Journal of ANDROLOGY

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Journal of Andrology, Vol. 23, No. 6, November/December 2002
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Exogenous Protein Kinases A and C, But Not Endogenous Prostasome-Associated Protein Kinase, Phosphorylate Semenogelins I and II From Human Semen

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Received for publication January 3, 2002; accepted for publication May 20, 2002.

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Semenogelins I and II are the quantitatively dominating proteins in human semen. They comprise the major part of the sperm-entrapping gel formed at ejaculation, which subsequently liquefies due to proteolysis of the gel-forming proteins by prostate-specific antigen (PSA). The mechanism behind gel formation and its physiological significance is not known. We have studied phosphorylation and dephosphorylation of human semenogelins. Both were phosphorylated by protein kinases A and C (PKA and PKC, respectively) at a rate about 5 times less than that of histone. For PKA, incorporated (^{32}P)phosphate into semenogelin approached a maximum above 1 mol/mol. Corresponding values for phosphorylation of the semenogelins with PKC were greater than 10. There was no change in the sensitivity of phosphosemenogelins to proteolysis by PSA. Serine (PKA) and serine and threonine (PKC) were the phosphate-accepting amino acid residues, and all incorporated (^{32}P)phosphate could be removed from the semenogelins with human acid phosphatase. Nil or very little phosphate could be detected in purified semenogelins isolated from seminal plasma. In vivo, about half the protein kinase activity in seminal plasma was bound to prostasomes. PKA but not PKC purified from prostasomes could phosphorylate specific substrates, but they could phosphorylate either of the semenogelins.

Key words: Acid phosphatase, histone, prostate-specific antigen, vasectomy

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Spermatozoa contain a protein kinase A (PKA) that may modulate sperm function (Hoskins et al, 1972; Lee and Iverson, 1976; Huacuja et al, 1977; Majumbder, 1978). The activity of this enzyme is apparently increased during epididymal maturation (Hoskins et al, 1974), and under experimental

conditions, which are conducive to both the induction of the acrosome reaction (Garbers et al, 1980) and motility reactivation (Ihsiguro et al, 1982; Tash and Means, 1982). More recently, a 55-kd protein has been described, whose tyrosine phosphorylation correlates closely to the motility state of spermatozoa (Vijayaraghavan et al, 1997). However, multiple forms of protein kinase activity are also present in seminal fluid (Majumder, 1978; Panyim, 1981; Stegmayr et al, 1982; Wilson et al, 1982), which are not stimulated by cyclic adenosine monophosphate (cAMP). Histone and phosvitin are excellent exogenous acceptor proteins of the protein kinases, and prostate secretion may be the predominant contributor for at least the histone kinase (Stegmayr et al, 1982; Wilson and Kaye, 1983). Approximately half the protein kinase activities in prostatic fluid are associated with membranous components (prostasomes) (Stegmayr et al, 1982; Wilson et al, 1984) and incubations of spermatozoa and prostasomes together resulted in a 10-fold increase in total protein phosphorylation compared with the level of phosphorylation achieved when either component was incubated alone (Stegmayr et al, 1982). Rat seminal vesicle secretion does not demonstrate protein kinase activity, either toward endogenous or exogenous proteins (Wilson, 1987). Hence, protein kinase activity is restricted to some of the secretions of the genital tract and selected sperm-binding proteins from accessory gland secretions are phosphorylated by protein kinases at the surface of the spermatozoa.

Semenogelins I and II constitute the predominant proteins both in human seminal vesicle secretion and in human semen. The semen gel liquefies within 5 to 15 minutes due to proteolysis of the gel-forming proteins into several soluble fragments by the quantitatively dominanting prostate-derived serine protease, prostate specific antigen (PSA) (Lilja et al, 1984, 1987). Semenogelins I and II are coded by 2 separate genes located on the long arm of chromosome 20 and share approximately 80% nucleotide sequence identity in the primary structure. The tissue expression of semenogelin I is exclusively restricted to the secretory epithelium of the seminal vesicles. This is also the major secretory origin of semenogelin II that is also to a minor extent expressed by the epididymal secretory epithelium (Lilja et al, 1989; Lilja and Lundwall, 1992).

Semenogelin I is composed of 439 amino acid residues (molecular weight 49 607 daltons), is not glycosylated, and contains six 60-residue regions that display extensive structural similarity. Semenogelin II is composed of 8 similar regions (559 amino acid residues, molecular weight 62 931 daltons) and occurs in 2 molecular forms, a larger glycosylated form and a smaller nonglycosylated form (Lilja et al, 1989; Lilja and Lundwall, 1992; Malm et al, 1996). Semenogelin I contains 1 cysteine residue and semenogelin II contains 2 cysteine residues, which may explain why the two semenogelins occur in high molecular mass complexes in the seminal clot and in seminal vesicle secretions (Lilja and Laurell, 1985). The disulfide bonds may be of some importance for gel formation (Chaistivanich and Boonsaeng, 1983), but the gel structure is most probably noncovalently assembled because it can be disrupted by denaturing agents (Lilja and Laurell, 1985). Neither the physiological significance of gel formation and liquefaction nor the molecular mechanism behind gel formation have been elucidated. Whether post-translational modifications of the semenogelins are involved in gel formation is not known. Apart from glycosylation of semenogelin II, no in vivo posttranslational modifications of the semenogelins have been described (Malm et al, 1996). However, data reported on the phosphorylation of human seminal proteins in vitro (Chantananukul and Panyim, 1982) suggest that abundant proteins with masses similar to those of the semenogelins may be substrates for protein kinases. This could suggest subsequent dephosphorylation of the proteins by the action of acid phosphatase, which is abundantly secreted by the prostate gland. This enzyme has been suggested to function as a phosphoprotein phosphatase, but the physiologic substrate remains unknown (Li et al, 1984; Chevalier et al, 1988).

We therefore studied the protein kinase-mediated phosphorylation of the semenogelins in vitro (ie, using exogenous, purified protein kinase A and C; PKA and PKC, respectively), and under more

physiological conditions using endogenous prostasome-associated protein kinase. Proceeding from the phosphorylated forms of semenogelins I and II we also investigated the possibility to dephosphorylate these phosphoproteins with prostate-secreted acid phosphatase. Finally, we studied the PSA-mediated proteolytic cleavage of native and phosphorylated semenogelins.

Materials and Methods

1,2-Diolein, L-α phosphatidylserine, and affinity-purified human prostatic acid phosphatase were all from Sigma Chemical Company (St Louis, Mo). The antibody directed to the PKA γ-isozyme was from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif). $\gamma(^{32}P)$ATP, Sephadex G25, PD-10, and CM-Sephadex were purchased from Amersham Biosciences (Uppsala, Sweden). DE-52, Whatman 3mm, and P-cellulose were from Whatman (Kent, England). All other reagents were of highest commercial quality.

Preparation of Prostasomes

Semen samples were obtained from men attending the fertility clinic for investigation of fertility. Some semen samples were from men who were previously normospermic, who had fathered one or more children, and who had undergone vasectomy and therefore exhibited azoospermia. Prostasomes were prepared and analyzed as described previously (Ronquist, 1987).

Seminal Protein Kinases

Seminal protein kinases were purified from prostasomes isolated from semen of men with normozoospermia and semen from men who had undergone vasectomy. The preparation was performed at 4 °C. The prostasomes (typically 8-10 mg of protein) were thawed, frozen in liquid nitrogen, and thawed in 3.5 volumes of 50 mM Tris-HCl buffer pH 7.4, 1 mM ethyleneglycotetraacetic acid (EGTA), 50 mM 2-mercapthoethanol, and 0.05% (w/v) polyethyleneglycol-400-dodecylether. The suspension was suspended using a Potter-Elvehjem homogenizer (Thomas, Swedesboro, NJ). At this step, 1 mM phenylmethylsulphonyl fluoride was added. The material was stirred for 45 minutes and centrifuged at 40 000 x *g* for 45 minutes. The supernatant was further processed by changing the buffer using a 60-mL Sephadex G25 equilibrated column and eluted in 5 mM potassium phosphate buffer pH 6.5, 0.1 mM ethylenediamine tetraacetic acid (EDTA), and 15 mM 2-mercaptoethanol (buffer A). The protein fraction was applied to 1 mL of DE-52 equilibrated in buffer A. After application the column was washed with 5 mL of buffer A and proteins were eluted with a gradient from 5 mM (9 mL) to 400 mM (9 mL) potassium phosphate in buffer A at a velocity of 0.3 mL/min. The fraction volume was 0.3 mL.

The proteins that did not bind to DE-52 were filtered on a PD-10 column, equilibrated, and eluted in buffer A also containing 0.1 mM cAMP, after which they were applied on a 0.5 mL CM-Sephadex column equilibrated in the same buffer. The column was washed with 8 mL of buffer A containing 0.1 mM cAMP, and the proteins were eluted in a linear gradient in buffer A from 0 to 300 mM NaCl (total volume, 5 mL). Finally, an elution step of 400 mM potassium phosphate buffer pH 6.5, 0.1 mM cAMP, 15 mM 2-mercapthoethanol, and 1 mM EDTA was used. The volume of the fractions was 0.2 mL.

The chromatograms were analyzed for protein, ionic strength, and protein kinase activity.

Alkaline phosphatase activity was measured as dinitrophenol absorbance at 410 nm with dinitrophenylphosphate as substrate.

ATPase activity was analyzed according to the method described by Nimmo et al (1976) with omission

of alkaline treatment. A 10-µL sample was incubated with 10 µL of 0.1 mM $\gamma(^{32}P)$ATP (specific radioactivity 2000 cpm/nmol) for 10 minutes at 30 °C.

Semenogelins I and II were purified from human semen obtained from a healthy volunteer. The sample was collected in a tube containing urea and dithiothreitol. After liquefaction and removal of spermatozoa, the semenogelins were purified as described in detail elsewhere (Malm et al, 1996).

PKA catalytic subunit type 2 was prepared essentially as described by Zoller et al (1979) from porcine heart. The specific activity was 600 000 units/mg.

PKC was prepared from porcine spleen by the method originally described by Parker et al (1984) and modified by Ferrari et al (1985). The specific activity was 80 000 units/mg. One unit of protein kinase activity is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of (^{32}P)phosphate into histone (1 mg/mL) in 1 minute under the conditions specified below.

Phosphorylation With PKA

Except for the protein kinase, RRASVA, histone HI, and semenogelins I and II in concentrations indicated, the incubations contained 25 mM morpholino-2-ethane sulfonic acid buffer pH 6.9, 10 mM magnesium acetate, 12.5 mM Tris-HCl buffer pH 8.7, 75 mM 2-mercaptoethanol, 40 mM NaCl, 0.25 mM EGTA, 2.5% (v/v) glycerol, 12.5 to 112.5 mM urea, and 0.2 mM $\gamma(^{32}P)$ATP (50 cpm/pmol). The reaction was carried out at 30 °C for indicated times. We found that urea did not influence the reaction velocity at the concentrations used.

Phosphorylation With PKC

The mixture contained the following reagents in a total volume of 40 µL: 50 mM Tris-HCl, 5-30 mM sodium chloride, 0.25 mM dithiothreitol, 0.7 mM calcium acetate, 0.01 mM EGTA, 10 mM magnesium acetate, 60 µg/mL L-α-phosphatidylserine, 1 µg/mL diolein, 0.2 mM $\gamma(^{32}P)$ATP (50 cpm/pmol), and substrate and kinase as indicated. The pH was 7.75, and the mixture was incubated at 30 °C for indicated times.

The phosphorylation reactions were terminated by applying aliquots of the mixture to pieces of Whatman 3MM papers (for PKC) and washed according to the method described by Humble et al (1984) or to P-cellulose paper (for PKA) that was washed with 50 mM NaCl 4 times, dried, and analyzed for radioactivity. When higher amounts of semenogelin were phosphorylated, the reaction was interrupted by chromatography on a Sephadex G50 column equilibrated and eluted with 50 mM sodium citrate buffer pH 5.0, or 50 mM Tris-HCl buffer pH 7.5. One unit was defined as the amount of enzyme that transferred 1 pmol of phosphate to mixed histone (1 mg/mL) in 1 minute under the conditions described.

Alkali-Labile Phosphate

Alkali-labile phosphate was determined according to the method described by Ekman and Jäger (1993), which determines phosphate content of 0.1 nmol or more in a sample. Semenogelins I and II (40 µg in each sample) were assayed with and without the heating step, and the values were determined from 2 standard curves (with and without the heating step).

Determination of (^{32}P)Phosphorylated Amino Acid

After phosphorylation, the sample (40 µL of semenogelin I or II) was precipitated by adding 2 mL of 10% (w/v) trichloroacetic acid that also contained 50 mM H_3PO_4. One milligram of bovine serum

albumin was added to increase the precipitate, which after centrifugation, was suspended in 0.5 mL of H_2O. The suspension was extracted twice with water-saturated ether to remove the acid, after which the ether was removed by heating the sample to 60 °C in a water bath for 30 minutes. Determination of phosphoserine and phosphothreonine was performed as described (Ljungström and Engström, 1974).

Dephosphorylation of (^{32}P)Phosphosemenogelin

After incubating the ^{32}P-labeled semenogelin (a mixture of I and II) with acid phosphatase in excess at 37 °C in 20 mM Tris-HCl buffer pH 7.5 at concentrations given in the "Results" section, the reaction was terminated by adding 1 volume of 65 mM Tris-HCl buffer pH 6.8, 10% (v/v) glycerol, 23% (w/v) sodium dodecyl sulfate (SDS), and 15% (v/v) 2-mercaptoethanol. The samples were separated by electrophoresis in polyacrylamide (see below), and the dried and stained gels were autoradiographed. The autoradiogram was densitometrically scanned and the degree of dephosphorylation was calculated.

Cleavage of (^{32}P)Phosphosemenogelin with PSA

PSA was added to semenogelins I and II in 1:4 (w/w) proportions. The control semenogelin was treated identically to the ^{32}P-labeled protein except that water instead of (^{32}P)ATP was added in the phosphorylation mixture. After incubation at 37 °C for the times indicated, the samples were processed for separation on polyacrylamide gels.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was conducted as described by Laemmli (1970) and modified by O'Farrell (1975) using an acrylamide concentration of 12%. The gels were stained with Coomassie brilliant blue and autoradiographed.

Results

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Protein Kinase Activity in Prostasomes

Intact prostasomes could not phosphorylate specific substrates of PKA, PKC, casein kinase (CK)1, or CK2 due to high ATPase activity at the outer surface of the prostasomes, nor could ATPase activity be inhibited by 0.4 mM SDS, which according to Ronquist et al (1978), should decrease ATPase activity by 99%.

When extract from prostasomes prepared from vasectomized men was separated on diethylaminoethyl-cellulose, there was almost no protein kinase activity toward PKA-specific peptide (RRASVA), but histone activity was eluted at 100 and 250 mM potassium phosphate (Figure 1). ATPase activity was found in the first half of the gradient, but this activity was not stable and could not be detected the day after the separation had been performed (data not given). The alkaline phosphatase activity, eluted at about the same position as ATPase in the chromatogram (see Figure 1), was stable. Protein kinase did not bind to CM-Sephadex either in the absence or presence of cAMP.

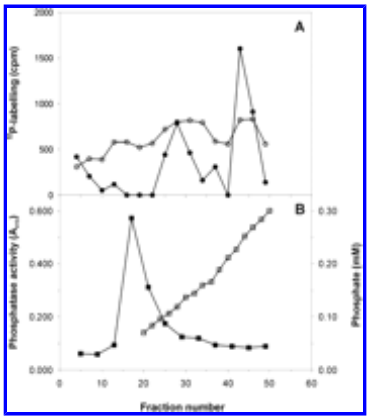

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Figure 1. The DE-52 chromatogram after separation of prostasomal extracts from vasectomized men. ○-○ Indicates protein kinase activity using RRASVA as substrate; •-• indicates protein kinase activity using histone HI as a substrate (shown in **A** as □-□ for potassium phosphate concentration; and as ■-■ for phosphatase activity in **B**). For details see "Materials and Methods." This separation was repeated 5 times with similar results.

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Characterization of two prostasomal protein kinases from normal semen[*]

A corresponding chromatogram from the same amount of prostasomes purified from men with normozoospermia revealed high activity toward the PKA-specific peptide RRASVA (100 µmol/min^{-1}/mg^{-1}). This enzyme, which was eluted at 100 mM potassium phosphate, was not activated by cAMP and was partially bound to CM-Sephadex in the absence as well as in the presence of cAMP (data not given). The ATPase and alkaline phosphatase patterns were similar to the chromatographed prostasomal extract from men with vasectomy (Figure 1).

The protein kinases in the DE-52 chromatogram from prostasomal extracts of men with normozoospermia were characterized kinetically (Table). The kinase activities eluted in two peaks, both resembling the corresponding protein kinase activities that were obtained from prostasomes from men with vasectomy (data not given). The activity was, however, much less when the latter material was used. The activity using mixed histones as substrate eluted in the first and second peaks was 3600 and 3000 pmol/min^{-1}/mL^{-1}, respectively, for prostasomes from men with normozoospermia and corresponding figures for prostasomes from men with vasectomy were 160 and 320. No activity was detected when a PKC-specific peptide (KRAKRKTAKKR) or casein were used as substrates. Semenogelin was not a substrate for any of the kinases that were both partially inhibited by a PKA-specific inhibitor. In addition, they were both detected in a Western blot in which antibodies directed to the γ form of PKA were used. They were not activated by cAMP. During this work, a prostasomal protein was purified, which was identified as lactoferrin by protein sequencing.

Determination of Covalently Bound Phosphate in Semenogelins I and II

When purified semenogelins I and II were assayed for covalently bound phosphate, the amount obtained was zero mol/mol for semenogelin I and 0.08 mol/mol for semenogelin II. It seemed possible that copurified acid phosphatase could have dephosphorylated semenogelin during purification and storage. Therefore, acid phosphatase activity was determined in the semenogelin preparation in 0.1 M malonate buffer at pH 6 and 30 °C with p-nitrophenylphosphate as substrate. A phosphatase activity corresponding to 0.06 µmol/min x mg of semenogelin was found.

A phosphate analysis on freshly ejaculated semen immediately precipitated with cold 10% (w/v) trichloroacetic acid was performed. The estimated amount of covalently bound phosphate was 0.01 mol/mol semenogelin.

The presence of phosphate in semenogelin I was assayed using a tandem electrospray mass spectrometer (Q-tof; Micromass, Manchester, United Kingdom) and equipped with a nanospray interphase after affinity absorption using immobilized metal-affinity chromatography (Posewitz and Tempst, 1999). No phosphorylated fragments were detected.

Phosphorylation of Semenogelin

Semenogelins I and II were phosphorylated with exogenous PKA and PKC. The time course of phosphorylation is shown in Figure 2. Under the conditions used, semenogelin II was more rapidly phosphorylated by PKA than semenogelin I was (Figure 2A). The maximal phosphorylation was 1.2 and 2.3 mol (^{32}P)phosphate/mol for semenogelins I and II, respectively. The reverse pattern was seen for phosphorylation with PKC. The phosphorylation rate was slightly higher with semenogelin I as substrate, but the maximal phosphorylation obtained was 10 mol of (^{32}P)phosphate/mol of semenogelin I, whereas this value became 14 for semenogelin II.

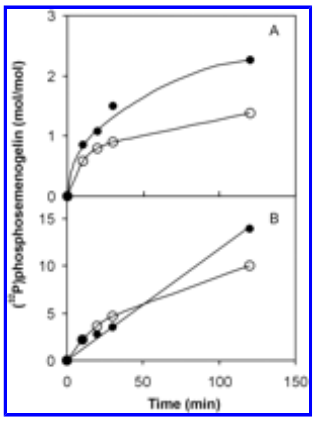

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Figure 2. Time course of the phosphorylation of semenogelins I (○-○) and II (•-•); 950 units of PKA **(A)** and 290 units of PKC **(B)** were used. The concentrations of semenogelins I and II were 0.54 µM. Conditions are described in "Materials and Methods." Values are means of 2 experiments that gave identical results.

In Figure 3, an SDS-PAGE gel and a corresponding autoradiogram are shown demonstrating a different semenogelin pattern in lane 4, in which a mixture of semenogelins I and II are phosphorylated with PKC compared to that phosphorylated with PKA (lane 3). Semenogelin I (54 and 52 kd) is phosphorylated to a higher degree after incubation with PKC than semenogelin II (76 and 71 kd), whereas the reverse pattern occurs for semenogelin phosphorylated with PKA.

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Figure 3. Phosphorylation pattern of a mixture of semenogelin separated by SDS-PAGE. The Coomassie brilliant blue—stained gel in which lane 1 shows the result of a 20-minute phosphorylation with PKA and lane 2 shows a 30-minute incubation with PKC. The corresponding autoradiogram is shown and lanes 3 and 4 correspond to lanes 1 and 2, respectively. The conditions for phosphorylation and electrophoresis are described in "Materials and Methods." The figure represents the result of an analysis that was performed at least 10 times with similar outcomes.

Determination of ^{32}P-Labeled Amino Acid

When the mixture of semenogelins was phosphorylated with PKA, serine was the only amino acid phosphorylated. Threonine was an additional minor target for the PKC phosphorylation of semenogelin (a mixture of both). The amount of (^{32}P) phosphothreonine was about 15% of the total phosphate incorporated in this case (data not given).

Dephosphorylation With Acid Phosphatase

Semenogelins I and II were separately phosphorylated with exogenous PKA and PKC. Control semenogelins were incubated with (^{32}P)ATP in the absence of PKA and PKC. The 6 samples were dialyzed to remove excess (^{32}P)ATP. After dialysis the protein was determined in the dialysis bags and 60%, 50%, and 5% of the unphosphorylated, PKA-phosphorylated and PKC-phosphorylated semenogelin remained in the solution, respectively. The ^{32}P-labeled samples were incubated with 250 units of acid phosphatase per milligram of protein in the absence or presence of protease inhibitors (3 mM benzamidine or 2 units of aprotinin) for different times. Semenogelin I, phosphorylated with PKA and PKC, respectively, was dephosphorylated to 15% and 22%, respectively. Corresponding values were 25% and 10% for semenogelin II (Figure 4). There was no degradation of the protein of these samples as seen after SDS-PAGE, and (^{32}P)orthophosphate was the only labeled product released (data not given). It was noted in these experiments that the commercial, affinity-purified acid phosphatase contained, depending on the batch, more or less of semenogelin and its related proteins, which could be seen after SDS-PAGE of the phosphatase batches (data not given).

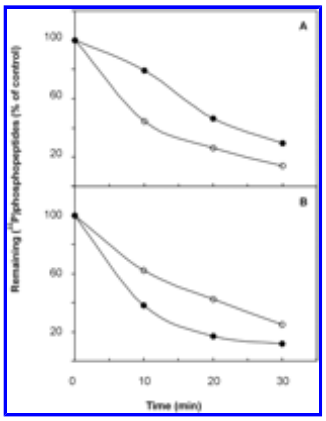

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Figure 4. Time course of dephosphorylation of semenogelins I and II with acid phosphatase. The phosphatase:semenogelin ratio was for PKA phosphorylated protein **(A)** and PKC phosphorylated protein **(B)** 750 and 12 500 units/mg, respectively. -◌-, semenogelin I and -•-, semenogelin II. Incubation was performed as described in "Materials and Methods." Semenogelins were phosphorylated to about 1 mol (^{32}P)phosphate/mol semenogelin irrespective of kinase used. The experiment is similar to dephosphorylation experiments performed at least 5 times but with other batches of acid phosphatase.

The phosphatase was shown to be somewhat more active at pH 5 than it was at higher pH values. Still, the activity at pH 7.5 was decreased by only 28% for the PKC phosphorylated semenogelin (a mixture of forms I and II). The corresponding figure for PKA phosphorylated semenogelin mixture was 30%. The pH used in the following experiments was 7.5, in conformity with the pH of semen. All tests were carried out in the absence of inhibitors. The activity of acid phosphatase is dependent on divalent cations, especially Zn^{2+}, and semen has a high concentration of Zn^{2+}. Therefore, Ca^{2+}, Zn^{2+}, and Mg^{2+} were tested as effectors during dephosphorylation of ^{32}P-labeled semenogelin (a mixture of both). The effectors had minor or no influence on phosphatase activity.

Cleavage of (^{32}P)Phosphosemenogelin With PSA

Semenogelins I and II phosphorylated with exogenous PKA or PKC for 60 minutes were used as substrates. PSA was added and the incubation was continued for 6 minutes (for PKA) or 10 minutes (for PKC). The resulting SDS-PAGE gels were densitometrically scanned and the resulting data showed no difference between the phosphosemenogelins and their unphosphorylated counter-parts in their sensitivity to PSA-mediated degradation (data not given). Semenogelin I was degraded into fragments with molecular weights of 46 and 27 kd according to the Coomassie-stained gel and its corresponding autoradiogram.

Semenogelin II phosphorylated with PKA was degraded, but the degradation products could not be detected in the gel (data not given). In the corresponding autoradiogram, labeled traces of degradation products of molecular weights of 65, 55, 25, and 20 kd were seen.

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Semenogelin could easily be phosphorylated to more than 2 mol of (^{32}P) phosphate/mol of semenogelin with exogenous PKA and PKC in vitro (Figure 2). Besides serine residues, which were the only phosphoamino acid detected after phosphorylation of a semenogelin mixture by PKA, PKC additionally phosphorylated threonine residues to 15%. Another observed difference was that PKA phosphorylated semenogelin II better than it phosphorylated semenogelin I. The phosphorylation pattern was similar for both semenogelin types phosphorylated with PKC.

When commercial acid phosphatase from prostate was used to determine whether phosphorylated semenogelin was a good substrate for this enzyme, it was found that ^{32}P-phosphorylated semenogelin could be dephosphorylated completely, although at a slow rate at physiological pH and using a high amount of phosphatase (data not given). There was a decrease by about 50% in dephosphorylation rate between phosphosemenogelins I and II if they had been phosphorylated with PKA. The dephosphorylation rate was the opposite for PKC-phosphorylated semenogelins, phosphosemenogelin II being a better substrate for the phosphatase than phosphosemenogelin I. It was observed during the preparation of phosphorylated semenogelins for this experiment that semenogelin precipitated in the dialysis bag and that the extent of precipitation differed between the different phosphoforms. The reason for this could not be determined, but because PKC-phosphorylated semenogelins were more prone to give a gel, this might indicate a physiological function perhaps, as suggested in the introduction, in gel formation in the presence of zinc. It is not only the presence of phosphate groups that is decisive, because the reverse is seen for fibrinogen, for which the unphosphorylated molecule is more difficult to keep in solution (Forsberg, 1989). In a wider perspective, it is interesting to note that tissue factor, which is a cell membrane—associated glycoprotein that serves as a receptor and an essential cofactor for factors VII and VIIa of the coagulation cascade (Nemerson, 1988), was found associated in very high levels to prostasomes (Fernández et al, 1997). As a matter of fact, tissue factor, with its true receptor properties, had the ability to mediate ligand-induced, kinase-dependent phosphorylation reactions (Rottingen et al, 1995). Therefore, intricate phosphorylation reactions exerted by prostasomes may be one facet of physiological expressions linked to these organelle structures in semen.

Apparently, semenogelins I and II can be phosphorylated in vitro provided that PKA or PKC is present. Human spermatozoa contain PKA and PKC (Li et al, 1985; Aanesen et al, 1998). It would

therefore be theoretically possible for a phosphorylation reaction to occur in vivo, especially because an interaction has to be anticipated between spermatozoa with their net negative surface charges (Ronquist et al, 1990) and the cationic properties of the two semenogelins. The extent and effect of such a phosphorylation are, however, uncertain in light of the finding that spermatozoa and the semenogelins (the latter contributed by the seminal vesicles) appear in different fractions of the split ejaculate (Stegmayr et al, 1980). In addition, when mixed in the total ejaculate, the abundant occurrence of acid phosphatase contributed by the prostate gland (appearing in the first fractions of a split ejaculate followed by the spermatozoa) will attend to the efficient dephosphorylation of any possible phosphosemenogelin (appearing in the last fractions of a split ejaculate). This could explain why no or very little phosphate could be found bound to purified semenogelin. Due to this we could not eliminate the possibility that the semenogelins were posttranslationally phosphorylated before secretion. It is alwo worthy of note in this context that prostasomes, despite their protein kinase content (Stegmayr et al, 1982; Wilson et al, 1984), were unable to phosphorylate the semenogelins.

V_{max} values could not be obtained, which suggests unspecific phosphorylation under the in vitro conditions we used. Another group of basic proteins, histones, behave similar to semenogelin in our phosphorylation assays (the rate is approximately 5 times higher with histones as substrates compared to that of semenogelin). Because the phosphate groups have physiological importance for histones, one could speculate on a biological relevance for the demonstrated in vitro phosphorylation of semenogelin as well.

It was apparent that there was more than one source for the endogenous protein kinase activity, because a 95% reduction in protein kinase activity was noted in prostasomes from seminal plasma of men subjected to vasectomy. It means that a contribution of enzymes or activators by testis, epididymis, or both was evident. The protein kinase activity might well be associated with spermatozoa. It has been reported (Wilson and Kaye, 1983) that the histone kinase activity in seminal fluid is correlated with the number of spermatozoa originally present in the ejaculate. Thus, the histone kinase activity may have been dissociated from spermatozoa during the maturation process in the epididymis. Another suggestion is that histone kinase may be the free catalytic subunit of a cAMP-dependent enzyme that originates in the sperm plasma membrane (Fabbro et al, 1982; Wilson et al, 1982).

Accordingly, prostasomes isolated from seminal plasma of men with normozoospermia differed quantitatively but not qualitatively from those of vasectomized men in protein kinase activity. The corollary is that all protein kinase activity is not genuinely associated with prostasomes, but rather, it is adsorbed to these extracellular organelles. This suggestion would align with that of a previous finding on the prostasomal association of 5' nucleotidase, in which prostasomes from seminal plasma of normozoospermic men contained about double the amount of enzyme activity compared with prostasomes from vasectomized men (Fabiani and Ronquist, 1995).

Our present finding of lactoferrin in prostasomes using protein sequencing may be in line with this reasoning because the seminal vesicles have been suggested to be the major source of this iron-binding glycoprotein in human seminal plasma (Hekman and Rumke, 1968; Roberts and Boettcher, 1969). However, a pure origin of some of the seminal lactoferrin in the prostasomes cannot be ruled out, because in neutrophil granulocytes, for example, lactoferrin is restricted to specific granules (Baggiolini et al, 1970), which are perhaps analogous to prostasomes.

Acknowledgments

The excellent assistance of Stefan Eriksson, Birgitta Frohm, and Åsa Haglund is acknowledged.

Footnotes

Supported by The Swedish Medical Research Council (K9903X0790313A), Foundation Federico S.A., The Crafoord Foundation, University Hospital Malmö, and Swedish Agricultural and Forestry Research Council (729.1181/97).

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