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Interaction of Mammalian Seminal Plasma Protein PDC-109 with Cholesterol: Implications for a Putative CRAC Domain

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ABSTRACT: Seminal plasma proteins of the fibronectin type II (Fn2) family modulate mammalian spermatogenesis by triggering the release of the lipids phosphatidylcholine and cholesterol from sperm cells. Whereas the specific interaction of these proteins with phosphatidylcholine is well-understood, their selectivity for cholesterol is unknown. To characterize the interaction between the bovine Fn2 protein PDC-109 and cholesterol, we have investigated the effect of PDC-109 on the dynamics of fluorescent cholesterol analogues in lipid vesicles by time-resolved fluorescence anisotropy. The data show that PDC-109 decreases the rotational mobility of cholesterol within the membrane and that the extent of this impact depends on the cholesterol structure, indicating a specific influence of PDC-109 on cholesterol. We propose that the cholesterol recognition/interaction amino acid consensus (CRAC) regions of PDC-109 are involved in the interaction with cholesterol.

Mammalian sperm cells undergo a complex maturation process, known as capacitation (*1*), in which they acquire the ability to fertilize an egg. This cellular genesis is characterized by an intensive remodeling of the sperm surface, e.g., because of changes in the plasma membrane composition caused also by binding of extracellular proteins to the sperm cell. Proteins bearing a fibronectin type II (Fn2)[1] domain have been shown to be involved in the capacitation process of sperm cells and in the formation of a female sperm reservoir (see refs 2 and 3). Fn2 proteins associate with the sperm during epididymal transit and at ejaculation. Binding to the sperm membrane is enabled by a specific interaction of the proteins with lipids of the plasma membrane. The best-characterized Fn2 proteins in seminal plasma are those from cattle (BSP proteins), and within this species, most studies have been performed using PDC-109 (also named BSP-A1/A2). The physiological role of bovine proteins has been associated with their capacity to remove lipids, mainly phosphatidylcholine (PC) and cholesterol from the sperm membrane (see refs 2 and 3). Although a change in the plasma membrane composition due to a release of lipids is an essential process of sperm capacitation, the molecular mechanism of Fn2-mediated lipid extraction is unknown. Very likely, the selectivity for extraction of PC is a consequence of the specific interaction of these proteins with this phospholipid (see refs 2 and 3). The crystal structure of PDC-109 revealed the sequences that interact with the choline group of PC (see ref 3). However, the interplay between PDC-109 and cholesterol is still unsolved. It has been reported that PDC-109 does not directly bind to cholesterol in an aqueous solution (*4*, *5*). However, the situation might be completely different when it is solubilized in a phospholipid bilayer. Indeed, several studies have shown that PDC-109 affects cholesterol in membranes (*5*, *6*). These studies, however, failed to discern whether PDC-109 interacts with cholesterol in membranes directly or indirectly via PC and how sterol structure influences the interaction.

Here, we have characterized the impact of PDC-109 on the dynamics of cholesterol in lipid vesicles by time-resolved fluorescence anisotropy using two fluorescent BODIPY−cholesterol analogues. Our data show that PDC-109 decreases the rotational mobility of cholesterol depending on the sterol side chain structure. We suggest that the CRAC (cholesterol recognition/interaction amino acid consensus) domain (*7*), which is typical for seminal plasma Fn2 proteins, mediates the interaction of PDC-109 with cholesterol.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL), and BODIPY-labeled PC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BPC), was from Molecular Probes (Invitrogen, Darmstadt, Germany). The BODIPY-labeled analogues of cholesterol, 23-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacen-8-yl)-24-norchol-5-en-3β-ol (BCh-1) and 22-[4-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacen-8-yl)-butyroxy]-23,24-bisnorchol-5-en-3β-ol (BCh-2), were synthesized

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[1]Abbreviations: BCh-1, 23-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacen-8-yl)-24-norchol-5-en-3β-ol; BCh-2, 22-[4-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacen-8-yl)butyroxy]-23,24-bisnorchol-5-en-3β-ol; BPC, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine; CRAC, cholesterol recognition/interaction amino acid consensus; HBS, HEPES-buffered solution; Fn2, fibronectin type II; LUVs, large unilamellar vesicles; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

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Table 1: Influence of PDC-109 on the Fluorescence Lifetime of BODIPY-Labeled Cholesterol Analogues BCh-1 and BCh-2 (250 nM) in POPC LUVs (250 μM) in the Absence and Presence of 50 μM PDC-109 (P) at 25 °C[a]

	τ_1 (ns)	α_1	τ_2 (ns)	α_2	$\langle\tau\rangle$ (ns)	χ_{tot}^2
POPC/BCh-1	5.88 ± 0.04	0.80 ± 0.10	1.62 ± 0.18	0.20 ± 0.10	5.73 ± 0.06	1.11
POPC/BCh-1 with P	5.98 ± 0.03	0.85 ± 0.01	2.02 ± 0.24	0.15 ± 0.01	5.75 ± 0.02	1.08
POPC/BCh-2	6.21 ± 0.04	0.81 ± 0.02	1.73 ± 0.16	0.19 ± 0.02	5.94 ± 0.02	1.12
POPC/BCh-2 with P	6.10 ± 0.02	0.82 ± 0.02	1.80 ± 0.11	0.18 ± 0.02	5.84 ± 0.02	1.11

[a]The experimental curves were fitted giving the fluorescence lifetimes (τ) and the relative amplitudes (α). From these values, an average lifetime ($\langle\tau\rangle$) was calculated. Means ± the standard deviation are given. Experiments were repeated three times for each set.

using the method described in ref 8. All other chemicals were from Sigma-Aldrich (Taufkirchen, Germany). The HEPES-buffered solution (HBS) contained 150 mM NaCl and 5 mM HEPES (pH 7.4).

PDC-109 was purified from the seminal plasma of reproductively active Holstein bulls by combination of affinity chromatography on heparin-Sepharose and DEAE-Sephadex chromatography as described previously (*9*).

Preparation of Large Unilamellar Vesicles (*LUVs*). Aliquots of POPC and fluorescent lipids (0.1 mol %) in an organic (2:1 chloroform/methanol) solution were transferred to a glass tube, dried under nitrogen, and resuspended in a small volume of ethanol and HBS at a final concentration of 1 mM [final ethanol concentration of < 1% (v/v)]. To prepare LUVs, five freeze−thaw cycles were performed followed by extrusion of the lipid solution 10 times at 40 °C through two 0.1 μm polycarbonate filters (mini-extruder from Avanti Polar Lipids; filters from Costar, Nucleopore, Tübingen, Germany).

Measurement of Fluorescence Spectra. Fluorescence spectra of LUVs containing BODIPY-labeled lipids were recorded using an Aminco Bowman Series 2 spectrofluorometer in the range of 480−550 nm (λ_{ex} = 470 nm; slit width for excitation and emission, each 4 nm) at 37 °C.

Measurement of Time-Resolved Fluorescence Anisotropy. Anisotropy decays were obtained by the single photon counting technique (TCSPC). For excitation at 440 nm of BODIPY-labeled lipids, a LDH pulsed diode laser (Picoquant, Berlin, Germany) was employed. According to the relative intensity of the different samples, slits providing bandwidths of 4 or 8 nm were adjusted. To further screen scattered excitation light, we added a cutoff filter (460, Shimadzu, Duisburg, Germany) in front of the collecting lens. For detection, a PMA-182 photomultiplier based on the Hamamatsu H5783 photosensor modules (Picoquant) was used, and the instrumental response function, IRF (30 ps), for reconvolution was generated from a solution containing unlabeled LUVs of the same composition. Emission at 505 nm was detected at the magic angle and at the parallel and perpendicular position relative to the vertically polarized excitation beam. The photon count rate was on the order of $6-8 \times 10^4$ counts/s for the probes and $\sim 1 \times 10^4$ counts/s for IRF acquisition. Data analysis was conducted using a nonlinear least-squares iterative procedure, and the goodness of fitting was judged by the distribution of the residuals and the χ^2 value. Fluorescence decays were analyzed with FluoFit (Picoquant). The correction factor G (factor ratio of the sensitivities of the detection system for vertically and horizontally polarized light) was estimated upon tail matching of the vertical and perpendicular decay of the standard probe. In this case, coumarin was employed having excitation and emission as for BODIPY analogues (see above). Anisotropy reconvolution fitting was performed according to

$$r(t) = \sum_j r_{0j}\, e^{-t/\varphi_j}$$

where r_{0j} values are the fractional anisotropies that decay with correlation times φ_j.

For lifetime measurements, decays were fitted with two or three exponential terms and the average lifetime $\langle\tau\rangle$ was calculated as

$$\langle\tau\rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i}$$

where τ_i is the lifetime of the component i and α_i represents the normalized pre-exponential relative to the fraction of molecules with decay time i.

RESULTS AND DISCUSSION

We employed two BODIPY-labeled cholesterol analogues, BCh-1 and BCh-2, both bearing the fluorescence moiety at the end of the sterol aliphatic side chain. BCh-1 has been shown to mimic the properties of native cholesterol. Like cholesterol, it partitions preferentially into liquid-ordered lipid domains (*10*). In contrast, BCh-2 with an artificial ester linkage within the side chain does not partition into those domains (*10*). The analogues were incorporated into POPC vesicles as it is known that PDC-109 effectively binds and interacts with membranes containing phospholipids with a phosphocholine headgroup (see refs 2 and 3).

Upon addition of PDC-109 to the vesicles, only a small decrease in the fluorescence intensities of analogues was observed (not shown). Fluorescence lifetime measurements revealed a long (τ_1) and a short (τ_2) component and similar average lifetimes ($\langle\tau\rangle$) for BCh-1 and BCh-2 (Table 1). The average lifetimes are in agreement with those recently reported (*10*). Binding of PDC-109 affected neither the single-lifetime components and relative amplitudes (α_i) nor the average lifetime for both analogues (Table 1), indicating that the protein does not influence significantly the environment of the BODIPY moiety linked to cholesterol.

Previous reports have shown that PDC-109 causes an effective reduction of the mobility of membrane phospholipids (see refs 2 and 3). Therefore, we analyzed its impact on the dynamics of BODIPY-labeled cholesterol analogues by time-resolved anisotropy, being highly sensitive to motion, flexibility, and wobbling of fluorescent molecules. The anisotropy decay of both analogues in POPC LUVs was fitted by two components having rotational correlation times (φ) of ~0.8 and ~5 ns (Figure 1). There were no differences in the short (φ_1) and long (φ_2) values of both analogues (Figure 1C,E). Likewise, the fractional amplitudes

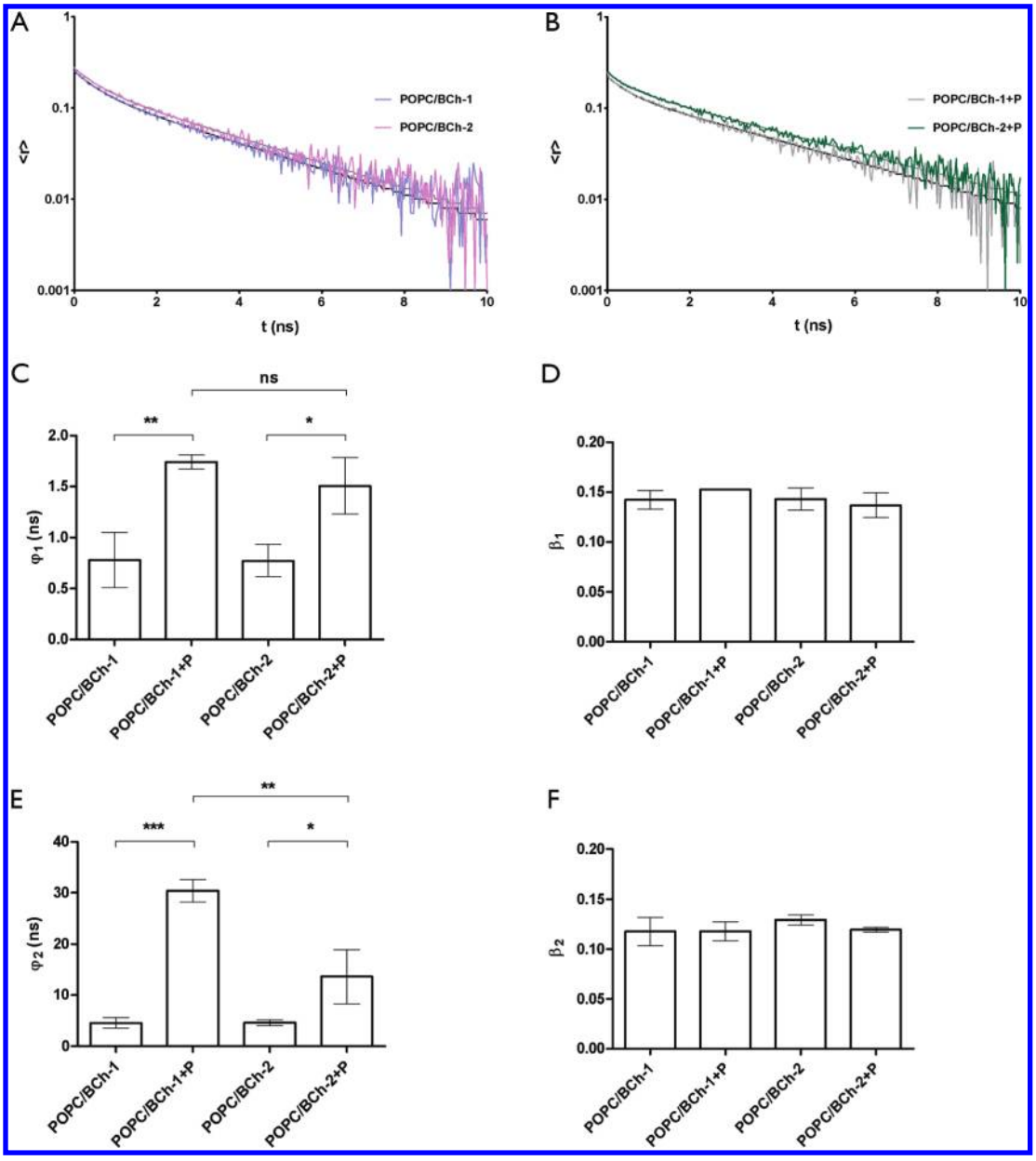

FIGURE 1: Influence of PDC-109 on the time-resolved anisotropy decay of BODIPY-labeled cholesterol analogues. Experimental curves show the anisotropy decay of BCh-1 or BCh-2 (each at 250 nM) in POPC LUVs (250 μM) in the absence (A) or presence (B) of PDC-109 (50 μM) at 25 °C. The curves of one representative experiment (colored) and the respective fitted curves (black) are shown. From the curves, rotational correlation times (C and E) and the respective amplitudes (D and F) were determined for POPC LUVs with BCh-1 or BCh-2 in the absence or presence of PDC-109 (P). Data represent means ± the standard deviation and were compared by the *t* test. (C) $^{**}p = 0.004$. $^{*}p = 0.016$. ns means not significant. (E) $^{***}p < 0.0001$. $^{**}p = 0.007$. $^{*}p = 0.043$.

(β) of both decay components were similar for BCh-1 and BCh-2 (Figure 1D,F). We surmise that the fast anisotropy component of < 1 ns is determined by wobbling of the BODIPY, whereas the slow component reflects the overall rotational diffusion of the analogue in the lipid bilayer (*10*).

Upon addition of PDC-109, for both analogues anisotropy decay curves could still be fitted by two components (Figure 1). The presence of the protein slowed the decay, as revealed from the increase in both rotational correlation times (Figure 1). However, whereas the effect of PDC-109 on the fast component [φ_1 (Figure 1C)] was similar for both analogues, the increase in the slow component (φ_2) was significantly larger for BCh-1 (Figure 1E). The fractional amplitudes (β) for both components did not change in the presence of the protein (Figure 1D,F). These data indicate that PDC-109 decreases the rotational mobility of cholesterol in POPC membranes depending on the structure of the cholesterol molecule: the influence of the protein is stronger for BCh-1, which mimics better the properties of endogenous cholesterol.

PDC-109 is known to interact with PC (see refs 2 and 3). Therefore, we also analyzed its effect on BODIPY-labeled PC (BPC). As for the cholesterol analogues, the protein had no influence on the fluorescence spectrum of BPC in POPC LUVs (not shown). Three fluorescence lifetime components were found: 5.8, 1.7, and 0.1 ns. Addition of the protein did not cause any significant change in these lifetimes (not shown). The decay of fluorescence anisotropy of BPC/POPC LUVs was fitted by a single component having a rotational correlation time of ~2 ns (Table 2). In the presence of PDC-109, this value increased to 8 ns. Moreover, a second decay component with an φ value of ~0.7 ns became

Table 2: Influence of PDC-109 (P) on the Time-Resolved Anisotropy Decay of BODIPY-Labeled PC[a]

	φ_1 (ns)	β_1	φ_2 (ns)	β_2	G	χ_{tot}^2
POPC/BPC	1.98 ± 0.04	0.18 ± 0.003	–	–	0.92	1.19
POPC/BPC with P	8.01 ± 3.68	0.11 ± 0.001	0.71 ± 0.19	0.12 ± 0.008	0.89	1.09

[a]The decay of the fluorescence anisotropy of BPC (250 nM) in POPC LUVs (250 μM) was measured in the absence and presence of 50 μM PDC-109 (P) at 25 °C. The experimental curves were fitted giving the rotational correlation times (φ) and the fractional amplitudes (β). Means ± the standard deviation are given. Experiments were repeated three times for each set.

Table 3: Identification of the Cholesterol Recognition/Interaction Amino Acid Consensus (CRAC) Pattern in Mammalian Fn2 Seminal Plasma Proteins[a]

name	Swiss-Prot entry	position	sequence										
PDC-109	P02784	74		L	D	A	D	**Y**[b]	V	G	R		
		79	V	G	R	W	K	**Y**[b]	C	A	Q	R	
		94	V	F	P	F	I	**Y**	G	G	K		
		121		L	S	P	N	**Y**[b]	D	K			
BSP-3	P044557	54	V	F	P	F	I	**Y**	G	N	K		
		79		L	D	A	D	**Y**	T	G	R		
		99	V	F	P	F	I	**Y**	E	G	K		
BSP-30	P81019	122		L	D	T	E	**Y**	Q	G	N	W	K
		142	V	F	P	F	I	**Y**	R	K	K		
		169		L	T	S	N	**Y**	D	R			
SP-1	Q70GG6	84		L	T	G	T	**Y**	S	G	S	W	K
pP1	P80964	69			V	T	T	**Y**	Y	M	K	R	
		116		V	T	P	N	**Y**	D	Q	D	R	
BSPH1	Q075Z2	70		L	N	K	T	**Y**	E	G	Y	W	K

[a]Amino acid sequences of various mammalian seminal plasma proteins containing the CRAC domain (L/V-X_{1-5}-Y-X_{1-5}-R/K) were aligned. L/V-X_{1-5}-Y-R/K or L/V-Y-X_{1-5}-R/K sequences were not considered. The data are given for searching the protein sequences from the N- to C-terminus. Searching in the reverse direction yielded one additional CRAC pattern for all proteins, except for pB1 (two patterns) and BSPH1 (no pattern). [b]Tyrosine residues that have been assigned to a pattern of invariant tyrosine and tryptophan residues to mediate the binding of the protein to the phosphorylcholine moiety of PC (see ref 3).

apparent (Table 2).[2] Addition of cholesterol to the LUVs at the same concentration used for BPC affected neither the fluorescence lifetime nor the decay of fluorescence anisotropy of BPC (not shown). These data confirm previous studies of the immobilizing effect of the protein on PC (see ref 3).

The changes in fluorescence anisotropy of cholesterol analogues in the presence of PDC-109 indicate a specific impact of the protein on cholesterol in bilayer membranes that could be caused by a direct and/or an indirect interaction via PC. We searched for putative protein regions that might be responsible for the interaction with cholesterol and identified the recently described CRAC domain that was found in some membrane proteins that interact with cholesterol (*7*, *11*). This domain consists of the sequence L/V-X_{1-5}-Y-X_{1-5}-R/K-, where X_{1-5} stands for one to five residues of any amino acid. The CRAC motif has been found to be localized in the vicinity of a transmembrane helix of respective proteins, thereby positioning the motif at the membrane surface for an effective association with cholesterol (see ref 7). It has been proposed that leucine or valine interacts with the hydrophobic side chain of cholesterol and tyrosine with the C3-hydroxy group of cholesterol, whereas arginine or lysine creates a pocket for the interaction with the sterol (see ref 12). While several studies highlight a role for the CRAC domain in the cholesterol-dependent process (see refs 7 and 12), the molecular mechanism(s) of protein−cholesterol interaction mediated by the CRAC domain is still an open issue.

We localized in the PDC-109 sequence four patterns that match the typical properties of the CRAC motif (Table 3). As the protein binds to the membrane surface, the localization of these sequences is comparable to that of the CRAC domains of respective membrane proteins (see above), enabling efficient interaction with cholesterol. Notably, three of these sequences are in regions that have been assigned to a segment of invariant tryptophan and tyrosine residues being responsible for the binding of PDC-109 to PC (Figure 2) (see ref 3). We hypothesize that at least some of these regions mediate the association of PDC-109 with PC and cholesterol.

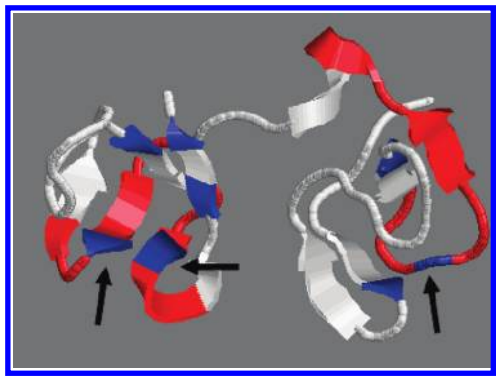

FIGURE 2: Structural view of PDC-109 (Protein Data Bank entry 1H8P, drawn with RasMol) showing the A chain, i.e., a monomer of PDC-109 that forms dimers upon association with phosphorylcholine. The CRAC domains (see Table 3) are colored red, and the tyrosine and tryptophan residues, which are part of the conserved amino acids involved in phosphorycholine binding of the protein (see ref 3), are colored blue. Three tyrosine residues of the lipid binding pocket are localized in the CRAC region (see arrows).

Sequence analysis revealed that seminal Fn2 proteins of various mammalian species accommodate a variable number of

[2]We surmise that this component may be also present in the absence of the protein. However, because of the overall rapid decay, we could not resolve this component from the 2 ns component.

CRAC patterns (Table 3). It is obvious that the bovine Fn2 proteins, especially PDC-109, contain the CRAC pattern at a significantly higher frequency than the proteins from the other species. BSP-3 and BSP-30, which have three CRAC sequences, extract cholesterol from membranes with an efficiency similar to that of PDC-109 (*13*). In contrast, the equine protein SP-1, which possesses only one CRAC sequence, is less able than PDC-109 to release cholesterol from membranes (*14*). However, any conclusion about the strength of a putative protein−cholesterol interaction solely on the basis of the number of CRAC patterns present in a protein has to be taken with care, because the affinity of a CRAC domain for cholesterol could be modulated, for example, by the specific conformation of the protein and its molecular membrane environment. In this regard, we note that the cholesterol:phospholipid ratio of sperm membranes and the concentration of Fn2 proteins in the respective seminal plasma differ markedly among various species (*15*, *16*).

In conclusion, we found that the CRAC domain is typical for seminal plasma Fn2 proteins. We postulate that CRAC domains in Fn2 proteins of bovine seminal plasma mediate the interaction of these proteins with cholesterol. Studies that did not find an interaction between the protein and cholesterol in solution (*4*, *5*) do not contradict our assumption; a putative interaction with pure cholesterol in solution is difficult to investigate because of the extremely low aqueous solubility of cholesterol (*17*). Future studies will help elucidate whether the CRAC motif is indeed involved in the impact of Fn2 proteins on sperm cell genesis. For that, the influence of sequence modifications on cholesterol, e.g., on cholesterol mobility or on release of cholesterol from membranes, should be investigated. An important advance is the ability to express recombinant Fn2 protein, which was achieved in human BSPHI by Lefebvre et al. (*18*).

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