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Research Paper

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

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Abstract

Objective: To study the molecular mechanism of epididymal protease inhibitor (Eppin) modulating the liquafication of semen. **Methods**: Human semenogelin cDNA (nucleotides 82-849) and Eppin cDNA (nucleotides 70-423) were generated by PCR and cloned into pET-100D/TOPO.Recombinant Eppin and Sg were produced by BL21 (DE3). The association of Eppin with Sg was studied by far-western and radioautography.In vitro the digestion of Sg by PSA in the presence or absence of recombinant Eppin 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 C-terminal Eppin (aa75-133).Recombinant Sg was digested with PSA ,many low molecular weight fragments were produced, when Eppin is bound to Sg,then digested by PSA ,producing incomplete digestion and a 14.5-14.8 kDa fragmen. Antibody binding to the N-terminal of Eppin did not affect Sg digestion. Addition of antibodies to the C-terminal of Eppin inhibited the modulating effects of Eppin. **Conclusion**: Eppin modulates the digestion activity of PSA through binding Sg.The active site locates at C-terminal.

Keywords: epididymal protease inhibitor; semenogelin; prostate specific antigen

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[1-2], which is involved in cogulum formation in the ejaculation.

Human seminal plasma spontaneously coagulates after ejaculation. The major component of this coagulum is semenogelin 1, a 52-kDa protein expressed exclusively in the seminal vesicles.Semenogelin (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. Sg is initially protected from proteolysis by protein C inhibitor (PCI) in the seminal vesicles and that PSA (prostate-specific antigen,a serine protease) in the prostatic secretions is inactive, inhibited by high concentration of zinc.PCI,a serine protease inhibitor [3]. During ejaculation, the mixing of Sg with prostatic secretions chelates most of the free zinc[4], which triggers release of PCI, aggregation of Sg and fibronectin, and activates PSA. PSA cleaves the coagulum proteins, resulting in the release of Sg proteolytic fragments[5].

In order to study if Eppin plays an important role in regulating the liquefacation of semen,This study investigated the digestion of Sg by PSA in the presence or absence of recombinant Eppin and the effect of antibody on Eppin-Sg binding and the hydrolysis of Sg by PSA *in vitro*.

MATERIALS AND METHODS

All chemicals and reagents used in this study were purchased from Sigma (St. Louis,USA).Plasmid PET100 was purchased from Invitrogen company. Purifications of plasmid and PCR cDNAs were performed using the respective kits from Qiagen (Va-

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lencia,USA). Immobilon-P and N transfer membranes were purchased from Millipore(Bedford,USA).Enzymatically active PSA was obstained from EMD Bioscience(San Diego,USA).

Recombinant Eppin and Sg 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 Platinium Polymerase (Invitrogen,USA) and cloned into pET-100 D/TOPO(Invitrogen,USA). In the similar manner, human semenogelin cDNA (nucleotides 82-849) was generated by PCR using human seminal vesicle cDNA library as template (a gift from Dr. Frank R. French, University of North Carolina, Chapel Hill, USA) and cloned into pET-100D/TOPO.

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

Antiserum Production

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

Western Blot Analysis

Proteins were separated on reducing 10%-20% gradient gels (Bio-Rad,USA) or on reducing NuPAGE 4%-12% Bis-Tris gels (Invitrogen,USA) and transblotted to Immobilon-P(Millipore,USA) and either stained for protein with amido black or blocked with Tris buffered saline(TBS)(50 mM Tris pH 7.4, 150 mM 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, USA) according to the manufacturer's instructions.

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

Labeling and Quantitative Binding Assay

Labeling of 20 μg of rEppin or rSg with ^{125}I was carried out with the Iodo-gen direct method (Pierce) according to the manufacturer's instructions and the unbinded ^{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,4ug of rEppin were immobilized on a nitrocellulose membrane(0.45 μm) using a Bio-Dot microfiltration apparatus (Bio-Rad) and the membrane was washed with Tris buffered saline-Tween (TBST)(50 mM Tris pH 7.4,150 mM 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℃,then washed in TBST,cut into 1-cm squares,each containing a single dot,and counted in a rcounter.To demonstrate the competition for binding,increasing amounts of unlabeled rSg were added to the ^{125}I -rSg Eppin biodot incubation mixtures.

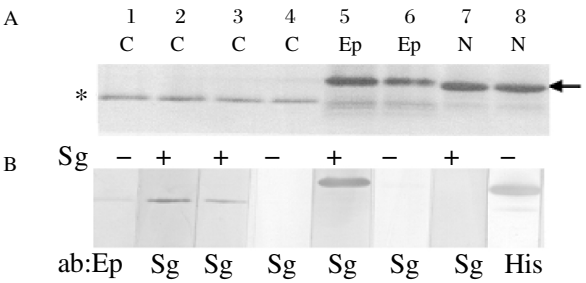
Semenogelin hydrolysis

All hydrolys is reactions of recombinant semenogelin (Sg) with commercial native PSA in the presence or absence of Eppin,were performed in 1M NaCl,0.1 M Tris-HCl,pH 8.3 at a 1∶50 enzyme/substrate ratio overnight at 37℃.Recombinant Eppin was incubated with Sg for at least 2 hours before PSA was added.The hydrolysis product was analyzed by 10%-20% precast SDS-PAGE (Criterion gels,Bio-Rad,Hercules,CA) and the gel stained overnight with 0.01% Bio-Rad R-250 Coomassie (Bio-Rad,Hercules,CA) in 10% acetic acid.Protein concentrations were determined micro BCA protein detection reagents(Pierce) using bovine serum albumin as a standard.To test the effects of specific anti-Eppin antibodies on the PSA hydrolysis of Sg,either anti-Q20E or anti-C-terminal Eppin was incubated with Eppin for 2 hours before Sg was added. After a further 2 hour incubation PSA was added for varying times at 37℃.

RESULTS

Recombinat Eppin and its C-terminal and N-ter-

minal were transferred onto PVDF membrane by Western-blot (***Fig. 1,A***).The membrane was incubated into recombinant Sg.Far-Western immunoblot analysis demonstrating that recombinant C-terminal Eppin binds rSg(***Fig. 1,B***).

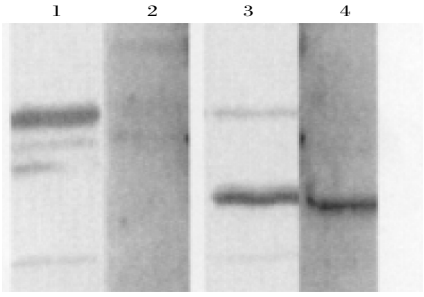


A:lane1-4:protein stain (amido black) of 10KDa recombinant C-terminal Eppin;Lane5-6:recombinant Eppin 18KDa;Lane7-8:recombinant N-terminal Eppin 12KDa.

B:Lane1:C-terminal Ep blot incubated without rSg for 1h,washed, and probed with anti-Eppin;Lane2-3:C-termoinal Eppin blot incubated in rSg for 1h,washed ,and probed with antisemenogelin. Lane4: C-terminal Ep blot incubated without rSg for 1h,washed, and probed with anti-Sg;Lane5: rEppin blot incubated in rSg for 1h,washed ,and probed with anti-Sg;Lane5: rEppin blot incubated without rSg for 1h,washed ,and probed with anti-Sg;Lane7: N-terminal rEppin blot incubated in rSg for 1h,washed ,and probed with anti-Sg;Lane8: N-terminal rEppin blot incubated without rSg for 1h,washed,and probed with anti-His tag;

Fig. 1 Far-Western immunoblot analysis demonstrating that recombinant C-terminal Eppin bound rSg.

Recombinant Sg and its N-terminal and C-terminal were transferred onto PVDF membrane by Western-blot.The membrane was incubated into ^{125}I labeled recombinant Eppin.Autoradiograph analysis demonstrated that recombinant Sg164-283 fragment bound 125I -Eppin (***Fig. 2***).



Lane1:protein stain (amido black)of recombinant Sg24-163;Lane2: autoradiograph of lane1 probed with 125I-rEppin;Lane3: protein stain (amido black) of recombinant Sg164-283;Lane4: autoradiograph of lane3 probed with 125I -Eppin.

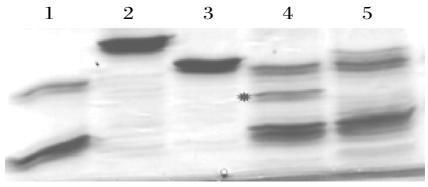
Fig. 2 Autoradiograph analysis demonstrating that recombinant Sg164-283 fragment bound 125I-Eppin.

Digestion of Sg by PSA initially produced several lower molecular weight fragments(< 10 Kda)(***Fig. 3, lane5***). In the presence of Eppin (***Fig. 3,Lane1***), Eppin was bound to Sg, then digested by PSA ,producing incomplete digestion and a 14.5-14.8 kDa fragment (asterisk, lane 4).Analysis of the protected fragments by MS/MS revealed that they contained cys239,the necessary residue for Eppin binding. Anti-Q20E (N-terminal) had no effect on Eppin-Sg binding as monitored by PSA digestion of Sg(data not shown).Antibody to C-terminal of Eppin made Eppin lose the modulating function and the protected 14.5-14.8 KDa fragments of Sg disappeared.

The complete semenogelin sequence was shown as follow:mkpniifvls lllilekqaa vmgqkggskg r/lpsefsqfp hgqkgqhysg qkgkqqtesk gsfsiqytyh vdandhdqsr ksqqydlnal hkttksqrhl ggsqqllhnk qegrdhdksk ghfhrvvihh kggkahrgtq npsqdqgnsp sgkgissqys nteerlwvhg lskeqtsvsg aqkgrkqggs qssyvlqtee lvankqqret knshqnkghy qnvvevreeh sskvqtslcp ahqdklqhgs kdifstqdel lvynknqhqt knlnqdqqhg r/kankisyqs ssteerrlhy gengvqkdvs qssiysqtee kaqgksqkqi tipsqeqehs qkankisyqs ssteerrlhy gengvqkdvs qrsiysqtek lvagksqiqa pnpkqepwhg enakgesgqs tnreqdllsh eqngrhqhgs hggldiviie qeddsdrhla qhlnndrnpl ft

When semenogelin was bound to Eppin, a semenogelin fragment with an approximate molecular weight of 14.5-14.8 kDa was protected from PSA digestion. Reduced and carboxymethylated semenogelin did not bind Eppin. When reduced and carboxymethylated semenogelin was digested with PSA, a semenogelin fragment with an approximate molecular weight of 14.5-14.8 kDa was also protected from PSA digestion. The sequence of the fragment protected from digestion was as follows:nteerlwvhg lskeqtsvsgaqkgrkqggs qssyvlqtee lvankqqret 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.While in the presence of antibody 9714 (an-



Lane 1: Recombinant Eppin; Lane 2: Recombinant semenogelin (Sg) Lane3: commercial native PSA.Lane 4: Recombinant Sg bound to Eppin digested with PSA producing a 14.5-14.8 kDa fragment (asterisk). Lane 5: Recombinant Sg digested with PSA producing low molecular weight fragments .

Fig. 3 Digestion of recombinant semenogelin(Sg) by PSA in the presence or absence of recombinant Eppin. SDS-PAGE gel stained with Coomassie Blue

ti-Eppin peptide epitope, amino acids 90-98) the fragments were not protected from PSA digestion.

DISCUSSION

During liquefaction of semen PSA cleaves semenogelin (Sg) bound to sperm surface, releasing the sperm motility inhibitory factor (amino acids 69-160)[5-8]. We now know that Sg on 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 Sg by PSA in the presence or absence of recombinant Eppin. As shown in figure3 , when recombinant Sg (Sg, lane 2) was digested with PSA many low molecular weight fragments were produced (lane 5). However, when Eppin was bound to Sg, digestion by PSA was modulated, producing incomplete digestion and a 14.5-14.8 kDa fragment(asterisk, lane 4). This experiment suggested that Eppin might be an inhibitor of PSA. It has important function for liquefaction of ejaculated semen, sperm capacitility and motility.

Our understanding of Eppin's essential role in sperm survival during transfer from male to female reproductive tract prior to fertilization stemed from an analysis of anti-Eppin antibody binding sites (epitopes) on Eppin. As described previously[9-12], sera from infertile male monkeys immunized with Eppin recognized two predominant epitopes, N-terminal (QGPGLTDWLFPRRCPKIRE; amino acids 20-38) and C-terminal (TCSMFVYGGCQGNNNNFQSKANCLN; amino acids 101-125). Production of antibodies to N-terminal amino acids 20-39 (anti-Q20E)[1-2], and to C-terminal recombinant Eppin have been described [1-2].To test the effect of specific anti-Eppin antibody 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 antibody to the C-terminal of Eppin resulted in blocking PSA activity modulation. Analysis of the protected fragment by MS/MS revealed that it contained cys239, the residue necessary for Eppin binding. Moreover, the N-terminal Sg sequence containing the sperm motility inhibiting peptide [13] had been cleaved from the cys239 containing fragment by PSA into a 10.4 kDa fragment, which would presumably no longer be anchored to Eppin. While sperm motility inhibiting peptide is bound to sperm they remain immotile and its removal is necessary for resumption of motilit y[14] and subsequent capacitation [7].

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