

Truncated Semenogelin I Binds Zinc and Is Cleaved by Prostate-Specific Antigen

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ABSTRACT: Semenogelins I and II are major coagulum-forming proteins in semen, and they are secreted mainly by the seminal vesicles. These proteins bind Zn^{2+} and act as substrates for prostate-specific antigen and transglutaminase. A variant semenogelin I lacking 60 amino acids has been described that occurs in different populations with an allele frequency of 1%–3%. To better understand the function of the semenogelins in vivo, our aim was to characterize the properties of the variant form and compare with the wild type. Recombinant proteins were synthesized in insect cells. Binding of Zn^{2+} was studied by titration of metal ions in the presence of a zinc (II) fluorophore chelator. SDS-PAGE was used to visualize the results of cleavage by prostate-specific antigen and cross-linking with transglutaminase. We found that the truncated and wild-type

semenogelin molecules had similar Zn^{2+} -binding properties (ie, a stoichiometry of at least 9–10 mol per mol of protein and an average dissociation constant of 5 $\mu\text{mol/L}$ per site), and they showed also similar susceptibility for degradation by prostate-specific antigen. Furthermore, like the wild-type form, the truncated semenogelin I was able to serve as a substrate for transglutaminase. These findings imply that the studied characteristics do not depend on a well-defined tertiary structure, or that the deletion has no major effect on the structure responsible for these features.

Key words: Transglutaminase, variant, semen, prostate-specific antigen, fertility, reproduction.

J Androl 2006;27:542–547

Semenogelins I and II (SgI and SgII) and fibronectin are secreted by the seminal vesicles, and they are the major coagulum-forming proteins in semen. During ejaculation, the secretions from the seminal vesicles and prostate are mixed with spermatozoa-rich epididymal fluid to form the high-molecular-weight protein network that traps the spermatozoa. As this coagulum is being produced, it also begins to undergo liquefaction. Prostate-specific antigen (PSA) is a human kallekrein-related protease with unique specificity (Malm et al, 2000); it is secreted by the prostate and plays a prominent role in the process of liquefaction by exerting its proteolytic effect on semenogelin. Breakdown of the coagulum releases the progressively motile spermatozoa so that they can be further transported to the ovum (Lilja et al, 1987; Lilja et al, 1989; Malm et al, 1996; Robert et al, 1997). In addition to participating in gel formation and acting as substrates for PSA, the semenogelins have been found to be the major zinc binders in semen and to be involved in modulating PSA activity by controlling the level of free Zn^{2+} in vitro

(Jonsson et al, 2005). SgI and SgII are also excellent substrates for transglutaminase, which catalyses the cross-linking of a primary amino group to a glutamine residue. The biological importance of structural modification of semenogelin by transglutaminase is not yet known (Peter et al, 1998).

The SgI molecule (439 amino acids, about 50 kd) is composed of 6 similar 60-amino-acid repeats, and SgII (559 amino acids, about 63 kd) consists of 8 such repeats. In contrast to SgI, SgII is also secreted in a glycosylated form (Lilja and Lundwall, 1992; Ulvsback et al, 1992; Malm et al, 1996). There is 78% amino acid identity between SgI and SgII (Lilja and Lundwall, 1992). Both proteins are synthesized primarily in the seminal vesicles, although more moderate expression has been observed in other reproductive and non-reproductive organs (Lundwall et al, 2002).

The 2 genes encoding SgI and SgII are located on chromosome 20. A variant semenogelin I gene lacking a 180-bp tandem repeat has been described by several groups (Jensen-Seaman and Li, 2003; Lundwall et al, 2003; Miyano et al, 2003), and it is present with an allele frequency of approximately 1%–3% in different populations. The gene product is a truncated form of semenogelin I (SgI₄₃) that lacks a repeat of 60 amino acids found in the wild-type form and has a molecular mass of approximately 43 kd. The deleted block of residues is located between residues 271 and 348 of the

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Received for publication October 17, 2005; accepted for publication February 2, 2006.

DOI: 10.2164/jandrol.05188

secreted wild-type protein, but because of the nature of the repeat structure, the exact location cannot be determined (Lundwall et al, 2003).

The semenogelins bind zinc and act as substrates for PSA and transglutaminase, functions that are probably important for the role these proteins play *in vivo* in semen. Accordingly, to learn more about the physiological effects of semenogelins, our objective was to characterize the product of the common 180-bp-lacking (ie, 14% of the secreted wild-type protein) variant of the semenogelin I gene and to compare it with the wild-type protein. We performed experiments using recombinant proteins synthesized in a baculovirus system. We employed a fluorescent Zn^{2+} chelator to investigate the Zn^{2+} -binding properties of the SgI₄₃, and we conducted enzymatic assays and gel electrophoresis to study the ability of that protein to serve as a substrate for PSA and transglutaminase.

Materials and Methods

Materials

PCR amplifications were performed using an Advantage 2 PCR kit from Clontech BD Biosciences (Stockholm, Sweden). Automated DNA sequencing was performed with a Big Dye Terminator Cycle Sequencing Ready Reaction Kit from Applied Biosystems (Foster City, Calif). Restriction enzymes and T4 DNA ligase were purchased from Fermentas (Helsingborg, Sweden). We also used a First-Strand cDNA Synthesis Kit from Amersham Biosciences AB (Uppsala, Sweden), and JetSorb and JetQuick extraction kits from Genomed (Bad Oeyenhausen, Germany). Fetal calf serum was obtained from Saveen (Malmö, Sweden), SF-900 II serum-free medium (SFM), DH10BAC competent cells, and CELLFECTIN were from Invitrogen AB (Stockholm, Sweden). Recombinant factor XIIIa was purchased from Zymogenetics (Seattle, Wash), and the Zn^{2+} -specific chelator Fluo-Zin 2 was from Molecular Probes Inc (Eugene, Ore). Mono-dansylthio-cadaverine (MDTC) was obtained from Larodan AB (Malmö, Sweden).

Proteins

Human semen specimens were collected from healthy volunteer sperm donors (through masturbation) at the Fertility Laboratory, Malmö University Hospital, Malmö, Sweden. The study was approved by the Ethics Committee of Lund University (LU 532-03). Sample collection and purification of SgI and SgII were performed essentially as previously described by Malm et al (Malm et al, 1996), with the following changes: instead of alkylating the proteins, a reducing agent (30 mmol/L dithiothreitol) was included in all buffers during purification, and the proteins were stored at $-70^{\circ}C$. PSA was purified as reported by Christensson et al (Christensson et al, 1990).

Construction of Baculovirus Donor Plasmids

Synthesis of seminal vesicle cDNA was performed as previously described (Lundwall and Clauss, 2002). A transcript encompassing the coding nucleotides of the SgI (wild-type) precursor was amplified using an Advantage 2 PCR kit and the primers 5'-CCT ACG TCG ACA TGA AGC CCA ACA TCA TCT TTG TA-3' and 5'-CAG GCT CTA GAG GTT TAT GTA AAT AAT GGG TTT C-3' under standard PCR conditions. After cleavage with Sall and XbaI, the transcript was cloned into the multiple cloning site of pFastBac1. To create a pFastBac1 construct encoding SgI₄₃, a 0.410-kb NcoI-SacI fragment of the SgI (wild-type) construct was replaced with the homologous 0.230-kb fragment of the variant allele, which was obtained by PCR using genomic DNA from a heterozygous carrier and primers flanking the NcoI and SacI sites. The PCR was run under standard conditions, with the primer pair 5'-TGC GCA CCA AGA CAA ACT CCA ACA TGG AT-3' and 5'-TGA CGA TCA CTG TCA TCT TCC TGC TCT AT-3'. Following restriction, the products of the 2 alleles were separated by electrophoresis on a 2% agarose gel, and the 0.230-kb fragment was isolated using JetSorb. The sequences of constructs were verified by DNA sequencing.

Expression of Recombinant Proteins

Recombinant baculovirus was produced in insect cells with a Bac-to-Bac baculovirus expression system (Invitrogen). The pFastBac1 transformation into DH10Bac (for transposition into the bacmid), isolation of recombinant bacmid, transfection of Sf9 cells and virus amplification were done according to the instruction manual. The virus was amplified in Sf9 cells grown in SF900 II medium with 10% fetal calf serum to titres exceeding 10^7 pfu/mL.

Recombinant protein expression was performed in suspension cultures of High Five cells grown at $27^{\circ}C$ in serum-free SF-900 II medium. At mid-log phase, the cells (10^6 /mL) were infected at a multiplicity of infection of 0.4–1. Forty-eight hours after infection, the cultivation was terminated and cells were removed by centrifugation at 1000 rpm for 10 minutes. The supernatant was mixed with an equal volume of a solution comprising 0.1 mol/L Tris (pH 8.7), 10 mmol/L EDTA, and 4 mol/L urea, after which the recombinant proteins were purified in the same manner as described for the seminal plasma proteins.

Characterization of the Semenogelins

Mass spectra were recorded with an API QSTAR Pulsar-I quadrupole/time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with a nanospray ion source (MDS Proteomics, Odense, Denmark). For analysis by nanospray ionization-mass spectrometry, samples of semenogelins (SgI and the recombinant proteins rSgI and rSgI₄₃) were desalted on a microcolumn of POROS R1 resin (Applied Biosystems) packed in a glass capillary (MDS Proteomics), and they were eluted with aqueous 50% (v/v) methanol containing 3% (v/v) formic acid directly into a nanospray capillary (New Objective Inc, Woburn, Mass). Spectra were acquired in the positive ion mode with an ion

spray voltage of 0.8 kV and a curtain gas (N₂) flow rate of 1.3 L/min. Analyst QS version 1.1 software (Applied Biosystems/MDS Sciex) was used to analyze the mass spectra.

The fluorescent zinc(II) chelator FluoZin-2 was used to quantify the specific binding of Zn²⁺ to SgI, rSgI, and rSgI₄₃. We performed the chelator study and interpreted the results by the CaLigator software essentially as previously described (Andre and Linse, 2002; Jonsson et al, 2005).

Purified and recombinant semenogelins were cleaved with PSA as related by Malm et al (Malm et al, 2000), and the products were visualized by SDS-PAGE (15%). The ability of factor XIIIa to separately cross-link SgI, rSgI, and rSgI₄₃ was assessed as reported by Peter et al (Peter et al, 1998).

Results

SgI, rSgI, and rSgI₄₃ were all purified by performing affinity chromatography on heparin-Sepharose and eluting with a NaCl gradient. All 3 proteins were eluted at a concentration of approximately 0.2 mol/L NaCl. SgI (Figure 1, lane 1) and rSgI (Figure 1, lane 4) had the same mobility in SDS-PAGE (run under reducing conditions), which corresponded to a mass of 50 kd. The rSgI₄₃ was visible as a band at 43 kd (Figure 1, lane 7). Mass spectrometric analysis indicated masses of 49 602, and 49 607, and 42 865 daltons for SgI, rSgI, and rSgI₄₃, respectively. By comparison, application of the ProtParam tool has predicted masses of 49 607 daltons for SgI and 42 797 daltons for rSgI₄₃ (Wilkins et al, 1999).

The zinc(II) fluorophore chelator FluoZin-2 was used to quantify the specific binding of Zn²⁺ to intact SgI, intact rSgI, and rSgI₄₃. Fluorescence was recorded during titrations of FluoZin-2 with Zn²⁺ in the absence or presence of the different semenogelins. The results indicate that as the concentrations of SgI, rSgI, and rSgI₄₃ were increased, more Zn²⁺ was needed to saturate FluoZin-2, and the slope of the plot of fluorescence versus Zn²⁺ concentration decreased (Figure 2). These observations suggest that the semenogelins competed with the chelator for the Zn²⁺, which could have occurred only if the proteins and the chelator had similar affinity for Zn²⁺. However, due to precipitation of the protein at high Zn²⁺ concentrations, it was not possible to obtain the complete titration curves that are needed to accurately determine the Zn²⁺ dissociation constant (K_D) for each protein. Nonetheless, the linear appearance of the curves for SgI, rSgI, and rSgI₄₃ suggests that all 3 proteins had a K_D similar to that of the Zn²⁺ chelator (ie, approx. 5 μmol/L) (Linse, 2002). We analyzed the first 9 titration points to estimate the stoichiometry of Zn²⁺ binding for the different forms of SgI. Only the lower limit of the stoichiometry could be determined, which was similar for the 3 proteins and was at least about 9–10 mol of Zn²⁺ per mol of protein.

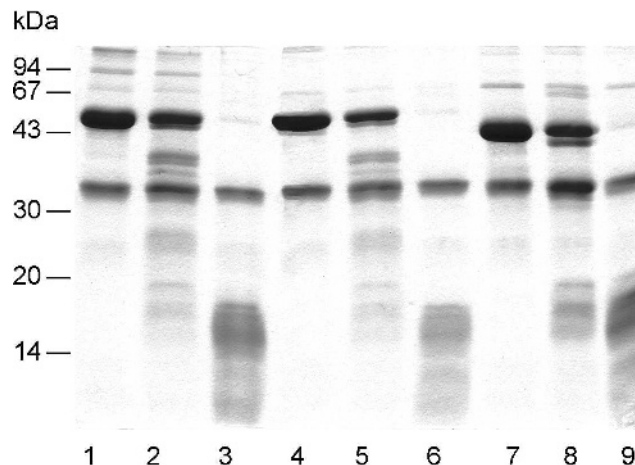


Figure 1. SDS-PAGE (15%) analysis of semenogelin cleaved by PSA. The gel was stained with Coomassie Brilliant Blue. The figure shows SgI (lanes 1–3), rSgI (lanes 4–6), and rSgI₄₃ (lanes 7–9) at initiation of cleavage, after 5 minutes, and after 4 hours, respectively.

According to the CaLigator analysis, the titration curve of rSgI₄₃ corresponded best to 9 mol of Zn²⁺ per mol of protein (Figure 2D).

To study susceptibility to digestion by PSA, we incubated SgI, rSgI, and rSgI₄₃ with PSA for different amounts of time and then analyzed the semenogelins by SDS-PAGE (Figure 1). We found that rSgI₄₃, rSgI, and SgI were essentially equally sensitive for PSA cleavage, as indicated by the observations that a smaller fraction of the bands representing intact semenogelin was degraded after 5 minutes, and after 4 hours no intact semenogelin was visible and mainly bands between 10–20 kd remained. PSA was visible at 33 kd in each lane.

To determine whether rSgI₄₃ and rSgI share the ability of SgI to function as a substrate for transglutaminase, the 3 proteins were incubated separately with mono-dansyl-thio-cadaverine (MDTC) and recombinant FXIIIa (a protein with transglutaminase activity) in the presence and absence of Ca²⁺. MDTC is a UV fluorescent and a substrate for transglutaminase, and it was included in the analysis to visualize the reactions on SDS/PAGE (Figure 3). SgI, rSgI, and rSgI₄₃ incubated in presence of MDTC and Ca²⁺ were all clearly visible when exposed to UV-light indicating that each of these proteins was able to incorporate MDTC. Therefore, both rSgI₄₃ and rSgI can indeed function as substrates for transglutaminase. Recombinant FXIIIa was visible in lanes 4–9 at approximately 80 kd.

Discussion

In this study, we have characterized rSgI₄₃ and demonstrated that it is similar to SgI with regard to its

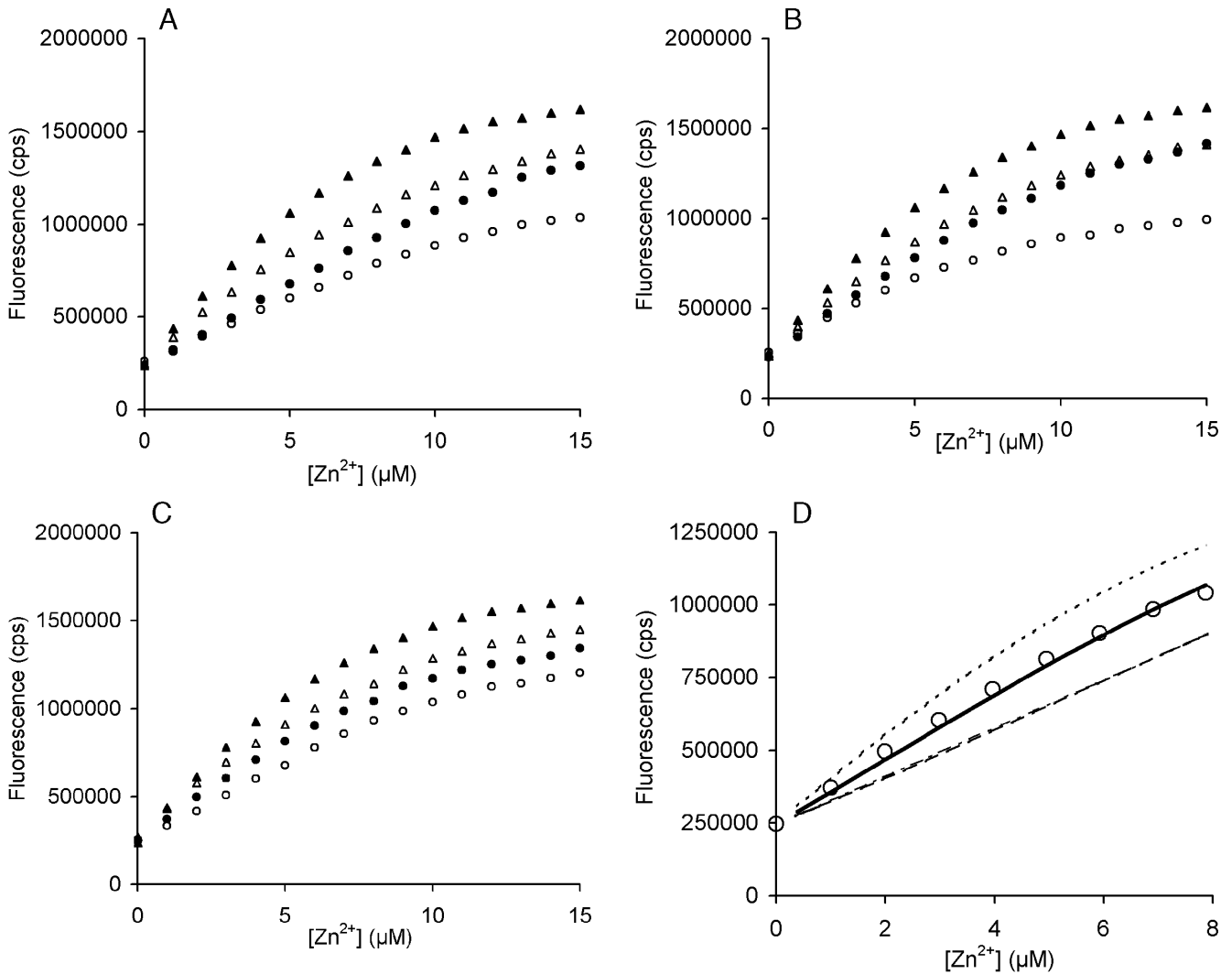


Figure 2. Fluorescence of 15 μmol FluoZin-2 titrated with Zn^{2+} in the presence of different amounts of semenogelins. (A) Zn^{2+} titrations with SgI at concentrations of 0 (\blacktriangle), 0.15 (\triangle), 0.30 (\bullet), and 0.60 (\circ) $\mu\text{mol/L}$. (B) Zn^{2+} titrations with rSgI at concentrations of 0 (\blacktriangle), 0.15 (\triangle), 0.30 (\bullet), and 0.60 (\circ) $\mu\text{mol/L}$. (C) Zn^{2+} titrations with rSgI₄₃ at concentrations 0 (\blacktriangle), 0.15 (\triangle), 0.30 (\bullet), and 0.60 (\circ) $\mu\text{mol/L}$. (D) Computer fitting to the first 9 steps of the Zn^{2+} titration with 0.30 $\mu\text{mol/L}$ rSgI₄₃. Using a K_D of approximately 5 $\mu\text{mol/L}$, the 3 fitting lines represent a stoichiometry of 1 (\cdots), 9 ($-$), and 20 ($---$).

capacity to bind Zn^{2+} and its ability to act as a substrate for PSA and transglutaminase.

So far, no human subject has been found to be homozygous for the variant semenogelin I gene, and no methods have been developed to separate the variant from the wild-type protein in heterozygous individuals. Therefore, to characterize the variant protein it was necessary to use recombinant proteins. SgI is a non-glycosylated protein, and we have shown that the properties of recombinant SgI are indistinguishable from those of SgI purified from human semen. Thus it seems reasonable to assume that the recombinant SgI₄₃ we studied and the SgI₄₃ found in seminal plasma have the same physicochemical characteristics.

SgI and rSgI had almost identical molecular masses according to the mass spectrometric results and theoretical values based on amino acid sequences. The smaller rSgI₄₃ deviated slightly from the mass indicated by ProtParam (ie, +68 Da). This difference cannot be explained by mass inaccuracy, which was in the range of 0.01% (Roepstorff, 1999). Therefore, it cannot be excluded that an amino acid substitution occurred during synthesis of the recombinant protein, or that there were some minor posttranslational modifications such as oxidation.

The block of 60 amino acids that is missing in SgI₄₃ is known to include 7 Glu, one Asp, and 2 His residues, all of which can coordinate Zn^{2+} (Auld, 2001). According-

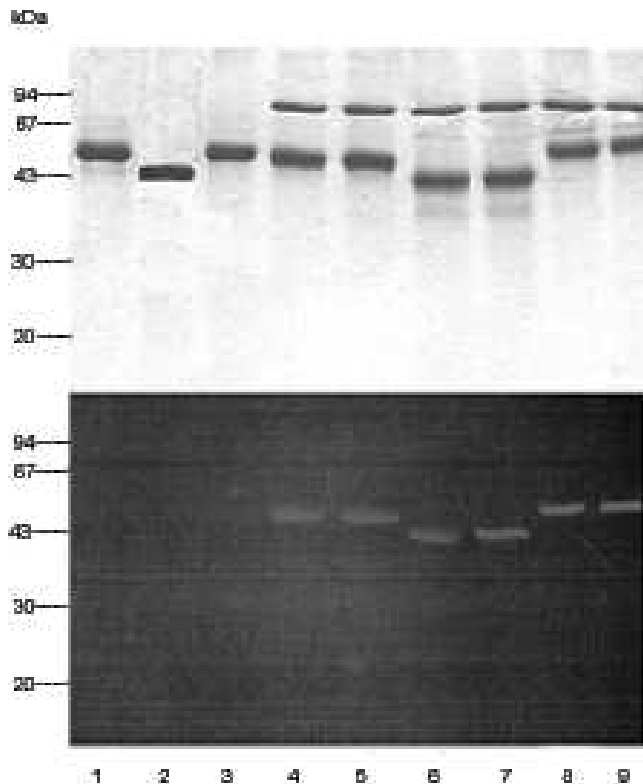


Figure 3. Cross-linking of semenogelin. **(A)** Gel stained with Coomassie Brilliant Blue. **(B)** Gel photographed in ultraviolet light. Lanes 1–3 respectively represent rSgI, SgI₄₃, and SgI (not cross-linked). Lanes 4–9 show duplicates of rSgI, SgI₄₃, and SgI, each at a final concentration of 2 $\mu\text{mol/L}$. To achieve cross-linking, 5 nmol thrombin, 40 mmol/L DTT, 40 $\mu\text{g/mL}$ FXIIIa, 0.7 mmol/L MDTC (mono-dansyl-thio-cadaverine), and finally 10 mmol/L CaCl_2 in 50 mmol/L TBS (pH 7.5) were added to each of the semenogelin samples, which were subsequently incubated for 2 hours at 37°C. The reaction was terminated with 20 mmol/L EDTA, and 15 μL of the reaction solution was applied on the gel.

ly, SgI₄₃ lacks potential Zn^{2+} -binding sites that may be found in the wild-type protein. However, although the truncated protein yielded a zinc titration curve that best corresponded to a stoichiometry of 9 mol of Zn^{2+} per mol of protein in our experiments, this result is not precise enough to claim that it differs significantly from the stoichiometry of 10 mol of Zn^{2+} or more per mol of protein observed for SgI and rSgI. Therefore, our finding suggests that the truncated protein can function in a manner similar to the wild-type molecule in processes that depend on the zinc-binding capacity of the semenogelins. Such an assumption calls for a discussion of the physiological role of zinc in this context. The high concentration of this metal ion in semen compared to other fluids or tissues, and the observations that SgI and SgII bind zinc and they are precipitated in the presence of zinc concentrations of around 100 $\mu\text{mol/L}$ support the idea that zinc is an important structural factor in the process of semen coagulation (Jonsson et

al, 2005). Consequently, it is interesting that the variant semenogelin I has the same high zinc-binding capacity as the wild-type protein, even though the stoichiometry may be slightly lower. This finding suggests that, when considering formation and stability of the semen coagulum, there is no major difference between individuals who are homozygous or heterozygous for the variant and those who are homozygous for the wild-type form.

The susceptibility of rSgI₄₃ to proteolytic degradation by PSA indicates that the liquefaction of semen samples is similar in men carrying the genetic variant and those not. This agrees with a previous study conducted in our laboratory (Lundwall et al, 2003), which showed that liquefaction of seminal plasma occurs in comparable ways in individuals heterozygous for the variant gene and those homozygous for the wild-type gene. The data showing that the semenogelins (including rSgI₄₃) can serve as substrates for transglutaminase in vitro implies that these proteins can be covalently linked to amino groups on the surface of a sperm. In humans, a prostate-specific transglutaminase has been cloned but not isolated (Grant et al, 1994; Dubbink et al, 1996).

Three features of the semenogelins, namely their repetitive primary structure, their tendency to precipitate, and their gel-forming ability, suggest that these proteins have more structural features in common with fibrous proteins than with globular proteins. This assumption is further supported by the observations that the deletion of 60 amino acid residues from SgI does not affect zinc binding or the ability to act as a substrate for PSA and transglutaminase. These findings imply that the studied characteristics do not depend on a well-defined tertiary structure, or that the deletion has no major effect on the structure responsible for these features.

We found that truncated and wild-type semenogelin I have similar properties, which agrees with the data published thus far that do not substantiate a correlation between heterozygosity for the variant gene and impaired fertility (Lundwall et al, 2003; Miyano et al, 2003). However, the absence of a provable physiological difference between the 2 gene products does not exclude the possibility that different genotypes do give rise to different phenotypes. To our knowledge, no human subject who is homozygous for the variant has yet been identified, but it would certainly be interesting to find and characterize such a person. That task should not be impossible, considering that, at an allele frequency of approximately 3% (as in Sweden) for a variant gene coding for a protein that seems to share most of its features with the wild-type molecule, it can be expected that one in 1100 individuals will be homozygous for the variant.

Acknowledgments

We are grateful to Margareta Persson, Department of Laboratory Medicine, Malmö, for skilful help with recombinant protein synthesis, and to Pål Stenberg, Malmö University Hospital, for supplying reagents and for good advice. We also acknowledge the staff of the Fertility Laboratory, Malmö University Hospital, for help with collection of semen samples.

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