sheared morphology (compare arrowheads). bar = 50 μm (B); = 25 μm (C)

Figure 6. Many proteins that regulate the composition of the luminal fluid are expressed and localized normally in the SED1-null epididymis. Immunoblot and immunocytochemical analyses are shown for some of the proteins in the initial segment that regulate luminal fluid composition. Principle distributions for each protein in the initial segment following submersion-based fixation are as follows. SED1: secretory pathway and stereocilia, AQP9: stereocilia, CIC3/5: narrow cells, VATPase: narrow cells and principal cells to a lesser degree, CAII: narrow and principal cells, Cx43: plaques adjacent to cell borders, NBC: basolateral membranes, EAAC: stereocilia. Expression levels for all are similar between wildtype (+/+) and SED1-null (-/-) tissue as judged by immunoblot, and show similar localizations in both genotypes (wildtype tissue shown). Some antibodies were only able to produce immunoblot (AE2,

CAXIV, NHE2, NHE3) or immunolocalization (EAAC) signals. PAS staining and SED1-immunoreactivity are illustrated for reference purposes. bar = 50 μ m

Figure 7.

The SED1-null pathology is not a secondary consequence of abnormal testis or efferent ductal function or of abnormal epididymal development. Testes and efferent ducts were isolated from SED1-null males containing epididymal granulomas, sections were prepared and stained with hematoxylin and eosin. Testes and efferent ducts of SED1-nulls are intact and without visible pathologies. A) The rete testis (arrowheads) appears normal and the seminiferous tubules contain the expected cell types, including Leydig cells, and Sertoli cells, and all spermatogenic stages. B) The efferent ducts (ED), which lie immediately upstream of the initial segment (IS), are also morphologically normal, containing a polarized ciliated epithelium, similar to wildtype. C) Sections of initial segment were immunolabeled with anti-AQP9 antibody and counterstained with hematoxylin to identify stereocilia and cell borders. Cell height and stereocilia length were quantified in 105 wildtype and 101 SED1-null cells using image analysis software; wildtype (+/+) and SED1-null (-/-) show similar cell heights and stereocilia lengths (n=6). D) The initial segment (IS) of wildtype (+/+) and SED1-null (-/-) epididymides exhibit comparable levels of endogenous β-galactosidase activity (arrowheads), suggesting the mutant is functionally differentiated. E) Epididymal sections were immunostained with syntaxin-3 and ZO-1 at the indicated stages of development. Both proteins are properly localized to the appropriate apical domains (arrowheads) in wildtype (+/+) and SED1-null (-/-) epithelial cells, suggesting the mutant epithelium is properly polarized and forms a blood-epididymal barrier. bars = 100 μm (A); = 100 μm (B, left); 20 μm (B, right); = 100 μm (D); = 25 μm (E, syntaxin-3); = 50 μm (E, ZO-1)

Figure 8.

SED1 is associated with 30–60 nm epididymosomes. Mouse epididymosomes were purified from luminal fluid by sucrose and glycerol velocity gradients, and fractions were immunoprobed for SED1 or Cx43. When resolved by sucrose gradient centrifugation, SED1 is found in fractions that contain small secretory-like vesicles (#11+) as well as larger endosome-like vesicles (#5–7). Soluble material remains at the gradient top (#17–18). Likewise, when the same material is resolved on glycerol gradients, SED1 partitions to fractions containing 30–60 nm vesicles (#7–11). Non-specific immunoreactivity is present in the starting sample (input), which remains at the top of both wildtype and SED1-null gradients. Cx43 is found coincident with SED1 in wildtype material, and equivalent fractions of SED1-deficient material also label with Cx43, suggesting SED1-null luminal fluid contains 30–60 nm epididymosomes.