Region-specific Expression and Secretion of the Fibrinogen-related Protein, fgl2, by Epithelial Cells of the Hamster Epididymis and Its Role in Disposal of Defective Spermatozoa*

Received for publication, September 13, 2004
Published, JBC Papers in Press, September 17, 2004, DOI 10.1074/jbc.M410485200

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The cauda epididymidis functions in the storage and protection of mature, fertile spermatozoa. We previously identified a region-specific secretory glycoprotein (termed HEP64) of the hamster proximal cauda epididymidis that specifically bound and coated the nonviable, but not the viable, spermatozoa within the epididymal lumen. In this study we employed expression screening of a hamster epididymal cDNA library to obtain the full-length sequence of HEP64 and to identify it as the fibrinogen-like protein fgl2. Northern blot analysis demonstrated that fgl2 mRNA is highly expressed by the proximal cauda epididymidis in comparison to other hamster tissues examined, and, in situ hybridization analysis of the epididymis revealed that fgl2 mRNA exhibited a region- and principal cell-specific expression pattern. Immunohistochemistry confirmed the association of fgl2 with abnormal spermatozoa in the cauda epididymidis and revealed smaller fgl2-containing particles. Immunoelectron microscopy revealed that fgl2 was distributed throughout an amorphous, “death cocoon,” complex assembled onto abnormal spermatozoa and that the smaller fgl2 aggregates consisted of the amorphous material with embedded sperm fragments, organelles, and membrane vesicles. A protocol was developed to isolate an enriched death cocoon fraction. SDS-PAGE and microsequence analyses revealed that the Mr 64,000 fgl2 monomer was assembled into two disulfide-linked oligomers of Mr 260,000 and 280,000. These data demonstrate that the epididymis possesses a specific mechanism to identify and envelop defective spermatozoa with a protein complex containing the fibrinogen-like protein fgl2. We propose that this represents an important protective mechanism not only to shield the viable sperm population from potentially deleterious enzymes released by dying spermatozoa but also to prevent the release of sperm proteins that could initiate an immune response if they escaped the epididymal environment.

Mammalian spermatozoa exiting the testis are functionally immature and must undergo a series of developmental modifications in the epididymis to acquire forward motility and fertilizing capacity (1, 2). In the species studied, spermatozoa from the proximal cauda epididymidis exhibit fertilizing capacity, and the distal cauda functions as a storage reservoir that maintains the viability of mature spermatozoa (1, 3). Both the sperm maturation and storage functions of the epididymis depend upon paracrine interactions between the epididymal epithelium and spermatozoa, which are mediated by a progressively modulated luminal fluid environment of region-specific ionic, organic solute, and protein composition (4, 5). The luminal fluid is produced by the secretory and absorptive activities of a lining epithelium whose major cell type, the principal cell, also displays striking regional differences in its gene expression and protein secretion patterns (6). Several principal cell secretory proteins appear to modify the sperm plasma membrane and promote their maturation (7, 8). However, other principal cell secretory proteins such as glutathione peroxidase, superoxide dismutase, and γ-glutamyl transpeptidase, which prevent oxidative damage, clusterin, a complement inhibitor (5, 9), and CRES (10) and HE4 (8), which are potential protease inhibitors, represent protective proteins thought to promote sperm viability (4, 11). Thus the luminal environment performs a dual function, promoting sperm maturation in the proximal and sperm survival in the distal regions of the epididymis, respectively.

Although the cauda epididymidis promotes their survival, the presence of dead spermatozoa in this region has been observed in many species (12–15). Degenerating spermatozoa release enzymes that may compromise the viability of neighboring cells and represent a source of autoantigens that could provoke an immune response if they escaped from the epididymal lumen. The protective strategies that the epididymis utilizes to prevent these negative impacts on male fertility are not clearly understood. We previously identified a 64-kDa polypeptide (termed HEP64) secreted by principal cells of the hamster cauda epididymidis that selectively binds to abnormal spermatozoa (16). In the present study we have identified this polypeptide as the fibrinogen-related protein fgl2, which was originally identified as a T-lymphocyte gene product (17) and subsequently as a procoagulant protein expressed during specific tissue pathologies (18). We demonstrate that fgl2 is polymerized into a cocoon-like complex enveloping both sperm fragments and defective spermatozoa in the hamster cauda epididymidis. We propose that this polymerized complex physiologically separates the defective from the viable sperm population and prevents release of sperm components with potential adverse effects on male fertility. This model is compared with other concepts proposed for the elimination of defective spermatozoa.

* This work was supported by National Institutes of Health Grant HD20419. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AY697779.
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EXPERIMENTAL PROCEDURES

Animales—Care and use of animals conformed to National Institutes of Health guidelines for humane animal care and use in research. Mature male golden hamsters were housed in an Association for Assessment and Accreditation of Laboratory Animal Care–approved animal facility. Female hamsters were caged individually and fed ad libitum. University veterinarians supervised animal care, and the Institutional Animal Care and Use Committee approved all animal protocols. Animals were sacrificed by CO2 asphyxiation, and tissues were immediately removed for the protocols described below.

Construction and Expression Screening of Epididymal cDNA Libraries—Hamster cauda epididymides were minced in ice-cold Tyrode's solution (145 mM NaCl, 5 mM Hepes, pH 7.4) to release spermatocytes and then frozen on dry ice. Poly(A)+ RNA was extracted and isolated with two sequential loadings on an oligo(dT) column (MessageRunner RNA Isolation kit, Stratagene). A cauda epididymal unidirectional cDNA expression library was constructed in the ZAP express Lambda phage vector (Stratagene); cDNAs were size-selected on Sepharose CL-2B columns prior to ligation into the ZAP express vector.

For expression screening, the cauda epididymal cDNA library was plated at 30,000 plaque-forming units/plate and incubated at 42 °C for 4 h. Nitrocellulose filters, treated with 10% (w/v) isoamyl alcohol, methanol, and 60% (v/v) deionized formamide, were layered on top of the plates that were then incubated for 16 h at 37 °C. The plates were cooled to 4 °C for 2 h, and the filters were removed for immunostaining. The filters were rinsed in TNT and blocked in TNT containing 5% goat serum and 2.5% BSA. The filters were then incubated in rabbit anti-fgl2 (16) in blocking solution and then incubated for 1 h in 1:500 dilution of goat anti-rabbit IgG (Sigma). The filters were then washed once with 0.1 M Tris-HCl, pH 7.5, and rehybridized (19 h), as described above, with a [α-32P]dATP-labeled probe for the constitutively expressed gene (625-bp fragment of CHO-B) encoding ribosomal protein S2 (25).

In Situ Hybridization—Sense and antisense riboprobes for nonspecific inhibitor of situ hybridization (26, 27) were prepared using 1 μg of linearized plasmid in 20-μl transcription reactions containing 40–50 units of SP6 (Promega, Madison, WI) or T7 (New England Biolabs, Waverly, MA) polymerase, 1× transcription buffer, 20 units of RNase inhibitor, 1 mM each of ATP, CTP, GTP, 0.65 mM UTP, and 0.35 mM Dig-UTP (Roche Applied Science). Unincorporated nucleotides were removed at room temperature with 0.1 M triethanolamine buffer, pH 8.0, and hybridized overnight at 60 °C with Dig-riboprobes. The slides were rinsed at room temperature for 5 min each in 2× SSC followed by STE (0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, and 1× EDTA), and then incubated for 30 min at 37 °C in STE containing 40 μg/ml RNase A. After hybridization the sections were sequentially washed for 5 min each with 2× SSC, 50% formamide at 60 °C, then with 2× SSC followed by 1× SSC, and then with 0.1× SSC, followed by 0.1× SSC, all wash solutions contained 10 mM mercaptoethanol and 1× EDTA.

To detect hybridized riboprobes, the slides were rinsed in TN buffer (0.1× Tris-HCl, pH 7.5, 0.15 M NaCl), blocked 1 h in TN containing 2% horse serum and 0.1% Triton X-100, and incubated 1 h in a 1:500 dilution of the alkaline phosphatase-conjugated anti-digoxigenin antibody in TN containing 1% sheep serum. Specimens were then sequentially rinsed in TN buffer followed by substrate solution (0.2 mg/ml nitroblue tetrazolium, 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 10 mM ηN-ethylmaleimide and 1 mM levamisole to inhibit endogenous alkaline phosphatase. Color development was stopped with 10× Tris-HCl, pH 8.0, and 1× EDTA, and specimens were mounted in Aquamount.

Isolation of a fgl2-enriched Fraction from the Cauda Epididymal Lumen Contents—Sperm suspensions were prepared by mincing freshly dissected cauda epididymides in calcium-free Tyrode's solution containing 0.5× EGTA and 2 mM benzamidine. Aliquots of the sperm suspension (6 ml) were layered over an isotonic discontinuous Percoll gradient composed of 2 ml of 20% Percoll and 2 ml of 40% Percoll in Tyrode’s solution. The gradients were centrifuged at 100,000 × g for 60 min. The particulate fraction that layered on top of the 20% Percoll was collected, diluted with ice-cold Tyrode's solution, and then sedimented at 100,000 × g for 60 min in a Beckman SW41 rotor. The pellet material was utilized for immunocytochemistry and SDS-PAGE. Because HEP-64 was originally identified as a contaminant of a sperm acrosomal fraction (16), cauda sperm suspensions were also used to prepare an enriched acrosomal fraction as previously described (28, 29).

Immunocytochemistry—Sperm suspensions and crossections of fresh-frozen epididymides were fixed for 15–30 min at 4 °C with 4% formaldehyde, 0.1% sodium phosphate buffer, pH 7.4, rinsed in TN buffer (0.15 M NaCl, 20 mM Tris-HCl, pH 8.0, 0.05% Tween 20), blocked in TN containing 5% normal donkey serum and 2.5% BSA, and then incubated in rabbit anti-hamster fgl2 or preimmune rabbit serum diluted 1:500–1:500 in 0.1% w/v gelatin (Jackson ImmunoResearch, West Grove, PA) diluted in blocking solution. For enzymatic detection on crossections, an affinity-purified peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) was utilized as a secondary antibody, and color was developed with diaminobenzidine and H2O2. All crossections were counterstained with hematoxylin. In some experiments crossections were subjected to antigen retrieval immediately following formaldehyde fixation; sections were incubated at 95 °C for 30 min in 25 mM Tris-HCl, pH 9.0, 5 mM EDTA, 0.1% dithiothreitol, and 0.1% Triton X-100 and then processed as described above.

Electron Microscopy—for post-embedding immunolabeling, spermatozoa were fixed at 4 °C with 4% formaldehyde, 0.25% glutaraldehyde

1 The abbreviations used are: BSA, bovine serum albumin; Dig, digoxigenin; PBS, phosphate-buffered saline; E2, ubiquitin-activating enzyme; E3, ubiquitin-converting enzyme; E2 ubiquitin carrier protein; E3 ubiquitin-protein isopeptide ligase.
in 0.1 \text{ M} sodium phosphate buffer, pH 7.4, dehydrated through an ethanol series, and embedded in LR White resin. Thin sections were mounted on nickel grids, blocked in Tris-saline (0.15 M NaCl, 25 mM Tris-HCl, pH 8.0, 0.1% Tween 20) containing 2.5% BSA, 5% goat serum, 0.1% fish gelatin, and then incubated in 1:100 dilution of rabbit anti-fgl2 or, for controls, nonimmune serum diluted in blocking solution for approximately 1 h. After three rinses in blocking solution, the grids were incubated for 1 hi n 10-nm gold-conjugated, affinity-purified, goat anti-rabbit IgG (Amersham Biosciences). The sections were then rinsed in PBS, fixed with 1% glutaraldehyde in 0.1 M sodium phosphate buffer, rinsed in \text{H}_2\text{O}, and stained with uranyl acetate and lead citrate. Pre-embedding immunogold labeling of formaldehyde-fixed spermatozoa with rabbit anti-fgl2 was performed as described previously (16).

Specimens for routine electron microscopy were fixed with 4% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, post-fixed in 1\% osmium tetroxide, and embedded in Embed 812. Thin sections were stained with uranyl acetate and lead citrate.

SDS-PAGE and Western Blotting—Polypeptides were separated by SDS-PAGE (30) on high porosity 3–19\% linear gradient separation gels prepared with a 30:0.4 acrylamide:piperazine diacrylamide ratio or on 7–15\% linear gradient gels prepared with a 30:0.8 acrylamide:bisacrylamide ratio. Protein loads were estimated by the method of Bradford (31). Polypeptides were either stained with Coomassie Blue (32) or electrophoretically transferred to polyvinylidene difluoride membranes (33) for immunoblot analysis or for N terminus sequencing using an Applied Biosystems 475S sequencer (Foster City, PA).

Immunoblots were blocked with PBS (0.15 M NaCl, 20 \text{ mM} sodium phosphate, pH 7.6) containing 0.1\% Tween 20, 5\% goat serum, 0.1\% fish gelatin, and then incubated with rabbit anti-fgl2 or, for controls, nonimmune serum diluted in blocking solution for 1 h. After three washes in PBS, the blots were incubated in a rabbit anti-fgl2 serum diluted in PBS, washed, and then incubated in a goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were visualized by chemiluminescence using ECL Western Blotting Detection Reagent and detection on Kodak BioMax film.
RESULTS

Identification of Hamster Epididymal fgl2 by Expression Cloning—A rabbit polyclonal antibody, prepared against a 64-kDa hamster epididymal glycoprotein (HEP64) that binds defective spermatozoa (16), was utilized for expression screening of a hamster cauda epididymal cDNA library. Seventeen independent clones were obtained and sequenced; all represented the hamster ortholog of human and mouse fgl2, a fibrogen-related protein with putative procoagulant protease activity (18, 34, 35). The 2946-bp cDNA encoding the full-length hamster fgl2 protein is shown in Fig. 1. The full-length protein is 430 amino acid residues in length and possesses a predicted signal peptide of 22 amino acid residues. The deduced molecular weight of the processed 408 amino acid containing polypeptide was 46,142, in close agreement to the reported molecular weight of 52,000 for deglycosylated HEP64 (16), and its theoretical pI was 7.95. Epididymal fgl2 possesses one predicted O-linked glycosylation site at threonine 199 and five consensus motifs for N-linked glycosylation. A fibrinogen beta- and gamma-chain C-terminal signature domain was identified, which encompasses amino acids 348–360 of the processed fgl2 protein. The hamster fgl2 amino acid sequence possesses 88.7% identity to mouse fgl2 (16) and 77.7% identity to human fgl2 (35, 36). These data demonstrate that the previously identified epididymal secretory glycoprotein HEP64 (16) represents epididymal fgl2.

Northern Blot Analysis of fgl2 Expression in Hamster Organs—Northern blot analysis of total RNA isolated from several hamster organs demonstrated that the highest fgl2 mRNA expression was detected in the proximal and distal cauda epididymides and that each exhibited a single transcript of ~3 kb. Only a low level of fgl2 mRNA was detected in the caput epididymidis, indicating region-specific fgl2 expression. Among other hamster tissues tested, the second highest expression level of fgl2 mRNA was detected in the spleen, and all other tissues displayed only very low expression levels of fgl2 mRNA (Fig. 2). All RNA samples exhibited similar levels of hybridization to the constitutive probe for ribosomal protein S2 demonstrating equivalent RNA loads in each lane (Fig. 2).

In Situ Hybridization Demonstration of Region-specific Epididymal fgl2 Expression—Cryosections of the caput and cauda epididymides were hybridized with antisense Dig-labeled fgl2 riboprobes to identify the cell- and region-specific fgl2 mRNA expression. No fgl2 expression was detected in the proximal caput epididymidis (Fig. 3a), but low expression was apparent in principal cells of the distal caput epididymidis (Fig. 3b). Highest expression of fgl2 mRNA was seen in the proximal cauda epididymidis and localized to the basal region of the principal cells (Fig. 3, c–e). No fgl2 expression was detected in periurubular or connective tissue cells. Adjacent lobules of the epididymis are separated by prominent connective tissue partitions, and the lobule immediately anterior to the proximal cauda epididymidis exhibited a decreased fgl2 mRNA expression in principal cells (Fig. 3c). Likewise, the distal cauda is separated from the proximal cauda by a connective tissue septum, and it is distinguished by an increased tubule diameter and reduced epithelial cell height. Compared with the proximal cauda, the principal cells of the distal cauda region also express significant, but reduced, levels of fgl2 mRNA (Fig. 3e). Control sections hybridized with sense Dig-labeled fgl2 riboprobes exhibited no detectable staining in any epididymal segment (Fig. 3f). These data demonstrate that fgl2 mRNA expression is both region- and principal cell-specific in the hamster epididymis.

Epididymal fgl2 Localization and Association with Defective Spermatozoa—Immunohistochemical staining of epididymal cryosections using anti-hamster fgl2 revealed a region- and cell-specific expression pattern identical to that detected for fgl2 mRNA by in situ hybridization. Principal cells of the proximal cauda epididymidis displayed a strong staining of the Golgi region (Fig. 4a). In addition focal accumulations of fgl2-positive material were apparent in the epididymal lumen (Fig. 4, a and b) that ranged in size from large sperm aggregates to small particulate elements (Fig. 4b). Parallel sections stained with preimmune serum exhibited no staining of the epididymal epithelium or lumen (Fig. 4c). Immunofluorescence analysis of cauda sperm suspensions revealed the association of fgl2 with specific domains of abnormal spermatozoa as reported previously (16) and with sperm fragments and small particulate elements (see Fig. 6a). Post- and pre-embedding immunoelectron microscopy on cauda epididymal sperm suspensions demonstrated that fgl2 localized to an amorphous electron dense coating material, or “death cocoon-like” complex, on abnormal spermatozoa. Post-embedding immunolabeling of cauda spermatozoa established that fgl2 was distributed throughout the full thickness of the cocoon-like coating (Fig. 5, a and b). Immunoelectron microscopy also revealed that fgl2 localized to aggregates of amorphous material containing embedded membrane vesicles and organelles of disintegrating spermatozoa (Fig. 5, c and d). fgl2 was not detected on morphologically normal spermatozoa (Fig. 5a) or on normal spermatozoa immunostained using a control nonimmune primary serum (not shown).

Isolation and Characterization of an fgl2-enriched Fraction from the Cauda Luminal Contents—Because the immunocytochemical data indicated that small fgl2-containing aggregates were present in the cauda epididymal lumen (Fig. 4, a and b) and in cauda sperm suspensions (Fig. 6a), a protocol was devised to isolate an enriched fraction of these particulate elements. Sperm suspensions obtained from tissue minces of the cauda epididymidis were centrifuged at 1,500 × g onto discontinuous Percoll gradients. The fraction that layed on top of the 20% Percoll was collected and then re-centrifuged at 100,000 × g. Phase contrast and immunofluorescence microscopy revealed that the pelleted material was highly enriched for amorphous particles that were intensely immunostained with anti-fgl2 (Fig. 6b). Electron microscopic analysis of the crude particulate fraction further revealed that it predominantly contained aggregates of electron dense, death cocoon material (Fig. 6c), which appeared identical to the material that coated abnormal spermatozoa (compare with Fig. 5, a–c); the fraction also contained a variety of membranous vesicles and occasional cytoplasmic droplets and sperm fragments.
SDS-PAGE and immunoblotting, using disulfide reducing and nonreducing conditions, were performed to identify the polypeptide composition of the fgl2-containing particulate fraction isolated from the cauda sperm suspensions. Because we previously identified fgl2 (HEP64) as a contaminant of a sperm acrosomal fraction isolated on Percoll density gradients (16), parallel lanes of acrosomal matrix were also included. The death cocoon-enriched fraction (Fig. 7, a and b, lane 1) and the acrosomal fraction (Fig. 7, a and b, lane 2) each revealed a discrete set of polypeptides after Coomassie Blue staining (Fig. 7, a and b, lanes 1 and 2). Immunoblots prepared from samples fractionated by SDS-PAGE in the absence of disulfide-reducing agents revealed that fgl2 was present in both fractions as oligomers of \( M_r \approx 260,000 \) and 280,000 (Fig. 7a, lanes 3 and 4).
but with disulfide bonds reduced fgl2 migrated as a monomer of M_r ~ 64,000 (Fig. 7b, lanes 3 and 4).

To verify that both the 260- and 280-kDa, disulfide-linked oligomers contained fgl2 subunits, they were subjected to N terminus microsequencing analysis, and 11 amino acids were obtained for the 280-kDa oligomer and 9 amino acids for the 260-kDa oligomer, respectively. Each protein exhibited identical amino acid sequences of YRLTQGLEDAS. The derived N terminus sequence agreed with the predicted N terminus obtained from the open reading frame of the fgl2 cDNA except at position two where the deduced sequence demonstrated an asparagine (N) rather than an arginine (R) residue (see Fig. 1).

**DISCUSSION**

This study identifies the regionally expressed secretory protein of the hamster cauda epididymis that specifically binds abnormal spermatozoa and that we previously termed HEP64 (16), as the fibrinogen-related protein fgl2 (18, 34). This finding demonstrates that the epididymis possesses a specific and previously unrecognized mechanism to coat defective spermatozoa with an fgl2-containing protein complex that segregates them from the normal sperm population. Principal cells of the proximal cauda epididymidis expressed the highest levels of fgl2 mRNA and protein. Its expression was somewhat reduced in the distal cauda, and only low expression was found in the caput region. The highest percentage of nonviable spermatozoa is also found in the cauda region (15), suggesting a role for fgl2 in the protection of mature spermatozoa. Previous histological studies of the guinea pig and rat have identified eosinophilic masses containing degenerating spermatozoa within the epididymal lumen (12, 13, 37). This finding was confirmed ultrastructurally in several mammalian species, where degenerating epididymal spermatozoa appeared embedded in an electron dense, amorphous material of unknown identity (14). Our data indicate that fgl2 is one component of these coating proteins, and collectively these observations suggest that fgl2 may represent a generalized mechanism for recognition of defective spermatozoa.

Epididymal fgl2 appears to confer its protective function by polymerizing into a cocoon-like complex on defective spermatozoa and sperm fragments. Our immunoelectron microscopic studies demonstrate that fgl2 is distributed throughout the death cocoon coating indicating it represents a structural protein of the assembled complex. The fgl2 complex may prevent...
defective spermatozoa from releasing hydrolytic enzymes, which could have detrimental effects on viable spermatozoa and the epididymal epithelium, and/or autoantigenic proteins, which could provoke an immune response if they escaped from the epididymal lumen. Unlike the cell elimination pathway employed during apoptosis in somatic tissues (38, 39), we have detected no phagocytosis of fgl2-coated spermatozoa by the epididymal epithelium. Instead they are likely eliminated with viable spermatozoa by outflow from the cauda. This direct polymerization of fgl2 into insoluble complexes coating compromised cells has not been reported in previous studies of somatic tissues. Although in the hamster, the highest expression was noted in the cauda epididymidis, fgl2 was also expressed in other organs at levels that were comparable to that detected in the caput epididymidis. Similarly other studies have shown that several organs of humans (40) and rats (41), but not the mouse (18), display constitutive fgl2 mRNA expression. fgl2 expression is induced both in vitro and in vivo by interferon γ (42, 43), and its expression is also up-regulated in specific pathological conditions such as hepatitis virus-promoted liver failure (18, 40) and spontaneous abortions both in humans and in mouse models (44–46). Thus it will be important to determine if the polymerization of an fgl2 complex on defective somatic cells also occurs in either normal or in pathological situations, indicating that it may represent a general mechanism to segregate defective cells when a phagocytic disposal pathway is lacking. In addition it will be of interest to determine if conditions that promote sperm death in the epididymis also increase fgl2 expression.

fgl2, originally termed fibroleukin, was first identified as a T-lymphocyte-specific gene product encoding a 64-kDa polypeptide (17) with a fibrinogen signature domain (47). T-cells, and fgl2-transfected cells, secrete fgl2 as a soluble, disulfide-linked, glycosylated oligomer (48). Soluble fgl2 has been suggested to perform immunomodulatory functions and suppress T cell proliferation and maturation in vitro (48); however, these findings have not been supported by in vivo studies of fgl2-deficient mice (36, 43, 49). In contrast, in pathological situations, including virus-dependent liver failure (18, 35, 50). Although these studies suggest fgl2 functions to catalyze pathological coagulative processes, our data suggest instead that fgl2 is a component of the polymerized matrix. Hamster epididymal fgl2 is also secreted as a soluble, disulfide-linked, glycosylated oligomer (16), but its subunit composition remains to be established, and it will be of interest to determine if fgl2 assembles into tissue-specific oligomers with distinct functions. We have yet to determine if epididymal fgl2 exhibits a pro-

**Fig. 6.** Matched phase contrast (a and b) and fluorescence (a’ and b’) photomicrographs of cauda spermatozoa (a and a’) and the death cocoon fraction isolated from cauda sperm suspensions using Percoll gradients (b and b’). In sperm suspensions (a and a’) fgl2 is associated with sperm flagellar (f) fragments, with detached heads (h), with specific domains of abnormal spermatozoa (ac = acrosomal segment) and with amorphous aggregates (ag). Normal spermatozoa are not labeled. In the death cocoon-enriched fraction amorphous aggregates of material are evident by phase contrast microscopy (b), and most stain intensely with anti-fgl2 (b’). c, electron micrograph of the isolated death cocoon fraction showing it contains a variety of membrane vesicles (v) and amorphous aggregates of death cocoon material (dc).
thrombinase-like proteolytic activity, however its function appears distinct from those proposed in other somatic tissues, in that it has both a recognition specificity for compromised cells and is a component of the polymerized death cocoon complex on defective spermatozoa.

Both the signal that initiates fgl2 binding to abnormal spermatozoa and the mechanisms regulating polymerization of the death cocoon complex remain to be defined. In apoptotic somatic cells the surface exposure of phosphatidylinerse functions as a recognition signal for their phagocytic elimination (38), and phosphatidylinerse has been demonstrated on the surface of oxidatively damaged mammalian spermatozoa (51). Moreover phosphatidylinerse stimulates the procoagulant activity of macrophage fgl2 (49, 52) indicating it could function in binding fgl2 to the cell surface. These observations suggest that phosphatidylinerse may represent a recognition signal to initiate fgl2 binding to abnormal spermatozoa and to promote a protease activity required for assembly of the sperm death cocoon, and these possibilities are being examined. It is emphasized however that the death cocoon complex is also assembled on several sperm-specific structural elements that become exposed by loss of the overlying plasma membrane, such as acrosomal components, the postacrosomal sheath, the mitochondrial sheath, and the fibrous sheath (16). This finding raises the possibility that fgl2 is a pattern recognition molecule that binds one or more common structural motifs in these organelles. Thus, like other fibrinogen-related proteins that function in the innate immune response by recognizing invading pathogens (53–55), fgl2 could recognize newly exposed sperm components via specific exposed domains and then initiate death cocoon polymerization to isolate these components.

We propose that the epididymal fgl2 oligomer plays a central role in protection of the epididymal sperm population by specifically targeting and physically enveloping defective spermatozoa. An alternative mechanism proposed for elimination of defective spermatozoa states that their surface ubiquitination leads to phagocytotic elimination by the epididymal epithelium (56–58). However, this proposal has been questioned by others, because there is little evidence supporting substantial phagocytic sperm elimination by the epididymal epithelium (59). Moreover ubiquitination of extracellularly oriented sperm surface proteins would require the presence not only of ATP but also the E1, E2, and E3 enzymes of the ubiquitination pathway, which are typically cytosolic (60, 61), and their presence in the epididymal luminal fluid remains to be established. We do not know if fgl2 may bind intracellular ubiquitinated proteins of defective spermatozoa that become exposed by loss of the overlying plasma membrane; if so, the fgl2 and ubiquitination pathways could co-operate in sperm elimination, but it would not likely be by a phagocytotic pathway, because we have found no evidence for epithelial uptake of fgl2-coated spermatozoa. We anticipate that detailed analysis of the ligand binding activity of the epididymal fgl2 oligomer will provide insights into the basis for its specific recognition of defective spermatozoa, define whether it contributes to their death, and reveal how it initiates polymerization of the death cocoon complex that segregates them from the healthy sperm population.

REFERENCES

1. Bedford, J. M. (1975) in Handbook of Physiology: Endocrinology, Male Reproductive System (Greep, R. O., and Astwood, E. B., eds) pp. 303–317, Waverley Press, Baltimore, MD
2. Orgebin-Crist, M. C., Danzo, B. J., and Davies, J. (1975) in Handbook of Physiology: Endocrinology, Male Reproductive System (Greep, R. O., and Astwood, E. B., eds) pp. 319–338, Waverley Press, Baltimore, MD
3. Jones, R. C. (1999) Int. J. Androl. 22, 57–67
4. Hinton, B. T., and Palladin, M. A. (1995) Microsc. Res. Tech. 36, 67–81
5. Hinton, B. T., Palladin, M. A., Rudolph, D., Lan, Z. J., and Labus, J. C. (1996) Curr. Topics Dev. Biol. 33, 61–102
6. Kirchhoff, C. (1999) Int. Rev. Cytol. 188, 133–202
7. Jones, R. (1998) J. Reprod. Fert. Suppl. 53, 73–84
8. Kirchhoff, C., Osterhoff, C., Pera, L., and Schroter, S. (1998) Andrologia 30, 225–232
9. Bailey, R., and Grieswold, M. D. (1999) Mol. Cell. Endocrinol. 151, 17–23
10. Cornwall, G. A., Orgebin-Crist, M. C., and Hann, S. R. (1992) Mol. Endocrinol. 6, 1653–1664
11. Jervis, K. M., and Robaire, B. (2001) Biol. Reprod. 65, 696–703
12. Simeone, F. A., and Young, W. C. (1931) J. Exp. Biol. 6, 163–175
13. Glover, T. D. (1961) Nature 190, 185–186
14. Cooper, T. G., and Hamilton, D. W. (1977) Am. J. Anat. 149, 93–110
15. Weissenberg, R., Yossefi, S., Oshory, Y., Madgar, I., and Lewin, L. M. (1994) Int. J. Androl. 17, 256–261
16. NagDas, S. K., Winfrey, V. P., and Olson, G. E. (2000) Biol. Reprod. 63, 1428–1436
17. Kayama, T., Hall, L. R., Haser, W. G., Tonegawa, S., and Saito, H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1609–1613
18. Ding, J. W., Ning, Q., Liu, M. F., Lai, A., Lebowitz, J., Peltekian, K. M., Cole, E. H., Fung, L. S., Holloway, C., Marden, P. A., Yeger, H., Phillips, M. J., and Levy, G. A. (1997) J. Viral. 71, 9225–9230
19. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) Protein Eng. 10, 1–6
20. Hansen, J. E., Lund, O., Engelbrecht, J., Bohr, H., Nielsen, J. O., and Hansen, J. E. S. (1995) Biochem. J. 308, 801–813
21. Hansen, J. E., Lund, O., Rapacki, K., and Brunak, S. (1997) Nucleic Acids Res. 25, 278–282
22. Jensen, J. E., Lund, O., Tolstrup, N., Gooley, A. A., Williams, K. L., and Brunak, S. (1998) Glycogen. J. 15, 115–130
23. Hofmann, K., Bucher, P., Falquet, L., and Bairoch, A. (1999) Nucleic Acids Res. 27, 215–219
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Cold Spring Harbor Laboratory Manual, 2nd Ed., Cold Spring Harbor University Press, Cold Spring Harbor, NY
25. Harpold, M. M., Evans, R. M., Salditt-Georgieff, M., and Darnell, J. E. (1979) Mol. Cell. Endocrinol. 11, 215–219
26. Panoskaltsis-Mortari, A., and Bucy, R. P. (1995) BioTechniques 18, 399–397
27. Olson, G. E., Winfrey, V. P., Matrisian, P. E., NagDas, S. K., and Hoffman, L. H. (1998) Cell Tissue Res. 293, 489–498
28. Olson, G. E., Winfrey, V. P., and Davenport, G. R. (1988) Biol. Reprod. 39, 1145–1158
