Oligomerization and Transglutaminase Cross-linking of the Cystatin CRES in the Mouse Epididymal Lumen

POTENTIAL MECHANISM OF EXTRACELLULAR QUALITY CONTROL^{*}\§

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CRES (cystatin-related epididymal spermatogenic), a member of the cystatin superfamily of cysteine protease inhibitors, is expressed in the epididymis and spermatozoa, suggesting specialized roles in reproduction. Several cystatin family members oligomerize, including cystatin C that forms amyloid deposits associated with cerebral amyloid angiopathy. Our studies demonstrate that CRES also forms oligomers. Size exclusion chromatography revealed the presence of multiple forms of CRES in the epididymal luminal fluid, including SDS-sensitive and SDS-resistant high molecular mass complexes. In vitro experiments demonstrated that CRES is a substrate for transglutaminase and that an endogenous transglutaminase activity in the epididymal lumen catalyzed the formation of SDS-resistant CRES complexes. The use of a conformation-dependent antibody that recognizes only the oligomeric precursors to amyloid, negative stain electron microscopy, and Congo Red staining showed that CRES adopted similar oligomeric and fibrillar structures during its aggregation as other amyloidogenic proteins, suggesting that CRES has the potential to form amyloid in the epididymal lumen. The addition of transglutaminase, however, prevented the formation of CRES oligomers recognized by the conformation antibody by cross-linking CRES into an amorphous structure. We propose that transglutaminase activity in the epididymal lumen may function as a mechanism of extracellular quality control by diverting proteins such as CRES from the amyloidogenic pathway.

As spermatozoa migrate through the long convoluted tubule known as the epididymis, they undergo maturation and acquire motility and fertility. Since sperm are synthetically inactive, the maturation process requires the interaction of sperm with proteins that are synthesized and secreted in a region-dependent manner by the epididymal epithelium. Following secretion, the fate of proteins in the epididymal lumen is varied. Some proteins bind to sperm and presumably affect sperm function directly, whereas others remain in the lumen throughout the length of the tubule (1, 2). Other proteins are present in the epididymal lumen for only a short time, suggesting that their continued presence may be detrimental to sperm maturation and/or epididymal cell functions, and thus selective mechanisms are in place for their removal.

CRES is the defining member of a reproductive subgroup of family 2 cystatins within the cystatin superfamily of cysteine protease inhibitors (MEROPS classification subfamily I25B) (3, 4). CRES is synthesized and secreted into the lumen by the epithelial cells in the most proximal part of the epididymis and then abruptly disappears from the lumen a short time later (5). In vitro CRES does not inhibit cysteine proteases but rather inhibited the serine protease prohormone convertase 2, suggesting an intracellular rather than an extracellular role for CRES (6). Although a function of CRES within the secretory pathway of the epididymal epithelial cells would make it dispensable once it was secreted into the luminal fluid, it is not clear why or how CRES is so rapidly removed from the lumen. One possibility is that an accumulation of CRES within the epididymal lumen may be inherently harmful to epididymal cellular function and/or sperm maturation.

The cystatins are one of several classes of proteins that are known to have an intrinsic propensity for cellular toxicity because of their ability to form amyloid (7–9). Most proteins fold into stable three-dimensional structures that allow their proper biological function. However, for a select group of proteins, if misfolding occurs, the proteins can oligomerize and form defined higher ordered structures that can lead to fibril formation and deposition of protein aggregates or amyloid that are associated with various diseases, including Alzheimer’s disease, Parkinson’s disease, and type II diabetes. The 20 or so proteins that are known to form amyloid seem to be unrelated, based on primary sequence, yet during their aggregation they will adopt common oligomeric and fibrillar structures that are detected with dyes such as Congo Red and thioflavin as well as with conformation-dependent antibodies (10, 11). Although amyloid fibrils were thought to be the causative form associated

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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with disease, more recent studies suggest that the oligomeric forms, precursors to the amyloid fibrils, are cytotoxic and may play the causal role in disease (12). Several cystatins have been shown to self-aggregate and form dimers and oligomers in vivo (13, 14). Moreover, cystatin C forms part of the amyloid deposits in patients with cerebral amyloid angiopathy (15), including that associated with Alzheimer disease (16), whereas the L68Q variant form of cystatin C forms amyloid deposits in the cerebral blood vessels of patients who suffer from hereditary cystatin C amyloid angiopathy and who die from hemorrhagic stroke by the age of 30 (17). There is a growing body of evidence suggesting that the tendency to aggregate under certain environmental conditions may be a common trait of all cystatins rather than a characteristic of individual family members, and therefore it is feasible that CRES may also have the propensity to form amyloid (18). To date, whether amyloid can form in the epididymal lumen and affect fertility is not known. However, amyloid fibrils have been found in the blood vessels of the testis and epididymis (19) and within the seminiferous tubules of the testis (20), and L68Q cystatin C amyloid deposits have been found in the testes of hereditary cystatin C amyloid angiopathy patients, suggesting the potential for reproductive pathologies as a result of amyloid formation (21).

Until recently, quality control mechanisms for removal of misfolded proteins have only been described intracellularly and include the rescue of proteins by chaperones and destruction by proteases. Presumably, mechanisms of protein folding control exist extracellularly as well; however, these have yet to be determined (22). A family of enzymes whose members are known to directly modulate protein aggregation and the formation of large molecular weight complexes of both soluble and particulate proteins and thus might contribute to protein quality control are the transglutaminases (TGases). 3 TGases are Ca\textsuperscript{2+} dependent enzymes that form stable protein cross-links through the formation of intra- and intermolecular ε-(γ-glutamyl) lysine bonds (23). The role of tissue-type TGase (tTGase) in the pathogenesis of amyloidogenic diseases is paradoxical in that some studies indicate a role for tTGase in enhancing protein aggregation, thus contributing to the disease, such as in Alzheimer disease (24, 25), whereas other reports suggest a protective effect of tTGase by virtue of its forming protein cross-links, thus preventing the self-assembly of an amyloidogenic protein into the characteristic oligomeric structure that is cytotoxic and associated with disease (26–28). Although the studies suggest that tTGase may act defensively against amyloidogenesis were carried out examining intracellular proteins (27), tTGase is also present extracellularly and as such may perform similar functions as a means of extracellular quality control.

Because of the active sperm maturation process that occurs within its lumen, the extracellular environment in the epididymis is unique from other organ systems and probably utilizes multiple surveillance/clearance mechanisms to protect against amyloid formation and the potential cytotoxicities that can be associated with these structures. In this report, we investigated whether CRES is present in the mouse epididymal lumen as high molecular mass oligomeric structures and thus may be a molecular model to examine amyloid formation and its control in the epididymis. We further determined whether an endogenous TGase activity in epididymal fluid may be involved in the oligomerization process and thus may be one mechanism of extracellular quality control.

**EXPERIMENTAL PROCEDURES**

**Materials**— Butyloxycarbonyl-L-(6-diazo-5-oxonorleucinyl)-Gln-Ile-Val-OMe (BOC-DON) and guinea pig liver tTGase were purchased from N-zyme BioTec GmbH (Darmstadt, Germany) and Sigma. Cystamine was from Sigma. All chromatography supplies and [1,4-\textsuperscript{14}C]putrescine dihydrochloride were purchased from GE Healthcare.

**Animals**— Mature retired breeder male CD-1 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Cres 129SvEv/B6 gene knock-out 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 National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

**Preparation of Mouse Epididymal and Testis Fluid Samples**— Epididymides were dissected into five segments as shown in Fig. 1, minced in cold 50 mM Tris-Cl, pH 6.8, and incubated on ice to allow sperm and luminal contents to disperse. The suspension was centrifuged at 2000 × g for 5 min to pellet the spermatooza, and the supernatant was centrifuged again to remove remaining spermatoza. The final supernatant was considered crude luminal fluid. Testes fluid samples were prepared in a similar manner. Protein concentrations were determined using the BCA assay (Pierce). Caput luminal fluid for size exclusion chromatography was prepared as described above, followed by centrifugation at 200,000 × g for 30 min to remove particulate material.

**Size Exclusion Chromatography**— Caput luminal fluid was loaded on a Superose 12 size exclusion column equilibrated with phosphate-buffered saline, pH 7.4, on an LCC 500 Plus fast protein liquid chromatography system (Amersham Biosciences). Fractions were either precipitated with 10% trichloroacetic acid, 0.5% Triton X-100 for separation on SDS-polyacrylamide gels or desalted by overnight dialysis against distilled water with four changes for vertical SDS-agarose gel electrophoresis.

[\textsuperscript{14}C]Putrescine Label Incorporation Assay—TGase activity was measured by incubation of [1,4-\textsuperscript{14}C]putrescine into N,N-dimethylcasein. One hundred microliters of luminal fluid from the testsis and each epididymal region were incubated for 2 h at 37 °C in the presence of 50 mM Tris-HCl, pH 6.8, 5 mM CaCl\textsubscript{2}, 10 mM DTT, 5 mg/ml N,N-dimethylcasein, and 0.5 μCi of [1,4-\textsuperscript{14}C]putrescine (specific activity 107 μCi μmol\textsuperscript{-1}). The reaction was terminated by trichloroacetic acid precipitation, and protein pellets were dissolved in 25% NaOH (w/v) for 30 min at room temperature, mixed with ScintiVerse® fluid
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(Fisher), and counted in a liquid scintillation counter (Beckman Coulter Inc., Fullerton, CA). All values were corrected for background dpm levels yielded by a blank control. For some samples, the TGase inhibitor BOC-DON dissolved in Me2SO or cystamine was included during the incubation at varying concentrations, whereas control samples contained equal volumes of vehicle alone.

Cross-linking of Caput Luminal Proteins by TGase—Luminal fluid from region I (50–70 µg) was incubated for the indicated times at 37 °C in 5 mM CaCl2 and 10 mM DTT in the presence or absence of 0.005 units of guinea pig liver tTGase. Some reactions also contained 10 mM cystamine or 20 µM BOC-DON or the appropriate vehicle. Reactions were stopped by the addition of EDTA to 25 mM and storage at −20 °C.

Recombinant Protein Studies—Recombinant CRES and cystatin C proteins were expressed in bacteria as previously described (5) with one modification. Linear MALDI-TOF mass spectrometry determined that CRES had a mass of 13 kDa compared with the expected 15.8 kDa. Mutation of lysine 115 to serine (CRES K115S) by site-directed mutagenesis (generated by Iris Lindberg, Ph.D., Louisiana State University Health Sciences Center) alleviated the site-specific cleavage that occurred during protein purification and enabled us to express a full-length CRES protein in Escherichia coli as confirmed by linear MALDI-TOF analysis using a Voyager DE time-of-flight mass spectrometer (PerSeptive Biosystems Inc). Recombinant mouse cystatin C was of an appropriate mass and did not require modification.

Guinea pig liver tTGase was incubated with 10 µg of CRES in 50 mM Tris-Cl, 100 mM NaCl, pH 7.4, containing 10 mM CaCl2, 10 mM DTT for the indicated times at 37 °C. Reactions were stopped by adding EDTA. Some samples included 10 mM cystamine or 20 µM BOC-DON or the appropriate vehicle. Reactions were allowed to proceed for 2 h. The reaction products were trichloracetic acid-precipitated, separated by reducing 15% SDS-PAGE, and examined under UV.

Fluorescent Peptide Mapping and Tandem Mass Spectrometry—Chemical proteolysis of CRES was done according to a previously published procedure (29). Briefly, 50 µg of recombinant CRES was incubated with 0.5 mM MDC for 2 h in the presence of guinea pig liver tTGase as described. The precipitated protein was dissolved in 200 µL of 80% aqueous acetic acid, 4 mM guanidine hydrochloride, o-iodosobenzonic acid (o-IBA) (20 mg/1.5 ml), and p-Cresol (30 µL/1.5 ml). The reaction mixture was incubated overnight for 18 h at room temperature in the dark. Resulting peptides were precipitated by trichloracetic acid for 30 min on ice. The pellet was washed twice with ice-cold acetone and then dissolved in Tricine-PAGE sample buffer while still slightly wet and resolved on 16.5% Tricine-polyacrylamide gels (30). The gels were washed with 10% acetic acid, 40% methanol for 30 min, and fluorescent bands were visualized with a UV transilluminator.

For mass spectrometry analysis, MDC-labeled recombinant CRES was digested with trypsin, and peptides were analyzed with a ThermoFinnigan LCQ Deca XP ion trap mass spectrometer. Mass spectra were analyzed by SEQUEST (Bioworks 3.1, ThermoFinnigan, San Jose, CA) software, which cross-correlates the experimentally acquired mass spectrometric data with theoretical idealized mass spectra generated from a data base of protein sequences. A mouse FASTA data base generated from the NCBI nonredundant mouse protein sequence database as well as an indexed protein data base containing the sequence of heterologous CRES sequences was used for the SEQUEST analysis. The search algorithm was modified to include permission of variable mass alterations of +318.16 Da for dansylated glutamine residues, +57 Da for carboxamidomethylated cysteines and +16 Da for oxidized methionines. Peptide precursors containing a modified glutamine residue displayed a 446.22-Da loss for Q-MDC as compared with the 128.05-Da loss observed for the native glutamine during collision-induced fragmentation. All of the peptides except for the proline-containing Gln-89 peptide ($X_{corr} = 1.85$) had acceptable $X_{corr}$ scores (4.3 (Gln-13), 2.61 (Gln-58), 3.05 (Gln-66) in the SEQUEST scoring system and were confirmed manually by analysis of their corresponding raw MS/MS spectra. The diminished score for the Gln-89 peptide resulted from its predominant fragmentation at the isoleucine-proline bond. The corresponding daughter ions y3 and b10 exceeded all other daughter ions in relative abundance (Fig. 5C). This unusual fragmentation behavior for proline-containing peptides has been observed previously and has been shown to have a lowering impact on correlation scores (31, 32).

Vertical SDS-Agarose Gel Electrophoresis—A glycine-NaOH/sodium sulfate buffer system was used as described (33, 34). Briefly, a 2.5% agarose gel was prepared by mixing Metaphor®-agarose (Cambrex Bioscience, Rockland, ME) with gel buffer (50 mM Na2SO4 and 0.05% SDS) and dissolving the agarose by boiling. Glass plates from the CBS Minigel System (CBS Scientific, Del Mar, CA) were assembled and heated by radiation with a 40-watt electric bulb placed at a distance of 5 cm for about 5 min. The warm liquid agarose was poured between the warmed glass plates, and a Bio-Rad mini-Protean 3 System 10-well comb (Bio-Rad) adapted to only insert 3 mm deep into the agar was inserted. The gel was left to solidify at room temperature for 20 min, and the comb was removed. The gel was then stored at 4 °C for 20 min in order to improve gelation. The gel cassette was placed in the gel box, and the whole apparatus was tilted backward to an angle of 45°. A sealant gel consisting of 20 mL of 1% agarose (UltraPureTM, Invitrogen) in anode buffer (50 mM Na2SO4) was poured underneath the gel into the tilted anode buffer tank in order to prevent the resolving gel from sliding through the plates. After the sealant gel had solidified, the tanks were filled with precooled anode and cathode buffers (100 mM glycine, pH 9.5, and 0.025% SDS). Samples were desalted by dialysis against distilled water, lyophilized, and redissolved in sample buffer (100 mM glycine-NaOH, pH 9.5, 50% (w/v) sucrose, 0.025% SDS, 2% 2-mercaptoethanol, trace of phenol red). Small sample volumes were dialyzed using a modification of the microdialysis method described by Sodhi and Rajput 2003 (35). The samples and marker were loaded, and the gel was electrophoresed at 70 V constant voltage at 4 °C. Gels were stopped when the phenol red dye front had migrated to a point 1 cm ahead of the gel bottom.

Western Blot Analysis—Samples were separated by 15% SDS-PAGE (5) or by 2.5% vertical SDS-agarose gels (33, 34). Gels...
were blotted onto polyvinylidene difluoride membrane (Immobilon P; Millipore Corp., Bedford, MA) in 25 mM Tris, 192 mM glycine transfer buffer containing 20% methanol and 0.01% SDS, blocked in 3% milk/TBS-T (0.2% Tween-20) for 1 h, and incubated overnight at 4 °C with affinity-purified polyclonal rabbit anti-mouse CRES antibody (1.3 μg/10 ml) in 3% milk in TBS-T (5). The blots were washed with TBST and incubated for 2 h with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (BioSource International, Camarillo, CA) at a 1:20,000 dilution in 3% milk in TBS-T. Blots were washed with TBS-T followed by incubation with chemiluminescence reagent (Western Pico or Femto ECL; Pierce) and exposure to film.

Dot Blot Analysis—Preexisting oligomeric complexes were removed from recombinant CRES and cystatin C proteins by Centricon (30 kDa cut-off) centrifugation. The filtrate was quantitated by BCA (Pierce), and 10-μg aliquots were either frozen immediately or incubated (0.2–0.5 mg/ml) for 1–2 days at 37 °C in 50 mM Tris, pH 7.4 (CRES) or 25 mM bis-Tris, pH 6.8 (cystatin C) to allow oligomerization. Protein samples were also incubated for 2 h in the presence of 0.005 units of tTGase, 10 mM DTT, and 5 mM calcium. The samples were removed from 37 °C at various times and stored at −20 °C. Samples (5 μl) were spotted on to Formvar/carbon-coated 200-mesh nickel grids (Ted Pella, Redding, CA) for 1 min, the liquid was removed with filter paper, and the samples were negatively stained with 2% uranyl acetate for 1 min. The samples were examined with a Hitachi 8100 electron microscope operating at an excitation voltage of 75 kV or a Hitachi 7650 electron microscope operating at an excitation voltage of 80 kV. The time of oligomerization required to detect the various amyloid structures varied between experiments and may reflect effects of protein concentration and the presence or absence of CRES seeds in the starting preparations.

CRES oligomers recovered from the retentate fraction after Centricon centrifugation and stored at 4 °C for prolonged periods of time (>4 weeks) or monomeric CRES prepared as described above were examined for amyloid fibrils by Congo Red staining (Amyloid Stain kit; Sigma). Briefly, samples were spread on a microscope slide and air-dried. The slides were then incubated in alkaline sodium chloride solution for 25 min, followed by incubation in alkaline Congo Red for 3 h. The slides were washed three times in 100% ethanol, followed by PBS, and then mounted with Fluoromount G (Southern Biotech) and examined with a Zeiss Axiosstar microscope equipped with an AxioCam MRC digital camera.

RESULTS

Presence of CRES Protein Complexes in Caput Epididymal Luminal Fluid—Based on the propensity of cystatins to oligomerize, we investigated whether CRES formed high molecular weight complexes following secretion into the epididymal lumen. We previously have determined that CRES is synthesized and secreted into the lumen by the proximal caput epididymis (region I), is present in the lumen of the midcaput (region II), and then abruptly disappears from the lumen of the distal caput (region III). Luminal fluid isolated from the caput epididymal regions I–III was centrifuged at high speed to remove insoluble material, and the supernatant was fractionated by size exclusion chromatography followed by SDS-agarose gel electrophoresis and Western blotting using an affinity-purified CRES antibody (33, 34). As shown in Fig. 1A, the void volume (fraction 13) contained multiple forms of CRES, including SDS-sensitive and SDS-resistant high molecular weight forms. Specifically, the detection of the 15-kDa and N-glycosylated 19-kDa monomeric forms of CRES proteins (5) in the void volume suggested their presence in large molecular mass complexes that reversed in the presence of SDS.
proteins in Fig. 1A did not migrate to their true molecular weight, since proteins resolved on agarose gels do not separate entirely based on molecular mass. The void volume also contained SDS-resistant high molecular mass CRES complexes that barely entered the gel, suggesting that CRES is also present in epididymal fluid as covalent protein complexes. No CRES complexes were detected in or near the sample well in the size exclusion fractions containing the bulk of monomeric CRES (fractions 19–21; Fig. 1A), demonstrating that the SDS-resistant high molecular mass complexes in the void volume were not due to artificial self-aggregation during electrophoresis. To confirm that the SDS-resistant complexes detected in the sample well of the void volume fraction truly represented CRES, we used size exclusion chromatography to fractionate caput epididymal fluid from CRES wild type (+/+) and gene knock-out (−/−) mice. We previously have established that the CRES mRNA and protein are not expressed in the CRES gene knock-out mouse. The absence of the SDS-resistant high molecular mass CRES complexes in the void volume of the fractionated epididymal fluid from the CRES knock-out mice further supported that the complexes contained CRES rather than representing a nonspecific sticking of the antibody to protein aggregates during the Western analyses (Fig. 1B).

To examine whether the complexes included self-aggregates of CRES, caput luminal fluid was incubated with the homobifunctional thiol-cleavable amine-reactive cross-linker dithiobis-succinimidylpropionate. The addition of dithiobis-succinimidylpropionate resulted in the detection of CRES immunoreactive complexes greater than 25 kDa, as indicated by Western blot analysis (see supplemental Fig. 1, horizontal gel). The cross-linked complexes were resolved by diagonal SDS-PAGE under nonreducing conditions in the first dimension followed by reducing conditions to reverse the cross-linking in the second dimension. When first dimension gels were resolved by SDS-PAGE in the second dimension and examined by Western blot analysis, the complexes reversed to the 15- and 19-kDa CRES isoforms. These observations suggest that CRES complexes, in part, consist of self-aggregates of CRES, including heterodimers and oligomers of the glycosylated and nonglycosylated isoforms.

Region-specific Localization of SDS-resistant CRES Complexes in the Epididymal Luminal Fluid—To examine the regional distribution of the high molecular mass SDS-resistant CRES complexes in the epididymis, luminal fluid from each epididymal region was fractionated by size exclusion chromatography and void volume (fractions 13 and 14) and monomeric fractions (fractions 19–21) examined by SDS-agarose and SDS-polyacrylamide gels, respectively, followed by Western blot analysis. As shown in Fig. 2, the high molecular mass SDS-resistant forms of CRES were present in the same regions as the CRES monomer, providing additional evidence that these complexes may represent higher ordered structures of CRES. The presence of high molecular mass CRES SDS-resistant complexes and the lack of monomer in epididymal region III suggest that the complexes may be more resistant to mechanisms responsible for CRES protein turnover. It is also possible that the loss of the monomer from region III may be due to its oligomerization into the high molecular mass CRES complexes.

Tissue-type Transglutaminase Activity in the Epididymis—TGases have been implicated in protein aggregation because of their ability to covalently cross-link proteins. Furthermore, TGases are structurally related to papain-like cysteine proteases and therefore likely to interact with cysteine protease inhibitors, such as cystatins. Thus, we investigated whether an active TGase in the caput epididymal luminal fluid could be responsible for the formation of the SDS-resistant high molecular mass CRES protein complexes. Studies were first carried out to measure TGase activity in luminal fluid prepared from various regions of the mouse epididymis using an established [14C]putrescine label incorporation assay. The highest TGase activity was detected in the luminal fluid from caput region I with decreasing activity in the remaining epididymal regions (Fig. 3A). This activity was significantly enhanced in the presence of calcium but could also be detected in its absence, whereas the activity was abolished by the addition of EDTA. To further characterize this enzymatic activity, [14C]putrescine label incorporation experiments were conducted using various TGase inhibitors, including the inhibitor cystamine (36) and the site-specific tissue-type TGase peptide inhibitor BOC-DON. The addition of cystamine or BOC-DON significantly

![Figure 2](image-url)
inhibited the TGase activity in epididymal fluid prepared from the caput (region I) compared with control samples incubated with (DMSO) or without (CON) the vehicle (Fig. 3B). Because the peptide inhibitor BOC-DON is specific for tissue-type TGase, its profound inhibition of the transamidating activity indicated a tTGase in the epididymal fluid (Fig. 3B). Since the plasma TGase factor XIIIa is not susceptible to BOC-DON inhibition, contaminating blood in the luminal fluid could be excluded as the source of the transamidating activity (see product sheet for N-Zyme and Ref. 37).

CRES Is a Substrate for a Tissue-type Transglutaminase—Experiments were next carried out to determine whether in vitro CRES is a substrate for TGase and contains both reactive lysine and glutamine residues that participate in the formation of an isopeptide bond. For these studies and subsequent studies, a recombinant CRES protein was used. Because bacterial expression resulted in a C-terminal truncation of the recombinant CRES protein, a single amino acid mutation (K115S) was introduced that prevented truncation and yielded a full-length CRES protein characteristic of that in the mouse. Recombinant CRESK115S was incubated with guinea pig liver tTGase, and the samples were examined by SDS-PAGE and Coomassie Blue staining. The CRES monomer disappeared in a time-dependent manner, suggesting the formation of high molecular mass CRES complexes that were too large to be resolved on the SDS-polyacrylamide gel (Fig. 4A). The addition of the TGase inhibitor BOC-DON prevented the formation of the CRES complexes, as evidenced by the levels of monomeric CRES similar to that in early time points. Similar results were obtained when the wild-type C-terminally truncated CRES protein was used (data not shown). The lack of detectable CRES monomer after treatment with TGase is probably not due to a loss of CRES during the

FIGURE 3. Tissue-type TGase activity in epididymal luminal fluid. A, luminal fluid obtained from the testis and five epididymal regions was analyzed for transamidating activity in the presence and absence of calcium and EDTA by a [14C]putrescine incorporation assay. Activity is reported as dpm/100 µg of protein and represents the mean ± S.E. of three independent experiments. B, caput luminal fluid from region I was incubated in the presence of calcium and increasing amounts (2.5–20 µM) of the TGase peptide inhibitor BOC-DON or 10 mM cystamine (Cy). Changes in TGase activity due to increasing concentrations of inhibitor were significant (p < 0.001) when compared with vehicle control (DMSO) as assessed by one way analysis of variance and Bonferroni’s multiple comparison test. No significant difference in transglutaminase was detected between the untreated blank control (CON) and the vehicle (DMSO).

FIGURE 4. CRES is a complete substrate for tissue-type TGase. A, recombinant CRES was incubated with guinea pig liver tTGase for increasing periods of time, followed by separation on SDS-PAGE and Coomassie Blue staining. The CRES monomer disappeared in a time-dependent manner, suggesting the formation of high molecular mass CRES complexes that were too large to be resolved on the SDS-polyacrylamide gel (Fig. 4A). The addition of the TGase inhibitor BOC-DON prevented the formation of the CRES complexes, as evidenced by the levels of monomeric CRES similar to that in early time points. Similar results were obtained when the wild-type C-terminally truncated CRES protein was used (data not shown). The lack of detectable CRES monomer after treatment with TGase is probably not due to a loss of CRES during the

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loading of the SDS-polyacrylamide gel, since in subsequent dot blot analyses, CRES samples treated with TGase showed equal amounts of CRES as untreated samples, yet SDS-PAGE demonstrated that most of the TGase-treated CRES did not enter the gel (Fig. 7). The formation of CRES complexes in the presence of TGase suggests that CRES is a complete substrate for TGase cross-linking and contains both reactive glutamine and lysine residues.  

To confirm the presence of reactive glutamine residues in CRES, experiments were next carried out in which recombinant CRES$_{K115S}$ was incubated with guinea pig liver tTGase in the presence or absence of the fluorescent substrate MDC, which served as the amine donor. Samples were then resolved by SDS-PAGE and examined under UV light. Although remaining in the monomeric state, CRES exhibited a time-dependent increase in fluorescence, representing TGase-mediated transfer of the MDC to presumably CRES glutaminyl residues (Fig. 4B). The TGase-mediated incorporation of the MDC fluorescence into CRES was inhibited by the calcium chelator EDTA as well as by the TGase inhibitor cystamine (Fig. 4B). Similar results were observed with the wild-type C-terminally truncated CRES protein, suggesting that reactive glutaminyl residues were upstream of the truncation and that the CRES$_{K115S}$ did not exhibit altered reactivity with TGase (data not shown).  

Identification of Target Glutamine Residues in CRES—Two approaches including fluorescent peptide mapping and LC-MS/MS were used to determine which glutamine residues in CRES were targets of TGase cross-linking. In the first experiment, the peptide fragments generated by o-IBA digestion of nonlabeled recombinant CRES$_{K115S}$ were compared with that of CRES$_{K115S}$ labeled with MDC in the presence of TGase. As shown in Fig. 5A, o-IBA digestion of nonlabeled CRES followed by Tricine-PAGE and Coomassie staining yielded a pattern of peptide fragments that resulted from complete and incomplete fragmentation of the protein at tryptophan residues. CRES labeled with MDC followed by o-IBA digestion and Tricine-PAGE yielded a similar pattern of fluorescent peptides as that obtained from nonlabeled CRES (Fig. 5B). Since both the 9.2-kDa C-terminal fragment III and the 4.9-kDa N-terminal fragment IV were labeled with MDC, this suggested that at least one glutamine residue in each of these fragments was a target residue for transamidation by TGase.  

In order to map the exact target glutamine residues, MDC-labeled CRES$_{K115S}$ was subjected to tryptic digestion, and the fragments were analyzed by nanospray LC-MS/MS. Four glutamine residues (Gln-13, Gln-58, Gln-66, and Gln-89) were identified as targets for TGase based on their altered molecular mass due to modification by MDC (Fig. 5C). The same glutamine residues were identified when the C-terminally truncated wild-type CRES protein was examined by LC-MS/MS (data not shown). Taken together, these data indicate that more than half of the total glutamine residues in CRES is utilized as substrate residues by the tissue-type TGase. It is of interest that one CRES glutamine target, Gln-58, is located in the one of the two inhibitory loops important for cystatin inhibition of cysteine proteases (38, 39).  

Transglutaminase Catalyzes the Formation of SDS-resistant High Molecular Mass Complexes of CRES in Caput Luminal Fluid—Experiments were next carried out to determine whether endogenous CRES is a substrate for TGase. Caput luminal fluid was incubated with guinea pig liver tTGase for varying lengths of time, followed by separation on SDS-agarose gels and Western blot analysis. As shown in Fig. 6 (left), the addition of exogenous TGase resulted in a rapid time-dependent loss in CRES monomers and an increase in the formation of SDS-resistant high molecular mass CRES complexes. The TGase-mediated formation of CRES complexes was inhibited by cystamine and the tissue-type TGase-specific inhibitor BOC-DON. Coomassie staining of the blotted membrane showed that the addition of TGase did not result in a nonspecific cross-linking of all proteins in the caput luminal fluid (data not shown).  

To determine whether endogenous TGase activity in the caput luminal fluid would also catalyze the formation of high molecular mass CRES complexes, caput luminal fluid was incubated in the presence of calcium. Although the formation of high molecular mass CRES complexes was not as rapid as with the exogenous TGase, a time-dependent formation of high molecular mass CRES complexes was observed (Fig. 6, right). The formation of the CRES complexes was prevented by the addition of BOC-DON, providing further evidence for a tissue-type TGase in the caput epididymal fluid. Coomassie staining of the blotted membrane showed that not all epididymal luminal proteins were cross-linked during the incubation period (data not shown). The longer incubation times to detect CRES complexes and the lack of defined intermediate molecular mass CRES complexes in this experiment may reflect either the profound dilution of epididymal TGase enzyme and thus activity that occurs during the preparation of the luminal fluid, an autocross-linking of the TGase over the time course thus diminishing its activity (40, 41), and/or different substrate utilization of the guinea pig liver tTGase compared with the mouse epididymal TGase. Such a variation in substrate utilization and complex formation pattern has been observed before for TGases 1 and 3 (42).  

TGase Prevents the Formation of Amyloid-type Aggregation of CRES—Amyloidogenic proteins are thought to utilize similar aggregation pathways and self-assemble into common structures, including soluble oligomers that are intermediate to fibril formation but may represent the primary toxic form of amyloids (10, 43–45). A conformation-dependent antibody (oligomer Ab in Fig. 7) that specifically recognizes oligomeric forms of amyloidogenic proteins independent of peptide sequence (10, 46) was used in a control dot blot analysis of monomeric (m), oligomeric (o), and fibrillar (f) forms of amyloid-β and oligomeric forms of α-synuclein, prion 106–126, and insulin (a generous gift from Charles Glabe). The conformation-dependent antibody detected only the oligomeric forms of these amyloidogenic proteins, confirming the specificity of the antibody for this common structure (10) (Fig. 7A). To determine whether CRES will self-assemble into oligomers with a structure common to the amyloidogenic proteins in the control dot blot, dot blot analysis was performed with recombinant CRES$_{K115S}$ that was allowed to oligomerize for extended times at 37 °C. Monomeric CRES (m) prepared fresh by ultrafiltration to remove large preexisting aggregates was not recognized by the oligomer antibody, whereas the same preparation of monomeric CRES allowed to oligomerize (o) at 37 °C was detected by the antibody
FIGURE 5. Fluorescent peptide mapping and LC/MS-MS analysis of MDC-labeled CRES protein. A, unlabeled recombinant CRES was digested with α-IBA, followed by separation on 16.5% Tricine-PAGE and Coomassie staining. The expected peptide fragments from α-IBA digestion are shown schematically. **, an additional fragment was detected that may result from oxidative cleavage at a noncanonical site. *, the expected 1.7-kDa fragment was not detected either due to it being present at levels below the sensitivity of Coomassie or cleavage at noncanonical sites resulting in peptides that did not resolve on the gel. All glutaminyl residues are indicated by the lollipop. Q13, Q58, Q66, and Q89, glutamine residues that were labeled with MDC. B, MDC-labeled recombinant CRES was digested with α-IBA and resolved by 16.5% Tricine-PAGE and examination under UV light. ucl, uncleaved. C, MS/MS spectra of the tryptic peptides generated from MDC-labeled CRES protein. The mass-to-charge ratio in Da is shown on the x axis, and the relative abundances (peak intensities) are shown on the y axis. Target glutamine residues were identified based on altered molecular mass due to MDC labeling.
CRES Transamidation and Oligomerization in the Epididymis

FIGURE 6. Cross-linking of endogenous CRES in caput luminal fluid by exogenous and endogenous tTGase. Left, representative Western blot analysis of CRES in caput luminal fluid incubated with guinea pig liver tTGase for increasing periods of time followed by separation on SDS-agarose gels. Right, representative Western blot analysis of CRES protein in caput luminal fluid incubated in the presence of calcium for increasing periods of time followed by separation on SDS-agarose gels. The TGase inhibitors BOC-DON and cystamine prevented the formation of high molecular mass CRES complexes formed by either endogenous or exogenous TGase.

A. 

amyloid-β

β-amyloid Ab

oligomer Ab

m o f

syn

prion

insulin

B. 

CRES

Myoglobin

Cystatin C

m o TG

m o

The addition of TGase for increasing periods of time followed by separation on SDS-agarose gels.

FIGURE 7. Amyloid-type aggregation of CRES. A, top panels, control dot blot containing monomeric (m), oligomeric (o), and fibrillar (f) forms of amyloid-β and oligomeric forms of α-synuclein (syn), prion 106–126, and insulin proteins were incubated with the conformation-dependent antibody (oligomer antibody). The blot was stripped and then reprobed with the β-amyloid antibody (6E10) to detect the amyloid-β protein. Bottom panels, monomeric, oligomeric, and TGase-treated (TG) CRES and cystatin C (CC) were spotted on nitrocellulose membrane and incubated with the conformation-dependent antibody (oligomer Ab) in dot blot analysis. The blots were stripped and reprobed with CRES and cystatin C antibodies. B, the same protein samples examined by dot blot analysis were separated by 15% SDS-PAGE followed by silver staining. Myoglobin was included as a negative control, since its structure does not predict amyloid formation.

(Fig. 7A). Similarly, cystatin C, a proven amyloidogenic protein, that was incubated at 37 °C to induce oligomerization also reacted strongly with the oligomer-specific antibody, whereas the monomeric form did not (Fig. 7A). When the dot blots were stripped and reprobed with the CRES and cystatin C antibodies, however, equal amounts of each protein were detected in the monomeric and oligomeric samples.

SDS-PAGE of the same monomeric and oligomerized CRES and cystatin C samples used for the dot blot studies confirmed that following incubation, a proportion of the monomeric forms had shifted to dimeric and larger molecular weight oligomeric structures (Fig. 7B). Myoglobin is not predicted to be amyloidogenic, and indeed, following incubation at 37 °C, it did not self-aggregate based on SDS-PAGE analysis (8). Myoglobin, however, could not be examined by dot blot analysis, since the protein exhibited nonspecific cross-reactivity with the chemiluminescence reagent, probably as a result of residual iron binding to the protein (data not shown). Together, these studies suggest that CRES, like cystatin C, is an amyloidogenic protein and can adopt a potentially cytotoxic, soluble oligomeric structure in its aggregation pathway.

To test whether TGase mediated cross-linking of CRES might prevent it from forming soluble oligomeric structures recognized by the antibody, the same preparation of monomeric CRES described above was allowed to oligomerize in the presence of guinea pig liver tTGase, after which samples were examined by dot blot analysis. As shown in Fig. 7, A and B, TGase-cross-linked (TG) CRES did not react with the oligomer-specific antibody, yet SDS-PAGE of the same sample clearly indicated that the majority of the monomeric CRES had shifted to larger molecular weight complexes, a portion of which were detected in the sample well. Similar results were also noted with cystatin C. Because the dot blot showed similar amounts of CRES and cystatin C proteins in the TG-treated as well as in the monomeric and oligomeric samples, this demonstrated that the reduced levels of detectable proteins in the gel were not due to a loss of protein during the experiment but rather due to the formation of complexes too large to enter the gel. These observations suggest that TGase-mediated cross-links in CRES and cystatin C proteins prevented these proteins from self-assembling into amyloid-type oligomeric structures, possibly by forming large aggregates in alternate conformations.

To establish whether CRES truly adopted amyloid-type structures during its oligomerization, monomeric samples prepared as described above were allowed to oligomerize at 37 °C for extended times, and samples were removed for analysis by negative stain electron microscopy. As shown in Fig. 8, during its oligomerization, CRES progressed from a monomeric state, characterized by an undefined structure (Fig. 8A), to soluble oligomers characterized by ball-like structures of 23–30-nm diameter (Fig. 8B), to short protofibrils (Fig. 8C), to fibrils (Fig. 8D). The addition of TGase to CRES during its oligomerization
caused the formation of large amorphous aggregates distinct from structures characteristic of the amyloid aggregation pathway, supporting that TGase cross-linking may prevent CRES amyloid formation (Fig. 8F). The use of the conformation-dependent dye Congo Red, which binds with high affinity to amyloid fibrils, was also used to establish whether CRES forms amyloid. Light microscopic analysis of CRES incubated for >4 weeks at 4 °C revealed the presence of fibrils that bound Congo Red (Fig. 8G), thus further supporting that CRES is an amyloidogenic protein. Similar analyses of monomeric CRES did not reveal fibrillar structures or Congo Red reactivity (data not shown).

**DISCUSSION**

The studies presented herein demonstrate that CRES is present in the caput epididymal lumen as monomeric as well as oligomeric forms. Using several approaches, including size exclusion chromatography and SDS-agarose gel electrophoresis, we showed the presence of noncovalent as well as SDS-resistant high molecular mass CRES complexes in the caput epididymal luminal fluid. Although it is likely that other proteins are associated with CRES and/or are present in these high molecular mass complexes, the propensity for CRES to oligomerize by the amyloid pathway in vitro and our ability to induce the formation of CRES oligomers in the presence of TGase strongly suggest that, at least in part, these complexes included self-aggregates of CRES. Our ability to detect CRES heterodimers and oligomers in an epididymal particulate fraction by using a chemical cross-linker further supports that CRES self-associates in the epididymal lumen.

The mechanism(s) by which CRES oligomerizes is not known and requires further study. In particular, studies are needed to determine whether CRES may undergo three-dimensional domain swapping, as has been demonstrated for several other cystatin family members, including the family 2 members cystatin C (47) and chicken cystatin (18, 48), and the family 1 member cystatin A (18) as well as other amyloidogenic proteins, including prion protein (49). In addition to the presence of noncovalent CRES complexes in the caput fluid, which is consistent with domain swapping, there are several lines of evidence to suggest that CRES has inherent properties like other cystatins to self-aggregate by this process. The predisposition for a protein to oligomerize by domain swapping can be predicted by algorithms that analyze the stability of protein mutations based on data base-derived potentials (50). Regions in the cystatin C sequence that were predicted by the PoPMuSiC algorithm to be structurally weak were within a flexible hinge loop region and indeed adopted alternate positions when the structure of human cystatin C, which crystallized as a domain-swapped dimer, was determined (47, 50). The cystatin C leucine 68 residue that is mutated in the heritable form of amyloid angiopathy, resulting in increased cystatin C oligomerization, by domain swapping is also located within one of these regions of structural weakness (50). Two similar regions of structural weakness were identified in the CRES sequence, including Arg-49 to Gln-51 and the region His-41 to Lys-43 that is predicted to be part of a flexible hinge loop, suggesting that CRES may also aggregate by domain swapping.

Alterations in environmental conditions, such as prolonged incubation at 37 °C and neutral pH, stimulated the self-aggregation of CRES and the subsequent formation of soluble oligomeric structures recognized by a conformation-dependent antibody as well as the eventual formation of Congo Red reactive amyloid fibrils. Although the precise conditions for stimulating cystatin C to oligomerize in vitro were slightly different than that for CRES, cystatin C also adopted the common structure recognized by the oligomer-specific antibody, suggesting that the mechanisms of aggregation are similar between the two proteins (13, 46). Indeed, our results of cystatin C oligomerization are similar to those of Wahlbom et al. (46). Although CRES formed dimers during its oligomerization in vitro, in vivo CRES dimers were more difficult to detect and required chemical cross-linking to trap the small proportion that existed as a heterodimer. In contrast, both wild type and L68Q cystatin C...
formed dimers in vivo (14, 51). The disparity between CRES and cystatin C to form dimers may reflect differences in the kinetics of their oligomerization. It is also possible that the dimer may represent a dead end product rather than a transition state from a monomeric to oligomeric form, as recently proposed for cystatin C (46).

Role of Transglutaminase in the Formation of SDS-resistant CRES Protein Aggregates—The presence of SDS-resistant high molecular mass CRES complexes in addition to the noncovalent complexes in caput luminal fluid suggested that several mechanisms may be involved in the formation and/or stabilization of soluble CRES aggregates in the epididymal lumen. Our studies presented herein suggest that the SDS-resistant complexes may arise, in part, from TGase-mediated cross-linking of CRES. Indeed, our studies demonstrate that both recombinant and endogenous CRES are substrates for TGase and that an endogenous tissue-type TGase activity is present in the epididymal fluid that will cross-link CRES protein into stable high molecular mass complexes.

TGase activities have previously been documented in the male reproductive tract, including testis, epididymis, prostate, and spermatozoa, and have been suggested to play a role in stabilization of follicle-stimulating hormone-receptor complexes (52), formation of the seminal coagulum (53), sperm motility (54), and suppressing sperm antigenicity (55, 56). Little is known regarding the expression or function of TGase in the epididymis or that sperm and fluid from the head of the epididymis exhibited higher levels of activity than the tail (57). Our studies suggest that the TGase activity present in the epididymal luminal fluid is a tissue-type TGase, as evidenced by inhibition of its activity in the presence of a tissue-type TGase-specific peptide inhibitor. Our studies also show that the highest activity was in the luminal fluid from caput regions I and II, which correlates well with the highest levels of luminal CRES protein, supporting a role for this enzyme in cross-linking CRES. In addition, although in vitro studies indicate that tissue-type TGase activity is optimal at basic pH (58), our [14C]putrescine incorporation studies to assess TGase activity in the epididymis were performed at pH 6.8, which closely approximates luminal pH in the caput epididymis,5 and under these conditions, considerable TGase activity was observed. Thus, our studies support a functional TGase within the caput epididymal lumen.

Additional support for a role of TGase in the formation of stable CRES protein complexes is provided by the fact that TGases are structurally related to papain-like cysteine proteases, and thus their catalytic site is highly accessible to cysteine protease inhibitors, such as the cystatins. Indeed, mapping of CRES-reactive glutamyl residues by fluorescent labeling and LC/MS-MS indicated that one of the four target glutamines was in an inhibitory loop domain conserved in all cystatins and important for interactions with cysteine proteases. The cystatins M/E and cystatin α have also been shown to be potent transglutaminase substrates in vitro (59, 60). Our studies suggest that cystatin C is also a TGase substrate.

5 T. Cooper, personal communication.
have broad implications toward understanding and preventing amyloidogenic diseases.

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