BIOLOGY OF REPRODUCTION **82,** 489–496 (2010)
Published online before print 4 November 2009.
DOI 10.1095/biolreprod.109.081331

Analysis of Recombinant Human Semenogelin as an Inhibitor of Human Sperm Motility[1]

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ABSTRACT

Eppin (epididymal protease inhibitor [SPINLW1]) is present in a protein complex on the human sperm surface that contains lactotransferrin, clusterin, and semenogelin (SEMG1). During ejaculation the presence of semenogelin inhibits sperm progressive motility until semenogelin is hydrolyzed by prostate-specific antigen (PSA). Although eppin binds all three components in its protein complex, the binding of semenogelin to eppin appears to be critical for the inhibition of progressive motility. The effect of the originally identified seminal plasma motility inhibitor fragment has not been clearly defined on live spermatozoa. Therefore, we have used recombinant semenogelin (rSEMG1) and its fragments, including a semenogelin mutant in which cysteine 239 was changed to glycine, coupled with a computer assisted sperm analysis assay to study the motility inhibitory properties of semenogelin. Each fragment and the mutant were tested for their effects on motility. Recombinant semenogelin significantly inhibited sperm progressive motility in a dose- and time-dependent manner. The C-terminal semenogelin fragment (amino acids 164–283) containing cysteine 239 significantly inhibited sperm progressive motility, whereas the N-terminal fragment (amino acids 24–163), a short C-terminal fragment (amino acids 172–215) without cysteine 239, and the mutant fragment (amino acids 24–283 with glycine 239) did not inhibit motility. After treatment with recombinant semenogelin, spermatozoa could be washed and treated with PSA, partially reversing the inhibition of progressive motility. Cysteine 239 of rSEMG1 appears to be the critical amino acid for both binding to eppin and inhibiting sperm motility.

contraception, eppin, semenogelin, seminal plasma, sperm, sperm motility and transport, spermatozoa

INTRODUCTION

Human seminal plasma has the interesting property of inhibiting sperm motility when it mixes with spermatozoa during ejaculation. In 1996, Robert and Gagnon [1] identified semenogelin (SEMG1) as the seminal plasma motility inhibitor (SPMI) protein in seminal plasma [2–8]. Upon ejaculation, SEMG1 is present in semen and on the sperm surface bound to eppin [9]. Immediately after ejaculation, activated prostate-specific antigen (PSA), a serine protease, cleaves Sg by hydrolysis, liquefying the semen coagulum [10] and removing SEMG1 from the sperm surface, allowing the spermatozoa to become motile and capacitation to proceed [2, 11]. The SPMI activity was identified in an N-terminal fragment of SEMG1 between amino acids 108–159 [10], based on a demembranated sperm motility assay [1]. Moreover, disulfide reduction of the single cysteine in Sg (Cys 239) did not alter the SPMI activity in this assay [1], and treatment of live spermatozoa with SEMG1 caused a concentration- and time-dependent reduction in motility [1]. Subsequently, Yoshida et al. [8] demonstrated that treatment of live spermatozoa with semenogelin I (SEMG1) purified from semen decreased the straight-line velocity and linearity of human spermatozoa.

Eppin is secreted by Sertoli cells and epididymal epithelial cells [12] and becomes localized on the surface of ejaculated spermatozoa in a complex of proteins containing lactotransferrin, clusterin and semenogelin [13]. The eppin protein complex [9, 13] modulates PSA protease activity [9, 14], provides antimicrobial protection for spermatozoa in the ejaculate coagulum [15], and is the critical binding site for the removal of semenogelin from spermatozoa in vivo during semen liquefaction and the initiation of progressive motility. In our previous study [16], examination of 38 individual spermatozoa treated with recombinant human Sg (rSEMG1) indicated that 65pg of rSEMG1/spermatozoon caused nonprogressive path lengths in some spermatozoa [16; Fig. 1E]. Both progressive straight trajectories and nonprogressive (very short) path lengths were observed, implying that individual spermatozoa must bind a critical amount of rSEMG1 in order to affect their swimming behavior.

The effect of the originally identified SPMI fragment (Sg, amino acids 108–159 [10]) has not been clearly defined on live spermatozoa. Therefore, we have used recombinant semenogelin and its fragments coupled with a computer-assisted sperm analysis (CASA) assay to study the motility inhibitory properties of semenogelin. We now report that, as expected, recombinant semenogelin significantly inhibited sperm progressive motility in a dose- and time-dependent manner. However, while the C-terminal semenogelin fragment (amino acids 164–283) containing cysteine 239 significantly inhibited sperm progressive motility, the N-terminal fragment (amino acids 24–163), containing the original SPMI fraction did not. An additional short C-terminal fragment (amino acids 172–215) without cysteine 239 and a mutant fragment in which cysteine 239 was changed to glycine (amino acids 24–283 with glycine 239) did not inhibit motility.

MATERIALS AND METHODS

Chemicals and reagents were molecular biology grade and purchased from Sigma-Aldrich. Immobilon-P transfer membranes were purchased from

[1]Supported by grant CIG-96-06 from the Consortium for Industrial Collaboration in Contraceptive Research of the Contraceptive Research and Development Program and by grants HD048843 and HD060494 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development.
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Received: 8 September 2009.
First decision: 1 October 2009.
Accepted: 22 October 2009.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

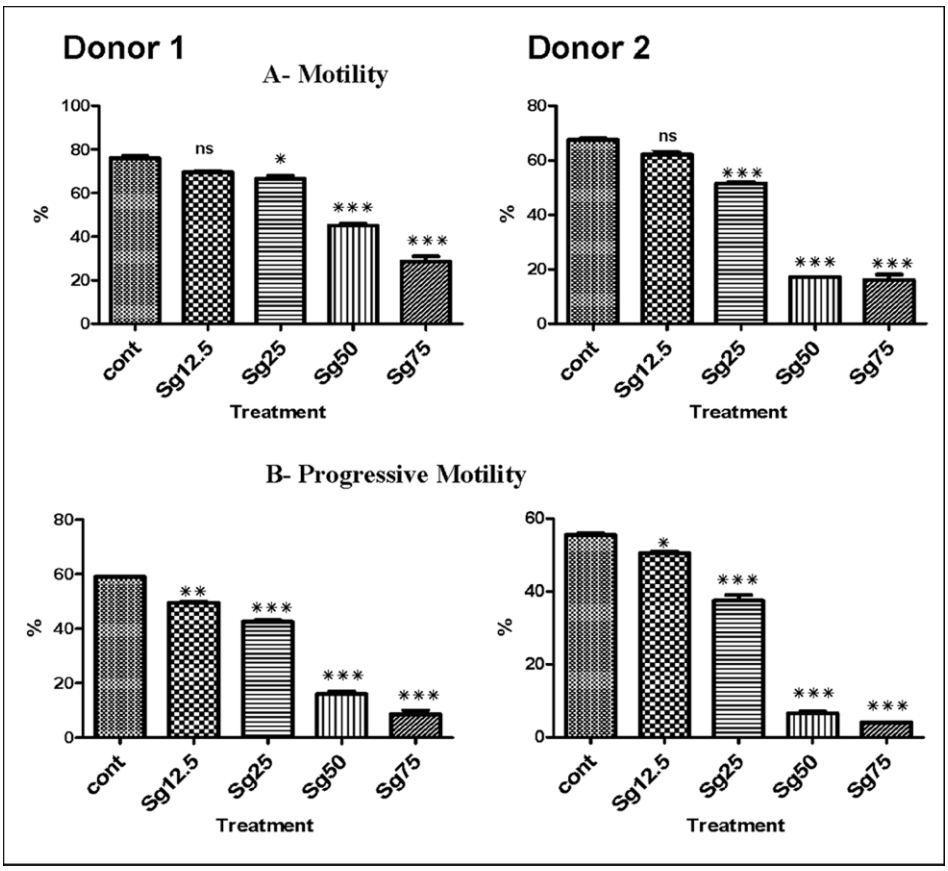

FIG. 1. The effect of different concentrations of recombinant semenogelin on human sperm motility. Values = mean ± SEM for multiple determinations from the same ejaculate at each concentration. Concentrations are given in picograms/sperm. **A**) Percent motility of spermatozoa from donor 1 and donor 2. **B**) Percent progressive motility of spermatozoa from donor 1 and donor 2. Abbreviations: Cont, control, buffer only; Sg12.5, rSEMG1$^{24–283}$ (12.5 pg/sperm); Sg25, rSEMG1$^{24–283}$ (25 pg/sperm); Sg50, rSEMG1$^{24–283}$ (50 pg/sperm); Sg75, rSEMG1$^{24–283}$ (75 pg/sperm). *P*-values in comparison to control: ***$P < 0.001$; **$0.001 > P < 0.01$; *$0.01 > P < 0.05$; ns = $P > 0.05$.

Millipore. Affinity-purified rabbit antibodies to the C-terminal of eppin (amino acids 103–123) were produced by Bethyl Laboratories, Inc. to the peptide SMFVYGGAQGNNNNFQSKANC (antibody S21C), in which alanine was substituted for cysteine 110 of the human eppin sequence.

Recombinant Proteins

Eppin. Eppin (nucleotides 70–423), lacking the N-terminal secretory sequence that was previously cloned into pFLAG-MAC [9], was expressed in BL-21 cells (Invitrogen) and purified on anti-Flag-M2 affinity columns (pFLAG-MAC; Sigma-Aldrich).

Semenogelin. Human recombinant semenogelin (rSEMG1$^{24–283}$, amino acids 24–283), semenogelin N-terminal, (N-ter$^{24–163}$, amino acids 24–163), and semenogelin C-terminal, (C-ter$^{164–283}$, amino acids 164–283) previously cloned into pET-100D/TOPO [9] were expressed in BL-21 cells (Invitrogen) and purified on Ni-NTA agarose columns. A short C-terminal recombinant fragment of semenogelin (short C-ter$^{172–215}$, amino acids 172–215) was generated by PCR using an LA Taq Kit (Takara Bio Inc.), cloned into pET-100D/TOPO (Invitrogen), expressed in BL-21cells, and purified on Ni-NTA agarose. The semenogelin Cys-mutant (Cys-mut$^{24–283}$, amino acids 24–283) produced by site-directed mutagenesis was expressed in BL-21cells and purified on Ni-NTA agarose. Recombinant protein concentrations were 0.2–0.4 mg/ml.

TABLE 1. The parameters of the Hamilton-Thorne Ceros 12.3.

Parameter	Value
Frame rate (Hz)	60
No. of frames acquired	60
Minimum contrast	80
Minimum size (pixels)	3
Default cell size (pixels)	6
VAP (μm/sec)	25
STR (%)	80
Slow cells	Static
VAP cut-off (μm/sec)	5
VSL cut-off (μm/sec)	11
Standard objective	10×
Chamber depth (μm)	20
Temperature (°C)	37

Site-Directed Mutagenesis

Site-directed mutagenesis of human semenogelin (rSEMG1$^{24–283}$) at the cysteine 239 residue was carried out using the Gene Tailor site-directed mutagenesis system (Invitrogen) according to the manufacturer's recommended protocol. SEMG1 plasmid DNA previously cloned in pET-100D/TOPO [9] was incubated with DNA methylase at 37° for 1 h. The methylated plasmid was amplified in a mutagenesis reaction with two overlapping primers: Forward-5′-GTAAAGTACAAACCTCACTCGGGCCTGCGCACC-3′ and reverse-5′-GAGTGAGGTTTGTACTTTACTTGAATGTTC-3′. The forward primer contained the GGG sequence coding for glycine to replace the TGT, which encodes cysteine at residue 239. Positive clones were selected by growth on ampicillin, followed by sequencing to confirm the mutation.

Affinity Purification

Affinity chromatography of the two recombinant fragments of SEMG1 N-terminal (N-ter$^{24–163}$) , C-terminal (C-ter$^{164–283}$), and short C-terminal (short C-ter$^{172–215}$) was carried out with polyclonal rabbit anti-SEMG1 (H-300, Santa Cruz Biotechnology Inc.) coupled to Reacti-Gel 6X beads (Pierce; coupling efficiency 80%). The protein samples were incubated with antibody-coupled beads overnight at 4°C. After washing with PBS, the bound proteins were eluted from the beads with ImmunoPure Elution Buffer (Pierce). The eluate was neutralized (pH = 7.0) and dialyzed against 1× PBS.

In Vitro Eppin Semenogelin Binding Assay

Magnetic Ni-NTA beads (40 μl suspension/assay) were preincubated with 1× casein (Vector laboratories Inc.) for 1 h to block nonspecific binding. Recombinant rSEMG1$^{24–283}$ (225 μg) and recombinant Cys-mut$^{24–283}$ (225 μg) proteins were added to separate bead aliquots in their respective tubes and incubated for 1 h. The beads were washed three times with 1× casein (Vector

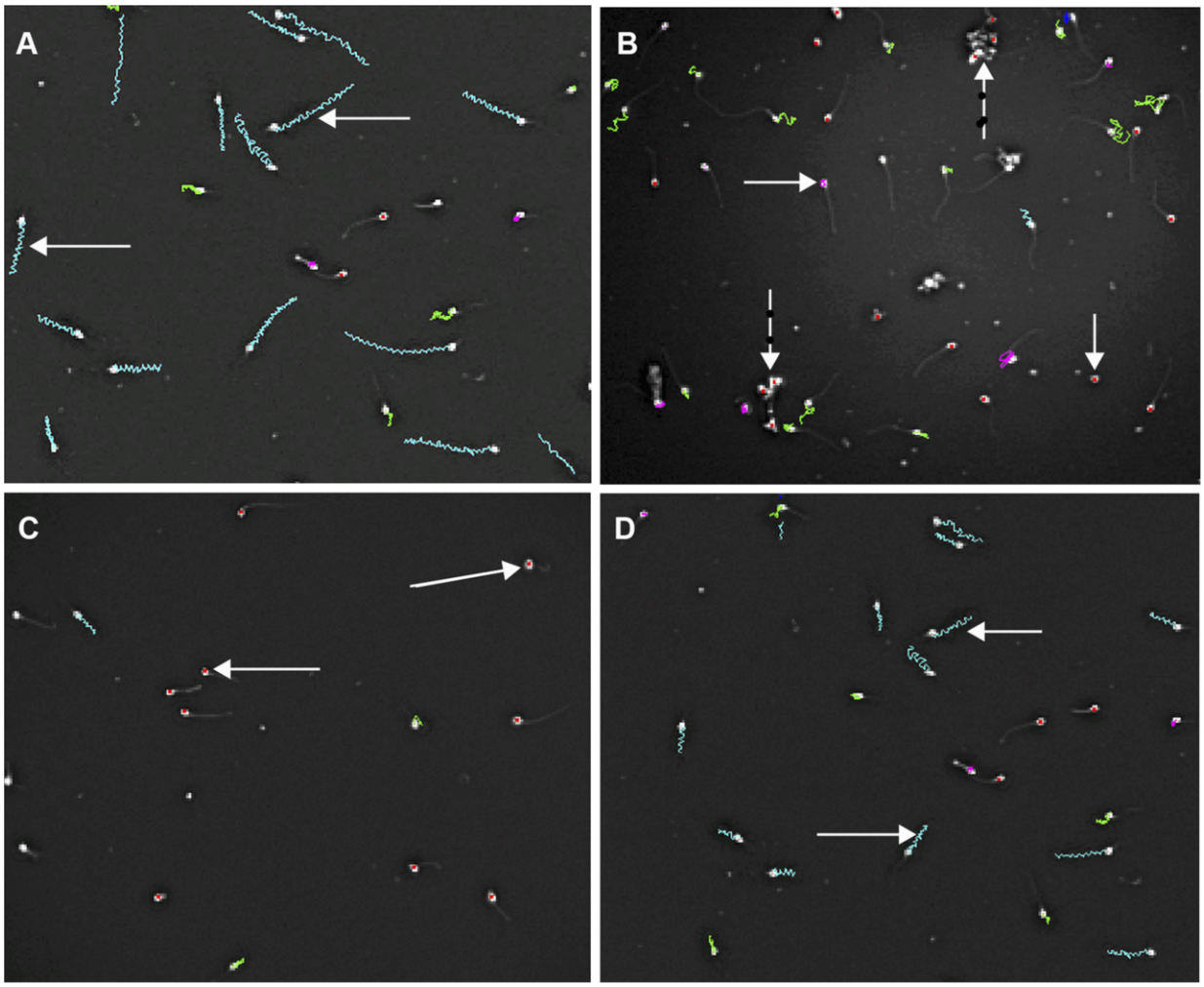

FIG. 2. Representative CASA tracks of spermatozoa after one hour of recombinant semenogelin treatment. **A**) Control spermatozoa, buffer only, arrows identify individual tracks. **B**) Spermatozoa treated with rSEMG1$^{24–283}$ (50 pg/sperm). Solid arrows identify spermatozoa without progressive motility; dashed arrows identify aggregates of spermatozoa. **C**) Spermatozoa treated with the C-terminal fragment of rSEMG1 (C-ter$^{164–283}$). Arrows identify spermatozoa without progressive motility. **D**) Spermatozoa treated with rSEMG1 Cys-mutant (Cys-mut$^{24–283}$). Arrows identify spermatozoa with progressive motility. Color codes: green or cyan = motile, red = static cells. Images represent individual frames recorded by the CASA system.

Laboratories) three times, and 300 µl of Flag-eppin (0.165 µg/µl) were added to the respective tubes and incubated for 1 h. Recombinant rSEMG1$^{24–283}$ added to the Ni-NTA beads without any addition of Flag-eppin served as one of the negative controls, and beads without rSEMG1$^{24–283}$ bound but, with the addition of Flag-eppin, served as the second negative control. The tubes were finally washed with 1× casein twice followed by wash buffer (20 mM imidazole). To each tube, 40 µl of elution buffer (250 mM imidazole) were added and incubated for 1 min. After adding 40 µl of sample buffer to the tubes and boiling for 5 min, the samples were analyzed by SDS-PAGE. Recombinant Flag-eppin (5 µg) was loaded on the gel as a positive control.

Western Blot Analysis

Two micrograms of recombinant semenogelin protein per lane were separated on reducing 10%–20% gradient gels (Bio-Rad), transferred to Immobilon-P (Millipore) and stained for protein with amido black. After destaining, the blot was blocked with 1× casein in Tris-buffered saline (50 mM Tris pH 7.4, 150 mM NaCl) for 1 h at room temperature and probed with primary antibodies. The recombinant semenogelin fragments were probed with rabbit anti-SEMG1 (1:2000; H-300; Santa Cruz Biotechnology). For the in vitro binding assay, mouse anti-flag antibodies (1:1000; Invitrogen) were used. Secondary antibodies were alkaline phosphatase-labeled goat anti-rabbit IgG (1:2000; Thermo Scientific) or goat anti-mouse IgG (ICN; Biomedical Inc.). The blots were developed by 1-Step NBT/ BCIP solution (Thermo Scientific) according to the manufacturer's instructions.

Preparation of Spermatozoa

Semen samples were collected from fertile donors at the UNC North Carolina Memorial Hospital infertility clinic, frozen in test-yolk buffer and stored in liquid nitrogen. This study was approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina, School of Medicine, Chapel Hill, North Carolina. Samples used for this study (four donors and 21 different ejaculates) were thawed at 37°C, washed twice in 37°C Sperm Washing Medium (modified HTF, human tubal fluid, Irvine Scientific) and prepared as previously described [16]. Aliquots of the ''swim-up'' population were taken to determine percent motility and concentration. The concentrations ranged from 1–10 × 10^6 sperm/ml.

Analysis of Sperm Motility by Computer-Assisted Sperm Analysis

Computer-assisted sperm analysis (CASA) was performed to determine the changes in parameters of the control and treated spermatozoa. The parameters measured were as follows: average pathway velocity (VAP: the average velocity of the smoothed cell path in µm/sec), the straight-line velocity (VSL: the average velocity measured in a straight line from the beginning to the end of track in µm/sec), the curvilinear velocity (VCL: the average velocity measured over the actual point-to-point track of the cell in µm/sec), the lateral head amplitude (ALH: amplitude of lateral head displacement in µm), the beat cross-frequency (BCF: frequency of sperm head crossing the sperm average path in

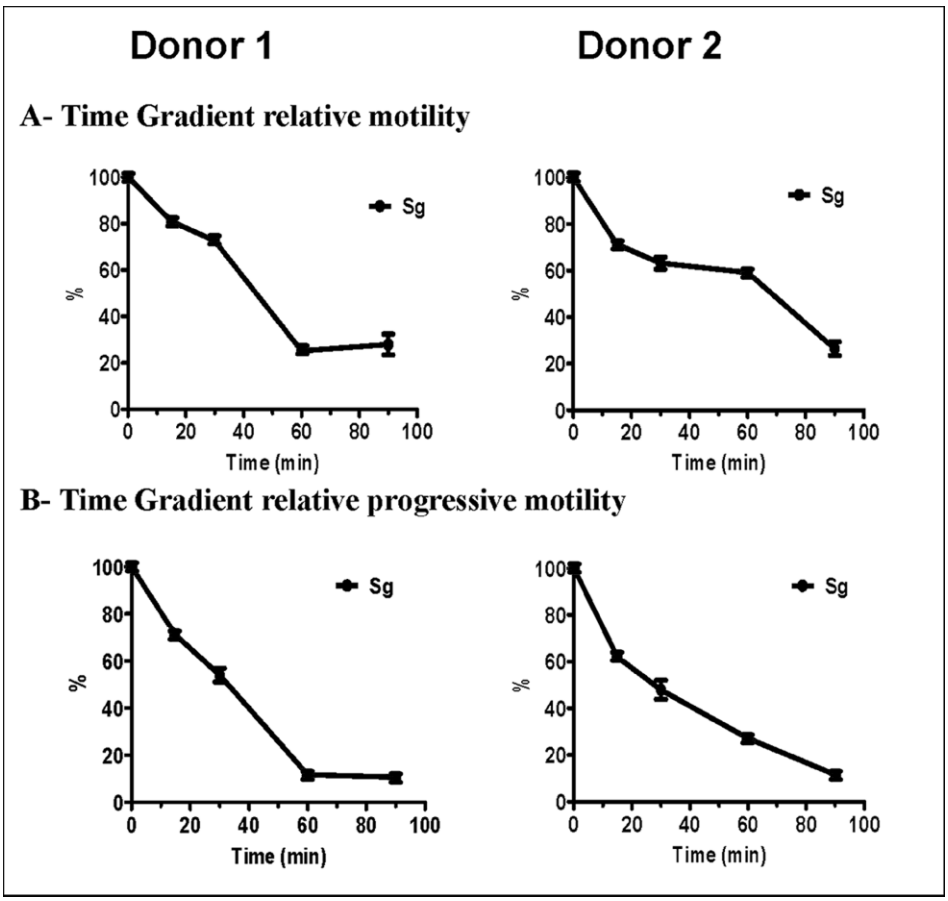

FIG. 3. The effect of treatment with 50 pg/sperm of rrSEMG1$^{24–283}$ over time on human sperm motility. Values = mean ± SEM for multiple determinations from the same ejaculate at each time point. **A**) Relative percent motility of spermatozoa treated with rSEMG1$^{24–283}$ in comparison to control in donors 1 and 2. Relative motility equals the motility of spermatozoa treated with rSEMG1$^{24–283}$ in comparison to the control at that point of time. **B**) Relative percent progressive motility of spermatozoa treated with rSEMG1$^{24–283}$ in comparison to control in donors 1 and 2. Relative progressive motility equals the progressive motility of spermatozoa treated with rSEMG1$^{24–283}$ in comparison to the control at that point of time.

Hz), the straightness (STR: the average value of the ratio VSL/VAP in %), and the linearity (LIN: the average value of the ratio VSL/VCL in %). The sperm motility parameters were assessed in prewarmed (37°C) two- or four-chambered 20-mm Leja slides (Leja), loading 5 or 2 μl, respectively, of the samples in each chamber and immediately using CASA. This computerized measuring device included a phase-contrast microscope (Olympus CX41), a camera, a minitherm stage warmer, an image digitizer, and a computer to store and analyze data. The software used for data analysis was the HTR Ceros 12.3

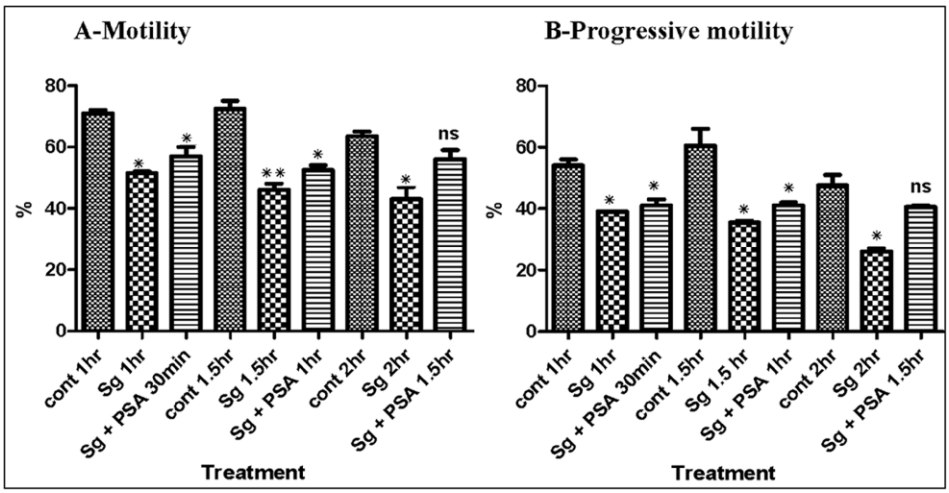

FIG. 4. The reversible effect of rSEMG1$^{24–283}$ protein on sperm motility. Spermatozoa were treated with rSEMG1$^{24–283}$ (50 pg/sperm) for 30 min and then washed and treated with PSA, and the reversibility of the effect of rSEMG1$^{24–283}$ was analyzed by CASA at different time points. Values = mean ± SEM for multiple determinations from the same ejaculate at each treatment. **A**) Percent motility of spermatozoa treated with rSEMG1$^{24–283}$ or with rSEMG1$^{24–283}$ followed by PSA treatment, in comparison to control (buffer only). **B**) Percent progressive motility of spermatozoa treated with rSEMG1$^{24–283}$ or with rSEMG1$^{24–283}$ followed by PSA treatment, in comparison to control (buffer only). Abbreviations: 1.0-h treatment: cont 1hr, control, buffer only after 1 h; Sg 1hr, treated with rSEMG1$^{24–283}$ for 1 h; Sg + PSA 30min, treated with rSEMG1$^{24–283}$ for 30 min, washed and treated with PSA for 30 min; 1.5-h treatment: cont 1.5hr, control, buffer only after 1.5 h; Sg 1.5hr, treated with rSEMG1$^{24–283}$ for 1.5 h; Sg + PSA 1hr, treated with rSEMG1$^{24–283}$ for 30 min, washed and treated with PSA for 1 h; 2.0-h treatment: cont 2hr, control, buffer only after 2 h; Sg 2hr, treated with rSEMG1$^{24–283}$ for 2 h; Sg + PSA 1.5hr, treated with rSEMG1$^{24–283}$ for 30 min, washed and treated with PSA for 1.5 h. **$0.001 > P < 0.01$; *$0.01 > P < 0.05$; ns = $P > 0.05$. The levels of significance of rSEMG1$^{24–283}$ and rSEMG1$^{24–283}$ + PSA treated samples were compared to the controls at that time.

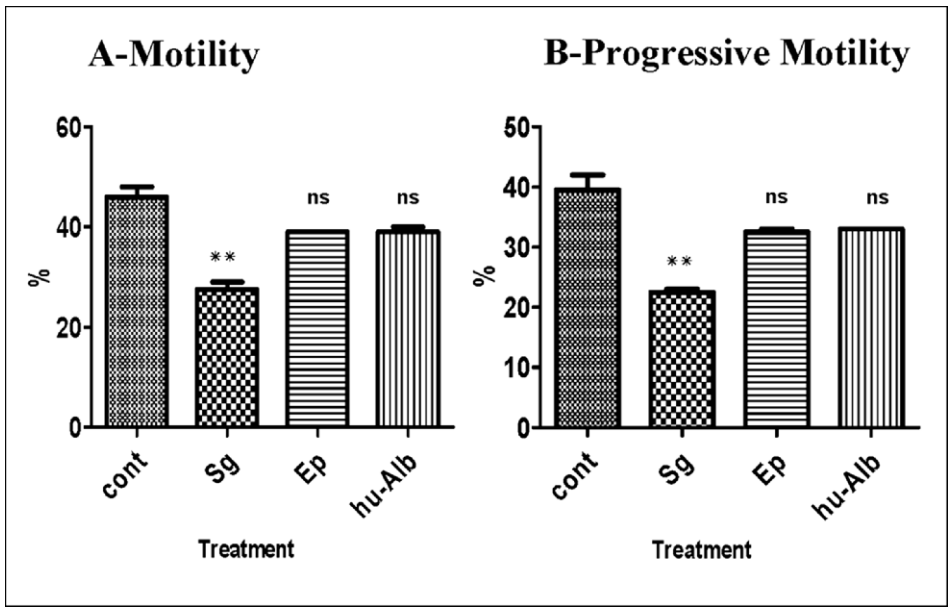

FIG. 5. The effect of control proteins on human sperm motility after 1 h of treatment. Values = mean ± SEM for multiple determinations from the same ejaculate at each treatment. **A**) Percent motility of spermatozoa treated with recombinant eppin, human albumin, or rSEMG1$^{24–283}$ at 50 pg/sperm. **B**) Percent progressive motility of spermatozoa treated with recombinant eppin, human albumin, or rSEMG1$^{24–283}$ at 50 pg/sperm. Abbreviations: cont, control, buffer only; Sg, rSEMG1$^{24–283}$; Ep, recombinant eppin; hu-Alb, human albumin. **0.001 > P < 0.01; ns = P > 0.05.

(Hamilton-Thorne Biosciences). A range of 13 to 20 fields were scanned from each chamber. Progressive cells had to have a minimum of 25 μm/sec VAP and 80% of STR. According to their velocities, the sperm were distributed into four categories: rapid—VAP > 25μm/sec; medium—25 μm/sec > VAP > 5 μm/sec; slow—VAP < 5μm/sec; and static—sperm not moving. Semen samples found to have untreated swim-up spermatozoa with a VCL <70 μm/sec were not used in this study. The parameters of the Hamilton-Thorne Ceros 12.3 are listed in Table 1.

Statistical Analysis

One-way analysis if variance with Tukey's multiple comparison test analysis was used to compare control and treated sperm samples (GraphPad Prism Software 5.01 for Windows; http://www.graphpad.com).

RESULTS

Recombinant human semenogelin (rSEMG1$^{24–283}$) inhibits human sperm motility in a concentration-dependent manner as observed by CASA. Samples from 21 different ejaculates from four different donors were analyzed by CASA (Supplemental Table S1, all Supplemental Data are available online at http://www.biolreprod.org). Typical CASA results from two different donors whose swim-up spermatozoa were incubated for 1 h with increasing concentrations of semenogelin are shown in Figure 1 (Table S1). Overall motility decreased significantly at ≥25 pgm/sperm (Sg25) for each donor, whereas progressive motility was significantly impacted with as little as 12.5 pgm/sperm. As illustrated in Figure 2, control spermatozoa (arrows, Fig. 2A) lose their characteristic progressive sperm trails when treated with rSEMG1$^{24–283}$ (solid arrows, Fig. 2B) and are recorded by CASA as nonprogressive. At higher concentrations of rSEMG1$^{24–283}$ (>50 pgm/sperm), the spermatozoa begin to agglutinate (dashed arrows, Fig. 2B) and lose motility, reminiscent of spontaneous agglutination seen in many mammalian species [17]. The effect of rSEMG1$^{24–283}$ on spermatozoon motility becomes more obvious over time as more spermatozoa lose their motility (Fig. 3). Although the effect was donor dependent, in most of the samples examined (n = 21) the maximum effect was reached in approximately 1 h.

To demonstrate the reversibility of rSEMG1$^{24–283}$ treatment, spermatozoa were incubated in rSEMG1$^{24–283}$ for 0.5 h and then treated with PSA for 0.5, 1.0, and 1.5 h. As shown in Figure 4, treatment with PSA allowed the spermatozoa to recover their motility and progressive motility. After 1.5 h of treatment the recovered spermatozoa demonstrated motility and progressive motility that was not significantly different from the untreated control (Fig. 4). In addition, to determine the extent of nonspecific effects of added protein on sperm motility, human recombinant eppin and human albumin were added to spermatozoa, and their motility was recorded by CASA. Neither eppin nor albumin had any effect on sperm motility at concentrations identical to those of rSEMG1$^{24–283}$ (Fig. 5).

To determine the domain of rSEMG1 responsible for the inhibition of motility, several recombinant fragments were constructed (Fig. 6 and Supplemental Fig S1) and motility and progressive motility recorded by CASA. As shown in Figure 7, only the C-ter$^{164–283}$ fragment significantly decreased both motility and progressive motility, showing the characteristic loss of progressive sperm trails (arrows, Fig. 2C). Neither the N-ter$^{24–163}$ fragment nor the short C-ter$^{172–215}$ fragment

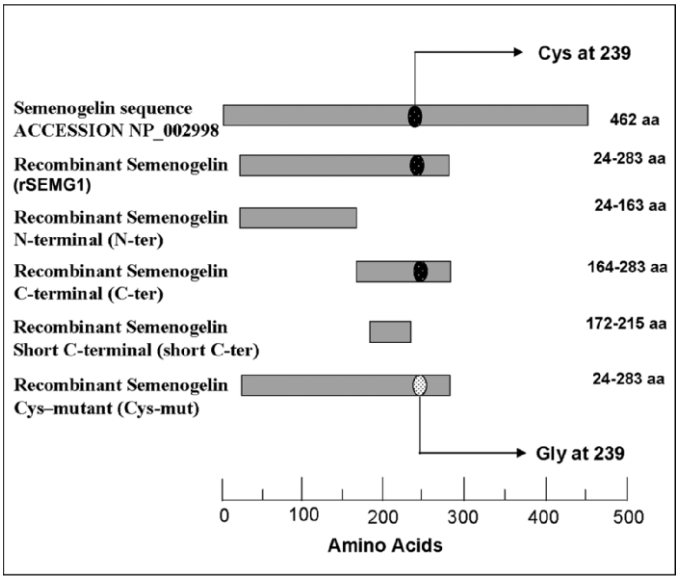

FIG. 6. Semenogelin recombinant fragments tested in this study, including the Cys-mutant in which cysteine 239 was mutated to glycine.

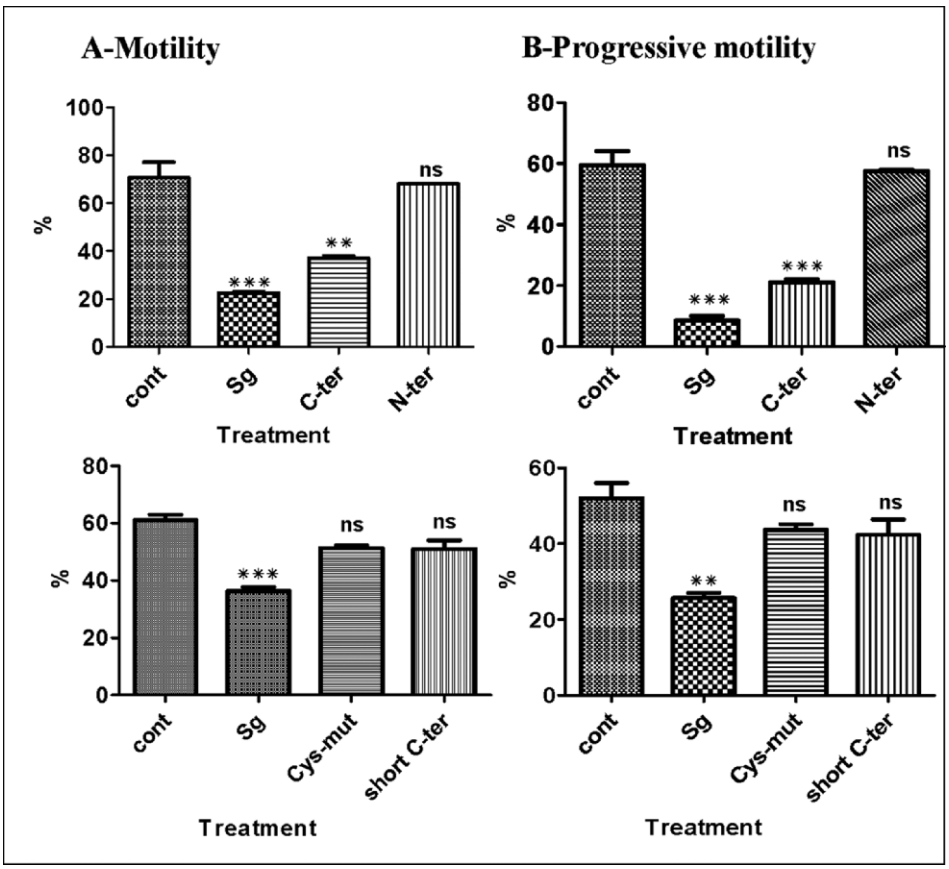

FIG. 7. The effect of rSEMG1 fragments on human sperm motility. Spermatozoa were treated with different rSEMG1 fragments (50 pg/sperm) and analyzed by CASA. Values = mean ± SEM for multiple determinations from the same ejaculate at each treatment. **A**) Percent motility of spermatozoa treated with different rSEMG1 fragments at 50 pg/sperm in comparison to control. **B**) Percent progressive motility of spermatozoa treated with different rSEMG1 fragments at 50 pg/sperm in comparison to control. Abbreviations: Sg, rSEMG1$^{24–283}$; C-ter, rSEMG1 C-terminal fragment$^{164–283}$; N-ter, rSEMG1 N-terminal fragment$^{24–163}$; Cys-mut, rSEMG1 cysteine-mutant fragment$^{24–283}$; short C-ter, rSEMG1 short C-terminal fragment$^{172–215}$. Fragments are described in Figure 6. ***$P <$ 0.001; **0.001 $> P <$ 0.01; ns = $P >$ 0.05.

significantly affected motility (Fig. 7). Previously we reported that reduction and carboxymethylation of rSEMG1 inhibited its binding to eppin [9]; therefore, we mutated cysteine 239 to glycine to test its effect on motility. Cys-mutant$^{24–283}$, that is, the rSEMG1$^{24–283}$ without cysteine 239, had no significant effect on motility or progressive motility (Fig. 7), giving the characteristic sperm trails seen in the control (arrows, Fig. 2D). To confirm rSEMG1$^{24–283}$'s requirement for cys239 in order to bind to eppin [9], we tested Cys-mut$^{24–283}$ in vitro for its ability to bind eppin. As shown in Figure 8, Cys-mut$^{24–283}$(lane 3) did not bind eppin, whereas rSEMG1$^{24–283}$ with cys239 did bind (lane 2).

DISCUSSION

Sperm motility is an important factor affecting fertility [18], and semenogelin is known to inhibit sperm motility [1–8]. In this study we have demonstrated that recombinant semenogelin (rSEMG1$^{24–283}$) inhibits sperm motility in a concentration- and time-dependent manner, confirming what has been previously reported for endogenous SEMG1 [1, 8]. Additionally we have demonstrated, using fragments of rSEMG1 and live spermatozoa analyzed by CASA, that the critical motility effect of semenogelin lies in its cysteine 239 amino acid. In our previous study of a small population of spermatozoa [16], progressive motility as measured by tortuosity (a curvilinear distance/straight-line distance ratio) was observed to increase in a concentration-dependent manner. Similarly, all the velocities (VCL, VAP, and VSL) measured in this report decreased in the rSEMG1$^{24–283}$-treated samples in comparison to the control (Table S1). In a study conducted by Hirano et al. [19], sperm motility and velocities (VCL and VAP) were found to be directly related to the fertilizing ability of human spermatozoa. In two proteomic analyses [20, 21], subfertile/asthenozoospermic men were shown to express more semenogelin precursors in comparison to their respective controls, implicating semenogelin in sperm motility and fertility.

Semenogelin forms a gelatinous mass at the time of ejaculation, which traps spermatozoa and restricts their movement. In this study we observed that at higher concentrations of rSEMG1$^{24–283}$, the spermatozoa agglutinated (Fig. 2B). Murakami et al. [22] observed a tendency of spermatozoa to aggregate when recombinant semenogelin expressed in insect cells was added. Consequently spontaneous agglutination of spermatozoa, which occurs in many mammalian species, might be the result of incomplete removal of semenogelin or similar proteins from the sperm surface [17].

The inhibitory effect of rSEMG1$^{24–283}$ on sperm motility and progressive motility increases with time, reaches an optimum after approximately 1 h, and becomes stable in some donors (Fig. 3, A and B, donor 1), whereas in other donors it continues to increase until all the spermatozoa become immotile (Fig. 3, A and B, donor 2). It is possible that as sperm surface eppin becomes saturated with semenogelin over time, the motility decreases proportionally. The reason of this interdonor and intradonor variance is unclear; however, it might be attributed to frequent variations in age and physiological status of spermatozoa within individual ejaculates.

Semenogelin is hydrolyzed by PSA after ejaculation, liberating motile spermatozoa [10]. The inhibitory effect of semenogelin was demonstrated in our studies to be partly reversible with addition of PSA (Fig. 4). Robert et al. [10] reported that PSA cleaves semenogelin into lower-molecular-weight fragments, considerably reducing its inhibitory effect. The results of the present study suggest that semenogelin has specific inhibitory effects on sperm motility. We assessed the

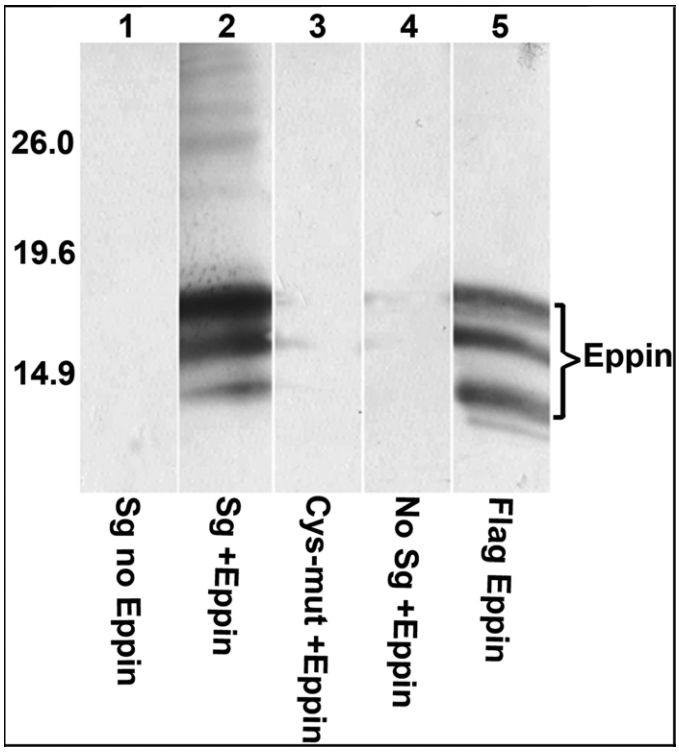

FIG. 8. In vitro binding of rSEMG1$^{24–283}$ and Cys-mutant rSEMG1$^{24–283}$ to eppin. Magnetic Ni-NTA beads were bound to rSEMG1$^{24–283}$ or Cys-mutant rSEMG1 (Cys-mut$^{24–283}$) and incubated with flag-eppin. The beads were washed and the eluate was analyzed by SDS-PAGE, and eppin was detected by Western blotting. Lane 1: rSEMG1$^{24–283}$ attached to the beads without eppin added; negative control. Lane 2: rSEMG1$^{24–283}$ attached to the beads with eppin added, eppin binds to rSEMG1$^{24–283}$. Lane 3: Cys-mut$^{24–283}$ attached to the beads with eppin added, eppin does not bind Cys-mut rSEMG1$^{24–283}$. Lane 4: Beads only, no rSEMG1$^{24–283}$ attached to the beads, with eppin added; no binding, negative control. Lane 5: Recombinant flag-eppin only on the gel (positive control).

effect of albumin and eppin as control proteins on sperm motility and found no significant inhibitory effect (Fig. 5), supporting the conclusion that semenogelin is a specific motility inhibitor.

In an effort to find the motility inhibiting domain of semenogelin, several domain fragments of rSEMG1$^{24–283}$ were tested (Fig. 6). The amino acids 284–462 were not tested, as it has been previously established that this domain is not the site of motility inhibition or involved in male infertility [23]. The C-ter$^{164–283}$ fragment of SEMG1 containing Cys 239 was found to have significant inhibitory effects on both motility and progressive motility (Fig. 7). The short C-ter$^{172–215}$ fragment without Cys 239 and the N-ter$^{24–163}$ did not have any significant effect on motility (Fig. 7), suggesting that Cys 239 plays an important role in the inhibition of sperm motility. We confirmed this hypothesis by using a Cys-mutant rSEMG1$^{24–283}$ and observed that the Cys-mut$^{24–283}$ did not affect either the motility or the progressive motility significantly (Fig. 7).

Contrary to earlier reports [1, 4, 23] in which demembranated spermatozoa were used as an assay for motility, the N-ter$^{24–163}$ fragment of SEMG1 in this study was not found to have an ''active site'' that would inhibit motility. The demembranated sperm assay, in which spermatozoa are reactivated with Mg^{++} ATP [1], apparently does not reflect the activity of domains of semenogelin on live (intact) spermatozoa as analyzed by CASA. Moreover, the production of a monoclonal antibody to the N-terminal active site (amino acids 108–159) [24] did not react with intact spermatozoa [25]. The original reports of semenogelin's effect on human sperm motility [1, 4], employing a live sperm assay, found that somewhat less than 100 human SPMI units/ml (~18 μg/ml) were necessary for motility inhibition. However, recent reports [8, 25] using reduced and acetylated semenogelin have necessitated much higher protein concentrations. Reduction and acetylation would render cysteine 239 inactive and require high, possibly toxic, concentrations in order to be effective (>5 mg/ml) [8, 25]. This is significantly more than the 0.2–0.4 mg/ml rSEMG1$^{24–283}$ used in this study. The present study demonstrated that cysteine 239 in the C-terminal of rSEMG1$^{24–283}$ is required for the inhibition of sperm motility when live spermatozoa are used in the assay (Fig. 7). Eppin is in a protein complex containing lactotransferrin and clusterin on the human sperm surface bound to semenogelin following ejaculation [9]. The Cys-mut$^{24–283}$ does not bind to recombinant eppin (Fig. 8), which is consistent with our previous report that carboxymethylated rSEMG1 does not bind eppin [4].

In conclusion we report that recombinant semenogelin inhibits human sperm motility in a dose- and time-dependent manner and that the inhibition is partially reversible by PSA hydrolysis. Cys 239 of rSEMG1$^{24–283}$appears to be the critical amino acid for both binding to eppin and inhibiting sperm motility.

ACKNOWLEDGMENTS

The authors thank Dr. D. O'Brien, Dr. P. Danshina, and S. Goodson for the use of their CASA system and their help in setting up the CASA measurements.

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