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Comparison of partial amino acid sequences of two protamine 2 variants from stallion sperm

Structural evidence that the variants are products of different genes

Arja Pirhonen, Annika Linnala-Kankkunen and Pekka H. Mäenpää

Department of Biochemistry, University of Kuopio, PO Box 6, SF-70211 Kuopio, Finland

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Protamine 1 and two protamine 2 variants were isolated from stallion sperm and separated by acetic acid-urea gel electrophoresis. After electroblotting onto polyvinyldifluoride filters, their amino-terminal amino acid sequences were determined by pulse-liquid peptide sequencing. The sequences of the two protamine 2 variants are homologous but slightly different in length and amino acid composition and indicate for the first time the existence of two different genes for this protamine species.

Protamine; Protein sequencing; Protamine gene; (Stallion)

1. INTRODUCTION

During sperm cell differentiation, histones in the cell nucleus are replaced by protamines, which are small arginine-rich proteins. The protamines have a role in packaging sperm DNA to a highly condensed, stable and inactive complex. All mammalian sperm examined thus far have been found to contain protamine 1. The complete amino acid sequences of protamine 1 from bull [1], boar [2], ram [3], stallion [4,5], human [6,7], and mouse [8] have been determined. In the different species studied, there exists a strong sequence homology in this protein.

Certain species including mouse [8], hamster [9], human [10], and horse [4] contain a second protamine variant, protamine 2, which differs in size and amino acid composition from protamine 1. In contrast to protamine 1, mouse protamine 2 is synthesized as a testicular precursor [11].

We have isolated the protamines from stallion spermatozoa and found three protamines: protamine 1 and two protamine 2 variants. When the amino-terminal amino acid sequences of the protamine 2 variants were determined, the sequences were found to be different in length and in amino acid composition indicating in stallion sperm the existence of different but homologous genes for these variants.

Correspondence address: A. Pirhonen, Department of Biochemistry, University of Kuopio, PO Box 6, SF-70211 Kuopio, Finland

2. MATERIALS AND METHODS

2.1. *Semen collection*

Semen samples were collected from Finnhorse stallions used for artificial insemination at the State Horse Breeding Institute, Ypäjä, Finland. Two to three first fractions of the ejaculates were obtained using an open-ended artificial vagina. Washed sperm cells were stored at −20°C.

2.2. *Isolation of basic nuclear proteins*

The isolation procedure was a modification of the methods described by Balhorn et al. [12] and Ammer and Henschen [5]. Approx. 8×10^8 sperm cells were extracted with 2 ml of 1% acetic acid for 15 min at room temperature and collected by centrifugation at $1500 \times g$. The pellet was extracted with 2 ml of 8 M guanidine hydrochloride (GuHCl) containing 0.1 M Li-acetate, pH 4, and 1 mM phenylmethylsulphonyl fluoride (PMSF) using sonication (Labsonic 2000) with two 10 s bursts at 50 W. After incubation for 15 min at room temperature, the nuclei were collected by centrifugation and washed twice with distilled water.

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The chromatin was solubilized by adding 1 ml of 5 M GuHCl containing 0.5 M Tris-HCl, pH 8. After incubation for 5 min at 37°C, the chromatin proteins were dissociated by adding urea, mercaptoethanol, NaCl, and PMSF to a final concentration of 0.5 M, 0.5 M, 2 M, and 1 mM, respectively. The solution was incubated for 1 h at 37°C and the DNA precipitated by adding HCl to 0.5 M. After 1 h at 4°C, the DNA was centrifuged for 15 min at 17000 × *g*. The supernatant was dialyzed overnight against 0.01 M HCl in Spectrapor-3 tubing (Spectrum Medical Industries, Los Angeles, USA). Protein concentration of the dialysate was measured using a protein assay kit from Bio-Rad Laboratories (Richmond, CA, USA) with bovine serum albumin as a standard.

The lyophilized protamines were reduced with a 38-fold molar excess of dithiothreitol (DTT) over protamine cysteine in 0.5 M Tris-HCl, pH 8, 5 M GuHCl, and 2 mM EDTA for 1 h at room temperature and alkylated with iodoacetamide (2-fold excess over DTT) for 1 h at dark. Alkylated protamines were dialyzed for 2 h against 0.01 M HCl in Spectrapor-3 tubing and lyophilized before electrophoresis. GuHCl, Li-acetate, PMSF, DTT and iodoacetamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. *Polyacrylamide gel electrophoresis, electroblotting and protein sequencing*

The alkylated protamines were separated by acetic acid-urea slab gels [13] containing 20% acrylamide, 0.13% bisacrylamide, and 2.5 M urea. The stacking gel and the sample buffer described by Spiker [14] were used. All reagents for the electrophoresis were purchased from Bio-Rad Laboratories. After electrophoresis, the protamines were electroblotted at 4°C onto polyvinyldifluoride (PVDF) filters (Immobilon, Millipore Inc., Boston, USA) for 1 h at 70 V using a Trans-Blot™-apparatus from Bio-Rad Laboratories. The transfer buffer was 2.5% acetic acid. The proteins were then stained with a solution containing 0.1% Amido Black, 45% methanol, and 10% acetic acid. The stained bands were cut out and stored at −20°C until sequencing. The amino-terminal sequences were determined directly from the filters using an Applied Biosystems 477A pulse liquid protein/peptide sequencer with a 120A PTH analyzer [15].

3. RESULTS

Total nuclear proteins from stallion sperm were isolated using three extraction steps. About 4 mg of protein was obtained from 8×10^8 sperm cells. The alkylated protamines were separated by acetic acid-urea gel electrophoresis. Three bands were seen on the gel (fig.1). Immediately after electrophoresis, the separated protamines were transferred onto a PVDF-filter. The major band (band 1) was further cut into 2 mm × 4 mm pieces and submitted to sequencing. Pieces from 3 to 6 bands were combined for analysis of the minor protamines (bands 2 and 3).

When the amino-terminal amino acids (20 residues) of the major band (band 1) were determined, the sequence was found to be identical with that of the major protamine (St1) from stallion sperm [4]. The amino-terminal sequences of the two other protamines (bands 2 and 3) were analyzed twice (25 cycles; fig.2) and the proteins were named protamine St2b and St2a, respectively. The St2 variants were found to be homologous proteins with a difference in amino-terminal length and amino acid sequence (three residues).

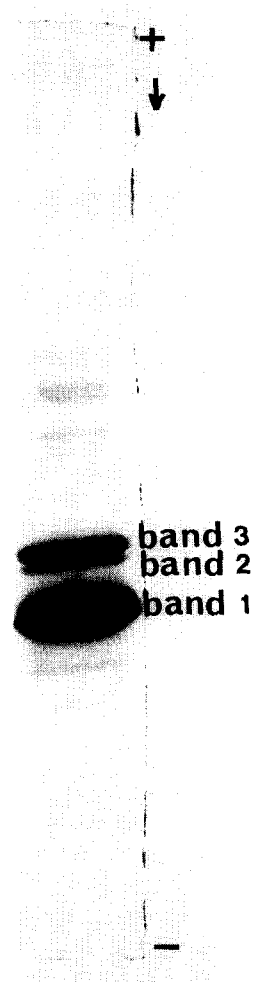

Fig.1. Acetic acid-polyacrylamide gel electrophoresis of alkylated stallion protamines. 20 μg of total protein was dissolved in the sample buffer and electrophoresed for 2 h at 200 V (stacking gel) and for 3 h at 150 V (running gel). The gel was stained with a solution containing 0.1% Serva Blau R, 50% methanol, and 10% acetic acid.

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St2a      A R T T A G S Y R R Y R R R C C S P R R L Y R L R - -
St2b            A G S Y Y S Y R R R R C S P R R L Y R L R R R R Y - -

HP2         R T H G Q S H - - Y R R R H C S R R R L H R I H R R Q H - -
HP3             G Q S H - - Y R R R H C S - -

pmP2 - - G R T H R G H H H - H R H R R C S R K R L H R I H K R - R - -
mP2             R G H H H - H R H R R C S - -
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Fig.2. The amino-terminal amino acid sequences of the two protamine 2 variants from stallion sperm. The sequences are aligned with corresponding sequences of human protamines (HP2 and HP3) [19], mouse protamine 2 (mP2) [8], and the testicular precursor of mouse protamine 2 (pmP2) (residues 40–67) [11]. Non-homologous regions of stallion protamines are underlined.

4. DISCUSSION

Recent studies on cDNAs of mouse protamine 1 (mP1) and protamine 2 (mP2) [16,17] as well as amino acid sequences of protamines from other species [1–7] indicate that most mammals have a protamine species with a strong homology to mP1 cDNA. In contrast, the expression of protamine 2 gene varies greatly among different species. For instance, protamine 2 has never been detected from rat by gel electrophoresis, although a gene sequence with strong homology to mP2 cDNA has been described [16]. The same situation occurs in bull and boar spermatozoa, both of which contain a single protamine but also appear to have a protamine gene homologous to mP2 cDNA. In stallion epididymal spermatozoa, no hybridization signal for mP2 cDNA has been detected [16].

Using polyacrylamide electrophoresis including 7.5% stacking gel and 20% running gel, a better resolution was achieved than with methods previously used for separation of basic nuclear proteins from sperm [13]. Three protamines from stallion spermatozoa were separated for sequence analysis. The major component of the basic proteins turned out to be protamine 1, since we found an amino-terminal sequence identical with that published previously [4].

A protamine 2 family with at least four variants (HP2, HP3, HPS1, and HPS2) differing only in the length of their amino-termini [19] has been detected by gel electrophoresis from mature human spermatozoa [18]. The intermediate proteins HPS1 and HPS2 are probably precursors of HP2 and HP3. There is a strong sequence homology between human protamine 2 variants and the testicular precursor of mouse protamine 2 [19]. These pro-protamines of mature human spermatozoa are probably products of one protamine 2 gene and represent different steps in the post-translational maturation process.

Sequence analysis of the two minor protamines from stallion sperm indicates that these proteins belong to the protamine 2 family. The 25 amino-terminal residues determined representing almost one half of the length of the proteins indicate a close structural relationship with human HP2 and HP3 and mouse mP2. Several conservative changes in the amino acid sequence of different species can be explained by single point mutations in protamine 2 gene. The lack of histidine and abundance of tyrosine are typical of the amino-terminal amino acid composition of the protamine 2 variants.

The two protamine 2 variants from stallion sperm differ from each other not only in their amino-terminal lengths but also in residues 9, 10, and 15 in St2a (5, 6, and 11 in St2b). In positions 10 and 15 in St2a (6 and 11 in St2b), the amino acid differences may have resulted from gene duplication followed by point mutations. The changes (Arg-Ser and Cys-Arg) are, however, not conservative in nature. Serine residues in protamines may participate in phosphorylation-dephosphorylation reactions, which seem to be involved in the process of packaging of chromatin and cysteine may participate in disulfide bridge formation. These non-conservative changes in the amino acid sequence of the protamine variants may thus cause differences in their binding characteristics and function. Finally, a change of Arg to Tyr in position 9 in St2a (5 in St2b) must have involved more than one point mutation.

In conclusion, according to the amino-terminal

amino acid sequence analysis of two protamine 2 variants, there are two different but homologous protamine 2 genes in stallion spermatozoa, which could have arisen by gene duplication and subsequent point mutations. The horse seems to be the only species thus far reported with two different genes for protamine 2.

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