Cystatin-related epididymal spermatogenic subgroup members are part of an amyloid matrix and associated with extracellular vesicles in the mouse epididymal lumen

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Submitted on September 22, 2015; resubmitted on June 6, 2016; accepted on July 13, 2016

STUDY QUESTION: Do the CRES (cystatin-related epididymal spermatogenic) subgroup members, including CRES2, CRES3 and cystatin E2, contribute to the formation of a nonpathological, functional amyloid matrix in the mouse epididymal lumen?

SUMMARY ANSWER: CRES2, CRES3 and cystatin E2 self-assemble with different aggregation properties into amyloids in vitro, are part of a common amyloid matrix in the mouse epididymal lumen and are present in extracellular vesicles.

WHAT IS KNOWN ALREADY: Although previously thought only to be pathological, accumulating evidence has established that amyloids, which are highly ordered protein aggregates, can also carry out functional roles in the absence of pathology. We previously demonstrated that nonpathological amyloids are present in the epididymis; specifically, that the reproductive cystatin CRES forms amyloid and is present in the mouse epididymal lumen in a film-like amyloid matrix that is intimately associated with spermatozoa. Because the related proteins CRES2, CRES3 and cystatin E2 are also expressed in the epididymis, the present studies were carried out to determine if these proteins are also amyloidogenic in vitro and in vivo and thus may coordinate function with CRES as an amyloid structure.

STUDY DESIGN, SAMPLES/MATERIALS, METHODS: The epididymides from CD1 and Cst8 (CRES)129SvEv/B6 gene knockout (KO) and wild-type mice and antibodies that specifically recognize each CRES subgroup member were used for immunohistochemical and biochemical analyzes of CRES subgroup proteins. Methods classically used to identify amyloid, including the conformation-dependent dyes thioflavin S (ThS) and thioflavin T (ThT), conformation-dependent antibodies, protein aggregation disease ligand (which binds any amyloid independent of sequence) and negative stain electron microscopy (EM) were carried out to examine the amyloidogenic properties of CRES subgroup members. Immunofluorescence analysis and confocal microscopy were used for colocalization studies.

MAIN RESULTS AND THE ROLE OF CHANCE: Immunoblot and immunofluorescence analyzes showed that CRES2, CRES3 and cystatin E2 were primarily found in the initial segment and intermediate zone of the epididymis and were profoundly downregulated in epididymides from CRES KO mice, suggesting integrated functions. Except for CRES3, which was only detected in a particulate form, proteins were present in the epididymal lumen in both soluble and particulate forms including in a film-like matrix and in extracellular vesicles. The use of amyloid-specific reagents determined that all CRES subgroup members were present as amyloids and colocalized to a common amyloid matrix present in the epididymal lumen. Negative stain EM, dot blot analysis and ThT plate assays showed that recombinant CRES2, CRES3 and cystatin E2 formed amyloid in vitro, albeit with different aggregation properties. Together, our studies demonstrate that a unique amyloid matrix composed of the CRES family of reproductive-specific cystatins and cystatin C is a normal component of the mouse epididymal lumen and may play a functional role in sperm maturation by coordinating interactions between the luminal fluid and spermatozoa.

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**Introduction**

CRES (cystatin-related epididymal spermatogenic) (CstB) is the defining member of a reproductive-specific subgroup within the family 2 cystatins of cysteine protease inhibitors (Turk and Bode, 1991; Cornwall and Hsia, 2003). All eight CRES subgroup members are primarily expressed in the testis and/or epididymis implying specialized functions during spermatogenesis and epididymal sperm maturation (Cornwall et al., 1992; Cornwall and Hsia, 2003; Frygelius et al., 2010). Subgroup members also lack consensus sites for cysteine protease inhibition suggesting their biological functions are likely distinct from the typical family 2 cystatins such as cystatin C. Indeed, CRES did not inhibit cysteine proteases in vitro but instead inhibited the serine proteases prohormone convertase 2 and prohormone convertase 4, suggesting a role in the regulation of proprotein processing (Cornwall et al., 2003; Mishra et al., 2012). In other studies, CRES was shown to exhibit antimicrobial activity suggesting protective functions against pathogens (Wang et al., 2012).

In the testis, CRES is expressed in round and elongating spermatids and is present in the sperm acrosome and associated with the outer dense fibers in the sperm tail, while in the epididymis CRES is synthesized and secreted into the lumen by the principal cells in the initial segment epithelium (Cornwall and Hann, 1995; Syntin and Cornwall, 1999; Ferrer et al., 2013). Mice lacking the CRES gene exhibit fertility defects due to an inability of sperm to undergo the acrosome reaction (Chau and Cornwall, 2011). The loss of CRES also resulted in an age-dependent lysosomal storage disease-like phenotype in the epithelial cells of the epididymis with an accumulation of dense amorphous material in the epididymal lumen of aged mice (Parent et al., 2011). Together, these studies indicate important roles for CRES in fertilization and epididymal function.

Cystatin C, a ubiquitously expressed family 2 cystatin, inhibited amyloid β fibril formation in vitro and in vivo inhibited the deposition of amyloid β in several amyloid precursor protein mouse models, suggesting it may play a protective role in Alzheimer’s disease (Sastre et al., 2004; Mi et al., 2007; Selenica et al., 2007; Tizon et al., 2010). Interestingly, cystatin C itself also self-aggregates and forms amyloid fibrils in vitro suggesting that the normal functioning of cystatin C may be in its aggregated state (Wahlbom et al., 2007). Similar to cystatin C, CRES is aggregation-prone and will self-assemble into oligomeric and fibrillar amyloid structures (von Horsten et al., 2007). Furthermore, within the epididymal lumen, CRES is present in a fibrillar-like amyloid matrix that is intimately associated with the maturing spermatozoa suggesting that CRES amyloid may carry out a biological role and thus is defined as a functional amyloid (Whelly et al., 2012).

Amyloids are proteins that self-assemble and form highly ordered cross-β-sheet fibrillar structures that typically are pathological entities associated with neurodegenerative diseases including Alzheimer’s and Parkinson’s disease and prionopathies such as Creutzfeldt-Jakob disease. Accumulating evidence, however, has established that some proteins self-assemble into amyloid fibrils that carry out biological roles in the absence of pathology and are known as functional amyloids (Fowler et al., 2007; Pham et al., 2014). In mammals, these include pre-melanosome protein (PMEL), whose amyloid functions as a scaffold for the synthesis of melanin, several hormones that are stored as amyloids in the pituitary gland, RIP1 and RIP3 (receptor-interacting proteins 1 and 3) amyloids involved in programmed necrosis and α-defensin that forms an amyloid net in the gut mucosa for trapping bacteria (Fowler et al., 2006; Maji et al., 2009; Chu et al., 2012; Li et al., 2012). In addition to our studies of CRES amyloid in the epididymal lumen, we have previously shown that the mouse egg zona pellucida is an amyloid, as is part of the mouse sperm acrosomal matrix, demonstrating that functional amyloids are a normal component of the reproductive tract with integral roles in sperm maturation and fertilization (Guyonnet et al., 2014; Egge et al., 2015). Although the mechanism(s) by which functional amyloids avoid pathology is not clear, presumably they form under controlled cellular conditions that minimize exposure of the cell to intermediate amyloid forms that can be cytotoxic (Bucciantini et al., 2002; Fowler et al., 2007).

Other members of the CRES subgroup include CRES2/cystatin E1 (Cst11), CRES3/TE-1 (Cst12), cystatin E2, cystatin T (Cst13), cystatin SC, testatin (Cst9) and CRES4/CstL1/Rcetv1/Rcetv2 (Tööhönen et al., 1998; Shoemaker et al., 2000; Li et al., 2002; Hsia and Cornwall, 2003; Xiang et al., 2008). Similar to CRES, CRES2, CRES3 and cystatin E2 are expressed by the principal cells in the mouse initial segment epididymal epithelium (Hsia and Cornwall, 2003; Li et al., 2003, 2005). The human

**LIMITATIONS, REASONS FOR CAUTION:** The structures examined in our studies were isolated from luminal fluid obtained by puncture of the epididymis and therefore we cannot rule out some contamination by epithelial cells. Although our studies show CRES family members are associated with extracellular vesicles, we have yet to determine if proteins are present on the surface or are within the vesicles. We also have not established if narrow/apical cells are the source of the CRES family extracellular vesicles. CRES and CRES2 have been previously found in the human epididymis and associated with spermatozoa; however, we have yet to determine if the human CRES subgroup proteins are amyloidogenic and if an amyloid matrix is present in the human epididymal lumen.

**WIDER IMPLICATIONS OF THE FINDINGS:** Understanding the regulation and biological roles of amyloids, such as the CRES subgroup amyloid matrix that functions without causing pathology, could have broad implications for understanding pathological amyloids including those associated with neurodegenerative diseases and prionopathies.

**LARGE SCALE DATA:** None.

**STUDY FUNDING AND COMPETING INTEREST(S):** This work was supported by NIH grants RO1HD033903 and RO1HD056182 to G.A.C. The authors declare there are no conflicts of interest.

**Key words:** cystatin / amyloid / epididymal protein / extracellular vesicles / sperm maturation
homologs of all CRES subgroup genes have been identified and the expression of four genes established, including CRES and CRES2 in the human testis and/or epididymis suggesting conserved functions in human reproduction (Wassler et al., 2002; Frygelius et al., 2010). However, except for studies showing that CRES2 and CRES3 are associated with human and mouse spermatozoa in the epididymis, respectively, little is known about the remaining CRES subgroup family members (Hamil et al., 2002; Li et al., 2005).

CRES2, CRES3 and cystatin E2 are coexpressed with CRES in the initial segment epididymal epithelium; therefore, we hypothesized that their functions may be interrelated and that they too are amyloid-forming proteins that contribute to the formation of the amyloid matrix in the epididymal lumen. Therefore, the objective of our studies was to characterize the CRES subgroup proteins CRES2, CRES3 and cystatin E2 in the mouse epididymis including examination of their amyloidogenic properties in vitro and in vivo. Our studies demonstrate that all CRES subgroup members form amyloid in vitro and in vivo colocalize in an amyloid matrix in the epididymal lumen suggesting they may carry out a coordinated biological role as a functional amyloid structure. We show also that a second population of each CRES subgroup member is present in extracellular vesicles in the epididymal lumen suggesting that CRES subgroup function in the epididymis may be multifaceted.

Methods

Animals

CD1 retired breeder male mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Cst8129SvEv/B6 gene knockout (KO) and wild-type mice were bred in house. Mice were maintained under a constant 12 h light/12 h dark cycle with food and water ad libitum. All animal studies were conducted in accordance with the NIH Guidelines for the Care and Use of Experimental Animals using a protocol approved by the Texas Tech University Health Sciences Center Institutional Animal Care and Use Committee.

Isolation of epididymal tissue and luminal fluid

Epididymides were sectioned into five regions following the designation by Jelinsky et al. (2007) with 1/2, initial segment/intermediate zone; 3/4, caput; 5, distal caput; 6/7, corpus and 8–10, cauda or into initial segment/caput (Segments 1–5) and corpus-cauda (Segments 6–10). The tissue sections were punctured in phosphate buffered saline (PBS) using a 26-G needle and luminal contents allowed to disperse for 15 min. The suspension containing spermatozoa and luminal proteins was centrifuged at 500 × g for 1 min to pellet spermatozoa and any epithelial cells (Pellet 1) and the supernatant removed and centrifuged again at 500 × g to remove any remaining cellular material. The supernatant representing the total luminal fluid protein (soluble and particulate) was either used directly in experiments or underwent differential centrifugation to separate out particulate material of varying molecular mass. This included centrifugation of the total luminal fluid first at 5000 × g for 10 min (Pellet 2), followed by centrifugation of the supernatant at 15 000 × g for 10 min (Pellet 3) followed by ultracentrifugation of the supernatant at 250 000 × g for 1 h (Pellet 4). All pellets were resuspended in PBS and stored on ice. In some experiments, after centrifugation to remove cellular material (Pellet 1), the supernatant was centrifuged at 250 000 × g for 1 h combining Pellets 2–4 into one high speed pellet. The final supernatant from the last centrifugation was designated as the supernatant fraction.

Epididymal tissue was washed with PBS and homogenized in radioimmunoprecipitation assay buffer (RIPA) (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 mM EDTA) using a polytron and centrifuged at 17 000 × g for 15 min to pellet insoluble material. The protein in the resulting supernatant was quantitated by bicinchoninic acid assay (BCA) assay (ThermoScientific, Rockford, IL, USA) and samples stored at −20°C until use.

Northern blot analysis

Total RNA was isolated from the epididymides from Cst8129SvEv/B6 and Cst8−/− mice using Trizol reagent (Invitrogen, Grand Island, NY, USA) following the manufacturer’s protocol. Equal microgram amounts of RNA were separated on a 1% agarose gel in borate buffer as described previously (Cornwall et al., 1992). Cst8 (CRES), Cst11 (CRES2), Cst12 (CRES3) and cystatin E2 cDNA probes were generated from purified cDNA insert using a random prime labeling method (Prime-It II; Agilent Technologies, Santa Clara, CA, USA). Cst3 (Cystatin C) served as a control. Equal loading of lanes was confirmed by ethidium bromide staining of RNA. After hybridization, the blots were washed twice in 1× SSC (0.15 M sodium chloride and 0.015 M sodium citrate) and 0.1% SDS at room temperature for 15 min and then twice at 65°C for 15 min before exposure to film.

Bacterial expression of recombinant proteins and amyloid formation in vitro

Recombinant His-tagged mouse CRES, CRES2, CRES3 and cystatin E2 proteins were expressed in bacteria and isolated from inclusion bodies in 6 M guanidine-Cl, 25 mM MES (2-(4-morpholino)-ethane sulfonic acid) as previously described (Cornwall and Hann, 1995). Following elution of the proteins from the nickel NTA agarose column, proteins were stored in 6 M guanidine-Cl, 25 mM MES, pH 4.5 at 4°C. Immediately prior to use, proteins were centrifuged through Centricon filters (30 kDa cut-off, Millipore, Billerica, MA, USA) to remove any preexisting aggregates. The filtrate was quantitated by BCA (ThermoScientific, Rockford, IL, USA) or nanodrop assay and diluted to 10 µM in 50 mM HEPES, 100 mM NaCl, pH 7.4. Samples were immediately examined for amyloid formation by thioflavin T (ThT) fluorescence, dot blot analysis and negative stain electron microscopy (EM), as described below.

ThT plate assay

Recombinant CRES subgroup proteins were diluted to 10 µM in 50 mM HEPES, 100 mM NaCl, pH 7.4, plus ThT (Sigma Chemical Co, St. Louis, MO, USA) added to 20 µM and fluorescence determined with a Synergy HT plate reader (BioTek, Winooski, VT, USA) with excitation at 450 nm and emission at 485 nm.

Dot blot analysis

To determine the specificity of antibodies raised against each CRES subgroup family member, 0.1 µg of each recombinant protein in 6 M guanidine-Cl, 25 mM MES, pH 4.5 were spotted on to polyvinylidene difluoride (PVDF, Millipore) membrane in a dot blot apparatus. Membranes were blocked with 3% milk in TBST (0.2% Tween-20) for 1 h at room temperature and incubated with 0.1 µg/ml affinity-purified rabbit anti-mouse CRES antibody previously generated in house (Cornwall and Hann, 1995), rabbit anti-mouse CRES2 and CRES3 antibodies commercially generated (Strategic Biosolutions, Newark, DE, USA) and used as whole serum at 1:10 000, protein A-purified rabbit
anti-human CD9 antibody (Sigma Chemical Co, St. Louis, MO, USA) were incubated with the membranes overnight at 4°C in 3% milk/TBST. The membrane strips were then washed in TBST and incubated with goat anti-rabbit horse-radish peroxidase (HRP) labeled secondary antibody (1:80 000, ThermoScientific, Rockford, IL, USA) for 2 h at room temperature followed by washing in TBST and exposure to SuperSignal West Pico chemiluminescent substrate (ThermoScientific, Rockford, IL, USA). Equal loading of proteins was confirmed by staining the membranes with colloidal gold.

To examine the amyloid populations in CRES subgroup proteins, 5 µg of each recombinant protein diluted into 50 mM HEPES, 100 mM NaCl, pH 7.4 buffer were spotted onto nitrocellulose (Biotrace, Pall Corp, Ann Arbor, MI, USA) in a dot blot apparatus. Membranes were blocked with 3% milk in TBST (0.05% Tween-20) for 1 h at room temperature and incubated with the anti-oligomer A11 and anti-fibrillar OC antibodies (Millipore, Billerica, MA, USA) at 1:3000 or 1:5000, respectively, in milk/TBST overnight at 4°C, as described previously (von Horsten et al., 2007). Membranes were washed in TBST and incubated with a goat anti-rabbit HRP-labeled secondary antibody (ThermoScientific, Rockford, IL, USA) (1:20 000) for 2 h at room temperature, washed 5x in TBST and incubated with chemiluminescent substrate (SuperSignal West Pico, ThermoScientific, Rockford, IL, USA).

**Negative stain EM**

Samples (5 µl) were spotted onto formvar/carbon coated 200 mesh nickel grids (Ted Pella, Redding, CA, USA) and stained with 2% uranyl acetate as previously described (Whelly et al., 2012).

**Immunoblot analysis**

Immunoblot was carried out as described previously after 15% Tris-glycine SDS-polyacrylamide gel electrophoresis (Criteron, Bio-Rad, Hercules, CA, USA) (von Horsten et al., 2007). An affinity-purified rabbit anti-mouse CRES antibody was used at 0.2 µg/ml, while the rabbit anti-mouse CRES2 and CRES3 antibodies were used as whole serum at 1:4000. The corresponding preimmune sera for CRES2 and CRES3 were also used at 1:4000. Protein A-purified rabbit anti-mouse cystatin E2 antibody, rabbit anti-human cystatin C antibody (Dako, Carpinteria, CA, USA) and rabbit anti-human CD9 antibody (Sigma Chemical Co, St. Louis, MO, USA) were used at 0.2 µg/ml.

**Filter trap assay**

Twenty micrograms of the high speed pellet (Pellets 2–4 combined) and supernatant generated from luminal fluid isolated from the different segments of the mouse epididymis were spotted onto prewet cellulose acetate membrane (Whatman OE66, 0.2 µm) in a dot blot apparatus and a vacuum applied. After several PBS washes, the membranes were placed in 3% milk/TBST (0.2% Tween-20) to block for 1 h at room temperature followed by incubation with rabbit anti-mouse CRES antibody (1 µg/ml), rabbit anti-mouse CRES2 antiserum (1:4000), rabbit anti-mouse CRES3 antiserum (1:4000) or rabbit anti-mouse cystatin E2 antibody (2 µg/ml) overnight at 4°C. The blots were washed 2x in TBST, incubated with goat anti-rabbit HRP-labeled secondary antibody (ThermoScientific, Rockford, IL, USA) (1:20 000) for 2 h at room temperature, washed 5x in TBST and incubated with SuperSignal West Pico chemiluminescent substrate (ThermoScientific, Rockford, IL, USA).

**Protein aggregation disease binding**

Luminal fluid isolated from the initial segment/caput epididymis (Segments 1–5) underwent differential centrifugation as described above. The supernatant from the 15 000 x g pellet was split into two tubes and spun at 250 000 x g for 1 h (Pellet 4). One pellet was resuspended in PBS and the other in 90% dimethylsulfoxide (DMSO) (100 µl) and extracted at room temperature for 90 min. Both samples were brought to 200 µl with PBS and samples incubated with the protein aggregation disease (PAD) ligands (Microsens Biotechnologies, London, UK) as previously described (Whelly et al., 2012).

**ThS/CRES subgroup colocalization in amyloid matrix**

Pellet 4 isolated from the initial segment/caput epididymis (Segments 1–5) was resuspended in PBS and 5 µl of sample were spread on to Superfrost Plus slides (ThermoScientific, Rockford, IL, USA) and dried overnight. Samples were stained with 0.05% ThS for 2 h in the dark followed by repeated water washes. For SDS treatment, Pellet 4 was incubated in solution with 1% SDS for 15 min at room temperature prior to drying onto slides and thioflavin S (ThS) staining. Slides were transferred to a humid plastic container and incubated with primary antibodies (1 µg/ml affinity-purified rabbit anti-mouse CRES antibody, 2 µg/ml protein A purified CRES2, CRES3 or cystatin E2 antibodies) and normal rabbit IgG (1–2 µg/ml) in PBST (0.1% Tween-20)/10% goat serum at room temperature for 1 h followed by incubation at 4°C overnight. The slides were washed with PBST 5x and incubated with a goat anti-rabbit Alexa Fluor 594 conjugated secondary antibody (Invitrogen, Grand Island, NY, USA) (1:250) in PBST/10% goat serum for 2 h at room temperature in the dark. Slides were washed 5x in phosphate buffered saline-Tween-20 (PBST). 1x water and mounted with VectaMount AQ (Vector Laboratories, Burlingame, CA, USA). Slides were examined using a Zeiss microscope equipped with epi-fluorescence with excitation at 425/40 nm and emission at 475 nm for ThS and 560/40 nm excitation and 610 nm emission for Alexafluor 594.

To optimize detection of the various CRES subgroup proteins, slight modifications of the protocol were carried out. For CRES, samples were fixed with 3.7% formaldehyde in PBS for 30 min at room temperature prior to addition of antibody. SDS treated samples also were fixed with 3.7% formaldehyde in PBS and then incubated with 1% Triton X-100 for 3 min after which the slides were washed three times (3x) in PBST prior to adding antibody. For CRES3, samples were exposed to 430 mM NaCl for 20 min at room temperature followed by two water washes prior to adding antibody. SDS treated samples were fixed with 3.7% formaldehyde in PBS prior to antibody. For cystatin E2, optimal detection was observed when the ThS staining was carried out after incubation with the cystatin E2 antibody.

For colocalization experiments, protein A purified rabbit anti-mouse CRES2 and CRES3 IgGs and corresponding preimmune IgGs were labeled with Alexa 594 and Alexa 488, respectively, using the Readlink labeling kit (Bio-Rad) following the manufacturer’s instructions. Alexa conjugated CRES2 and CRES3 antibodies were then incubated with nonconjugated CRES and cystatin E2 antibodies in double-staining immunofluorescence analysis. Briefly, Pellet 4 from the initial segment/intermediate zone (Segment 1/2) was exposed to 1% SDS for 15 min prior to drying on a slide overnight. Slides were washed with water followed by PBS and PBST containing 0.1% Tween-20 and incubated with the first primary antibody (2.5–5 µg/ml anti-CRES, anti-CD9, anti-cystatin C or anti-cystatin E2) in 10% heat-inactivated goat serum in PBST for 4–5 h at 4°C. Slides were incubated with anti-cystatin E2 overnight at 4°C. Normal rabbit IgG served as a control antibody. Slides were then washed 5x in PBST and incubated with Alexa 594 or Alexa 488 goat anti-rabbit secondary antibody in solution.
10% heat inactivated goat serum at 1:750 for 2 h at 4°C. Slides were washed 5x in PBST and then incubated with the second primary antibody (5 µg/ml Alexa 594-conjugated CRES2 or Alexa 488-conjugated CRES3) overnight at 4°C followed by 5x PBST washes, 1x water and mounting with Mowiol. Images were captured with a Nikon Ti-E confocal microscope. Controls included slides incubated with the first primary antibody followed by Alexa conjugated IgG as second primary antibody; normal rabbit IgG as first primary antibody followed by Alexa conjugated CRES2 or CRES3 as second primary antibody; and normal rabbit IgG and Alexa conjugated IgG as first and second primary antibodies, respectively.

**Immunohistochemical analysis of epididymal tissue sections**

Mouse epididymides were fixed with 4% paraformaldehyde or 10% formalin overnight at 4°C and embedded in paraffin. Sections were deparaffinized in xylene followed by rehydration in 100, 95 and 70% ethanol and endogenous peroxidase activity quenched by incubation in 3% hydrogen peroxide in methanol for 10 min at room temperature. Antigen retrieval was carried out by microwaving the tissue sections in 10 mM citrate buffer, pH 6 for 15 min on high followed by cooling at room temperature for 1 h. Slides were transferred to a humid plastic container and blocked in 10% goat serum/PBS for 20 min at room temperature followed by incubation with rabbit anti-mouse affinity-purified CRES antibody (2 µg/ml), rabbit anti-mouse CRES2 serum (1:500), rabbit anti-mouse CRES3 serum (1:500) or rabbit anti-human CD9 antibody (2 µg/ml) (Sigma Chemical Co., St. Louis, MO, USA) for 2 h at room temperature in 10% goat serum/PBS. Sections that were incubated with rabbit anti-mouse protein A purified cystatin E2 antibody (2 µg/ml) went overnight at 4°C. Control antibodies were normal rabbit IgG or preimmune sera. Sections were then washed 3x in PBS and incubated with a biotinylated goat anti-rabbit secondary antibody (1:200, Vector Labs, Burlingame, CA, USA) in 10% goat serum/PBS for 45 min. Sections were washed again 3x in PBS and the antibody detected using the Vectastain ABC kit (Vector Labs, Burlingame, CA, USA) with development of the signal using the 3,3′-diaminobenzidine chromogen (BioGenex, Fremont, CA, USA). Coverslips were mounted with Vectamount AQ.

For CRES subgroup and CD9 colocalization with H+V-ATPase, following antigen retrieval without cooling, slides were washed in water followed by PBS and then blocked in 10% goat serum for 1 h at room temperature followed by incubation with anti-CRES subgroup antibodies in 10% goat serum/PBS (5 µg/ml CRES and cystatin E2 and 1:1000 CRES2 and CRES3 antisera) or rabbit anti-human CD9 antibody (5 µg/ml Sigma) for 1 h at room temperature (cystatin E2 was incubated at 4°C overnight). Controls received normal rabbit IgG or preimmune serum. The slides were washed 3x in PBS and incubated with a goat anti-rabbit Alexa 488 secondary antibody (1:750 in 10% goat serum/PBS) for 1 h at room temperature. The slides were washed 3x in PBS and incubated with a chicken anti-human H+V-ATPase antibody (raised against amino acids 608–617, C-MQNAFRSLED of the V-ATPase subunit A, a generous gift from S. Breton, Harvard Medical School, MA, USA) at 1:200–1:400 for 2 h at room temperature, washed 3x in PBS then incubated with a goat anti-chicken Alexa 594 secondary antibody (1:750 in 10% goat serum/PBS) for 1 h at room temperature. Slides were washed 3x in PBS, followed by DAPI (0.72 µg/ml) for 5 min at room temperature, washed 3x in PBS, 1x in water and mounted with Mowiol. Images were captured with a Nikon Ti-E confocal microscope.

**Isolation of extracellular vesicles**

Extracellular vesicles were isolated from initial segment/caput epididymal luminal fluid (Segments 1–5) using the ExoQuick-TC precipitation solution (SBI, Mountain View, CA, USA). Briefly, luminal fluid was centrifuged at 3000 x g for 15 min to remove spermatozoa and other cellular material. The protein concentration of the resulting supernatant was determined by BCA assay (ThermoScientific, Rockford, IL, USA). Equal amounts of protein from CstB+/+ and CstB−/− samples were adjusted to 1 ml with PBS, 333 µl of ExoQuick reagent was added and extracellular vesicles isolated following the manufacturer’s protocol. The extracellular vesicles were resuspended in RIPA buffer and stored at −20°C until use.

**AmylPred2**

Primary sequences of mouse CstB (CRES) (UniProt P32766), Cst11 (CRES2) (UniProt Q9D629), Cst12 (CRES3) (UniProt Q9DAN8), cystatin E2 (NCBI NP_084236.1) and Cst3 (cystatin C) (UniProt P21460) were analyzed by AmylPred2, an algorithm for consensus prediction of amyloidogenic determinants in polypeptide sequences (http://aais.biol.uoa.gr/AMYLPRED2) (Tsolis et al., 2013). Briefly, AmylPred 2 uses the consensus, defined as the hit overlap of 5 of 11, different methods that are known or specifically designed to predict features related to the formation of amyloid fibrils. Included in the 11 methods are several established web-based tools for predicting amyloid including AGGRESCAN (Conchillo-Sole et al., 2007), Pafig (Tian et al., 2009) TANGO (Fernandez-Escamilla et al., 2004) and Waltz (Maurer-Stroh et al., 2010).

**Results**

**CRES subgroup expression in the mouse epididymis**

To examine the distribution of CRES2, CRES3 and cystatin E2 proteins along the epididymis, immunoblot analysis was carried out on tissue and luminal fluid isolated from different segments of the epididymis using rabbit anti-mouse CRES2 and CRES3 antibodies that were commercially generated and a rabbit anti-mouse E2 antibody (Fig. 1A–D). Both anti-CRES2 and anti-CRES3 antibodies recognized proteins of the expected molecular weight (~14 kDa) that were not detected with their respective control preimmune serum (PI) (Fig. 1B). Furthermore, except for a very slight binding of anti-CRES2 antibody to CRES, anti-CRES3, anti-cystatin E2 and anti-CRES antibody specifically recognized their antigens and did not cross-react with other family members as shown by dot blot analysis of recombinant proteins (Fig. 1C). The CRES subgroup antibodies also did not cross-react with cystatin C (Fig. 1C).

Similar to what we previously observed with CRES, which is shown for comparison, CRES2, CRES3 and cystatin E2 were predominantly in the tissue and luminal fluid from the initial segment/intermediate zone with little to no protein detected in regions distal to the caput epididymis (Segment 5) (Fig. 1D) (von Horsten et al., 2007). In addition to the 14 kDa cystatin E2 protein, an ~20 kDa isof orm was also detected which may represent cystatin E2 that is posttranslationally modified or is translated from an alternatively spliced mRNA (Li et al., 2003). For comparison, cystatin C, a ubiquitously expressed family 2 cystatin, was examined and exhibited a pattern that was very different from the CRES subgroup members with levels increasing from the caput to the cauda epididymis (Fig. 1D). The almost identical distribution of CRES, CRES2, CRES3 and cystatin E2 in the epididymis suggests a common or related function among subgroup members.

To examine this further, we performed immunoblot analysis of CRES subgroup members in luminal fluid isolated from the epididymides of the CRES wild-type (CstB+/+) and CRES KO (CstB−/−) mice.
In addition to the expected loss of CRES, the absence of the CRES gene also resulted in an almost complete disappearance of CRES2 and CRES3 and a substantial reduction in cystatin E2 in the epididymal fluid from Cst8\(^{-/-}\) mice (Fig. 2A). In contrast, cystatin C levels were unaffected in the Cst8\(^{-/-}\) epididymis. Northern blot analysis of total RNA isolated from the epididymides from Cst8\(^{+/+}\), Cst8\(^{+-}\) and Cst8\(^{-/-}\) mice showed a large decrease in CRES2, CRES3 and cystatin E2 mRNA levels in Cst8\(^{-/-}\) epididymides indicating the loss of the corresponding protein from the epididymal fluid was due to the downregulation of gene expression and not a result of altered protein expression or localization (Fig. 2B).

**Cellular localization of CRES subgroup members**

Immunohistochemical analysis was next carried out to examine the localization of the CRES subgroup members in the epididymis. In addition to staining in principal cells, CRES was present in the initial segment lumen in densely stained aggregates (Fig. 3 CRES a), while in other tubules CRES was present in the lumen in a seemingly nonaggregated form (Fig. 3 CRES b). In the adjacent intermediate zone, a transition region between the initial segment and caput, CRES was detected within apical blebs that contained numerous microvesicles and that extruded from the surface of what appeared to be narrow/apical cells (Fig. 3, CRES c, arrow). Within the lumen of these tubules, CRES was associated with numerous small extracellular vesicles, \(\sim 1000\) nm in size, that extruded from the surface of what appeared to be narrow/apical cells (Fig. 3, CRES c–e). Similar results were observed with CRES2, CRES3 and cystatin E2 (Fig. 3). All three CRES subgroup proteins were primarily localized...
to the initial segment and intermediate zone epithelium with strong
immunostaining in aggregate structures in the initial segment lumen
and in extracellular vesicles in the lumen of the intermediate zone and
caput (Fig. 3, CRES2 a–f, CRES3 a–f, cystatin E2 a–f). While CRES2
and CRES3 were also present in apical blebs of putative narrow/apical
cells, cystatin E2 was only occasionally found in these cells (Fig. 3,
cystatin E2 c, arrow). CRES3 also displayed an unusual checkerboard
pattern in the intermediate zone due to strong immunostaining in
some principal cells (Fig. 3). Some CRES2, CRES3 and cystatin E2 was
also present within the principal cells from the caput through to the
cauda, including localization in the nucleus; however, in the lumen, all
three proteins were primarily associated with extracellular vesicles
~1000 nm in size that were present along the epididymal tubule (Fig. 3).

The presence of extracellular vesicles is well established within the
epididymal lumen including a distinct CD9 positive population that
preferentially transfers molecules to maturing spermatozoa (Caballero
et al., 2013). Therefore, we examined CD9 localization in the epididy-
mos including determining if the apical blebs of the narrow/apical cells
in the intermediate zone contained CD9. As shown in Fig. 3, CD9
immunoreactivity was present within the epithelium from the initial
segment to the cauda region. In the intermediate zone CD9 localized
to apical vesicle-containing blebs in possible narrow/apical cells, as
was observed for the CRES subgroup proteins (Fig. 3, CD9 c). A
checkerboard staining of CD9 was also observed in the caput region.
CD9 positive extracellular vesicles of varying size (~1000 nm) were
also detected in the lumen from the caput to the cauda.
Colocalization of CRES subgroup members in H^+V-ATPase expressing cells

Immunofluorescence analysis using an anti-H^+V-ATPase antibody, a marker for the narrow/apical/clear cells in the epididymis, was next carried out to establish if the cells that showed CRES subgroup immunostaining in apical microvesicle-containing blebs were indeed narrow/apical cells (Breton et al., 1999; Hermo et al., 2000). As shown in Fig. 4, using confocal microscopy we determined that CRES, CRES2 and CRES3 were detected in cells that showed H^+V-ATPase immunostaining at the cell surface and that possessed apically localized nuclei, both of which are characteristics of epididymal narrow cells and apical cells. In many of these cells, we also observed the typical narrowing of the cell as it extended down to the basement membrane, another distinctive feature of narrow cells (Hermo et al., 2000). H^+V-ATPase was primarily associated with the narrow/apical cell surface, as expected for a membrane-associated protein, while the CRES subgroup proteins were distributed throughout the cell. Examination of cells in the initial segment, intermediate zone and caput regions showed that while the majority of H^+V-ATPase positive cells contained CRES, CRES2 and CRES3, fewer H^+V-ATPase cells contained cystatin E2. Similarly, few H^+V-ATPase positive cells contained cystatin C. Colocalization experiments showed that CD9 was also present in the H^+V-ATPase positive cells including in the apical microvesicle containing blebs suggesting that a population of CD9 positive extracellular vesicles may originate from the narrow/apical cells. Indeed, for all the CRES subgroup antibodies examined, immunoreactivity was also associated with extracellular vesicles in the epididymal lumen.

Presence of CRES subgroup members in isolated extracellular vesicles

To confirm the presence of the CRES subgroup members in vesicles, we isolated extracellular vesicles from the initial segment and caput (Segments 1–5) epididymal luminal fluid from Cst8^{+/+} and Cst8^{-/-} mice using the ExoQuick reagent followed by immunoblot analysis to detect CRES subgroup proteins. As shown in Fig. 5, CRES, CRES2 and cystatin E2 were detected in vesicles isolated from the epididymal fluid from Cst8^{+/+} but not Cst8^{-/-} mice. CRES3, however, was not detected in the isolated vesicles by immunoblot. CD9 was present in extracellular vesicles isolated from both the Cst8^{+/+} and Cst8^{-/-} epididymal fluid.

Distribution of CRES subgroup members in luminal particulate fractions

To examine the distribution of CRES subgroup members between the soluble and particulate fractions, luminal fluid from segments 1/2 underwent ultracentrifugation and supernatant and pellet fractions were examined by immunoblot and filter trap assays. As shown in Fig. 6A by immunoblot, CRES, CRES2 and cystatin E2 were present in the luminal fluid in both the soluble and particulate fractions, while CRES3 was only present in the particulate fraction. Filter trap assays revealed that in the pellet (P), a proportion of each CRES subgroup protein was present in large structures that were trapped on the filter and that these structures were present in the lumen from the caput to the cauda epididymidis (Fig. 6B). Very little protein in the supernatant (S) was trapped on the filter suggesting that in this fraction most of the protein is soluble. Although the filter trap assay showed that CRES

Figure 4 CRES subgroup members are present in H^+V-ATPase expressing narrow/apical cells. Mouse epididymal tissue sections were incubated with anti-CRES subgroup antibodies (green) followed by anti-H^+V-ATPase antibody (red) for double-labeled immunofluorescence analysis and images captured using a Nikon Ti-E confocal microscope. Blue: DAPI staining of nuclei. CRES3 arrow, principal cell. Arrowheads, narrow/apical cells containing cystatin E2 and cystatin C. Scale bar, 20 µm. The intermediate zone is shown for all antibodies except for cystatin E2 staining, which is shown in the initial segment.
subgroup members were present in the lumen of the corpus and cauda, our inability to detect them by immunoblot (Fig. 1D) may be because the proteins are in high-molecular-mass structures that are too large to enter standard SDS-PAGE gels, as was previously shown for CRES (von Horsten et al., 2007).

CRES subgroup proteins form amyloid in vitro and in vivo

Using approaches that are classically used in the field to identify amyloid, we carried out studies to determine if CRES2, CRES3 and cystatin E2 were amyloid-forming proteins. Recombinant CRES2, CRES3 and cystatin E2 in 6 M guanidine-Cl were diluted into aqueous buffer which immediately caused the proteins to self-assemble into large aggregate structures. Although Centricon filtration should have removed most of the preexisting aggregates we cannot rule out that some aggregates may have reformed prior to dilution of proteins into aqueous buffer. Incubation of the aggregated proteins with ThT, a conformation-dependent dye that exhibits a spectral shift upon binding to the characteristic cross-β-sheet structures of amyloid, showed that all CRES subgroup proteins possessed amyloid structures, with CRES3 being the most amyloidogenic and possessing the most cross-β-sheet structure (Fig. 7A). The same aggregated protein samples used for the ThT assays were also spotted on to grids and examined by negative stain EM. As shown in Fig. 7B, all CRES subgroup proteins possessed structures characteristic of amyloid including bundles of fibrils, protofibrils with a beads-on-a-string appearance (CRES), large branched polygons (CRES3) and bundles of fibrils/films assembling into higher ordered structures (CRES2, cystatin E2). Recombinant proteins diluted into aqueous buffer were also examined for amyloid by spotting the samples on to nitrocellulose and carrying out dot blot analysis using conformation-dependent antibodies that recognize the immature forms of amyloid (anti-oligomer A11 antibody) and the mature forms of amyloid (anti-fibrillar OC antibody) (Kayed et al., 2010). The anti-fibrillar OC antibody bound to all CRES subgroup proteins suggesting the presence of amyloid fibrils, while A11 bound only to CRES and cystatin E2 suggesting they also contained earlier, less mature, forms of amyloid including protofibrils and oligomers (Fig. 7C).
We next analyzed the primary sequences of the mouse CRES subgroup in comparison with cystatin C, using the algorithm Amylpred2 to identify potential amyloidogenic sites throughout each protein. All CRES subgroup members possessed several sites that were predicted to be amyloidogenic, as indicated by blue highlighting and that were present in similar locations within each protein, supporting the in vitro data that all epididymal CRES subgroup members are amyloid-forming proteins (Fig. 7D). Similarly, cystatin C also possessed sites that were predicted to be amyloidogenic.
Studies were performed to determine if CRES2, CRES3 and cystatin E2 were also part of the epididymal luminal fluid amyloid matrix. Pellet 4 fraction generated from ultracentrifugation of luminal fluid from the initial segment/caput (Segments 1–5) was stained with ThS, a conformation-dependent dye that exhibits a strong yellow-green fluorescence upon binding to cross-β-sheet amyloid structures, followed by immunofluorescence analysis with the CRES subgroup antibodies. Pellet 4 was also exposed to 1% SDS for 15 min to help expose epididymal amyloids prior to ThS and immunofluorescence analysis. As shown in Fig. 8, all CRES subgroup proteins colocalized with the ThS positive film-like matrix in Pellet 4. Furthermore, pretreatment of the amyloid film with SDS caused it to unwind into fibrillar matrices/fibrils that exhibited a large increase in ThS staining and immunoreactivity with the CRES subgroup antibodies, further supporting that CRES subgroup proteins are part of a highly ordered amyloid structure in the epididymal lumen.

CRES subgroup members colocalize in the luminal amyloid matrix

Double-staining immunofluorescence analyzes and confocal microscopy were performed to determine if the CRES subgroup members were present in the same film-like amyloid matrix in the epididymal lumen. To enhance detection of the CRES subgroup, the amyloid film was first exposed to 1% SDS to partially unwind the film into fibrils. As shown in Fig. 9, by pairing of different Alexafluor-conjugated primary antibodies, we determined that all CRES subgroup members as well as cystatin C were present in the same population of amyloid matrix in the epididymal lumen.

A second approach that was used to establish the presence of the CRES subgroup in the epididymal amyloid was to use the PAD ligand that will bind any amyloid independent of protein sequence followed by immunoblot analysis to detect the CRES subgroup members. In this experiment, Pellet 4 fraction was first treated with 90% DMSO for 90 min to disrupt hydrogen bonds and help unwind and expose amyloid structures prior to incubation with the PAD ligand (Loksztejn and Dzwolak, 2009). Following exposure to DMSO, all CRES subgroup proteins bound to the PAD ligand suggesting they are present in highly ordered amyloid structures within the epididymal lumen (Fig. 10). The presence of some CRES, CRES2 and cystatin E2 in the final supernatant obtained after ultracentrifugation to obtain Pellet 4 and the absence of CRES3 in the same supernatant, supports our earlier conclusion that CRES3 is the most highly ordered amyloid protein in the epididymal lumen.

Figure 8 CRES, CRES2, CRES3 and cystatin E2 colocalize with a ThS-positive amyloid matrix in the mouse epididymal lumen. Pellet 4 generated from the initial segment/intermediate zone LF was spread onto slides and stained with 0.05% ThS (yellow-green fluorescence) to detect amyloid followed by incubation with rabbit anti-mouse CRES, CRES2, CRES3 and cystatin E2 antibodies and a goat anti-rabbit Alexafluor 594 secondary antibody (red fluorescence). Samples of Pellet 4 were also incubated with 1% SDS and then spread onto slides and incubated with ThS and CRES subgroup antibodies as described above. Inset, negative control slides were incubated with normal rabbit IgG. Scale bar: all images, 5 um.
aggregation prone subgroup member and appears to be present solely in particulate form in the luminal fluid.

**Discussion**

**A functional amyloid matrix in the epididymal lumen**

The studies presented herein demonstrate that the epididymal CRES subgroup members are a family of amyloidogenic proteins that, together with cystatin C, contribute to the formation of a nonpathological, presumably functional, amyloid matrix within the epididymal lumen. In support of the hypothesis that amyloids are a normal component of the epididymal lumen are our previous studies showing by EM that 10 nm fibrils, typical of amyloids, were present in the particulate fraction isolated from epididymal fluid, as well as the observation that EM micrographs of mouse epididymis the lumen was filled with a fibrillar matrix that surrounded the maturing spermatozoa (Whelly et al., 2012; Cornwall and Hermo, unpublished observations). Furthermore, X-ray diffraction of a high-speed pellet containing the amyloid matrix revealed diffraction rings at 4.7 and 10 Å, characteristic of the cross-β-sheet structures of amyloid (Whelly et al., 2012). Although all methods that were used in our previous and current studies strongly indicate the epididymal structure is amyloid, it remains to be determined if it possesses unique features that distinguish it from pathological amyloids. Indeed, because functional amyloids appear to be structurally indistinguishable from pathological amyloids, it is not clear how similar structures can elicit such diverse biological outcomes. This has raised the question of whether functional amyloids are truly amyloid or instead amyloid like. Solid state nuclear magnetic resonance (NMR), which is well adapted for the study of amyloids and a powerful method to study fibrils at atomic resolution, however, has revealed comparable β-sheet-rich structures in both functional and pathological amyloids suggesting that for an amyloid to be functional it may not need to have a unique structure (Petkova et al., 2005; Van Melckebeke et al., 2010; Hu et al., 2011; Li et al., 2012; Raveendra et al., 2013). Preliminary circular dichroism and solid-state NMR analysis of CRES3 suggested a soluble form with mixed secondary structure that became β-sheet rich in the aggregated state, consistent with a true amyloid (Wylie and Cornwall, unpublished observations).

Finally, because the amyloid matrix was isolated from luminal fluid obtained from the epididymis by tissue puncture, we cannot rule out the possibility of some cellular contamination. However, our ability to detect amyloid matrix in luminal fluid samples that showed little to no cellular contamination (Whelly et al., 2012), as well as the presence of fibrillar material in EM micrographs of the epididymal lumen, strongly suggest the amyloid matrix is a normal component of the epididymal lumen and is not a cellular contaminant or formed during its isolation.

**Mechanisms to control functional amyloid formation**

Proteins that form functional amyloids follow similar aggregation pathways to those that form pathological amyloids and therefore it is...
thought that functional amyloids form under controlled cellular conditions while pathological amyloidogenesis is uncontrolled. Several cellular mechanisms have been proposed to regulate functional amyloid assembly to avoid or minimize exposure of the cell to intermediate, oligomeric forms of amyloid that can be cytotoxic (Bucciantini et al., 2002). These mechanisms include restricting amyloidogenesis to within organelles such as secretory granules or melanosomes, the requirement for proprotein processing to trigger amyloidogenesis or interactions of related proteins to control fibril assembly, as occurs in Escherichia coli (Fowler et al., 2006; Wang and Chapman, 2008; Maji et al., 2009). The bacterial curli proteins determine when and where amyloidogenesis occurs since the CsgB amyloid functions as a nucleator on the cell surface that triggers the soluble CsgA protein to assemble into amyloid allowing the formation of an extracellular matrix (Chapman et al., 2002; Wang and Chapman, 2008). Our studies of both recombinant and in vivo protein showed that the CRES subgroup members exhibited different aggregation properties, with CRES being the least and CRES3 being the most amyloidogenic of the four proteins examined. It is possible that, similar to that which occurs in bacteria, interactions between CRES subgroup members could be a mechanism by which the epididymis controls the formation of amyloid in its lumen with CRES3 functioning as a nucleator to facilitate matrix assembly.

The rapid kinetics of CRES subgroup amyloidogenesis may also be a mechanism to control epididymal amyloid formation and avoid prolonged exposure of epididymal and sperm cells to cytotoxic amyloids. In vitro, CRES subgroup members rapidly formed fibrillar forms of amyloid within minutes of dilution from 6 M guanidine into nondenaturing buffer, times that were comparable to the aggregation rates of other functional amyloid forming proteins including PMEL, RIP1/RIP3 and CPEB (cytoplasmic polyadenylation element-binding protein) (Fowler et al., 2006; Li et al., 2012; Raveendra et al., 2013). In contrast, proteins/peptides such as Aβ and α-synuclein that form pathological amyloid often require days/weeks to form fibrils in vitro which in vivo could translate into increased exposure of cells to cytotoxic amyloid intermediates (Conway et al., 2000).

CRES subgroup members are in extracellular vesicles

A second population of each CRES subgroup protein was detected in extracellular vesicles ∼1000 nm in size that we propose are released from apical blebs or multivesicular bodies extruded from the narrow/apical cells that are prevalent in the intermediate zone. Narrow/apical cells were also present, but to a lesser degree, in the initial segment and caput epithelium. We refer to the narrow/apical cells collectively since it is not clear whether these cells represent distinct cell types or are one and the same (Adamali and Hermo, 1996; Breton et al., 1999). The extracellular vesicles containing CRES subgroup members persisted along the epididymal tubule into the cauda lumen. Although CRES3 was detected in extracellular vesicles by immunohistochemical and immunofluorescence studies, we were unable to detect CRES3 in isolated vesicles by immunoblot. This may be due to CRES3 being in higher ordered structures that are too large to enter standard SDS-PAGE gels or is present in a population of vesicles that were not isolated with the method used. Studies are currently ongoing to determine if the CRES family members colocalize in the same vesicle.

Exosomes, called epididymosomes in the epididymis, are a distinct population of extracellular vesicles that typically range from 30 to 120 nm and arise from the release of apical blebs from the epididymal principal cells (Hermo and Jacks, 2002). In other cell systems, exosomes are usually formed in the endosomal compartments that contain multivesicular bodies which then fuse with the plasma membrane to release the vesicles (Yanez-Mo et al., 2015). Larger vesicles up to 1000 nm have also previously been described in the rodent epididymal lumen, as were multivesicular bodies, suggesting there may be several populations of extracellular vesicles in the epididymal lumen that are released from distinct cell types by different mechanisms and that this may vary between species (Rejraji et al., 2006; Caballero et al., 2013). Our studies showing that the apical bleb in narrow/apical cells contained the tetraspanin CD9, a marker for exosomes and that CD9 positive vesicles were observed distal to the intermediate zone suggests that narrow/apical cells may also release exosomes but possibly by the more conventional mechanism involving endosomes and multivesicular bodies and that these vesicles in the epididymis may be larger in size. We cannot rule out at this time, however, that the presence of CRES subgroup members and CD9 in narrow/apical cells could also be a result of endocytosis. Finally, cystatin E2 and cystatin C, although present in luminal extracellular vesicles, were not routinely observed in the narrow/apical cells, in contrast to CRES, CRES2 and CRES3. It is possible these proteins may be in a distinct population of extracellular vesicles released from other epididymal cells such as the principal cells.

Figure 10 CRES subgroup proteins are present in epididymal amyloid. Pellet 4 isolated from mouse initial segment/caput epididymal LF (Segments 1–5) was resuspended in PBS (−) or 90% DMSO (+) and incubated for 90 min at room temperature followed by incubation with the PAD ligand. Proteins were eluted with 1× Laemmli buffer containing β-mercaptoethanol, separated by SDS-PAGE and examined for CRES subgroup proteins by immunoblot. Start, 10 μg LF prior to isolation of Pellet 4; End, 10 μg LF after isolation of pellet. DMSO, dimethylsulfoxide.
Functionally, extracellular vesicles transfer selected proteins to spermatozoa as part of the sperm maturation process (Sullivan, 2015). While the role of the extracellular vesicles in the transfer of CRES subgroup members to spermatozoa has not been established, both CRES2 and CRES3 have been detected in epididymal spermatozoa (Hami et al., 2002; Li et al., 2005; Muthusubramanian and Cornwall, unpublished observations). Also, although we have yet to establish if CRES subgroup members in extracellular vesicles are amyloid, it is possible that sequestering them in the vesicles could be a means to safely deliver amyloids to spermatozoa. Recently, it was shown that PMEL formed a functional amyloid in the intraluminal vesicles in endosomes and was found associated with the surface of exosomes in pigment cells suggesting that vesicles served as amyloid nucleating platforms and as a means to avoid toxicity inherent to PMEL amyloidogenesis (van Niel et al., 2015). We have yet to determine if the CRES subgroup proteins are associated with the extracellular vesicle surface or within the vesicle itself.

Functional role of CRES amyloids in the epididymal lumen

The significance of multiple CRES subgroup proteins contributing to the formation of an amyloid matrix and the function of the amyloid matrix itself has yet to be determined. Furthermore, it is not clear why most CRES subgroup members are present in both the amyloid matrix and extracellular vesicles as well as in a soluble form in the epididymal lumen. In bacteria, the amyloidogenic curli proteins assemble into an extracellular amyloid matrix that ultimately forms a biofilm. It is tempting to speculate that the CRES subgroup amyloid matrix in the epididymal lumen similarly functions as an interactive and protective infrastructure for the maturing spermatozoon, possibly organizing them into a community of cells to facilitate delivery of maturation-associated molecules via extracellular vesicles. Taken together, our studies show that functional amyloids are an integral part of the male reproductive tract and that further study of the normal function and assembly of these structures could provide an important insight into possible mechanisms of amyloid-associated pathologies including neurodegenerative diseases and prionopathies.

Acknowledgements

The authors would like to acknowledge Aveline Hewetson, Ph.D. for her assistance with the mouse colony, Sylvie Breton, Ph.D., Harvard Medical School, for the H⁺ V-ATPase antibody and Mike Griswold, Ph.D., Washington State University for the cystatin E2 antibody.

Authors’ roles

S.W. and G.A.C. conceived and designed the experiments. S.W., A.M., J.P., S.J., M.K.H. and G.A.C. performed the experiments. S.W., A.M. and G.A.C. analyzed the data and G.A.C. wrote the article.

Funding

National Institutes of Health (grants RO1HD033903 and RO1HD056182 to G.A.C.). The content of this report is solely our responsibility and does not necessarily represent the official views of the NIH. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Conflict of interest

None declared.

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