Interaction of Fibronectin Type II Proteins with Membranes: The Stallion Seminal Plasma Protein SP-1/2[†]

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Received September 12, 2003; Revised Manuscript Received November 17, 2003

ABSTRACT: Seminal plasma of mammalians contains, among others, proteins that are characterized by the fibronectin (Fn) type II module. Our knowledge about the structure and the physiological function of seminal Fn type II proteins mainly originates from studies on PDC-109, the bovine representative of this protein family. The present work focuses on the equine protein SP-1/2 (also named HSP-1/2) with particular emphasis on its interaction with lipid membranes by employing the intrinsic protein fluorescence and a number of spin-labeled and fluorescent lipid analogues. The results indicate that the interaction of SP-1/2 with (lipid) membranes is similar to that of PDC-109 which can be explained by homologous amino acid sequences of both proteins. Like PDC-109, SP-1/2 has a specificity for phospholipids with the phosphocholine headgroup. Upon binding to lipid vesicles, the protein intercalates into the hydrophobic membrane core, resulting in a rigidification of the lipid phase and, at higher concentration, in a perturbation of membrane structure. However, compared with PDC-109, the impact of SP-1/2 on membranes is less intense in that the degree of protein-mediated immobilization of lipids was lower. Furthermore, different to PDC-109, SP-1/2 was not able to extract lipids from human red blood cells. The data are discussed with regard to similarities and species-specific differences of the function of seminal Fn type II proteins in the genesis of sperm cells.

During passage through the male genital tract and upon ejaculation mammalian sperm interact sequentially with the secretory components of the tissues that altogether form the seminal plasma, the fluid portion of the semen. Seminal plasma contains a number of proteins that modulate the genesis of sperm cells during their subsequent transit through the female genital tract (*1*). Some of these proteins have been suggested to be involved in the processes of capacitation, acrosome reaction, and fusion of the sperm cell with the oocyte (*2*). Due to structural characteristics of seminal plasma proteins, various protein families have been classified such as CRISP proteins, spermadhesins, and Fn type II proteins (*3*−*6*). Differences between species in the relative abundance of these proteins and in the expression pattern along the male genital tract may contribute to the species-specific physiology of mammalian spermatozoa.

Fn type II proteins are characterized by the fibronectin type II module (*7*). The most prominent representative of this protein family in seminal plasma is PDC-109 (also called BSP-A1/A2 or "major protein"), which is present in a large quantity in bovine seminal plasma (*8*, *9*). Proteins of this family have also been found in the horse (HSP-1/2 or SP-1/2), pig (pB1), dog, and human (*10*−*12*). So far, our knowledge about seminal Fn type II proteins is essentially obtained from studies on PDC-109. This protein interacts with the bovine sperm cell by binding to its plasma membrane via phospholipids bearing a phosphocholine headgroup, such as phosphatidylcholine (PC)[1] and sphingomyelin (SM) (*13*, *14*). A cluster of hydrophobic, surface-exposed amino acids seems to be responsible for this binding of the protein to membrane lipids (*15*, *16*). Upon interacting

[†] This work was supported by grants from the Deutsche Forschungsgemeinschaft to P.M. (Mu 1017/2) and to E.T.-P. (To 114/4).

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[1] Abbreviations: C5-SL-PC, 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-glycero-3-phosphocholine; C10-SL-PC, 1-palmitoyl-2-(10-doxylstearoyl)-*sn*-glycero-3-phosphocholine; C16-SL-PC, 1-palmitoyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphocholine; ESR, electron spin resonance spectroscopy; FRET, fluorescence resonance energy transfer; HBS, HEPES-buffered saline; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; lc-SL-PL, long-chain spin-labeled phospholipid(s); L/P, lipid/protein molar ratio; LUV, large unilamellar vesicle(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid(s); PS, phospatidylserine; pyrenePC, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; pyrenePE, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine; pyrenePL, pyrene-labeled phospholipid(s); RBC, human red blood cells; sc-SL-PC, 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine; sc-SL-PE, 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoethanolamine; sc-SL-PL, short-chain spin-labeled phospholipid(s); sc-SL-PS, 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoserine; SL-A, spin-labeled androstane (3-doxyl-17β-hydroxy-5α-androstane); SL-C, spin-labeled cholestane (3β-doxyl-5α-cholestane); SL-Chol, spin-labeled cholesterol (25-doxylcholesterol); SL-SM, *N*-(4-doxylpentanoyl)-*trans*-sphingosyl-1-phosphocholine; SM, sphingomyelin.

10.1021/bi035647l CCC: $27.50
Published on Web 12/20/2003

with membranes PDC-109 affects the membrane structure in that the mobility of lipids is decreased (*17*, *18*) and the phase transition of pure lipid membranes is changed (*19*, *20*). At higher concentrations, the protein causes a permeabilization of membranes (*19*). Evidence for a physiological role of PDC-109 was given by studies showing that epididymal sperm cells, which are difficult to capacitate, can be stimulated to undergo this process more rapidly by preincubation of the cells with PDC-109 (*21*). The influence of PDC-109 on sperm cell capacitation could be explained by its ability to modulate membrane stability. It has been shown that the protein mediates an efflux of lipids, selectively PC and cholesterol, from the plasma membrane of cells (*22*, *23*). The efflux of cholesterol from the sperm cell membrane is a crucial process for the capacitation of spermatozoa and is believed to be triggered by the foregoing hydrolysis of cholesterol sulfate, a prominent cholesterol species in the membrane overlaying the acrosome (*24*−*27*).

For other mammalian species such a detailed knowledge on the interaction of respective seminal Fn type II proteins with membranes is lacking. Of particular interest is whether the pattern of membrane interaction as found for the bovine protein is preserved in other species. To answer these questions, we have focused in the present study on the interaction of equine Fn type II proteins SP-1 and SP-2 (formerly named HSP-1 and HSP-2) with membranes. Horse seminal plasma contains eight different fractions of proteins (*28*) from which the homologous SP-1 and SP-2 represent the major fraction (70−80% of the total protein content). Like PDC-109, the equine proteins consist of two Fn type II domains. SP-1 and SP-2 differ from each other by the degree of glycosylation and by the number of an N-terminal peptide sequence which is present either as single (SP-2) or as double copy (SP-1) (*10*). So far, the separate isolation of both proteins under nondenaturating conditions has failed. Here, we have characterized the interaction of SP-1/2 with lipid membranes by applying various spectroscopic methods that have been previously used for the analysis of the bovine protein PDC-109 (*17*, *29*). The data obtained for the equine protein are compared with those of the bovine protein in order to identify similarities and species-specific differences in the impact of these proteins on membranes.

EXPERIMENTAL PROCEDURES

Chemicals. PC, SM, phosphatidylethanolamine (PE), each from egg, and phosphatidylserine (PS) from brain as well as the spin-labeled steroids 3-doxyl-17β-hydroxy-5α-androstane (SL-A) and 3β-doxyl-5α-cholestane (SL-C) were purchased from Sigma (Deisenhofen, Germany). Short-chain spin-labeled phospholipids (sc-SL-PL) 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphocholine (sc-SL-PC), *N*-(4-doxylpentanoyl)-*trans*-sphingosyl-1-phosphocholine (sc-SL-SM), 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoethanolamine (sc-SL-PE), and 1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoserine (sc-SL-PS) were prepared as described previously (*30*). 25-Doxylcholesterol (SL-Chol) was synthesized according to the protocol of Maurin et al. (*31*). Long-chain spin-labeled phospholipids (lc-SL-PL) 1-palmitoyl-2-(5-doxylstearoyl)-*sn*-glycero-3-phosphocholine (C5-SL-PC), 1-palmitoyl-2-(10-doxylstearoyl)-*sn*-glycero-3-phosphocholine (C10-SL-PC), and 1-palmitoyl-2-(16-doxylstearoyl)-*sn*-glycero-3-phosphocholine (C16-SL-PC) were purchased from Avanti Polar Lipids (Birmingham, AL), and pyrene-labeled phospholipids (pyrenePL) 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (pyrenePC) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine (pyrenePE) were from Molecular Probes (Leiden, The Netherlands).

All other chemicals were obtained from Sigma (Deisenhofen, Germany) at the highest quality available.

All experiments were performed in HEPES-buffered saline (HBS; 150 mM NaCl, 5 mM HEPES, pH 7.4) at 37 °C unless stated otherwise.

Isolation of SP-1/2. SP-1 and SP-2 were isolated from the heparin-binding fraction of horse seminal plasma by affinity chromatography as described previously (*11*). Purity of the proteins was verified by polyacrylamide gel electrophoresis and HPLC (*10*, *11*). For the measurements a 2.5 mM stock solution of SP-1/2 in HBS was prepared.

Preparation of Large Unilamellar Vesicles (LUV). Unlabeled and labeled LUV were prepared using the extrusion technique as described previously (*17*). LUV were labeled either symmetrically, i.e., the analogues were present on both membrane leaflets, or asymmetrically, i.e., the analogues were solely on the outer membrane leaflet.

Fluorescence Measurements. Spectra were recorded using an Aminco Bowman spectrometer, series 2 (Rochester, NY). Measurement of the intrinsic fluorescence of SP-1/2 (2.5 μM) in the absence and in the presence of LUV was performed as described for PDC-109 (*29*).

For measuring fluorescence resonance energy transfer (FRET), PC LUV were symmetrically labeled with pyrenePC or pyrenePE at 1 mol %. Fluorescence spectra of labeled LUV (final lipid concentration 20 μM) between 300 and 500 nm were recorded in the absence and in the presence of SP-1/2 (lipid/protein molar ratio, L/P = 10) with an excitation wavelength of 280 nm and slit width of 4 nm for excitation and emission. Since, even at this excitation wavelength, we observed some pyrene fluorescence for labeled LUV in the absence of protein, i.e., without FRET, we subtracted this spectrum from that measured in the presence of protein to extract the component solely caused by FRET (see Results). For comparison, we also recorded the fluorescence spectra of protein without vesicles and with unlabeled PC LUV.

ESR Measurements. ESR measurements were performed as described for PDC-109 (*17*, *18*). Briefly, LUV symmetrically labeled with phospholipid analogues (lc-SL-PL, sc-SL-PL) or steroid analogues (SL-A, SL-C, SL-Chol) or asymmetrically labeled with sc-SL-PL were mixed with SP-1/2 and incubated for 2 min at 25 °C (lc-SL-PL) or on ice (sc-SL-PL, SL-A, SL-C, SL-Chol). ESR spectra in the absence and in the presence of the protein (L/P = 5 or 10) were recorded at 25 °C (for lc-SL-PL, if not stated otherwise) or at 4 °C (for sc-SL-PL, SL-A, SL-C, and SL-Chol) using a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany) with the following parameters: modulation amplitude, 2.5 G; power, 20 mW; scan width, 100 G; accumulation, eight times. To better resolve the protein-mediated immobilization, all spectra shown in the Results section were recorded at L/P = 5. Extraction of the immobilized component from the ESR spectra and quantification of the extent of immobilization were performed as described previously (*17*). For a better presentation, spectra of the immobilized

component shown in the figures were enlarged by a factor of 3−6.

Ascorbate Assay. The reduction of the spin-labeled moiety by ascorbate was measured at 4 and 37 °C as described in ref *17*.

Measurement of Lipid Extraction. Citrate-stabilized blood samples of healthy human donors were purchased from the local blood bank (Berlin, Germany). Red blood cells (RBC) were washed three times in HBS (once at 500*g* and twice at 2000*g* for 10 min at 4 °C). Washed cells (30% volume concentration) were incubated with and without SP-1/2 or PDC-109 (L/P = 14) for 30 min at 37 °C. Subsequently, RBC were centrifuged (2000*g*, 10 min) at 4 °C, and the supernatant was withdrawn for isolating the lipids according to Bligh and Dyer (*32*). Phospholipid content was quantified by measuring the phospholipid phosphorus after destruction of lipids in an aliquot with 70% perchloric acid for 30 min at 180 °C (*33*). From another aliquot the cholesterol content was determined by using the Boehringer kit (Mannheim, Germany). Lipid contents measured in the supernatant were related to the amount of phospholipid and cholesterol of RBC not incubated with protein.

RESULTS

Intrinsic Fluorescence of SP-1/2. By measuring the intrinsic fluorescence of SP-1/2, we investigated whether the environment of aromatic amino acids is changed upon addition of lipid membranes (*19*, *29*). SP-1/2 contains three tryptophan residues which mainly contribute to its intrinsic fluorescence. LUV of different composition [PC, PC/SM (2:1), PC/PE (2:1), PC/PS (2:1)] were prepared, and the fluorescence of SP-1/2 (excitation at 280 nm) was measured in the absence and in the presence of lipid vesicles. In the absence of LUV, the fluorescence spectrum of SP-1/2 was characterized by a broad peak with a maximum at 343 nm (Figure 1A, spectrum a; see arrow). Upon addition of lipid vesicles to the protein solution an increase in fluorescence intensity and a blue shift of the fluorescence maximum were observed (Figure 1A; see arrows; only shown for PC/PE LUV, spectrum b, and PC LUV, spectrum c). These changes of fluorescence were dependent on the L/P (Figure 1B). Increasing L/P up to 10 enhanced fluorescence intensity whereas at higher L/P fluorescence intensity kept almost constant for the respective lipid vesicles. Likewise, the maximum blue shift of fluorescence (336 nm) was obtained at a L/P of about 10 (data not shown). Protein fluorescence depends on the lipid composition of vesicles as well (Figure 1B). LUV composed of PC or PC/SM had larger effects on SP-1/2 fluorescence compared with PC/PE and PC/PS vesicles, suggesting a lipid specificity of SP-1/2 toward phospholipids bearing the phosphocholine headgroup. The changes of intrinsic fluorescence indicate that, in the presence of LUV, at least some tryptophan residues of SP-1/2 enter a more hydrophobic environment, which could be explained by a conformational change of the protein and/or by intercalation of (part of) the protein into the membrane phase.

Fluorescence Resonance Energy Transfer onto Pyrene-Labeled PL. To prove an intercalation of SP-1/2 into the hydrophobic lipid phase upon binding to LUV, we recorded the fluorescence resonance energy transfer (FRET) between tryptophan and the membrane-embedded probe pyrene linked to a fatty acid chain of the phospholipid PC or PE (Figure 2). This pair of fluorophores fulfills the criteria for measuring FRET (*34*). The spectra in the presence of labeled LUV reveal that part of the SP-1/2 fluorescence energy is transferred to the pyrene moiety as seen from a decrease in tryptophan fluorescence around 340 nm and an increase of pyrene (monomer) fluorescence between 380 and 400 nm (Figure 2; compare unlabeled and labeled LUV in the presence of protein, curves b and c, respectively). FRET was more efficient to pyrenePC than to pyrenePE (Figure 2A,B; compare curves c), giving further support for a lipid specificity of this protein. To exclude that FRET occurs from protein dissolved in the extravesicular buffer onto pyrenePL, we also searched for an energy transfer between bovine serum albumin and pyrenePC or PE. Upon mixing bovine serum albumin with labeled vesicles, we detected no FRET-dependent pyrene fluorescence at all (spectra not shown). From these data it can be concluded that upon binding of SP-1/2 to lipid membranes (part of) the protein bearing tryptophan residues intercalates into the membrane phase.

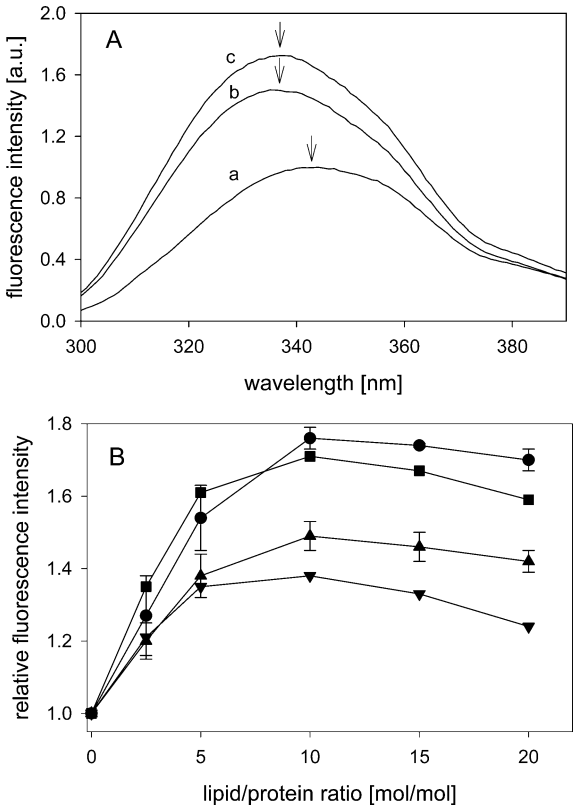

FIGURE 1: Influence of the lipid composition on the intrinsic fluorescence of SP-1/2. (A) Fluorescence spectra (excitation at 280 nm) of 2.5 μM SP-1/2 in HBS were recorded in the absence (line a) and in the presence of 25 μM PC/PE (2:1) LUV (line b) and PC LUV (line c) at 37 °C. The arrows designate the fluorescence maxima. (B) Fluorescence spectra of 2.5 μM SP-1/2 mixed with different amounts of LUV were recorded at 37 °C. Fluorescence intensities measured at 336 nm normalized to the protein fluorescence in the absence of LUV are given for PC LUV (●), PC/SM (2:1) LUV (■), PC/PE (2:1) LUV (▲), and PC/PS (2:1) LUV (▼). Data are given as the means ± standard error of estimate (only for PC and PC/PE LUV) of at least three independent experiments.

Interaction of SP-1/2 with Spin-Labeled Phospholipids in LUV Membranes. By using spin-labeled phospholipids, we studied the impact of SP-1/2 on the mobility of lipids in LUV membranes. In a first set of experiments we labeled the outer leaflet of LUV consisting of PC, PC/SM (2:1), PC/PE (2:1),

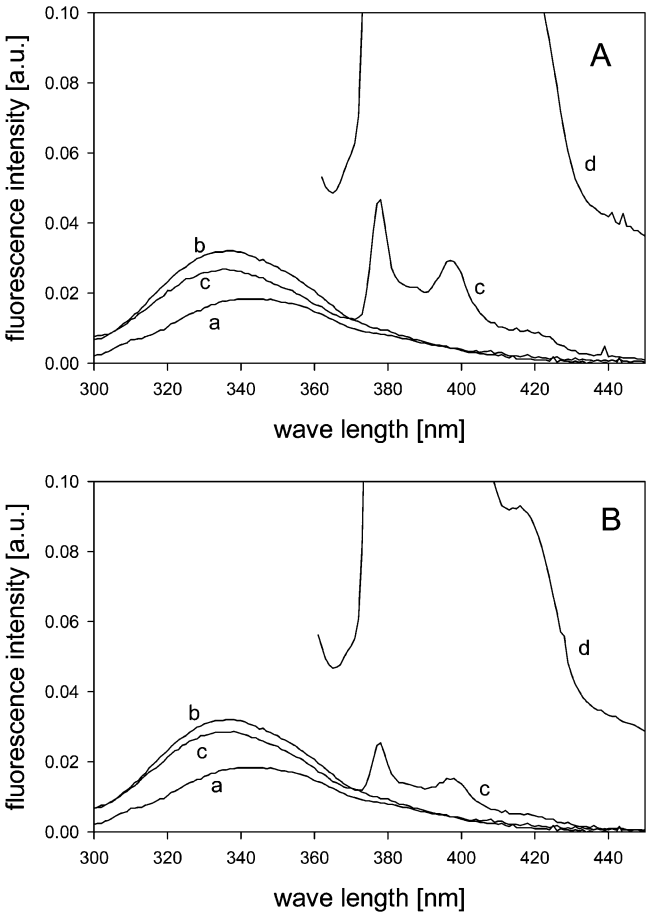

FIGURE 2: Fluorescence energy transfer (FRET) from the tryptophan residues of SP-1/2 to pyrene-labeled phospholipids. PC LUV were labeled with pyrenePC (A) and pyrenePE (B) at 1 mol %. Fluorescence spectra of SP-1/2 were recorded at 37 °C in the absence (line a) and in the presence of unlabeled (L/P = 10, line b) and labeled LUV (L/P = 10, line c) with an excitation of 280 nm as well as in the presence of labeled LUV (L/P = 10, line d) with an excitation of 345 nm.

and PC/PS (2:1) with short-chain spin-labeled phospholipids sc-SL-PC, sc-SL-SM, sc-SL-PE, and sc-SL-PS. ESR spectra of labeled vesicles were recorded in the absence and in the presence of the protein at 4 °C. The respective spectra for PC LUV labeled with sc-SL-PC and PC/PS LUV labeled with sc-SL-PS are shown in Figure 3A,B. In the absence of the protein, for both analogues typical membrane spectra were obtained (spectra I). In the presence of SP-1/2 (shown for L/P = 5) for both kinds of vesicles an additional component in the ESR spectra was observed, which indicates an immobilization of part of the analogue molecules (spectra II; see arrows). Total signal intensities as measured by the double integrals of spectra were similar in the absence and in the presence of protein. The spectra of the immobilized analogues (spectra III) were extracted and quantified by spectra subtraction as described (*17*). The fraction of immobilized analogues in LUV of different composition at L/P = 10 is given in Figure 3C. It demonstrates that the presence of the aminophospholipids PS and PE in membranes reduced the immobilized component compared with that in PC and PC/SM LUV. Moreover, we measured the effect of SP-1/2 (L/P = 10) on sc-SL-PC incorporated symmetrically into PC LUV (spectra not shown). Under these conditions, 26.3 ± 2.7% (n = 3) of the sc-SL-PC molecules were immobilized, which agrees well with the immobilization degree of 47.0 ± 5.0% (n = 7) found for sc-SL-PC in outer leaflet (asymmetrically) labeled vesicles (Figure 3C). These data indicate that binding of SP-1/2 on the outer leaflet does not affect mobility of lipids on the opposite leaflet of the membrane.

To explore the effect of SP-1/2 binding to lipid vesicles on membrane properties in various depth, we used long-chain spin-labeled PC bearing the label moiety at different positions of the *sn*-2 fatty acid chain (C5-SL-PC, C10-SL-PC, C16-SL-PC). PC LUV were symmetrically labeled with these analogues. In the absence of protein the spectra of lc-SL-PC recorded at 25 °C reflect the well-known gradient of flexibility along the membrane normal with a low mobility in the headgroup region and a higher one in the methyl group region (Figure 4A,B, spectra I; only shown for C5- and C10-SL-PC). In the presence of SP-1/2 ESR spectra revealed a second component especially obvious from a broadening of the low-field peak (Figure 4A,B, spectra II; see arrows; shown for C5- and C10-SL-PC at a L/P = 5). This component is reflecting an immobilization of part of the analogue molecules and could be extracted and quantified (Figure 4A,B, spectra III). From a comparison of the effect of the protein on these two analogues, the degree of immobilization was higher for C5-SL-PC than for C10-SL-PC (Figure 4C). For C16-SL-PC, the protein-mediated immobilization at 25 °C was too small to perform spectra subtraction. Therefore, the influence of SP-1/2 on this analogue in LUV membranes was investigated at 4 °C where we found a measurable immobilization of 10.3 ± 0.9% (n = 3) at L/P = 10. This result could reflect that the protein does not affect C16-SL-PC at 25 °C or that a protein-mediated immobilization of the analogue could not be deconvoluted at the higher temperature due to a rapid exchange of analogue molecules between the bulk phase and the protein neighborhood.

Interaction of SP-1/2 with Spin-Labeled Steroid Analogues in LUV Membranes. We also investigated the influence of SP-1/2 on steroids in vesicle membranes by using spin-labeled analogues of androstane (SL-A), cholestane (SL-C), and cholesterol (SL-Chol). ESR spectra of labeled PC LUV were recorded in the absence and in the presence of SP-1/2 at 4 °C. Since the influence of the protein at L/P = 10, especially on SL-Chol, was too low (spectra not shown), we recorded spectra and quantified the extent of immobilization at the higher protein concentration of L/P = 5 (Figure 5). In the presence of SP-1/2 part of the steroid analogues became immobilized as seen from an additional broader component in the ESR spectra (Figure 5, spectra II; see arrows). This component was extracted and is shown in Figure 5 (spectra III). Under these conditions SP-1/2 immobilized about half of the SL-A analogues whereas the effect was lower on SL-C (about 30% immobilized) and on SL-Chol (about 12% immobilized) (Figure 5D).

Influence of SP-1/2 on the Membrane Integrity of LUV Membranes. For investigating whether the impact of SP-1/2 on lipid membranes described above might also cause a perturbation of membrane structure affecting its permeability properties, we applied the following approach (*17*): PC LUV were symmetrically labeled with sc-SL-PC, and the reduction of the analogue by the water-soluble ascorbate was measured at 4 and 37 °C. In the absence of protein upon addition of

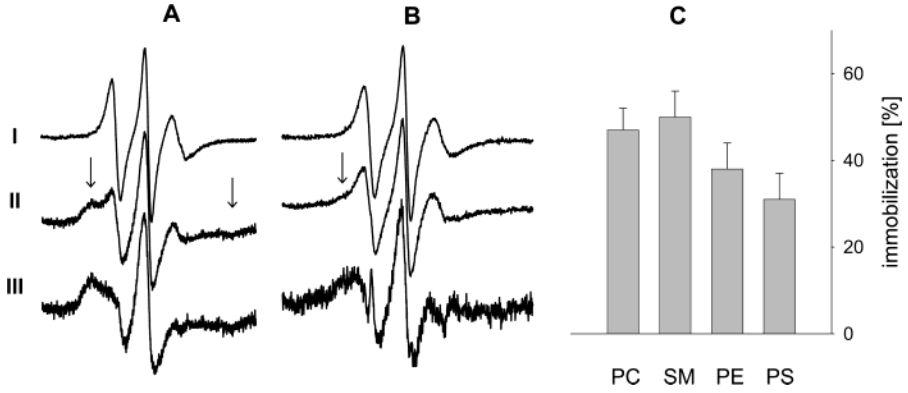

FIGURE 3: Influence of SP-1/2 on short-chain spin-labeled phospholipids incorporated into the outer leaflet of LUV membranes (i.e., asymmetrically labeled). LUV consisting of 2 mM PC (A) and PC/PS (2:1) (B) were labeled with 50 μM sc-SL-PC (A) and sc-SL-PS (B), respectively, as described in Experimental Procedures. ESR spectra of labeled vesicles were recorded at 4 °C in the absence (I) and in the presence (II) of SP-1/2 (L/P = 5). The immobilized component observed in the presence of the protein (see arrows) was extracted by spectra subtraction, i.e., spectra II − I, yielding spectra III. (C) Spectra were recorded as described above but at L/P = 10. From spectra subtraction the extent of lipids immobilized was estimated for PC, PC/SM (2:1), PC/PE (2:1), and PC/PS (2:1) LUV labeled with sc-SL-PC, sc-SL-SM, sc-SL-PE, and sc-SL-PS, respectively. Data represent the means ± standard error of estimate of at least three independent experiments.

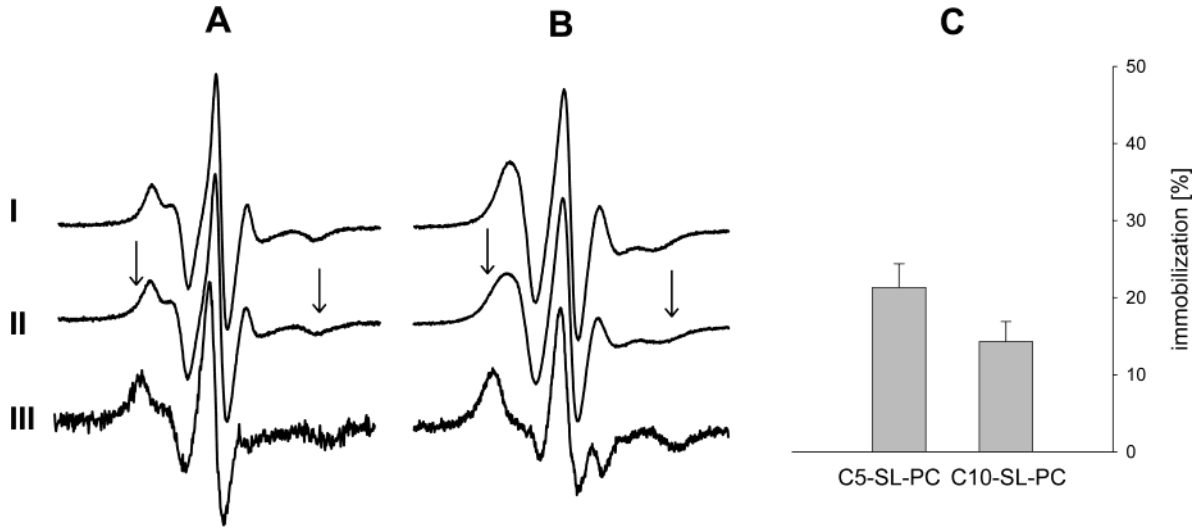

FIGURE 4: Influence of SP-1/2 on long-chain spin-labeled PC incorporated into both leaflets of PC LUV (i.e., symmetrically labeled). PC LUV (2 mM) were labeled with 50 μM C5-SL-PC (A) and C10-SL-PC (B) as described in Experimental Procedures. ESR spectra of labeled vesicles were recorded at 25 °C in the absence (I) and in the presence (II) of SP-1/2 (L/P = 5). The immobilized component observed in the presence of the protein (see arrows) was extracted by spectra subtraction, i.e., spectra II − I, yielding spectra III. (C) Spectra were recorded as described above but at L/P = 10. From spectra subtraction the extent of immobilized lipids was estimated. Data represent the means ± standard error of estimate of at least three independent experiments.

ascorbate, at both temperatures a rapid decrease of signal intensity to about 50% was detected which, subsequently, remained almost constant (Figure 6A,B, circles). This observation indicates that solely analogues in the outer membrane leaflet are accessible to ascorbate whereas those localized in the inner layer are shielded from the reducing substance. When the reduction assay was performed in the presence of SP-1/2 (L/P = 10) at 4 and 37 °C, no or only a minor influence of the protein on membrane integrity was observed in the first minutes of incubation. Like the control curves, signal intensities at both temperatures rapidly decreased to about half of the initial value (Figure 6A,B, triangles). While the signal intensity at 4 °C remained almost constant, at 37 °C a gradual decrease of the ESR signal was seen with prolonged incubation time. Increasing L/P to 5, at 37 °C the ESR signal almost completely disappeared within 10 min (Figure 6B, squares). Even at 4 °C signal intensity decreased rapidly to about 20% but afterward remained almost constant (Figure 6A, squares). Solubilization of LUV membranes in the absence or in the presence of protein by addition of Triton X-100 caused a complete loss of signal intensity (data not shown). These data indicate that SP-1/2 is able to affect the structure and stability of PC membranes. Due to this protein-mediated perturbation of membrane structure ascorbate is able to permeate across the membrane, thereby also reducing analogues in the inner leaflet. This effect of the protein on lipid membrane is supported by rapid leakage of carboxyfluorescein from LUV in the presence of SP-1/2 (P. Müller and A. Herrmann, unpublished results).

Lipid Extraction by SP-1/2. To assess whether SP-1/2 is able to extract lipids from membranes, we used RBC as a model. Cells were incubated with the protein for 30 min at 37 °C, and subsequently, the amount of phospholipids and cholesterol in the supernatant was quantified (see Experimental Procedures). In parallel we performed this experiment with PDC-109 for which an extraction of lipids from

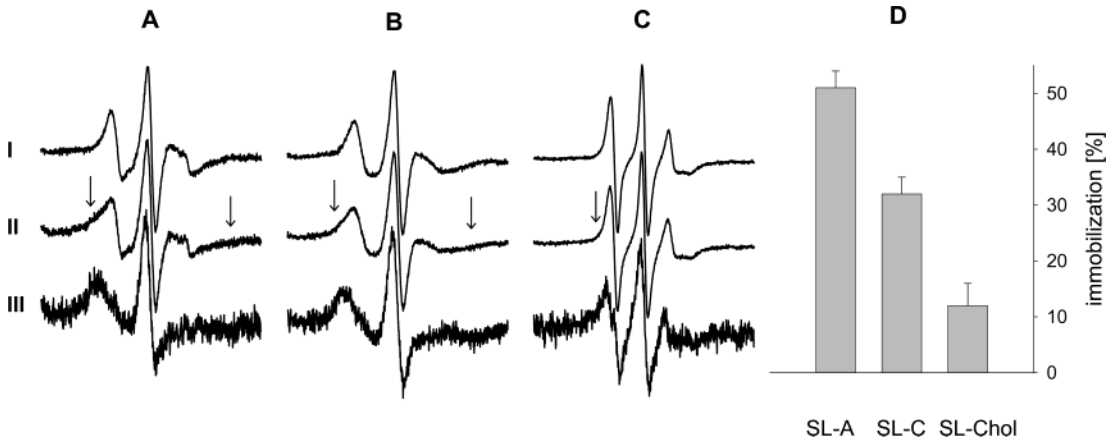

FIGURE 5: Influence of SP-1/2 on spin-labeled steroids incorporated into PC LUV. PC LUV (2 mM) were labeled with 50 μM SL-A (A), SL-C (B), and SL-Chol (C) as described in Experimental Procedures. ESR spectra of labeled vesicles were recorded at 4 °C in the absence (I) and in the presence (II) of SP-1/2 (L/P = 5). In the presence of the protein an immobilized component was observed (see arrows) which was extracted by spectra subtraction (spectra II − I) yielding spectra III. (D) From spectra subtraction the extent of lipids immobilized in the presence of SP-1/2 was estimated. Data represent the means ± standard error of estimate of at least three independent experiments.

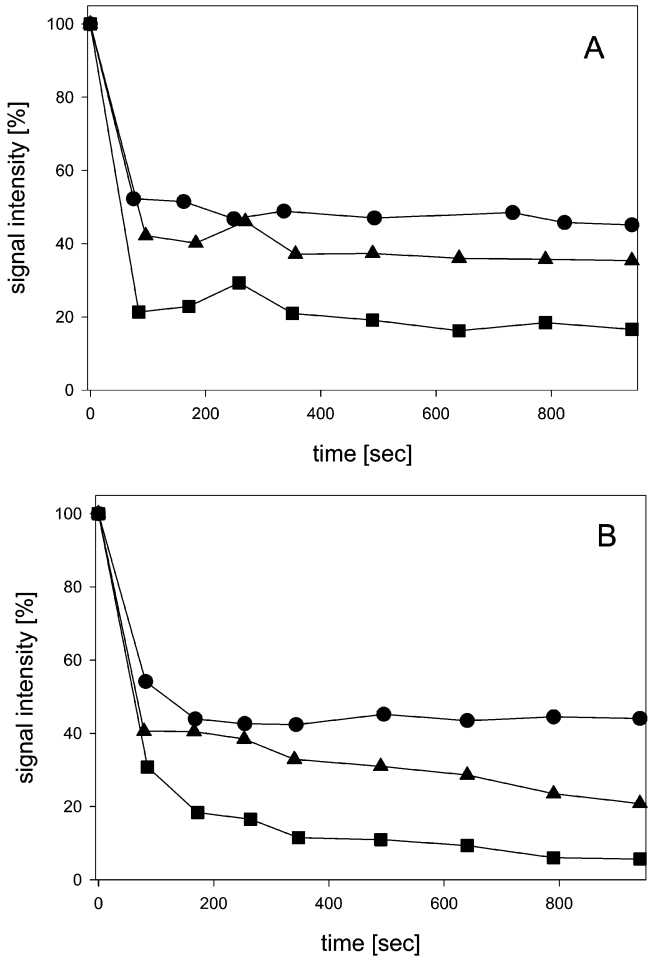

FIGURE 6: Kinetics of ascorbate-mediated reduction of sc-SL-PC in PC LUV. PC LUV (2 mM) were symmetrically labeled with 50 μM sc-SL-PC. Labeled LUV were mixed with ascorbic acid to give a final concentration of 20 mM at time zero and transferred to an ESR capillary. ESR spectra were recorded at the given time points at 4 °C (A) and at 37 °C (B) in the absence (●) and in the presence of SP-1/2 at a L/P of 10 (▲) and 5 (■). ESR signal intensities were estimated by double integration of spectra and were normalized to those in the absence of ascorbate. Spectra were corrected for baseline, i.e., spectrum of HBS.

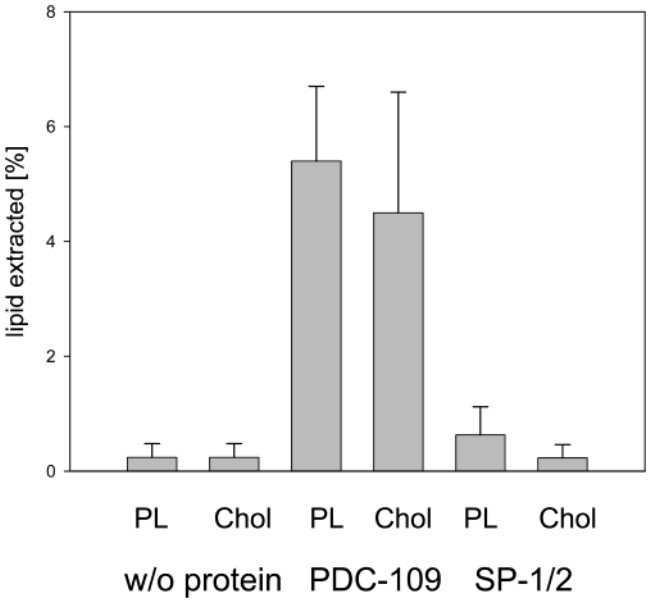

FIGURE 7: Extraction of lipids by seminal plasma proteins. Washed human red blood cells were incubated without and with PDC-109 or SP-1/2 (L/P = 14) for 30 min at 37 °C. After centrifugation of the cells, the amount of phospholipids (PL) and cholesterol (Chol) in the supernatant related to the lipid concentration of control cells was quantified as described in Experimental Procedures. Data represent the means ± standard error of estimate of at least three independent experiments.

membranes has been originally described (*22*, *23*). Whereas we found under these conditions a substantial quantity of phospholipids and cholesterol in the supernatant of cells incubated with PDC-109, for SP-1/2 only a very low amount of lipids was detected which was especially for cholesterol at the detection limit of the assay and similar to the values in the absence of protein (Figure 7).

DISCUSSION

In the present study we asked whether the pattern of interaction between membranes and seminal Fn type II proteins might be conserved in mammalian species. Answering this question will help to better understand the physiological role of this protein family for fertilization. Here, we characterized the interaction of the equine seminal plasma protein SP-1/2 with membranes and compared its impact on LUV with that of the bovine protein PDC-109. On the basis

of several studies an impact of PDC-109 on the plasma membrane of sperm cells during their genesis has been suggested (see the introduction). However, the interaction with membranes as well as the physiological function of seminal Fn type II proteins from other species is not well examined.

Our results show that SP-1/2 interacts with membranes in a manner similar to that of PDC-109 in that (i) it interacts preferentially with PC and SM, lipids bearing a phosphocholine headgroup, (ii) upon binding to lipid vesicles (part of) SP-1/2 molecules intercalate into the hydrophobic region of the membrane, (iii) binding of SP-1/2 to LUV results in a rigidification of the lipid phase, and (iv) SP-1/2 causes a perturbation of the membrane structure. However, our data also indicate that the influence of SP-1/2 on membranes is lower in comparison to PDC-109, in that the extent of SP-1/2-mediated immobilization of lipids was smaller and SP-1/2 had a low capacity, if at all, to extract lipids from RBC membranes.

We provide here the first evidence for a PC/SM selectivity of SP-1/2 upon interacting with intact membranes. First, we found for SP-1/2 that the changes of intrinsic fluorescence were larger in the presence of PC and PC/SM than of PC/PS- and PC/PE-containing vesicles. From the dependence of intrinsic protein fluorescence on the lipid/protein ratio it can be deduced that one protein molecule interacts with about 10 lipid molecules, which is similar to the data given for PDC-109 (*19*, *20*, *29*). Second, immobilization of spin-labeled analogues of PC and SM was stronger in comparison to sc-SL-PS and sc-SL-PE. Third, FRET from the protein was more intense to pyrenePC than to pyrenePE. The lipid specificity of SP-1/2 found in this study is in accordance with the isolation protocol of the protein by using phosphocholine-containing columns (*11*).

The changes of protein fluorescence in the presence of lipid vesicles indicate a penetration of SP-1/2 into the lipid bilayer. This is proven by the observed FRET between tryptophan residues of the protein and pyrenePL incorporated into LUV. Previously, we have shown that PDC-109 intercalates into lipid membranes by a similar approach, employing fluorescent fatty acids as the acceptor (*29*). In the present study we used pyrenePL as acceptors having two long-chain fatty acids, thereby providing a more faithful analogue of endogenous, nonlabeled lipids. For PDC-109, we recorded also FRET from the protein to pyrenePL with similar differences between pyrenePC and pyrenePE as observed for SP-1/2 (P. Müller and A. Herrmann, unpublished results). However, energy transfer was less efficient for SP-1/2 in comparison with PDC-109, indicating a deeper penetration of the latter into the lipid bilayer (see below).

Similar to PDC-109, upon binding to lipid membranes SP-1/2 caused a rigidification of the membrane as sensed by spin-labeled lipids (*17*, *18*). The immobilizing effect of SP-1/2 was larger for a PC analogue bearing the label moiety in the upper part of the fatty acid chain (C5-SL-PC) compared with C10-SL-PC, suggesting a stronger interaction of the protein with the region near the headgroups of the membrane. SP-1/2 also impaired the mobility of spin-labeled steroids incorporated into PC membranes. The effect of the protein on steroids decreased in the range SL-A > SL-C > SL-Chol, similar to what has been found previously for PDC-109 (*18*). This sequence suggests that the effect of both proteins on steroids depends on the structure of the steroid molecule. We are aware that SL-A and SL-C may differ in their properties with respect to cholesterol. However, it has been proven that SL-Chol is a trustworthy reporter of endogenous cholesterol (*35*).

Besides the similarities in the interaction of PDC-109 and SP-1/2 with lipids/membranes, we obtained several indications for a different impact that both proteins have on membranes. The blue shift of the intrinsic fluorescence in the presence of LUV is consistent with an intercalation of both proteins into the membrane (see above). However, we observed for the bovine protein a shift of the maximum fluorescence intensity to 333 nm (*29*) whereas the respective value for the equine protein was 336 nm. This difference argues for a deeper penetration of PDC-109 into the hydrophobic core of the bilayer compared with SP-1/2, which agrees with results of the FRET assay (see above).

When the immobilization of spin-labeled lipids induced by both proteins was compared on a quantitative level, the motional restriction of spin-labeled analogues as measured by the outer hyperfine splitting ($2A_{max}$) was found to be similar. For example, $2A_{max}$ of the immobilized component of sc-SL-PC in the outer leaflet labeled PC LUV at 4 °C in the presence of PDC-109 (*17*) and SP-1/2 (see Figure 3) was 61.4 G and 63.1 ± 2.5 G ($n = 9$), respectively. However, the degree of immobilization, i.e., the amount of analogues affected by the protein, was lower for SP-1/2 compared with PDC-109. For example, by labeling PC LUV symmetrically and asymmetrically with sc-SL-PC, we found for SP-1/2 at 4 °C a degree of immobilization of 26% and 47%, respectively, whereas for PDC-109 these values were 41% and 86%, respectively (*17*). Likewise, this difference was detected with the long-chain analogue C5-SL-PC at 25 °C; 21% and 48% of the analogue molecules were immobilized in the presence of SP-1/2 (see Figure 4) and PDC-109 (*17*), respectively. From these data it can be concluded that the equine protein has a reduced immobilizing effect on membrane lipids compared with the bovine protein. The higher immobilization of lipids in the presence of PDC-109 could be caused by a larger number of PDC-109 molecules binding to/interacting with the membrane and/or by a more effective impact of PDC-109 on membrane lipids. The measurement of intrinsic fluorescence indicating a similar number of PC molecules which interact with PDC-109 and SP-1/2 (see above) argues for the latter hypothesis.

A less intense membrane interaction of SP-1/2 compared with that of PDC-109 could rationalize the differences found in protein-mediated lipid extraction. Whereas for the bovine protein we observed a significant release of phospholipids and cholesterol from human RBC as already described for sperm cells (*22*, *23*), the amount of lipids extracted by the equine protein was very low and similar to that measured in the absence of protein. We are well aware that RBC may only serve as a model system since the lipid and protein compositions of RBC and mammalian (epididymal) sperm cell membranes are different. For example, PL in sperm cells contain a large quantity of highly unsaturated fatty acids. However, Manjunath and co-workers have shown that also mammalian nonspermic cells, i.e., fibroblasts, can be used to characterize the PDC-109-mediated extraction of lipids (*36*, *37*).

What is the relation of our data to the structure of Fn type II proteins? The crystal structure of the PDC-109−phospho-

choline complex has been recently solved at a resolution of 1.8 Å, providing the structural basis for the specific protein−PC interaction (*16*). In each Fn type II domain five aromatic residues form a solvent-exposed hydrophobic cluster, which line the lipid-binding pocket of the protein. These residues bind to phosphocholine through a cation−π interaction between a tryptophan and the quaternary ammonium and hydrogen bonding between hydroxyls of tyrosines and the phosphate group of PC. Due to the homologous amino acid sequences of SP-1/2 and PDC-109 (*5*, *8*, *10*, *38*) it can be assumed that the equine protein contains similar surface-exposed clusters of hydrophobic residues explaining the PC specificity of this protein (*15*, *16*).

On the other hand, which difference(s) between the structure of PDC-109 and SP-1/2 could explain the quantitative differences in the impact of both proteins on membranes? First, the N-terminal peptide of PDC-109 is shorter and contains only one glycosylation site compared with that of SP-1, which has four glycosylation sites in this region. So far, it is not known whether this segment and the different glycosylation degree/pattern have an influence on the interaction of the proteins with membranes. It might be speculated that the higher glycosylation of SP-1 interferes with the lipid interaction of this protein in membranes by steric hindrance and/or polarity. Moreover, protein−lipid/membrane interaction could be affected indirectly by the aggregation state of the proteins. It has been suggested that the type and extent of glycosylation influence the heparin-binding capability of SP-1/2 via a modulation of protein oligomerization (*10*, *11*). We note that SP-1 and SP-2 differ from each other in the N-terminal region with a different glycosylation degree of both proteins (*5*). The proportion of SP-1 and SP-2 in our preparations is between 1:0.5 and 1:1 (*11*).

Second, a hydrophobicity plot of PDC-109 and SP-1 reveals some differences in the hydrophobicity of the Fn type II A and B domains, respectively, i.e., the segments that are responsible for lipid binding (not shown). It might be possible that solely the different hydrophobicity in one of the two Fn type II domains causes a different lipid binding. For PDC-109, it has been shown that the isolated Fn type II B domain is still able to bind PC but is unable to extract lipids from membranes (*39*), suggesting that both Fn type II domains are essential for mediating a lipid efflux. However, from our measurements we cannot assign the protein segments that are responsible for the different impact of PDC-109 and SP-1/2 on membranes.

Although further studies are warranted, our data provide some insight into the physiological function of PDC-109 and SP-1/2. Both proteins exhibit conserved properties in that they interact with membranes via a preferential binding to PC/SM, resulting in a rigidification of the membrane. Bovine and equine sperm plasma membranes contain a large amount of PC, thereby allowing an effective binding of the proteins to the sperm cell (*40*). Changes of membrane fluidity have been described during sperm cell capacitation (*41*, *42*). The efflux of cholesterol from sperm cells is another crucial process of membrane remodeling during capacitation (*25*−*27*). In line with this, several studies have shown that the sole extraction of cholesterol from sperm cells using the artificial cholesterol-binding substance cyclodextrin is sufficient to capacitate those cells (*43*−*45*). Lipoprotein, serum albumin, and, recently for the bull, PDC-109 have been suggested to function as physiological extracellular acceptors for cholesterol of sperm cells. Although for stallion a decreased cholesterol content of spermatozoa during their capacitation has not been shown so far, an involvement of cholesterol efflux could be assumed also in this species since Pommer et al. (*46*) observed an enhanced capacitation of equine spermatozoa by incubation of the cells with methyl-cyclodextrin. Our data may indicate that SP-1/2 is not the main physiological acceptor for sperm cholesterol, and other substances have to be considered for that in stallion. However, at the present stage we cannot exclude an involvement of SP-1/2 in the efflux of cholesterol (and PC) from stallion spermatozoa since the interaction of SP-1/2 with other factors in the seminal plasma might modulate its ability to extract lipids. In this context it should be noted that seminal plasma of stallion contains cholesterol-rich vesicles, so-called prostasomes, which block the cholesterol efflux from sperm cells (*47*). Very recently, it has been proven that, in contrast to PDC-109, SP-1/2 is already bound to sperm cells in the epididymis (S. Kirchhoff and E. Töpfer-Petersen, unpublished results). On the basis of those data other functions of the equine protein could be surmised, e.g., a regulation of sperm cell motility or hyperactivity.

In summary, Fn type II proteins found in the seminal plasma of stallion and bull interact with membranes in a comparable manner, which is probably based on their similar protein structure. However, there are also indications for different properties of these proteins that might direct to their species-specific influence in the course of sperm cell genesis. Future studies also involving Fn type II proteins from other species will help to clarify their physiologial role.

ACKNOWLEDGMENT

We are grateful to Mrs. S. Schiller and Mrs. M. Ploog (both at Humboldt-Universität zu Berlin) for the synthesis of spin-labeled lipids and for technical assistance, respectively.

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BI035647L