

·Original Article·

Molecular mechanism of epididymal protease inhibitor modulating the liquefaction of human semen

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Abstract

Aim: To study the molecular mechanism of epididymal protease inhibitor (Eppin) modulating the process of prostate specific antigen (PSA) digesting semenogelin (Sg). **Methods:** Human Sg cDNA (nucleotides 82–849) and Eppin cDNA (nucleotides 70–423) were generated by polymerase chain reaction (PCR) and cloned into pET-100D/TOPO. Recombinant Eppin and Sg (rEppin and rSg) were produced by BL21 (DE3). The association of Eppin with Sg was studied by far-western immunoblot and radioautography. *In vitro* the digestion of rSg by PSA in the presence or absence of rEppin was studied. The effect of anti-Q20E (N-terminal) and C-terminal of Eppin on Eppin-Sg binding was monitored. **Results:** Eppin binds Sg on the surface of human spermatozoa with the C-terminal of Eppin (amino acids 75–133). rSg was digested with PSA and many low molecular weight fragments were produced. When rEppin is bound to rSg, then digested by PSA, incomplete digestion and a 15-kDa fragment results. Antibody binding to the N-terminal of rEppin did not affect rSg digestion. Addition of antibodies to the C-terminal of rEppin inhibited the modulating effect of rEppin. **Conclusion:** Eppin protects a 15-kDa fragment of rSg from hydrolysis by PSA. (*Asian J Androl* 2008 Sep; 10: 770–775)

Keywords: epididymal protease inhibitor; semenogelin; prostate specific antigen

1 Introduction

Epididymal protease inhibitor (Eppin) is a testis/epididymis-specific protein. Human ejaculated spermatozoa are coated with Eppin over both head and tail regions before and after capacitation [1–4], which is involved in

cogulum formation in the ejaculation. Human seminal plasma spontaneously coagulates after ejaculation. The major component of this coagulum is semenogelin (Sg), a 52-kDa protein expressed exclusively in the seminal vesicles. Sg is the major protein involved in gelatinous entrapment of ejaculated spermatozoa, which plays an important role in the regulation of sperm motility and fertilization. The protein is rapidly cleaved after ejaculation by the chymotrypsin-like protease prostate-specific antigen (PSA), resulting in liquefaction of the semen coagulum and the progressive release of motile spermatozoa. PSA cleaves the coagulum proteins, resulting in the release of Sg proteolytic fragments [5]. Cleavage of Sg by

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PSA during liquefaction removes Sg from the sperm surface and results in the motility and capacitation of spermatozoa.

During human ejaculation, Eppin binds Sg before PSA digestion. To determine if Eppin plays an important role in regulating the hydrolysis of recombinant Sg (rSg) by PSA, we investigated the digestion of rSg by PSA in the presence and absence of recombinant Eppin (rEppin) and the effect of antibodies on Eppin-Sg binding and the hydrolysis of rSg by PSA *in vitro*.

2 Materials and methods

All chemicals and reagents used in the present study were obtained from Sigma (St. Louis, MO, USA). Plasmid PET100 was purchased from Invitrogen (CA, USA). Purifications of plasmid and polymerase chain reaction (PCR) cDNAs were performed using the respective kits from Qiagen (Valencia, CA, USA). Immobilon-P and -N transfer membranes were purchased from Millipore (Bedford, MA, USA). Enzymatically active PSA was obtained from EMD Bioscience (San Diego, CA, USA).

2.1 rEppin and rSg production

An Eppin cDNA (nucleotides 70–423) lacking part of the N-terminal secretory sequence was generated by PCR using the eppin-1/Bluescript clone [1] as template. PCR was performed with Pfx Platinum Polymerase (Invitrogen) and cloned into pET-100D/TOPO (Invitrogen). In a similar manner, a human Sg cDNA (nucleotides 82–849) was generated by PCR using a human seminal vesicle cDNA library as template (a gift from Dr Frank R. French, University of North Carolina, Chapel Hill, NC, USA) and cloned into pET-100D/TOPO.

All constructs were verified by sequencing and expressed in DH5- α . Bacterial lysates were purified on Ni-NTA agarose (pET-100D/TOPO) or anti-FLAG-M2 affinity gels (pFLAG-MAC; Siana).

2.2 Antiserum production

Affinity-purified rabbit antisera to N-terminal amino acids 20–39 of mouse Eppin were made by Bethyl Laboratories (Montgomery, TX, USA). Cysteine residue 33 was changed to an alanine. These antisera (anti-Q20E) reacted with both mouse and human Eppin.

2.3 Western blot analysis

Proteins were separated on reducing 10%–20 % gra-

dient gels (Bio-Rad, Hercules, CA, USA) or on reducing NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and transblotted to Immobilon-P (Millipore) and either stained for protein with amido black or blocked with Tris buffered saline (TBS) (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl) containing 3% BSA for 60 min at room temperature and probed with primary antibodies as described [2]. Two micrograms of recombinant protein were loaded per lane. Primary antibodies were used at a 1:2 000 dilution and secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG, 1:2 000) were either alkaline phosphatase labeled and developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate or peroxidase labeled and developed with chemiluminescence using Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

For far-western blots, proteins were immobilized on Immobilon-P, blocked as above, incubated for 1–2 h or overnight in protein probes, washed and detected with primary and secondary antibodies as described above. The protein concentrations were determined using Micro BCA Protein Detection Reagents (Pierce).

2.4 Labeling and quantitative binding assay

Labeling of 20 μ g of rEppin or rSg with 125 I was carried out using the Iodo-gen direct method (Pierce), according to the manufacturer's instructions, and the unbound 125 I was removed with a micro Bio-spin 6 chromatography column (Bio-Rad). Proteins were immobilized on Immobilon-P, blocked as above, incubated for 1–4 h in either 125 I-rEppin or 125 I-rSg, and exposed for autoradiography overnight. *In vitro* 125 I-rSg binding assay and 4 μ g of rEppin were immobilized on a nitrocellulose membrane (0.45 μ m) using a Bio-Dot microfiltration apparatus (Bio-Rad) and the membrane was washed with TBS-Tween (TBST) (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl with 0.05% Tween 20) and blocked with 5% BSA in TBST. Triplicate bio-dots on a membrane with or without Eppin (control) were incubated in increasing amounts of 125 I-rSg overnight at 4°C, then washed in TBST, cut into 1 cm squares, each containing a single dot, and counted in a γ -counter. To demonstrate the competition for binding, increasing amounts of unlabeled rSg were added to the 125 I-rSg and 125 I-Eppin bio-dot incubation mixtures.

2.5 Sg hydrolysis

All hydrolysis reactions of rSg with commercial native PSA in the presence or absence of rEppin, were performed in 1 mol/L NaCl, 0.1 mol/L Tris-HCl, pH 8.3 at a 1:50 enzyme/substrate ratio overnight at 37°C. rEppin was incubated with rSg for at least 2 h before PSA was added. The hydrolysis product was analyzed using 10–20% precast SDS-PAGE (Criterion gels, Bio-Rad) and the gel stained overnight with 0.01% Bio-Rad R-250 Coomassie (Bio-Rad) in 10% acetic acid. Protein concentrations were determined using micro BCA protein detection reagents (Pierce), using BSA as a standard. To test the effects of specific anti-Eppin antibodies on the PSA hydrolysis of rSg, either anti-Q20E or anti-C-terminal Eppin was incubated with rEppin for 2 h before rSg was added. After a further 2-h incubation, PSA was added for varying times at 37°C.

3 Results

3.1 rEppin

rEppin and its C-terminal and N-terminal were trans-

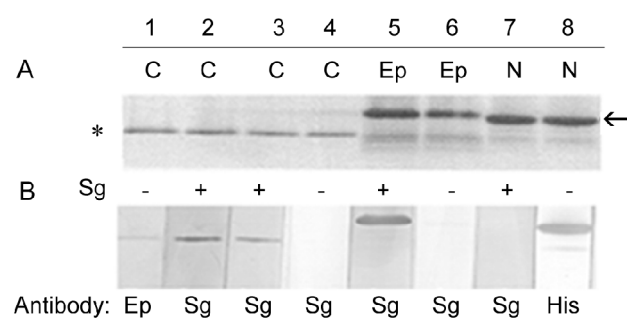


Figure 1. Far-western immunoblot analysis demonstrating that the C-terminal of recombinant epididymal protease inhibitor (rEppin) binds to recombinant semenogelin (rSg). (A): Lanes 1–4: protein stain (amido black) of the C-terminal of rEppin (asterisk, C, 10 kDa). Lanes 5–6: rEppin (arrow, Ep, 18 kDa). Lanes 7–8: the N-terminal of rEppin (N, 12 kDa). (B): Lane 1: C-terminal of rEppin blot incubated without rSg for 1 h, washed and probed with anti-Eppin antibody. Lanes 2–3: C-terminal of Eppin blot incubated with rSg for 1 h, washed and probed with anti-Sg antibody. Lane 4: C-terminal of Eppin blot incubated without rSg for 1 h, washed and probed with anti-Sg antibody. Lane 5: rEppin blot incubated with rSg for 1 h, washed and probed with anti-Sg antibody. Lane 6: rEppin blot incubated without rSg for 1 h, washed and probed with anti-Sg antibody. Lane 7: N-terminal of rEppin blot incubated with rSg for 1 h, washed and probed with anti-Sg antibody. Lane 8: N-terminal of rEppin blot incubated without rSg for 1 h, washed and probed with anti-His tag.

ferred onto Immobilon-P Polyvinylidene Difluoride (PVDF) membrane by Western blot (Figure 1A). The membrane was incubated into rSg. Far-western immunoblot analysis demonstrates that the C-terminal of rEppin binds to rSg (Figure 1B).

3.2 rSg

rSg and its N-terminal and C-terminal were transferred onto PVDF membrane by Western-blot. The membrane was incubated into 125 I-rEppin. Autoradiograph analysis demonstrates that rSg164–283 fragment binds 125 I-rEppin (Figures 2 and 3).

3.3 Digestion of rSg by PSA

Digestion of rSg by PSA initially produces several lower molecular weight fragments (< 10 kDa) (Figure 4, lane 5). In the presence of rEppin (Figure 4, lane 1), rEppin was bound to rSg, then digested by PSA, producing incomplete digestion and a 15-kDa fragment

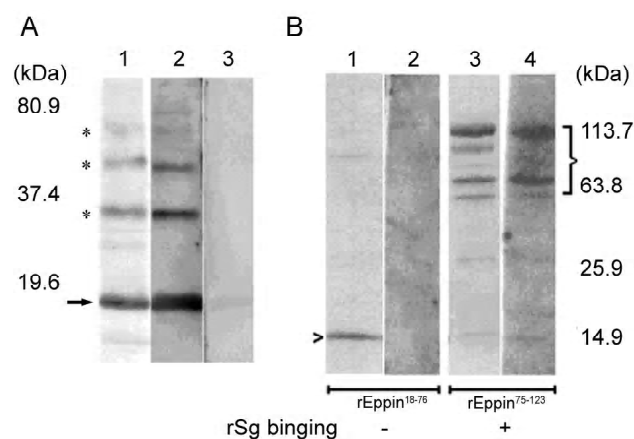


Figure 2. Autoradiograph analysis demonstrating that only the C-terminus of recombinant epididymal protease inhibitor (rEppin) binds 125 I-recombinant semenogelin (rSg). (A): Autoradiograph analysis demonstrating that rEppin binds 125 I-rSg. Lane 1: protein stain (amido black) of rEppin on Western blot; 18 kDa rEppin (arrow) and 2 ×, 3 × and 4 × multimer forms (*). Lane 2: autoradiograph of lane 1 probed with 125 I-rSg. Lane 3: autoradiograph of lane 1 probed with 125 I-rSg in the presence of 10 × unlabeled Sg. The position of molecular weight standards (kDa) is indicated on the left. (B): Autoradiograph analysis demonstrating that only the C-terminus of rEppin binds 125 I-labeled rSg. Lane 1: protein stain (amido black) of recombinant N-terminus of Eppin (>), amino acids 18–76. Lane 2: autoradiograph of lane 1 probed with 125 I-rSg; N-terminal of Eppin (>) does not bind Sg. Lane 3: recombinant C-terminal Eppin (}), amino acids 75–133. Lane 4: autoradiograph of lane 3 probed with 125 I-rSg; C-terminus of rEppin binds rSg.

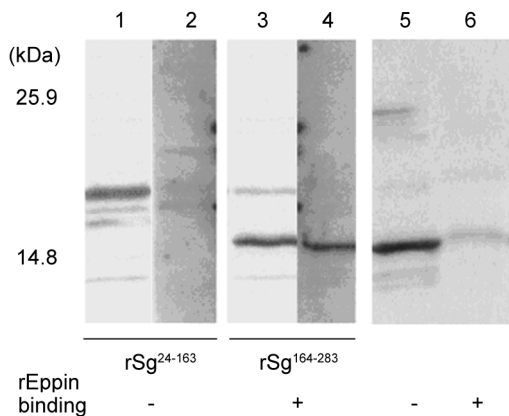


Figure 3. Autoradiograph analysis demonstrating that recombinant semenogelin fragment (rSg^{164–283}) binds ¹²⁵I-recombinant Eppin (rEppin) and that non-reduced cys²³⁹ is necessary for binding. Lane 1: protein stain (amido black) of rSg^{24–163}. Lane 2: autoradiograph of lane 1 probed with ¹²⁵I-rEppin. Lane 3: protein stain (amido black) of rSg^{164–283}. Lane 4: autoradiograph of lane 3 probed with ¹²⁵I-rEppin. Lane 5: autoradiograph of Sg^{164–283} probed with ¹²⁵I-rEppin. Lane 6: autoradiograph of reduced and carboxymethylated cys²³⁹ rSg^{164–283} probed with ¹²⁵I-rEppin. Lanes 5 and 6 were probed with a different preparation of ¹²⁵I-rEppin from that in lanes 2 and 4. The position of molecular weight standards (kDa) is indicated on the left.

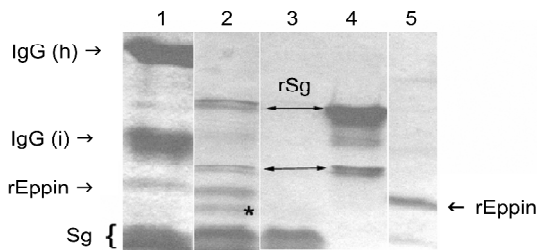


Figure 4. Digestion of recombinant semenogelin (rSg) by prostate specific antigen (PSA) in the presence or absence of recombinant Eppin (rEppin). SDS-PAGE gel stained with Coomassie Blue. Lane 1: rEppin incubated with anti-Eppin antibodies, rSg and PSA, no protected Sg fragment. Lane 2: rEppin incubated with rSg and PSA, producing a 15-kDa fragment (asterisk). Lane 3: rSg digested by PSA; Lane 4: rSg only; Lane 5: rEppin only.

(Figure 4, lane 2, asterisk). Analysis of the protected fragment by MS/MS revealed that it contained cys²³⁹, the necessary residue for rEppin binding. Anti-Q20E (N-terminal) had no effect on Eppin-Sg binding, as monitored by PSA digestion of rSg (data not shown). Antibodies to the C-terminal of rEppin make rEppin lose the

modulating function and the protected 15-kDa fragment of rSg disappears. MS analysis of the protected fragment of rSg by rEppin.

The complete Sg sequence was showed as follows:
 mkpniifvls llilekqaa vmgqkggskg r/lpsefsqfp
 hgqkqghysg qkgkqtesk;
 gsfsiqtyyh vdandhdqsr ksqqydlal hkttksqrh
 ggsqllhkn qegrhdhksk;
 ghfhrvvihh kggkahrqtq npsqdqgnsp sggkissqys
 nteerlwvhg lskeqtsvsg;
 aqkgrkqggs qssyvlqtee lvankqqrret knshqnkghy
 qnvvevreeh sskvqtslcp;
 ahqdklqhgs kdifstqdel lvynknqhqt knlnqdqqhg r/
 kankisyqs ssteerrlhy;
 gengvqkdvs qssiysqtee kaqgksqkqi tipsqepehs
 qkankisyqs ssteerrlhy;
 gengvqkdvs qrsiysqtek lvagksqiqq pnpkqepwhg
 enakgesgqs tnreqdllsh;
 eqngrhqhgs hggldiviie qeddsdrhla qhlndrnpl ft.

When rSg was bound to rEppin, an Sg fragment with an approximate molecular weight of 15 kDa was protected from PSA digestion. Reduced and carboxymethylated rSg did not bind rEppin. When reduced and carboxymethylated rSg was digested with PSA, an Sg fragment with an approximate molecular weight of 15 kDa was also protected from PSA digestion. The sequence of the fragment protected from digestion was as follows:

nteerlwvhg lskeqtsvsgaqkgrkqggs qssyvlqtee
 lvankqqrret knshqnkghy qnvvevreeh sskvqtslcpahqdklqhgs
 kdifstqdel lvynknqhqt knlnqdqqhg r

In the presence of anti-Eppin antibody Q20E (anti-Eppin peptide, amino acids 20–39) bound to Eppin, the fragments were still protected from PSA digestion. In the presence of antibody 9714 (anti-Eppin peptide epitope, amino acids 90–98), the fragments were not protected from PSA digestion (Figure 4, Lane 1).

4 Discussion

Recombinant protein purification is facilitated using high expression systems. The solubility and yield of pure protein are highly dependent on various combinations of chemical additives, ionic and non-ionic detergents and salts, with solubilizing agents followed by refolding of denatured protein into its active form. As the extraction of the purified protein from high expression systems requires denaturation and a subsequent refolding step, care-

ful balancing steps were needed to develop under different controlled conditions. Here the purified fragments of refolded proteins were screened to select the conditions that yield the activity having native conformation. The refolded recombinant protein was analyzed by RP-HPLC, showing a purity of 99%. The size exclusion chromatography profile shows that there are minimal aggregates in the active protein and the percentage of renaturation is approximately 99%.

During liquefaction of semen, PSA cleaves Sg bound to the sperm surface, releasing the sperm motility inhibitory factor (amino acids 69–160) [5–8]. We now know that Sg on the sperm surface is bound to Eppin and, therefore, the cleavage of Sg by PSA must occur while Sg is bound to Eppin. Consequently, we compared *in vitro* the digestion of rSg by PSA in the presence or absence of rEppin. As shown in Figure 4, when rSg (Sg, lane 4) is digested with PSA, many low molecular weight fragments are produced (lane 3). However, when rEppin is bound to rSg, digestion by PSA is modulated, producing incomplete digestion and a 15-kDa fragment (asterisk, lane 2). This experiment suggests that Eppin has an important function in ejaculated semen liquafication, sperm capacility and motility.

Our understanding of Eppin's essential role in sperm survival during transfer from male to female reproductive tracts prior to fertilization stems from an analysis of anti-Eppin antibody binding sites (epitopes) on Eppin. As described previously [9–12], sera from the infertile male monkeys immunized with Eppin recognizes two predominant epitopes: N-terminal (QGPGLTDWLFPRRCPKIRE; amino acids 20–38) and C-terminal (TCSMFVYGGCQGN-NNNFQSKANCLN; amino acids 101–125). Production of antibodies to N-terminal amino acids 20–39 (anti-Q20E) [1, 2], and to C-terminal rEppin have been described [1, 2]. To test the effect of specific anti-Eppin antibodies on the PSA hydrolysis of Sg as it might occur *in vivo*, either anti-Q20E or anti-C-terminal Eppin was incubated with Eppin. Incubation continued with the addition of Sg, and finally PSA was added for a final incubation period. Addition of anti-Q20E had no effect on Eppin-Sg binding, as monitored by PSA digestion of Sg. Therefore, antibody binding to the N-terminal of Eppin did not affect Sg digestion. However, addition of antibodies to the C-terminal of Eppin resulted in blocking PSA activity modulation. Consequently, digestion with PSA produced many low molecular weight fragments and, notably, the protected 15-kDa fragment (Figure 4,

lane 2, asterisk) was absent (Figure 4, lane 1). Analysis of the protected fragment by MS/MS revealed that it contained cys²³⁹, the residue necessary for Eppin binding. Moreover, the Sg N-terminal sequence containing the sperm motility inhibiting peptide [13] had been cleaved from the cys²³⁹ containing fragment by PSA into very small fragments, which would presumably no longer be anchored to Eppin. Although sperm motility inhibiting peptide is bound to sperm, it remains immotile and its removal is necessary for resumption of motility and subsequent capacitation [14–16].

We can hypothesize from our analysis of anti-Eppin epitopes on Eppin that when anti-Eppin antibodies in the infertile male monkeys entered the epididymal fluid and bound to Eppin on the sperm surface, they blocked the binding site for Sg [10, 11]. Blocking the binding of Sg had two consequences. First, as a result of not being bound to Eppin, Sg in the ejaculate was quickly hydrolyzed into small fragments; no modulation of PSA activity and no semen coagulum was observed. Second, having anti-Eppin bound to Eppin on the sperm surface mimicked the physiological effect of having sperm motility inhibiting peptide bound to the surface, namely, a loss of forward motility, which was observed in semen from infertile men. The second consequence predicts that the removal of anti-Eppin antibodies from the sperm surface would allow spermatozoa to recover their motility. Further studies are underway to verify it.

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