Fibronectin type II–module proteins in the bovine genital tract and their putative role in cell volume control during sperm maturation.

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Structural and Molecular Characterization of Equine Sperm-Binding Fibronectin-II Module Proteins

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ABSTRACT Phospholipid-binding proteins in the male genital tract are characterized by differing numbers Fn-2 modules (B-domain) carrying N-terminal extensions (A-domain) of variable length. In the stallion, three different proteins were identified, SP-1, SP-2, and EQ-12. SP-1 and SP-2 of the AA′BB′- and ABB′-type, respectively, are major proteins of the seminal plasma. Here we report the cDNA sequences of SP-1, and of a new member of the SP-2 family (SPnew) and the partial characterization of their iso- and glycoforms. The phosphorylcholine (PC)-binding ability of the long Fn-2 protein, EQ-12, with four tandemly arranged Fn-2 modules was determined by PC-affinity chromatography. Expression patterns of EQ-12, and the SP-proteins were studied by means of RT-PCR, Northern blot analysis and immunological approaches indicating differential expression along the male reproductive tract. The vast majority of the short SP-1 and SP-2 proteins are produced by the ampulla whereas EQ-12 originates from the epididymis. Indirect immunofluorescence microscopy of sperm isolated from different regions of the epididymis and Western blot analysis indicate that both, the long and the short Fn-2 proteins associate to the sperm surface during post-testicular maturation. Sperm binding of Fn-2 proteins at the post-acrosome and midpiece was at first detected in the corpus epididymis. Enhanced fluorescence intensity after ejaculation point to an increased number of molecules bound to the sperm surface. The function of these proteins is discussed in regard to their structure–function relationships. *Mol. Reprod. Dev. 70: 45–57, 2004.*

Key Words: male genital tract; phosphorylcholine-binding proteins; Fn-2 module; glycosylation; tissue expression; sperm maturation; capacitation

INTRODUCTION

Sperm binding proteins secreted post-testicularly by the male genital tract have been shown to be involved in the establishment of the oviductal sperm reservoir (Gwathmey et al., 2003), in the control of sperm capacitation (Manjunath and Therien, 2002) and also in gamete interaction (Töpfer-Petersen, 1999b; Cuasnicu et al., 2001). The expression of these functional important proteins corresponds to the site within the male genital tract where sperm acquire full fertilizing competence (Yanagimachi, 1994). Dependent on their regionalized secretion, their relative abundance may vary considerably across species. The seminal plasma of ungulates, for example, pig, horse, and cattle, is a rich source of mainly three protein classes which have been shown to interact with the sperm surface and participate in sperm function (Töpfer-Petersen, 1999a). These are the spermadhesins (Töpfer-Petersen et al., 1998), cysteine-rich secretory proteins (CRISPs, Schambony et al., 1998), and Fn-2 type proteins, which represent prototypes of widely occurring protein modules. Whereas the spermadhesin family seems to be restricted to ungulates, members of the CRISP and Fn-2 protein families are expressed in the male genital tract of most mammalian species pointing to a common role in fertilization (Cuasnicu et al., 2001; Manjunath and Therien, 2002).

The major seminal plasma proteins in cattle (Manjunath and Sairam, 1987), horse (Calvete et al., 1995a,b; Menard et al., 2003), goat (Villemure et al., 2003), and bison (Boisvert et al., 2003) are characterized by their conserved gelatin-binding fibronectin II (Fn-2) type module (Smith et al., 2000). Related proteins have been also identified as minor components in porcine seminal plasma (Calvete et al., 1997). Fn-2 type proteins of the male genital tract are characterized by two (Manjunath and Sairam, 1987; Calvete et al., 1995a,b, 1997) or four (Saalmann et al., 2001) tandemly arranged

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Fn-2 type modules carrying N-terminal polypeptide extensions of variable length. Continuous investigations from P. Manjunath's laboratory and others make the major bovine seminal plasma proteins (collectively designated as BSP) the most intensely studied members of the male Fn-2 protein family. Bovine Fn-2 proteins have been first characterized as phospholipid- and heparin-binding proteins involved in the heparin-stimulated capacitation in bovine sperm and thereby potentiating the agonist-induced acrosome reaction (Therien et al., 1995). They are small (15–30 kDa) acidic proteins consisting of two Fn-2 modules (also designated as BB′-domains) with a short highly acidic N-terminal polypeptide extension (A-domain; BSP-1/BSP-A2; BSP-A3) (Manjunath and Sairam, 1987) or a repeatedly *O*-glycosylated 71-residue N-terminal polypeptide extension (BSP-30) (Calvete et al., 1996). BSP-A1 and BSP-A2 representing different glycoforms (Calvete et al., 1994; Gerwig et al., 1996) with identical polypeptide chains are also collectively termed PDC-109 (Esch et al., 1983).

At ejaculation some million BSP-A1/A2 molecules bind to the sperm surface (Calvete et al., 1994). Part of these molecules remain adhered to the plasma membrane during sperm transport through the female genital tract to the fallopian tube (Manjunath and Therien, 2002) and appear to be involved in the establishment of the oviductal sperm reservoir (Gwathmey et al., 2003). The strong binding of BSP-A1/A2 to the sperm surface is mediated by their specific interaction with phospholipids bearing a phosphorylcholine (PC) head group (Desnoyer and Manjunath, 1992, 1993). Interaction of the Fn-2 proteins with lipid membranes and with epididymal sperm causes an effective rigidification of the membrane (Müller et al., 1998, 2002; Greube et al., 2001; Ramakrishnan et al., 2001).

At higher concentrations the proteins induce a permeabilization of lipid membranes (Gasset et al., 1997, 2000) and stimulate an efflux of cholesterol and phosphatidylcholine from cell membranes resulting in the formation of protein–lipid particles (Therien et al., 1998, 1999; Moreau and Manjunath, 1999). This release of lipids which can be enhanced by factors secreted by the oviduct as heparin-like glycosaminoglycans (Moreau and Manjunath, 1999) and high density lipoprotein (HDL) (Therien et al., 1997, 2001) is a crucial process of sperm cell capacitation. The number of interactions of the bovine Fn-2 proteins with impact on the sperm cell membrane may account for the observed alterations of membrane properties that may prime the capacitation machinery in bull sperm (Manjunath and Therien, 2002). Thus, in the bovine system the Fn-2 proteins of the ABB′-type seem to be multifunctional in that they are involved in the establishment of the sperm reservoir and in modulating sperm genesis.

SP-1 and SP-2 (previously named HSP-1 and HSP-2 for *h*orse *s*eminal *p*roteins) have been characterized as the major heparin- and PC-binding proteins of equine seminal plasma (Calvete et al., 1995a, 1997) and are structurally related to the BSP proteins with A-domains (Calvete et al., 1995a). The primary structure and post-translational modifications, for example, glycosylation and disulfide bonds of SP-1 have been studied by protein chemical methods indicating that it belongs to the AA′BB′-type (Swiss-Prot: P81121). A 26-residue N-terminal amino acid sequence of SP-2 is also known documenting that it belongs to the ABB′-type (Calvete et al., 1995a).

Very recently, it has been shown that the pattern of interactions of SP-1/2 with membranes is similar to that of BSP-A1/A2 (Greube et al., 2004). Still, these homologous proteins of different species may display distinct biological properties (Calvete et al., 1997; Greube et al., 2004). Here we report the molecular cloning, expression and extended structural analysis of the equine Fn-2 proteins SP-1 and SP-2 by a combination of molecular and protein chemical approaches. Special attention was focussed on the differential distribution of Fn-2 type proteins along the equine male genital tract and on the sperm surface to understand their contribution to the species-selective modulation of sperm function leading finally to fertilization.

MATERIALS AND METHODS

Unless otherwise stated, chemicals were purchased from Sigma Chemical Company (Steinhausen, Germany), Merck (Darmstadt, Germany), or Roth (Karlsruhe, Germany) and were of analytical grade or higher purity.

Semen and Tissues

Semen samples were collected from healthy and fertile stallions of Hannover breed, 6–12 years old, using an artificial vagina. Only ejaculates of sufficient quality meeting the criteria of normospermia were used as judged by conventional semen analysis according to Sieme et al. (2001). Sexual organs were collected from five fertile stallions, 17–19 years old, which were sacrificed for nonreproductive reasons. Immediately after the slaughter, tissue samples were dissected and prepared for RNA extraction using standard protocols. Alternatively, tissue samples were prepared for immunohistochemistry about 60 min after dissection. For protein extraction, the tissue samples were minced and homogenized in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, (TBS) containing 2 mM PMSF (phenylmethylsulfonylfluoride) using a UItra-Turrax homogenizer, followed by centrifugation at 12,000*g* for 10 min (TBS-extract). Resulting pellets were extracted with TBS–1% CHAPS and complete inhibitor solution (Roche, Mannheim, Germany) and designated as CHAPS-extracts. Protein concentrations were determined using the Bradford assay (Bio-Rad, Munich, Germany) according to the manufacturer's instruction. Alternatively, sperm were recovered from testis, caput, corpus, and cauda regions of the epididymis by flushing the ducts with 10 mM sodium phosphate, 135 mM NaCl, pH 7.4, (PBS). Sperm were washed three times in PBS (centrifugation at 150*g*, 15 min) and prepared for immunofluorescence microscopy. Alternatively, sperm (50×10^6 cells/ml) were extracted with TBS–1% CHAPS (1 hr at 4°C) in the

presence of 2 mM PMSF. Extracts were centrifuged at 13,000*g* for 30 min. The sperm free supernatant was used for SDS–PAGE.

RNA Extraction, Standard RT-PCR, and Northern Blot Analysis

Total cellular RNA was extracted from frozen tissue samples by standard procedures and complementary DNA (cDNA) synthesized from 2 to 5 μg of total RNA per reaction by oligo-(dT)-primed reverse transcription (RT) using Superscript™ reverse transcriptase (Gibco-BRL, Karlsruhe, Germany) as described (Saalmann et al., 2001; Ekhlasi-Hundrieser et al., 2002). Amplification of fragments was carried out using a standard PCR protocol employing 5 μl of cDNA, 10 pmol each of degenerated primers designed by reverse translation of peptide sequences (degenerate sp1F-sense: 5′-AA(CT)CC(CT)GACCA(GA)CA(GA)CT(GACT)ATCATG-3′; degenerate sp-1R-antisense: 5′-TA(CT)TTCCA(GA)-GC(TGAC)CC(GA)TG(GA)TGGTC-3′; specific sp1F-sense: 5′-TTTTCCATTCAACTATCGAG-3′; specific sp1R-antisense: 5′-CGTGATGGTCATAGTTAGGA-3′, and 1 U of *Thermophilium aquaticum* (*Taq*) polymerase (Perkin Elmer Applied Biosystems, Weiterstadt, Germany) in a 50 μl reaction volume. RT-PCRs were run in a thermocycler (Omnigene; Hybaid, Heidelberg, Germany) using the following conditions: after 5 min at 95°C the first two cycles were held for 30 sec at 95°C (denaturation), 60 sec at 37°C (annealing), and 60 sec at 72°C (extension). The following cycles were carried out under the same conditions, except for the annealing temperature (cycles 3–4 for 90 sec at 45°C and the last 30 cycles at 55°C) and the extension for 90 sec. When specific primers were employed, the cycling conditions were 30 sec at 95°C, 60 sec at 55°C, and 60 sec at 72°C.The amplified fragments obtained by RT-PCR were separated in 2% TAE agarose/ethidium bromide gels (Gibco-BRL), purified (DNA Clean, Hybaid, Heidelberg, Germany), and ligated into pCRII cloning vector (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. Supercoiled plasmid DNA was isolated using Nucleobond AX columns (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol and sequenced.

Partial cDNAs and digoxigenin (DIG)-labeled probes were prepared according to the instructions of the supplier (Boehringer, Mannheim, Germany) using the following sets of oligonucleotide primers: SP-related 304-bp product: 5′-CTGACCAACAGCTAATC-3′/5′-CG-TGATGGTCATAGTTAGG-3′; SP-related 264-bp product: 5′-ATGGCACTGCAGTTGGGAGTC-3′/5′-TTTG-GCGTAGTCCTCAGCAACA-3′; EQ-12, 752-bp-product: 5′-GGGTCTTCTTACTTCTCTTG-3′/5′-TCCTTTATTG-GTGGTTATGCCAC-3′ (compare Saalmann et al., 2001). Amplicons were separated and visualized on 1% TAE agarose/ethidium bromide gels. Identity of inserts was confirmed by subcloning into the TA-cloning vector pGEM-T Easy (Promega, Mannheim, Germany) followed by DNA cycle sequencing (MWG-Biotech, Ebersberg, Germany). Northern blot analysis was performed as described (Pera et al., 1996). Briefly, 5–10 μg of total RNA per lane, depending on the type of experiment, were separated by denaturing agarose gel electrophoresis and transferred to Hybond N^+ nylon membranes (Amersham Biosciences, Freiburg, Germany). A DIG-labeled probe prepared from rat-18S ribosomal RNA using the following primers: 5′-AGGACCGCGGTTC-TATTTTGTTG-3′/5′-CGGGCCGGGTGAGGTTT-3′ and a 60–50°C "touch-down" PCR-program was employed as a blotting control.

Generation of 5′- and 3′-Ends and Completion of cDNAs

Partially cloned cDNA fragments were completed at their 5′-ends by inverse RT-PCR as described (Gebhardt et al., 1999; Saalmann et al., 2001). Specifically, antisense primers located in the known sequences of partial cDNA clones were used for reverse transcription (SP-1 cDNA synthesis primer: 5′-GATTTCTTGA-TTTTCTTACCGCAG-3′; SP-2 cDNA synthesis primer: 5′-CAGTGGGAACAAAGTGGAG-3′) of 5 μg each of total epididymal RNA employing Superscript™ reverse transcriptase (Gibco-BRL). Inverse PCR was carried out on the circularized cDNA using sense and antisense primers as follows. SP-1: 5′-AAGTGCATCTAACC-CATGG-3′; SP-2: 5′-CAGTGCATCTAACCCATGG-3′) and corresponding antisense primers (SP-1: 5′-(GAGT-GACTGAGCACCAAGAA)-3′; SP-2: 5′-TTTGGCGTA-GTCCTCAGCAACA-3′). Generation of 3′-ends was performed according to standard techniques as described (Kirchhoff et al., 1994). Sequences of the gene-specific oligonucleotide primers employed were: RACE1-Primer: 5′-GACTCGAGTCGACATCGATTTTTTTTTTTT-TTTTT-3′; RACE2-primer: 5′-GACTCGAGTCGACAT-CG-3′. Sequences were compiled using the DNASTAR™ software package and the following pairs of primers employed for subsequent confirmation of "full-length" cDNA fragments: SP-1: 5′-AAGGCAAGCTGCCATG-GC-3′/5′-GCAGAGAAAGTGGTTTATTG-3′; SP-2: 5′-A-TGGCACTGCAGTTGGGAG-3′/5′-GGCTTTCAGGCA-CAGTGGG-3′.

Isolation of Equine Fn-2 Proteins, SP-1 and SP-2

Within 60 min after collection of the ejaculates the seminal plasma was freed from sperm by centrifugation at 150*g* for 15 min followed by an additional centrifugation step of the supernatant at 1,000*g* for 15 min. Seminal plasma was subjected to Heparin-Sepharose CL-6B (Amersham Biosciences) chromatography (column dimension 2.8×12.5 cm) in TBS supplemented with 0.025% sodium azide and 1 mM phenylmethysulfonylfluoride). The column was extensively washed with TBS to remove non-binding fractions. Heparin-binding Fn-2 proteins were eluted with 10 mM PC in TBS. After extensive dialysis (MWCO 6,000–8,000) against TBS, the heparin-binding protein fraction were subjected to affinity chromatography on PC-agarose (column dimension 0.5 × 1.5 cm; Pierce, Rockford, IL). Contaminating weakly lipid-binding proteins were released by washing

the column with 10 mM PC in TBS, whereas the desired proteins were eluted with 100 mM PC in TBS and dialyzed against water before further analysis. Protein purity was tested by SDS–polyacrylamide electrophoresis.

Fn-2 protein were separated by reverse-phase HPLC on Eurosil 300 C18 (Knauer, Berlin, Germany) column (250×2 mm, 5 μm particle size) as described (Calvete et al., 1995a). Proteins were detected at 220 nm and collected for further analysis.

Alternatively, CHAPS-extracts of the caput epididymis were dialyzed against TBS and subjected to PC-agarose affinity chromatography as described above. The flow through and the fraction eluted with 100 mM PC were tested by SDS–PAGE and Western blotting for the occurrence of Fn-2 proteins as described below.

MALDI-ToF Mass Spectrometry Analysis

HPLC purified proteins were analyzed by mass spectrometry using a MALDI-TOF (Kratos Analytical V5.2, Manchester, UK) before and after chemical desialylation. Proteins were co-crystallized with α-cyano-4-hydroxy cinnamomic acid on the target according to Kussmann et al. (1997).

Edman Degradation

N-terminal amino acid sequencing was performed using a Procise 492 protein sequencer (Applied Biosystems, Darmstadt, Germany) according to Hunkapiller et al. (1983).

Antibody Production

A polyclonal antibody was generated by immunizing a pathogen free cockerel with the purified SP-1 protein. The antigen (200 μg/1.5 ml) was emulsified with Freund's complete Adjuvant (Difco, Detroit, MI) and injected into several subcutaneous sites. A second immunization (200 μg/1.5 ml) was performed 4 weeks later. Blood was collected approximately 14 days after the second immunization. Preimmune serum was collected about 3 weeks before the first immunization. The antibody was designated as anti-SP-1. Specificity and cross reactivity was tested by SDS–PAGE and Western blots (see below).

Electrophoretic and Western Blot Analysis

Seminal plasma fractions, tissue extracts and sperm extracts (50×10^6 cells/ml) were diluted in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% Brom Phenol Blue, 10% glycerol), heated for 5 min at 95°C. Each sample (~10 μg/well) were subjected to gel electrophoresis on a 15 or 18% polyacrylamide gel according to Laemmli (1970). For immunological analysis, gels were blotted onto PVDF membranes and sequentially probed with anti-SP-1 (1:3,000 v/v) and anti-chicken IgY-alkaline phosphatase (1:5,000 v/v; Dianova, Hamburg, Germany) in blocking buffer (Roche). Washing steps were performed with TBS, 0.1% Tween as described recently (Ekhlasi-Hundrieser et al., 2002). The reaction was developed for 15–30 min in NBT/BCIP-solution (Roche) according to the manufacturer's instruction. Specificity of the antibody reaction was tested by preabsorption of the specific antibody with purified SP-1 protein (about 100–500 μg/ml anti-SP-1, 15 min at room temperature) before usage. Alternatively, the protein blots were probed with the polyclonal anti-peptide antibody for Ce-12 (amino acid 103–122; Saalmann et al., 2001) in guinea pig (1:1,000 v/v) as described by Schäfer et al. (2003), followed by detection with anti-guinea pig-alkaline phosphatase (1:5,000 v/v: Dianova).

Immunohistochemistry

Tissue samples were fixed in 4% formaldehyde for 12 hr and embedded in paraplast plus (Shandon, Frankfurt, Germany). Sections of 3–4 μm were deparaffinized using standard protocols and blocked for endogenous peroxidases as recently described (Ekhlasi-Hundrieser et al., 2002). Immunoreaction was performed by sequential treatment of the slides with avian anti-SP-1 antibody (1:3,000 v/v in PBS, 60 min), biotinylated anti-chicken-IgY anibody (1:200 v/v in PBS, 60 min; Vector-Alexis, Grünberg, Germany), and streptavidin–peroxidase (1:150 v/v in PBS; 60 min; Dianova). Sections were stained for 10 min with 3,3′-diaminobenzidine following the FAST DAB protocol, counterstained with Mayers's haematoxylin solution (15 sec), dehydrated and mounted in DePeX. Controls were performed by: (1) omission of the specific antibody, (2) replacement of the specific antibody with heat-inactivated preimmune serum (1:1,000 in PBS), (3) preabsorption of the specific antibody with SP-1 (about 100–500 μg/ml serum overnight).

Immunofluorescence Microscopy

Smears of washed sperm were air dried and blocked by incubation in PBS containing 5% BSA overnight at 4°C. Immunoreaction was performed by sequential incubation with anti-SP-1 (1:3,000), biotinylated anti-chicken IgY (1:500), and streptavidin-FITC (4 μg/ml; Boehringer). All incubation steps were done in PBS containing 0.5% BSA for 60 min at room temperature in a humid chamber. Between each incubation a washing step (3×5 min in PBS, 0.5% BSA) was performed. Smears were rinsed in water, mounted in PBS–glycerin (1:9) and observed under a Zeiss Axioscope microscope equipped with epifluoresence. Controls were the same as for the immunohistochemistry.

RESULTS

Cloning and Sequence Analysis of SP-Related cDNAs

Degenerate oligonucleotide primers (see Materials and Methods) were designed based on the published SP-1 peptide sequence (Calvete et al., 1995a; accession no. P81121) and employed in an initial RT-PCR analysis of equine ampulla cDNA. Subcloning and sequencing of amplicons revealed a whole family of closely related nucleic acid sequences, their deduced amino acid sequences showing significant similarity to SP-1 and SP-2 (Calvete et al., 1995a). By completion of the partial

cDNAs (see Materials and Methods) it became obvious that SP-1 (accession no. AJ582012) represented a clearly defined equine gene product. All nucleic acid sequences in this contig were collinear, predicting an amino acid sequence congruent with the published SP-1 peptide sequence (Fig. 1a). Each of the four amino acid positions which appeared blank during peptide sequencing (compare Calvete et al., 1995a) were represented by threonine codons predicted to be subject of *O*-glycosylation (NetOGlyc, Hansen et al., 1998).

A second contig comprised a heterogeneous group of closely related but highly variable SP-like cDNA sequences, designated as SPnew (variant 1, accession no. AJ582013). The predicted amino acid sequences showed a greater similarity to SP-2 than to SP-1 (Fig. 1b; compare Calvete et al., 1995a). Among this group of heterogeneous cDNAs, two novel sequences were identified most probably representing mRNA splice variants originating from a distinct gene. These variants predicted two SP-2-like isoforms of different lengths, one of them with a prolonged C-terminus (variant 2, accession no. AJ562014). Additionally, partial cDNA sequences were obtained predicting yet different SP-related, novel equine gene products (data not shown).

Isolation and Characterization of SP-1 and SP-2 Isoforms

In order to identify Fn-2 isoforms, SP-1 and SP-2 proteins were purified from the heparin-binding pool of seminal plasma by PC-agarose-affinity chromatography eluting from the column at 100 mM PC. Reverse-phase HPLC led to the resolution of four protein fractions, designated P1–P4 (Fig. 2). Purity of the protein fractions were tested by SDS–PAGE (Fig. 3, inset a). P1 and P2 exhibited molecular masses approximately at 18 kDa, whereas P4 appeared as a band at 14 kDa. The minor peak P3 seems to be a mixture of proteins and were therefore not further analyzed. Peaks eluting from

a

```
                                                    cagcaag gcaagctgcc  17
atg gca cca cgt ttg gga atc ttt ctg att tgg gct ggc gct tgt  62
 M   A   P   R   L   G   I   F   L   I   W   A   G   A   C
-25                                                     -11
atc ttt ctc caa ctg gac cat gtg gat gga gat ctg cag acg acg 107
 I   F   L   Q   L   D   H   V   D   G ⇓ D   L   Q   T   T
-10                                   -1  +1                +5
ggg gct gac cat tct gct aca gtg aac cca gat cag cag ctg att 152
 G   A   D   H   S   A   T   V   N   P   D   Q   Q   L   I
+6                                                      +20
atg acc aaa cat tct gct aca gtg aca cca gaa aat aaa tgt gtt 197
 M   T   K   H   S   A   T   V   T   P   E   N   K   C   V
+21                                                     +35
ttt cca ttc aac tat cga ggc tat cgg tat tac gat tgc act agg 242
 F   P   F   N   Y   R   G   Y   R   Y   Y   D   C   T   R
+36                                                     +50
act gat tcg ttt tac cgt tgg tgt tcc tta act ggc act tat tca 287
 T   D   S   F   Y   R   W   C   S   L   T   G   T   Y   S
+51                                                     +65
ggg agt tgg aaa tat tgt gct gcc aca gac tat gcc aaa tgt gcc 332
 G   S   W   K   Y   C   A   A   T   D   Y   A   K   C   A
+66                                                     +80
ttt ccc ttt gtc tac cgt ggc caa aca tat gac cgt tgc aca act 377
 F   P   F   V   Y   R   G   Q   T   Y   D   R   C   T   T
+81                                                     +95
gat gga agt ctt ttt cgc att tct tgg tgc tca gtc act cct aac 422
 D   G   S   L   F   R   I   S   W   C   S   V   T   P   N
+96                                                    +110
tat gat cat cat gga gct tgg aaa tat tgc tag taataaaaag tgca 469
 Y   D   H   H   G   A   W   K   Y   C   *   *   *
+111                                  +120
tctaac ccatggccat gaatgcaatg aagcctttga aatctgcgtg aagaaaat 523
ca agaaatctca agcataaagg tgatgctctg tgcaacagag gtgcaaaact t 576
tccctgaaa ctccactttg ttcccactgt gcctgaattc aatcaataaa ccact 630
ttctc tgc(a)n                                               638
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Fig. 1. a, **b**: Nucleotide and cDNA derived amino acid sequences of Fn-2 proteins. a: Nucleotide and amino acid sequences of SP-1 (GenBank accession number: AJ582012). The four *O*-glycosylation sites (Calvete et al., 1995a) are highlighted in gray. b: Nucleotide and amino acid sequences of SPnew (GenBank accession number: AJ582013, variant 1). The potential *N*-glycosylation site is highlighted in gray. Amino acids are shown as one-letter-code. The cleavage site between the signal peptides and the mature proteins is indicated (⇓), the AATAAA polyadenylation signal is underlined. An asterisk indicates the stop codon.

b

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atg gca ctg cag ttg gga gtc ttt ctg att tgg gct ggt gtt tgt  45
 M   A   L   Q   L   G   V   F   L   I   W   A   G   V   C
-25                                                     -11
atc ttt ctc caa ctg gac cat gtg gac gga gat cag cag cag att  90
 I   F   L   Q   L   D   H   V   D   G ⇓ D   Q   Q   Q   I
-10                                   -1  +1                +5
gta aat gac cat tca tct aca agg aaa cca gat aac aaa tgt gtc 135
 V   N   D   H   S   S   T   R   K   P   D   N   K   C   V
+6                                                      +20
ttc cca ttc aag tat caa ggc aga cag tac tac gat tgc act agg 180
 F   P   F   K   Y   Q   G   R   Q   Y   Y   D   C   T   R
+21                                                     +35
act gat tcc ttt cat cgt tgg tgt tcc tta act gaa aat tac tct 225
 T   D   S   F   H   R   W   C   S   L   T   E   N   Y   S
+36                                                     +50
ggg aaa tgg aga tat tgt gtt gct gag gac tac gcc aaa tgt ttc 270
 G   K   W   R   Y   C   V   A   E   D   Y   A   K   C   F
+51                                                     +65
ttc ccc ttt gtc tac cgt ggc cga aca tac cac act tgc aca act 315
 F   P   F   V   Y   R   G   R   T   Y   H   T   C   T   T
+66                                                     +80
gat ggg agt ttt ttt ctc att cct tgg tgc tca gtc act ccc aac 360
 D   G   S   F   F   L   I   P   W   C   S   V   T   P   N
+81                                                     +95
tat gac cgt gat gga ggt tgg aaa tat tgc atg tga  taaaagcaac 406
 Y   D   R   D   G   G   W   K   Y   C   M   *
+96                                    +106
accgtgttaa attgctctcc agtgcatcta acccatgacc ataaatgtga tcaa 460
gtcttt gaaacataac tgaagaaaat caagaaatct caagcatgaa ggtgatgt 513
tc tgtgcaacac gggtgcaaaa ctttccctga aactccactt tgttcccact g 566
tgcctgaaa gccaatcact agt                                   589
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Fig. 1. (*Continued*)

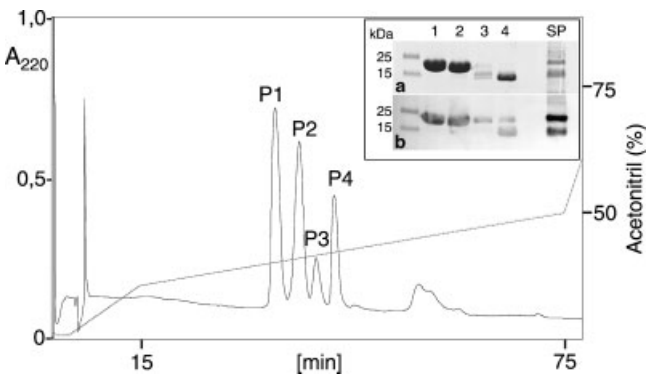

Fig. 2. Reverse-phase HPLC of the phosphorylcholine (PC)-binding proteins of equine seminal plasma. PC-binding proteins (~250 μg) were separated on a Eurosil 300 C18 column and eluted with 0.8 ml/min with a gradient of water (**A**) and 100% acetonitrile (**B**), both containing 0.1% trifluoroacetic acid, first isocratically (25% B) 5 min followed by gradient from 25 to 35% (B) for 15 min and then 35–50% (B) for 60 min (dashed line). Proteins were detected at 220 nm. Fractions were designated as P1–P4. Inset a: SDS–PAGE of the isolated fractions P1–P4 (~10 μg/lane) after staining with Coommassie Blue (**lanes 1–4**). Inset b: Western blot analysis of P1–P4 (**lanes 1–4**) probed with anti-SP-1 and anti-chicken IgY-alkaline phosphatase.

the column at 45–48% acetonitril concentration (Fig. 2) did not cross-react with anti-SP-1 and Ce-12 antipeptide antibody (not shown). The analysis of the profile of seminal Fn-2 proteins does not indicate the major occurrence of products of the *SPnew* gene.

MALDI-ToF mass spectrometry analysis of the proteins before and after chemical desialylation confirmed the acidic glycoprotein nature of the proteins (Table 1). P1 showed a broad peak with the centroid at m/z ~16,500 that split into m/z 15,370 and 15,660 (n = 3) after desialylation which is in excellent agreement with the calculated structure of the mature SP-1 representing a polypeptide chain of 13,905 Da with four sialylated O-linked Gal–GalNAc disaccharide chains (m/z 16,500) and the desialylated forms with no (m/z 15,370) or one *N*-acylneuraminic acid (m/z 15,660).

The broad signal of P2 at m/z ~15,900 changed into m/z 15,010 (n = 3) after chemical treatment pointing to the presence of three *N*-acylneuraminic acid residues. The average mass difference of 13,912 Da between the desialylated P2 and three Gal-GalNAc disaccharide chains corresponds to the length of the polypeptide chain of SP-1. The N-terminal sequence, DLQMT is homologous to SP-1 with the N-terminal sequence, DLQTX, whereby threonine is exchanged against methionine in position four and X in position five stands

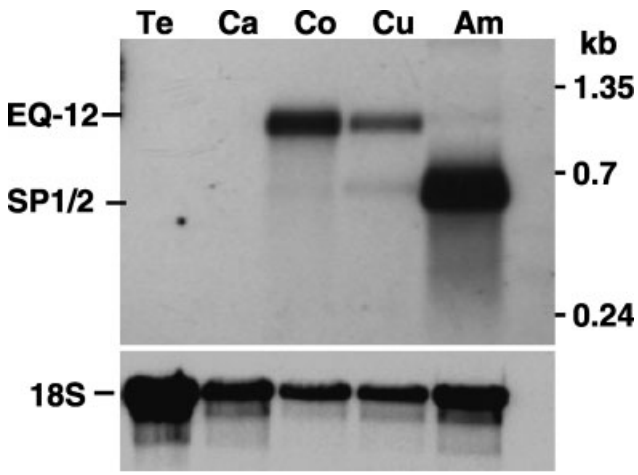

Fig. 3. Northern blot analysis of mRNAs encoding "long" and "short" Fn2-module proteins. Simultaneous hybridization with DIG-labeled EQ-12 and SP probes (**upper panel**) revealed differential expression in tissues of the equine male genital tract. (10 μg total RNA/lane from the different tissues) Te, testis; Ca, caput epididymis; Co, corpus epididymis; Cu, cauda epididymis; Am, ampulla of vas deferens. To control for RNA intactness and equal loading, the blot was subsequently hybridized with a DIG-labeled 18S RNA probe (**lower panel**).

for the first glycosylated threonine residue of SP-1 (Calvete et al., 1995a). The corresponding position in P2 is an unmodified threonine residue indicating that this threonine is not glycosylated. Altogether these data indicate that P2 might represent an isoforms of SP-1 carrying three *O*-glycan side chains.

The N-terminal sequence of P4, $D^{M}/_{K}QPI$ is homologous to the N-terminal sequence of the SP-2 protein, $D^{M}/_{Q}QPI$ (Calvete et al., 1995a). In position two methionine and lysine are present in equimolar amounts. The molecular masses of about *m/z* 13,820 for the untreated protein and *m/z* 13,290 (n = 3) after desialylation indicate the presence of two *N*-acylneuraminic acid residues pointing to the membership of P4 to the SP-2 family with two *O*-glycosylation sites in the single A-module (Calvete et al., 1995a).

Tissue Distribution of Fn-2 Type mRNAs in the Equine Male Genital Tract

Northern blot analysis was performed including total RNA extracts of equine male genital tract tissues, namely testis, three subsequent regions of the epididymis (caput, corpus, cauda), and the ampulla. Employing two different DIG-labeled SP probes (see Materials and Methods) a broad prominent band of approximately 0.7 kb was observed in RNA extracts of the ampulla (Fig. 3). A faint band was also observed in the cauda epididymis while the other samples were negative. In comparison, a DIG-labeled EQ-12 probe (Saalmann et al., 2001) employed on the same blot showed maximum hybridization with an approximately 1.2 kb mRNA in RNA extracts of corpus and cauda regions of the equine epididymis.

The expression pattern along the equine male reproductive tract was investigated by the more sensitive method of RT-PCR using specific primers. SP-1-specific amplicons were detected in all regions of the epididymis, seminal vesicle, and ampulla of vas deferens (Fig. 4a, upper panel). In each case, PCR products of the expected length of approximately 238 base pairs were obtained showing identical sequences. No PCR products, however, were generated in testis, rete testis, prostate, and glandula bulbourethralis as well as in liver which was included as a negative control (Fig. 4a).

Tissue Distribution of Fn-2 Proteins in the Equine Male Genital Tract

An antibody against the purified, homogenous SP-1 protein was raised in chicken and designated as anti-SP-1. Anti-SP-1 showed comparable immunoreactions with P1 and P2, whereas P4 showed less but distinct cross-reactivity when probed by Western blot analysis indicating that the anti-SP-1 recognizes both, SP-1, and SP-2 type proteins (Fig. 2, inset b). Preabsorption of anti-SP-1 with SP-1 protein completely abolished the signals on the membranes (not shown) indicating the specificity of the antibody.

The distribution of Fn-2 proteins along the male genital tract was studied by immunohistochemisty (Fig. 5a–f). Strong immunostaining was observed in the cytoplasm of the principal cells and at the apical epithelial surface along the entire epididymal duct and the ampulla of the vas deferens. The basal cells showed no staining reaction. Particularly prominent signals were seen in the long stereocilia lining the epithelium of caput and cauda regions (Fig. 5a,c). In the duct lumen of corpus and cauda regions, positive staining was asso-

TABLE 1. N-Terminal Amino Acid Sequences and MS Analysis of Fn-2 Proteins of Equine Seminal Plasma

	N-terminal amino acid sequence*	Molecular mass [m/z] native	Molecular mass [m/z] desialylated	Number of N-acylneuraminic acids (calculated)	Polypeptide backbone (calculated)**
P1	DLQTX	16500 Da	15370/15660 DA	4	13910 Da
P2	DLQMT	15900 Da	15010 Da	3	13912 Da
P4	$D^{M}/_{K}QPI$	13800 Da	13290 Da	2	12560 Da

N-terminal sequences as determined by Edman degradation and molecular masses as determined by MALDI-ToF analysis (n = 3) of HPLC-isolated Fn-2 proteins. *For calculation of the number of sialic acids an average mass of 300 Da and **for calculation of the polypeptide backbone the trisaccharide NeuNAc/Gc-GaI-GaINAc were underlain.

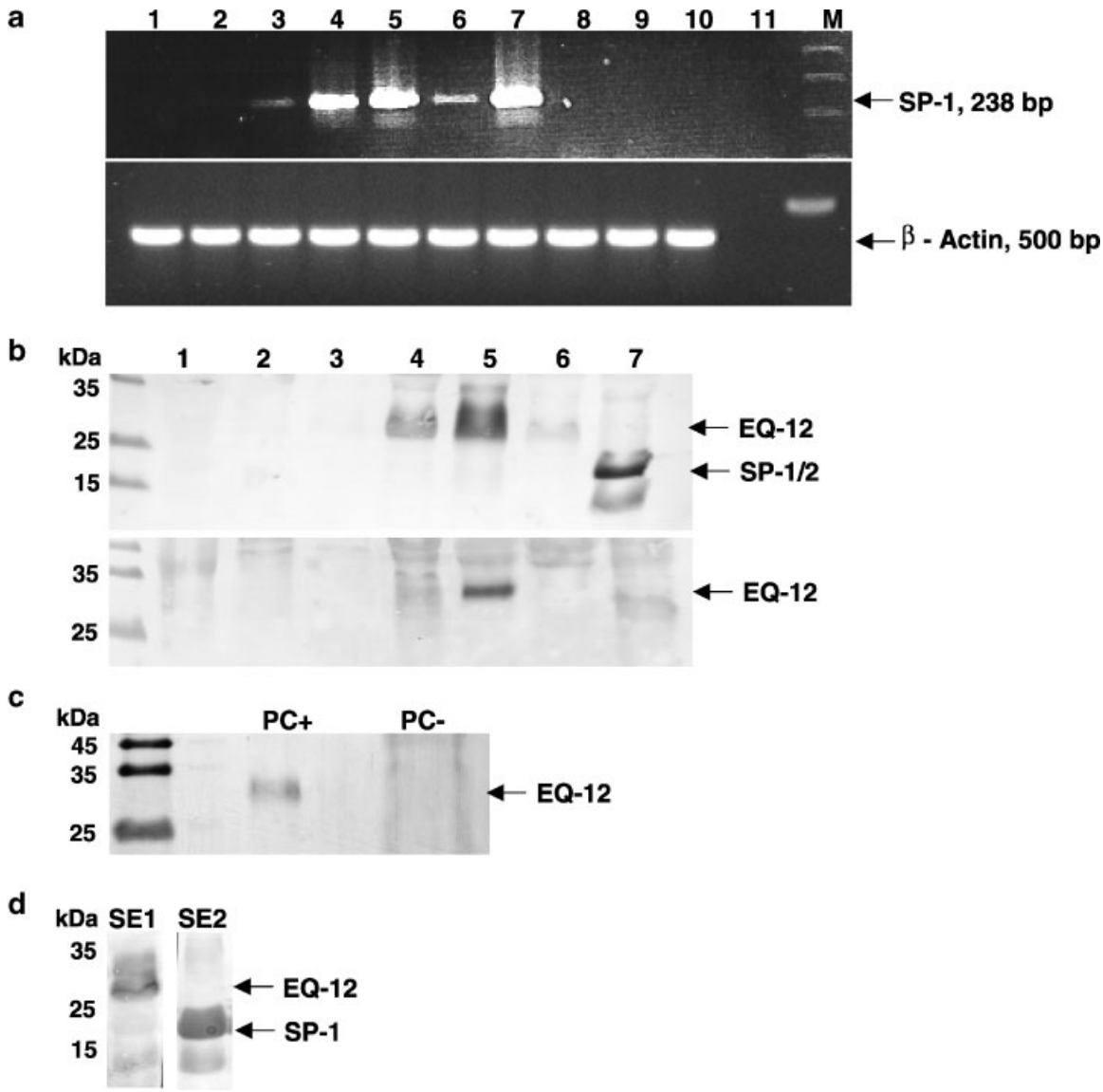

Fig. 4. a–d: RT-PCR analysis of SP-1 transcripts and Western blot analysis of Fn-2 proteins along the male genital tract and of sperm. **a**: Ethidium bromide stained gel showing the amplified transcripts of SP-1 (**upper panel**) and β-actin as control for equal loading (**lower panel**) from tissues of the equine male genital tract (10 μl PCR-reaction/lane as specified in Material and Methods): testis (**lane 1**), rete testis (**lane 2**), caput (**lane 3**), corpus (**lane 4**), cauda epididymis (**lane 5**), seminal vesicle (**lane 6**), ampulla of vas deferens (**lane 7**), prostata (**lane 8**), glandula bulbourethralis (**lane 9**), and liver (**lane 10**). In a control assay cDNA was omitted (**lane 11**), as length standard a 100 bp ladder (M) was used. **b**: Western blot analysis of the CHAPS extracts (10 μg protein/lane) of the corresponding tissue regions probed with anti-SP-1 (**upper panel**) and anti-Ce-12 (**lower panel**). Liver (**lane 1**), testis (**lane 2**), caput (**lane 3**), corpus (**lane 4**), cauda epididymis (**lane 5**), seminal vesicle (**lane 6**), ampulla of vas deferens (**lane 7**). **c**: Western blot analysis with anti-Ce-12 of the PC-binding fraction isolated from the CHAPS extract of cauda epididymis by PC-agarose affinity chromatography. **d**: Western blot analysis of the SDS-extract (SE) of washed ejaculated equine sperm probed with anti-SP-1 (**lane SE2**) and anti-Ce-12 (**lane SE1**).

ciated with the fluid and sperm present in the lumen (Fig. 5b,c). In the seminal vesicle epithelium only a few scattered areas were weakly positive (Fig. 5f). Control tissues like liver and muscles were completely negative as were the genital tract tissues after probing with the preadsorbed antibody (not shown).

Accompanying these studies, Western blots of TBS and CHAPS tissue extracts from the same organs were probed subsequently with two antibodies, one raised against the purified SP-1 protein and another raised against a 20 mer oligopeptide (amino acid 103–122) of CE-12, a novel epididymal Fn-2 type protein of approximately 30 kDa containing four Fn-2 modules (Saalmann et al., 2001). This peptide sequence is highly conserved in the homologous equine EQ-12 protein.

Using anti-SP-1, a strong signal of ~18 kDa and a weaker signal of ~14 kDa was observed in TBS and CHAPS protein extracts of the ampulla, the immunostained proteins most probably representing SP-1 and SP-2 (Fig. 4b, upper panel). In protein extracts of the

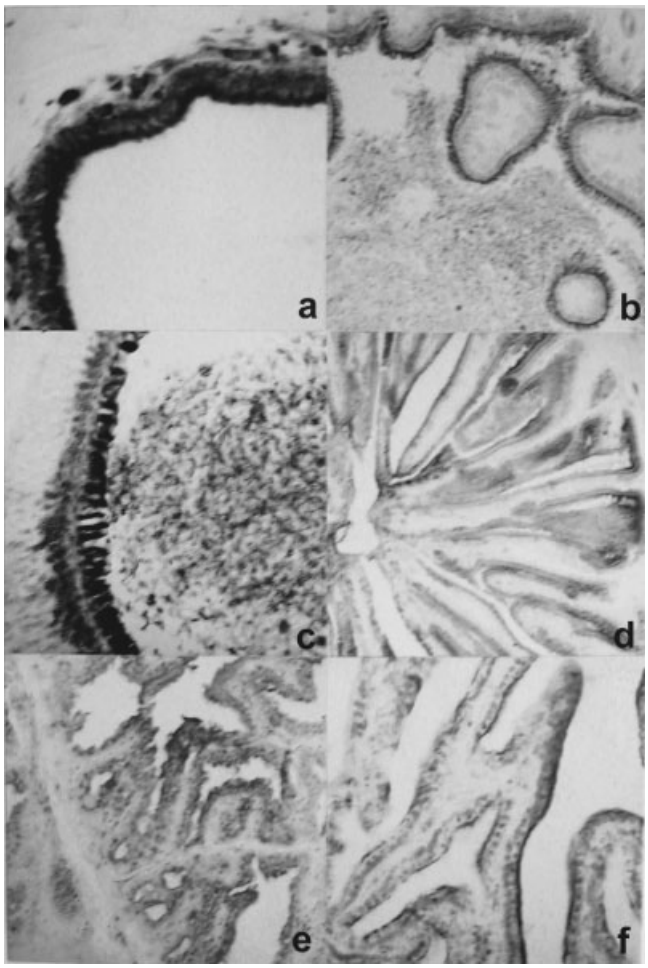

Fig. 5. a–f: Immunohistochemical analysis of Fn-2 protein expression along the equine male genital tract. Paraffin sections of tissues were reacted with anti-SP-1, visualized using the FAST DAB protocol and counterstained with haematoxylin. a: Caput, (b) corpus, (c) cauda epididymis, (d) vas deferens, (e) ampulla of vas deferens, (f) seminal vesicle.

epididymis, on the other hand, a signal was observed at ~30 kDa. Occasionally, a faint signal was seen at 18 kDa as well. The monospecific CE-12 antipeptide antibody, in comparison, cross-reacts solely with the 30 kDa protein band in the extracts of the epididymis (Fig. 4b. lower panel), most probably corresponding to EQ-12, the equine counterpart of the Fn-2 proteins containing four Fn-2 modules (Saalmann et al., 2001). These results also indicate that the antibody raised against purified SP-1 recognizes Fn-2 proteins of the SP-1 and SP-2 family but also reacts with the novel family having four Fn-2 modules (Saalmann et al., 2001). Anti-SP-1 is therefore a tool to detect generally equine Fn-2 proteins. No or only very weak signals were seen with both antibodies in the corpus region of the epididymis, vas deferens, and the accessory glands as seminal vesicles, prostate, and bulbourethral gland (Fig. 4b). Control tissue as liver was completely negative.

As an alternative approach, the PC-binding properties of EQ-12 were tested by isolating PC-binding proteins from CHAPS extracts of cauda epididymis using PC-affinity chromatography. After Western blotting with the Ce-12 antipeptide antibody a strong signal at ~30 kDa was detected in the 100 mM PC eluent (Fig. 4c, PC+); no signal was found in the flow through (Fig. 4c, PC−) indicating that EQ-12 exhibits PC-binding properties as described for the members of the SP-1 and SP-2 families. Western blot analysis of proteins extracted from ejaculated sperm using anti-SP-1 and anti-Ce-12 antibody revealed both types of Fn-2 proteins, EQ-12 (~30 kDa) and the small SP-1 and SP-2 proteins (~18 and 14 kDa) (Fig. 4d).

Uptake of Fn-2 Proteins by Sperm During Passage Through the Epididymis and at Ejaculation

Testicular sperm and sperm isolated form the caput region showed no immunoreaction with anti-SP-1 (Fig. 6a), whereas sperm from the corpus region of the epididymis showed a weak but distinct immunofluorescence pattern for Fn-2 proteins at the post-acrosome and midpiece (Fig. 6b). The labeling was enhanced in sperm isolated from the cauda epididymis (Fig. 6c). The fluorescence pattern did not change substantially from caudal to ejaculated sperm, except that the fluorescence becomes more prominent (Fig. 6d) indicating an increased number of molecules bound to the sperm surface.

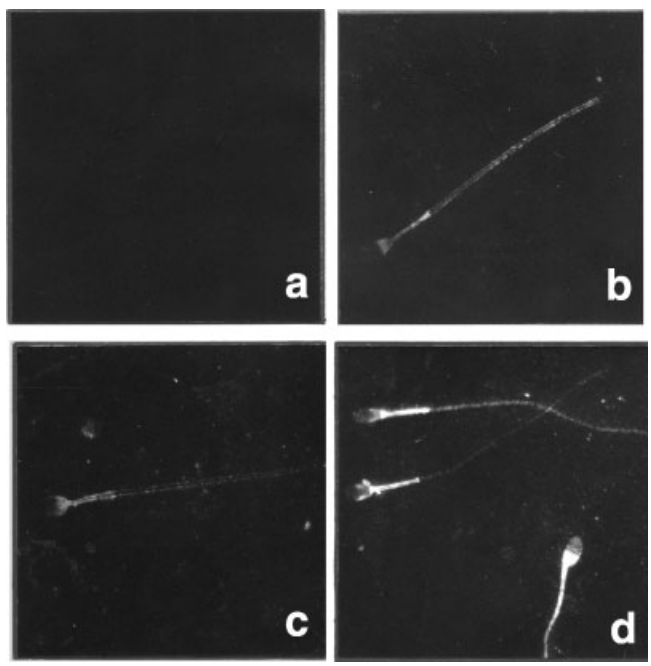

Fig. 6. a–d: Immunofluorescence images of sperm isolated from different regions of the male genital tract. Ejaculated sperm and sperm flushed from the epididymal duct were reacted with anti-SP-1. Immunoreaction was visualized by reacting with anti-chicken-FITC. a: Caput epididymis. No sperm showed fluorescent labeling. b: Corpus epididymis. Increasing numbers of sperm showed labeling at the post-acrosome and midpiece. Most sperm (~80%) from the cauda epididymis (c) and ejaculated sperm (d) showed fluorescent labeling at the same regions.

DISCUSSION

Tissue Expression and Structure of Equine Fn-2 Proteins

Fn-2 module proteins of the male genital organs were characterized in the stallion, our results corroborating previous suggestions that these proteins are ubiquitously expressed in the mammalian male genital tract. At least three different proteins were identified, named SP-1, SP-2, and EQ-12 (Saalmann et al., 2001). They differ in their numbers of Fn-2 modules (B-modules) as well as in their N-terminal extensions (A-modules) and are produced in subsequent post-testicular regions, ranging from the epididymis to the ampulla of the vas deferens. The vast majority of the smaller SP-1 and SP-2 proteins with only two Fn-2 modules are produced by the ampulla. Inter-species comparisons showed only 50–60% sequence identity making it difficult to define species orthologues of the smaller Fn-2 proteins. The larger proteins, in comparison, which are represented by EQ-12 in the stallion showed a much higher degree of sequence conservation (>80% inter-species similarity). They are produced in the more proximal parts of the genital tract, mainly the epididymis.

In the stallion as in the other mammals investigated, Fn-2 proteins occur as multiple forms encoded by several closely related genes. Moreover, each single gene may give rise to multiples protein products representing iso- or glycoforms generated by alternative mRNA splicing and/or differential glycosylation. The SP-1 protein sequence (=HSP-1; Calvete et al., 1995a) is identical to that predicted from the cDNA sequence (this paper).

SP-2 (=HSP-2), on the other hand, appeared to represent a mixture of at least two closely related proteins (Calvete et al., 1995a). The predicted N-terminus was similar to the partially known SP-2 (=HSP-2) protein (Calvete et al., 1995a), suggesting that we have indeed cloned one representative of this heterogeneous protein family. The minor sequence differences between the proteins predicted by the SPnew cDNA family and the SP-2 protein (Calvete et al., 1995a) may be explained by genotype differences between different horse strains, but could also result from the cloning of a different, but closely related family member of SP-2 type protein-encoding genes.

Comparison of the amino acid sequence of SPnew and SP-1 revealed that the BB′-region containing the two Fn-2 modules are 66% identical whereas the A-domains showed a sequence identity of only about 50%. Thr27 of SP-1 corresponding to Thr12 of the SP-2, both being *O*-glycosylated in the native proteins (Calvete et al., 1995a), is conserved in SPnew whereas the likewise *O*-glycosylated Thr22 and Thr7 in SP-1 and SP-2, respectively is exchanged against an asparagine residue in SPnew (Fig. 7).

Mass analysis of the native and desialylated SP-1 proteins are in agreement with a AA′BB′ module pattern carrying four (P1) and three (P2) *O*-glycan whereas P4 belongs to the SP-2 family showing an ABB′-module pattern (Fig. 7). The 26-residue (Calvete et al., 1995a) and the short five-residue (P4, this work) N-terminal amino acid sequences document that SP-2 represents a bundle of isoforms and particularly the amino acid sequences of the A-domain vary considerably between species and protein isoforms (Fig. 7). Since these A-domains are so diverse, they may be just a scaffold for the presentation of the carbohydrate side chains showing unfolded and extended structures. The peptide backbone of another highly glycosylated sperm surface antigen, HE5/CD52, is also poorly conserved between species (Kirchhoff and Hale, 1996).

Our results document on the mRNA as well as on the protein level that the Fn-2 proteins are expressed in subsequent regions of the male genital tract. As already

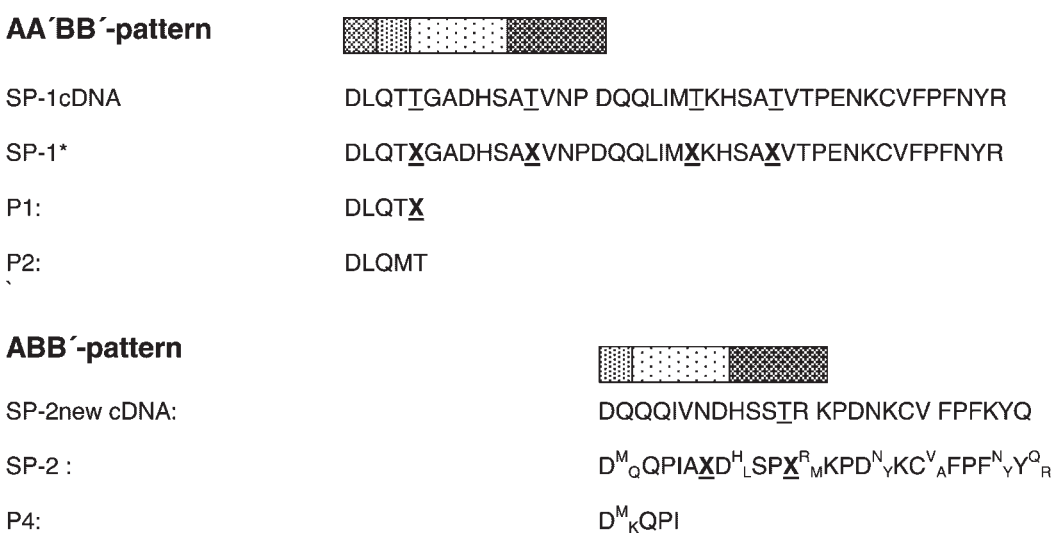

Fig. 7. Alignment of the N-terminal amino acid sequences of SP-1 and SP-2 isoforms. N-terminal sequences were determined by Edman degradation or derived from the cDNA sequences of SP-1 and SPnew (this work). *SP-1 sequence according to Calvete et al. (1995a).

described for the canine and human counterparts of EQ-12 (Saalmann et al., 2001, Schäfer et al., 2003), the larger proteins are produced in the epididymis, specifically in the corpus and cauda regions. The smaller Fn-2 proteins are produced more distally, largely by the ampulla of the vas deferens, thereby accounting for the vast majority of Fn-2 proteins in the equine seminal plasma. In the stallion, sperm are stored in the ampulla of the vas deferens. The higher secretory activity of this organ compared to the seminal vesicles may be related to this sperm storage function.

Still, a small but distinct fraction of SP-1 transcripts was observed in the epididymis, suggesting that small amounts of SP-1 proteins may be secreted already in the cauda epididymidis and may bind to the sperm surface prior to ejaculation.

The antibody raised against purified SP-1 appeared to recognize SP-1/2 proteins as well as EQ-12 and may react with all different types of equine Fn-2 proteins. The prominent epithelial immunostaining in the caput epididymis (Fig. 5a), however, is not reflected by the distribution of the encoding transcripts. It thus remains to be investigated whether still unknown Fn-2 proteins other than SP-1 and EQ-12 may be expressed in this part of the epididymis. The staining of sperm in the lumen of the epididymal duct was confirmed by indirect immunofluorescence of sperm isolated from subsequent regions of the duct. Sperm-binding of Fn-2 proteins particularly at the post-acrosomal and midpiece region was at first detected in the corpus epididymidis. The post-testicular uptake of Fn-2 proteins with four and two Fn-2 modules was corroborated by Western blot analysis of ejaculated sperm. Binding of EQ-12 to the sperm surface during epididymal transit would explain the occurrence of the prominent band at 30 kDa in the extract of ejaculated sperm.

The putative functions of Fn-2 proteins have to be considered with regard to their survival on the sperm surface during their journey through the female genital tract to the oviduct. Preliminary insemination studies in horse and immunoflorescence microscopical survey of sperm collected from the female tract provide evidence that Fn-2 proteins remain associated to the sperm surface during the passage through the female genital tract (Hess, 1998) and may play a role during the initial events occurring in the oviduct.

Structure–Function Relationships of Equine Fn-2 Proteins

The phospholipid-combining sites (Moreau et al., 1999) and the basic amino acids involved in heparin-binding of the bovine Fn-2 protein, PDC-109; Calvete et al., 1999) have been mapped to the BB′-region of the molecule. The crystal structure of the PDC-109–PC complex gives detailed insight into the topology of secondary structure elements and provides the structural basis for understanding the biological role of seminal Fn-2 proteins (Wah et al., 2002). The (1) Fn-2 and the (2) Fn-2 modules are clustered in that way that the choline binding sites are placed at the same face of the molecule. The disulfide bonds between cysteines 1–3 and 2–4 help to stabilize the interacting surfaces of the two domains (Wah et al., 2002). This conserved pattern of disulfide bonds has been shown in the bovine (PDC-109/BSP-30), equine (SP-1) and porcine (pB1) seminal proteins (Calvete et al., 1995a, 1996, 1997). The Fn-2 domains of PDC-109 bind phospholipids with a PC head group, for example, phosphatidylcholine and sphingomyelin. This specific interaction is realized via a cation–π interaction between the quaternary ammonium group of PC and certain tryptophan residues supported by hydrogen bonding between the phosphate of the lipid and the hydroxyls of tryrosine residues lining the binding pocket. The disulfide bridges and the key residues involved in PC-binding of PDC109, Tryp47, Tyr30, and Tyr54 of the (1) Fn-2 module and Trp108, Tyr 75, and Tyr100 of the (2) Fn-2 module of monomer A as well as Trp93 and Tyr108 of monomer B, respectively (Wah et al., 2002) are conserved in the equine proteins, SP-1 and SPnew (Fig. 8). Due to these structural features, a similar architecture of the phospholipid-binding pocket can be assumed for the seminal Fn-2 proteins of the BB′-type explaining also the observed phoshorylcholine-binding activity of the native SP-1/SP-2 mixture (Calvete et al., 1995a; Greube et al., 2004, and this study). Very recent data argue for a less intensive interaction of the equine proteins with membranes compared with bovine PDC-109 which may reflect species-specific structural differences giving the basis for species-selective influences of Fn-2 proteins in the course of germ cell genesis (Greube et al., 2004).

Although the three-dimensional organization of Fn-2 proteins with four modules [(1) Fn-2 (2) Fn-2 (3) Fn-2 (4) Fn-2] is not yet known, the Fn-2 modules (1) Fn-2 and (2) Fn-2 of EQ-12 have the invariant amino acids which are responsible for the phospholipid-binding activity (Fig. 8). That EQ-12 can be isolated from epididymal tissue extracts by PC-affinity chromatography and is detected on the sperm surface. Additionally argues for a binding of this proteins to lipids having a PC head group.

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          (1)Fn-2 domain
PDC-109:  CVFPFVYRNRKHFDCTVHGSLFPWCSLDADYVGR-WKYCAQRDYAK
SP-1:     CVFPFNYRGYRYYDCTRTDSFYRWCSLTGTYSGS-WKYCAATDYAK
SPnew:    CVFPFKYQGRQYYDCTRTDSFHRWCSLTENYSGK-WRYCWAEDYAK
EQ-12           GSSYFSCTKTNSFFPWCATRAIYDRQ-WKNCLTEDYPR

          (2)Fn-2 domain
PDC-109   CVFPFIYGGKKYETCTKIGSMWM-SWCSLSPNYDKDRAWKYC
SP-1      CAFPFVYRGQTYDRCTTDGSLFRISWCSVTPNYDHHGAWKYC
SPnew     CFFPFVYRGRTYHTCTTDGSFFLIPWCSVTPNYDRDGGWKYC
Eq-12     CIFPFIYRGKSHDNCITEGSFFGKLWCSVTSSFDEKQQWKYC
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Fig. 8. Alignment of the amino acid sequences of the (1) Fn-2 domain and (2) Fn-2 domain of Fn-2 proteins. Amino acid sequences according to Manjunath and Sairam (1987) (PDC-109), this work (SP-1, SPnew) and Saalmann et al. (2001) (EQ-12). According to Wah et al. (2002) the conserved key amino acids involved PC binding (see also text) are labeled with bold characters. The α-helical stretches in PDC-109 are underlined.

From that we surmise that EQ-12 has a comparable architecture of at least the first two modules like the Fn-2 proteins with two modules and belongs to the lipid-binding proteins of the equine male genital tract.

In conclusion, the cDNA cloning and protein chemical data accumulate evidence that SP-proteins of the ABB′-type originate most probably from a SP-2 gene family whereas the proteins of the AA′BB′-type are derived from one single *SP-1* gene. Fn-2 proteins with four and two Fn-2 modules are differentially expressed along the male genital tract and bind to the sperm surface during post-testicular maturation via their PC-binding sites. Structural differences between these proteins may account for distinct properties and hence for their selective roles on sperm function during fertilization.

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