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# Characterization of boar sperm cytoskeletal cylicin II as an actin-binding protein

Roselyne Rousseaux-Prévost,[a,*] Christophe Lécuyer,[a] Hervé Drobecq,[b] Christian Sergheraert,[b] Jean-Louis Dacheux,[c] and Jean Rousseaux[a]

[a] *FRC3 CNRS, Institut de Biologie de Lille-BP 447, Lille 59021, France*
[b] *UMR 8525 CNRS-Lille 2, Institut de Biologie de Lille-BP 447, Lille 59021, France*
[c] *UMR INRA-CNRS 6073, Institut National de la Recherche Agronomique, Nouzilly 37380, France*

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**Abstract**

The presence of actin-binding proteins in the perinuclear theca of boar spermatozoa has been investigated, using stepwise extractions of proteins from sperm heads. Proteins extracted with the alkaline buffer 1 M $Na_2CO_3$, pH 11, were found to contain a 66 kDa protein that binds F-actin in actin pelleting assays. Sequence studies and immunological characterization with antibodies specific for human cylicin II identified the 66 kDa protein as the homologue of bovine and human cylicin II. Immunocytochemical studies showed the presence of porcine cylicin II in the acrosomal region of round spermatids and in the postacrosomal region of late spermatids and spermatozoa, in agreement with the previously described localization of cylicins. Taken together, the results suggest that cylicin II, a protein of the sperm perinuclear cytoskeleton, is a novel actin-binding protein, which probably plays a role in the actin-related events that occur during spermiogenesis and the early events of fertilization.


*Keywords:* Sperm; Spermatid; Acrosome; Cytoskeleton; Perinuclear theca; Actin

The perinuclear theca is a rigid cytoskeleton that almost covers the entire nucleus of mammalian spermatozoa [1–3]; it is composed of two regions: the subacrosomal layer or perforatorium and the postacrosomal sheath or calyx [2,4,5]. The protein composition of the perinuclear theca is complex and variable between species. The sequence of some proteins that make up perinuclear cytoskeleton is known: calicin [6]; cylicins I and II [7,8], both members of the multiple band polypeptide (MBP) group [2,9]; a histone variant, subH2Bv [10]; CPα3 [11,12] and CPβ3 [13], testis-specific isoforms of the alpha and beta subunits of capping protein; two novel actin-related proteins Arp-T1 and Arp-T2 [14]; protein PERF 15 or TLBP [15,16]; and the transcription factor Stat 4 [17]. Other proteins have been described, but their molecular structure is still unknown: three proteins of the perinuclear theca of mouse spermatids of 75, 77, and 80 kDa, named thecins [18], a 90 kDa protein of the subacrosomal layer of human sperm [19] and about 20 polypeptides of 15–60 kDa in bull sperm perinuclear theca [20,21]. Works performed by the group of Oko [21] have shown that, in the round spermatid, the perinuclear theca coats the acrosomal vesicle before its attachment to the anterior region of the nucleus. After attachment, the subacrosomal layer of mammalian spermatids is also filled with F-actin. F-actin remains during the cap phase of spermiogenesis; in late spermatids and spermatozoa of many species, most or all the F-actin is depolymerized to G-actin and seems to be redistributed in a species-specific pattern (for review, see [22–24]). The role of F-actin and of its interaction with other perinuclear theca proteins is unknown. Possible roles are anchorage of the acrosome [21,22,25,26] or shaping of the head by capping the nuclear membrane [27]. A better understanding of actin function implies a search for the actin-binding properties of proteins of the

* Corresponding author. Fax: +33-320871233.
*E-mail address:* roselyne.rousseaux@ibl.fr (R. Rousseaux-Prévost).

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perinuclear cytoskeleton. Recently, we have shown that calicin, a protein of the kelch family, binds F-actin [28]. We report here the characterization in boar sperm heads of a 66 kDa actin-binding protein identified by biochemical and immunological studies as the porcine homologue of bovine and human cylicin II.

## Materials and methods

*Materials.* Spermatozoa from cauda epididymis and testes from freshly slaughtered boars were collected at the Station de Physiologie des Mammifères Domestiques (INRA, Nouzilly, France). Spermatozoa were stored at −80 °C and testes were treated immediately after collection. Human semen from fertile donors with normal semen characteristics according to WHO criteria was obtained from the CECOS-Nord (Lille, France) and stored, until use, in liquid nitrogen.

*Antibodies.* Anti-peptide antibodies were prepared as described in a previous report [28]. The following synthetic peptides were used: a peptide with the amino-terminal 1–24 sequence of human calicin; a peptide with the sequence 270–284 of human cylicin II; and peptide P2 of porcine cylicin II (see below, results section). Antibodies to actin were a mouse monoclonal antibody to α-smooth muscle actin from Amersham-Pharmacia (Les Ulis, France). For immunoblotting, goat anti-rabbit IgG or goat anti-mouse IgM conjugated either with peroxidase or with alkaline phosphatase (Sigma, Saint-Quentin Fallavier, France) was used. Secondary antibodies for immunofluorescence were fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma).

*Isolation of spermatozoa and of sperm heads.* All procedures were carried out at 4 °C, with all buffers containing the following proteinase inhibitors: aprotinin, 10 μg/ml; bestatin, 5 μg/ml; leupeptin, 50 μg/ml; p-aminobenzamidine, 0.1 mM; pepstatin A, 50 μg/ml; and phenylmethylsulfonyl fluoride (PMSF), 1 mM. Preparations of human ejaculated spermatozoa, boar epididymal spermatozoa, and boar sperm heads were performed as reported previously [28].

*Extraction of proteins from sperm heads.* Successive extractions of proteins from sperm heads were performed essentially as described by Oko and Maravei [20] with the following modifications: after the step of extraction with 1 M NaCl, an additional step was applied before extraction with 0.1 M NaOH, including treatment with 1 M $Na_2CO_3$, pH 11, for 1 h at 4 °C. The supernatants containing the proteins were dialyzed against PBS and concentrated by filtration on Diaflo PM 10 membrane (Amicon Bioseparation; Millipore, Saint Quentin en Yvelines, France) followed by filtration on Nanosep (10-kDa cutoff) centrifugal membrane (Pall Filtron, Northborough, MA). Protein concentration was determined by Coomassie blue protein-dye binding assay [29] and the material was stored as aliquots at −80 °C.

*Purification of proteins.* Proteins extracted from boar sperm heads with 1 M $Na_2CO_3$, pH 11, were dialyzed against 20 mM sodium phosphate, 50 mM NaCl, 1 mM dithiothreitol, and 0.01% sodium azide, pH 7.4 (buffer A) and loaded onto a 1 ml Hi-Trap SP column (Amersham-Pharmacia) equilibrated with buffer A. Elution was performed at 0.7 ml/min with a 20 ml linear gradient of NaCl from 50 mM to 3 M. Fractions of 0.7 ml were collected.

*Gel electrophoresis and immunoblotting.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using either a linear gradient (8–16%) polyacrylamide gel or a 12% polyacrylamide gel, and the Laemmli buffer system [30]. For two-dimensional gel electrophoresis, the protein extracts were treated as described in [2] and the first dimension was performed by nonequilibrium pH gradient gel electrophoresis (NEPHGE) [31]. Proteins were stained either with Coomassie blue or with the silver staining procedure [32]. Immunoblotting was performed as described previously [33]. Densitometric analyses were made with the Kodak Digital Science 1D System (Eastman Kodak, Rochester, NY, USA).

*Cosedimentation assay with F-actin.* The Actin Binding Protein Biochem kit from Cytoskeleton (Denver, USA) was used, essentially according to instructions of the manufacturer. Prior to the assays, purity of actin was controlled by SDS–PAGE and was found to contain less than 3% of protein contaminants. Polymerized F-actin was stored ($\leqslant$ 1 wk) on ice in F-buffer (5 mM Tris–HCl, 50 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 1 mM ATP, pH 8). Proteins were centrifuged at 150,000*g* for 30 min at 4 °C immediately before each actin-binding assay. Incubation with F-actin (final concentration 4 μM) of proteins extracted from sperm heads with 1 M $Na_2CO_3$, pH 11 (5 μg), was performed under a volume of 50 μl for 30 min at 4 °C. Incubation of purified protein (final concentration 18–150 nM) was performed for 1 h at 4 °C, under a volume of 25 μl, using 1 μM (final concentration) F-actin. The mixtures were pelleted by centrifugation at 150,000*g* for 30 min. Controls without actin were performed for each protein concentration. Equal volumes of solubilized pellets, and supernatants were loaded on gels for SDS–PAGE.

*Protein sequence analysis.* Internal amino acid sequences of the 66 kDa protein were determined as described in [34]. Protein was separated by SDS–PAGE. After Coomassie blue staining, the 66 kDa band was excised from the gel and subjected to in-gel proteolysis with trypsin [34]. Peptides were purified by reverse-phase HPLC on a Beckman ultrasphere ODS column 2 × 200 mm and eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Isolated peptides were sequenced in a Perkin–Elmer Procise 492. Sequences were compared against all non-redundant databases.

*Immunofluorescence microscopy.* Immunofluorescence microscopy was performed on boar sperm heads and on testicular tissue. Sperm heads were spread as droplets on glass slides, air-dried, and fixed with methanol at −20 °C for 5 min and then with acetone at −20 °C for 2 min. Tissue sections were prepared as described previously [28]. Immunofluorescent labeling was performed essentially as reported in [33]; an incubation for 1 h at room temperature in PBS with 0.2% Triton X-100 was made before immunostaining.

## Results

### *Characterization of a porcine homologue of human and bovine sperm cylicin II*

Boar sperm heads were prepared from epididymal spermatozoa. Successive protein extractions were performed with: 0.2% Triton X-100 (extract E1); 1 M NaCl (extract E2); 1 M $Na_2CO_3$, pH 11 (extract E3); and 0.1 M NaOH (extract E4). Several proteins were obtained at each step (Fig. 1A). Treatment with sodium carbonate removed a lower amount of proteins than extraction with 0.1 M NaOH (from $50 \times 10^9$ sperm heads: 0.9–3 mg for extract E3; 7–16 mg for extract E4). Nevertheless, SDS–PAGE profile of extract E3 (Fig. 1A, lane 3) showed a restricted protein pattern, with a component of $M_r$ 66 kDa, representing, according to the experiment, 5–30% of the proteins (as determined by densitometric scanning). Fractionation of the proteins of extract E3 on cation-exchange column Hi-Trap SP (Fig. 1B) showed that a 66 kDa protein was eluted at the end of a 0.05–3 M NaCl gradient, indicative of a high basic charge. These basic properties were also found by two-dimensional gel electrophoresis of the purified protein (Fig. 1C),

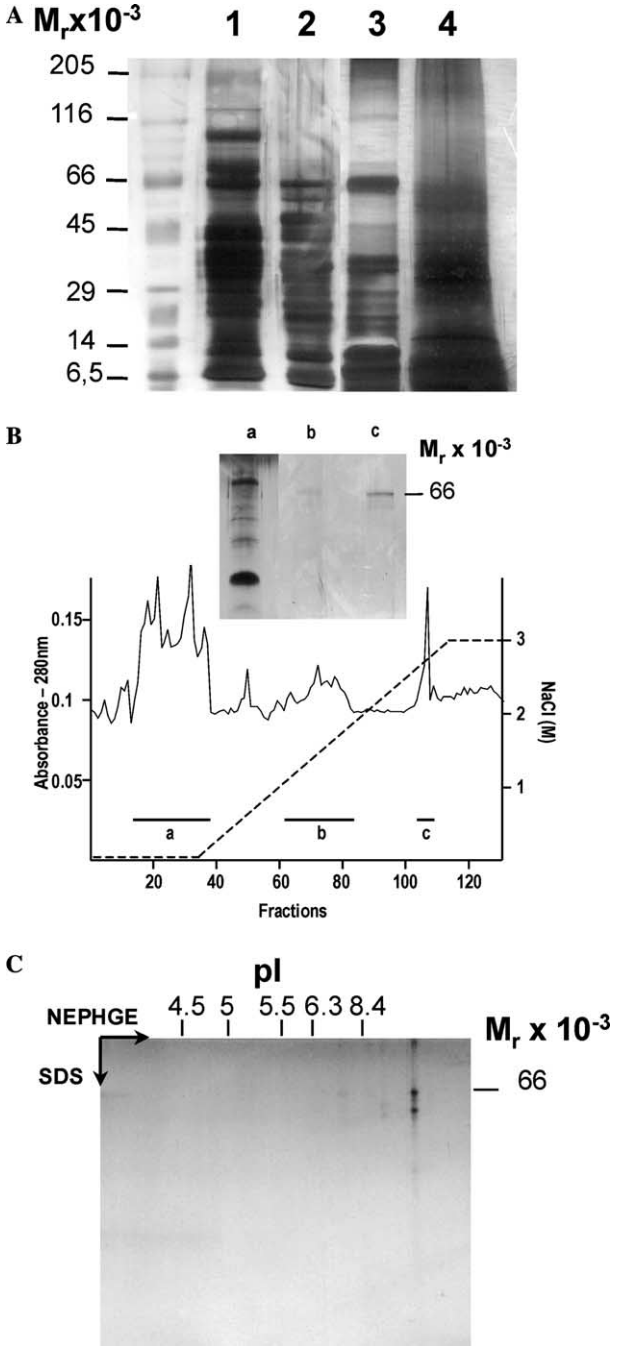


Fig. 1. Purification and characterization of a 66 kDa protein extracted from boar sperm heads with 1 M sodium carbonate, pH 11. (A) Silver stained SDS–PAGE (8–16% linear polyacrylamide gradient) of proteins obtained by successive extractions from boar sperm heads with 0.2% Triton X-100 (extract E1, lane 1); 1 M NaCl (extract E2, lane 2); 1 M $Na_2CO_3$, pH 11 (extract E3, lane 3); and 0.1 M NaOH (extract E4, lane 4). Left side: molecular weight markers. (B) Elution curve of proteins from extract E3 on a 1 ml Hi-Trap SP column, with a linear 0.05–3 M NaCl gradient. Inset: controls of peaks a, b, and c by SDS–PAGE (12% acrylamide). The molecular weight of the protein present in peak c is indicated on the right side of the gel. (C) Two-dimensional gel electrophoresis of protein from peak c (NEPHGE in the first dimension, SDS–PAGE in the second dimension). Apparent isoelectric points, as deduced from the position of marker proteins (on a gel run in parallel), are indicated at the top.

which estimated an isoelectric point of about 10, a basic p*I* only reported in sperm heads for cylicins [7,8].

The 66 kDa protein was digested with trypsin and resulting peptides were separated by reversed-phase HPLC: several peaks were obtained, most of them corresponding to mixtures of small basic peptides rich in lysine. Two main peptides were isolated and sequenced. Their sequence is shown in Fig. 2. Sequences of peptides P1 and P2 match with those of human and bovine cylicin II [8]: 99% homology (91% identity) for peptide

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Peptide P1                  I S E K P S V Y L A A R
Human, bovine cylicin II  84 I S E R P S V Y L A A R 95
                             * * * + * * * * * * * *

Peptide P2                  K K P S E A D T E S K D T K
Human cylicin II          275 K K P S S T D S D S K D D V 288
                             * * * *   * + + * * *
Bovine cylicin II         447 K K P G E A E S E P K D S A 460
                             * * *   * * + + *  * *  +
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Fig. 2. Sequences of two peptides (P1 and P2) isolated from the 66 kDa protein of boar sperm heads and corresponding sequences of human and bovine cylicin II. Identical residues are indicated by an asterisk, conservative changes by a +, and non-conservative changes are printed in bold type.

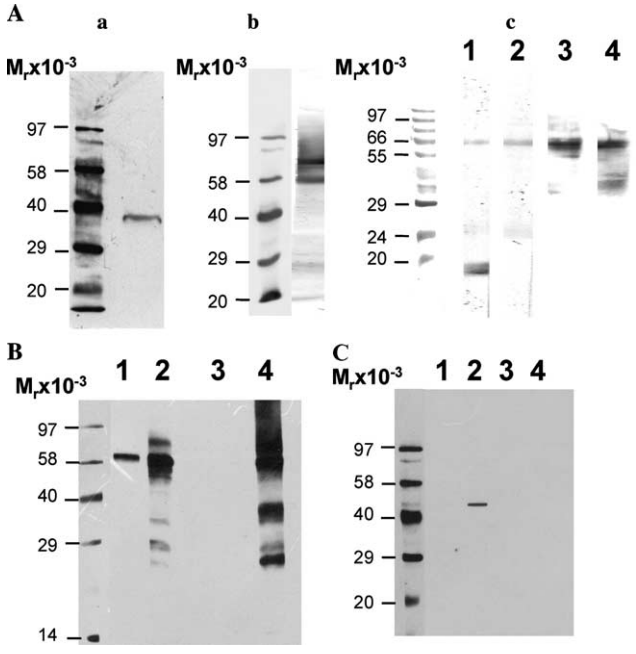


Fig. 3. Immunological characterization of cylicin II (A), calicin (B), and actin (C). (A) Immunoblot analysis with anti-peptide antibodies specific for human cylicin II. (a) Whole proteins from human sperm (12% SDS polyacrylamide gel); (b) whole proteins from boar sperm heads (12% SDS polyacrylamide gel); and (c) proteins successively extracted from boar sperm heads with 0.2% Triton X-100 (extract E1, lane 1); 1 M NaCl (extract E2, lane 2); 1 M $Na_2CO_3$, pH 11 (extract E3, lane 3); and 0.1 M NaOH (extract E4, lane 4) (8–16% linear gradient SDS polyacrylamide gel). (B) Immunoblot analysis of extracts E1–E4 (lanes 1–4) with anti-calicin antibodies. (C) Immunoblot analysis of extracts E1–E4 (lanes 1–4) with anti-actin antibodies.

P1; 82% homology (66% identity) for peptide P2. Homology of the 66 kDa protein with human and bovine cylicin II was confirmed by the use of antibodies specific for human cylicin II (see the reactivity of these antibodies with 39 kDa human cylicin II [8], in Fig. 3A, part a). In a protein extract from boar sperm heads (Fig. 3A, part b), these antibodies reacted with a main 66 kDa band and with another component (55 kDa), probably corresponding to a proteolytic product (also present in the purified protein preparations, see Fig. 1C). The proteins obtained by successive extractions from boar sperm heads were also probed with anti-human cylicin II antibodies (Fig. 3A, part c). A strong reaction with the main 66 kDa component of the 1 M sodium carbonate extract E3 was found (Fig. 3A, part c, lane 3). The 66 kDa protein purified from extract E3 by ion-exchange chromatography was found to react with anti-human cylicin II antibodies (not shown). Taken together, the results showed that the 66 kDa protein is the porcine homologue of human and bovine cylicins II, with a size more closely related to that of bovine cylicin II [7,8]). The presence of calicin, a main protein of boar sperm heads with actin-binding properties [27], and the presence of actin were also analyzed by immunoblotting. Neither calicin nor actin was found in extract E3. Calicin (Fig. 3B) was mainly present in the proteins removed from sperm heads with 0.1 M NaOH (Fig. 3B, lane 4), but also found in the proteins extracted either with Triton X-100 or with 1 M NaCl (Fig. 3B, lanes 1, 2), in agreement with previous results [27]; actin was only found in the 1 M NaCl extract (Fig. 3C, lane 2).

### *Actin-binding ability of porcine cylicin II*

Prior to actin-binding studies, protein fractions removed from boar sperm heads in alkaline conditions (extracts E3 and E4, expected to be perinuclear theca proteins) were analyzed for their solubility in the buffer used for cosedimentation assay, in the absence of F-actin. Proteins removed with 0.1 M NaOH were insoluble in cosedimentation buffer and thereby cosedimentation assay with F-actin could not be performed with proteins from extract E4. Most of the proteins from extract E3 (i.e., proteins removed with 1 M sodium carbonate, pH 11) were found soluble in the absence of F-actin (Fig. 4A, part a, -actin); pellets obtained in the presence of F-actin contained most of the 66 kDa band (Fig. 4A, part a, +actin). This band reacted with

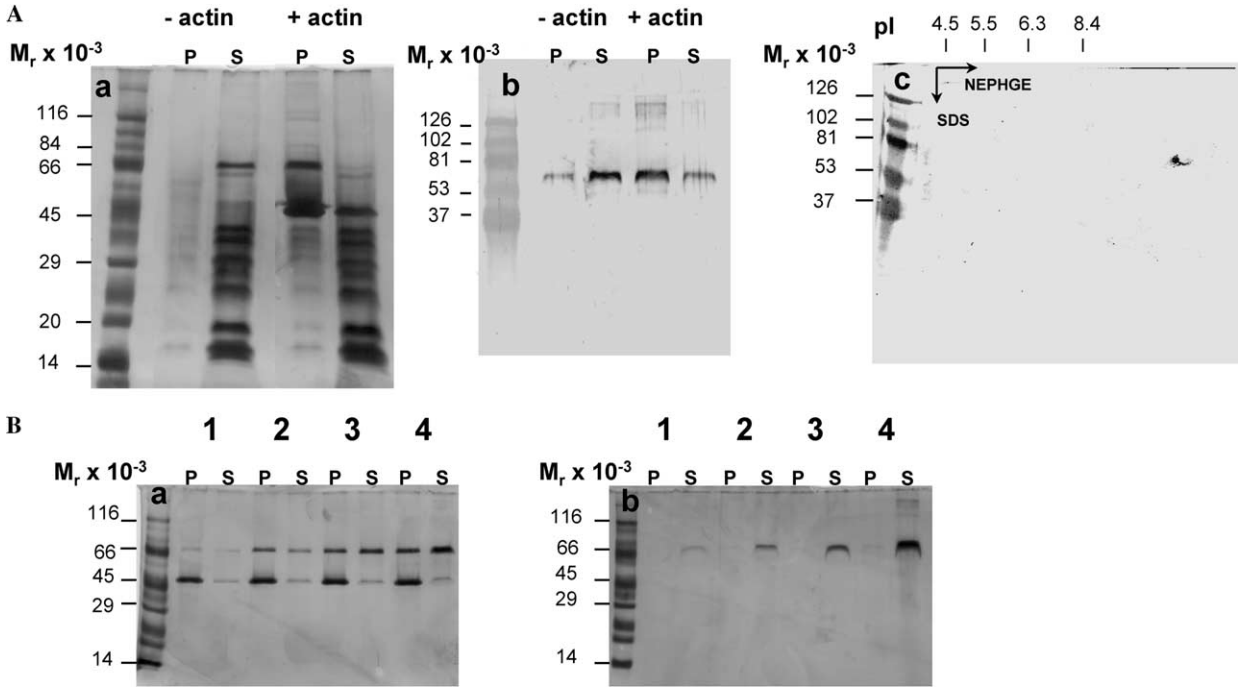


Fig. 4. Actin-binding studies of porcine cylicin II. (A) Cosedimentation with F-actin of proteins extracted from boar sperm heads with 1 M sodium carbonate buffer, pH 11. (a) Silver stained SDS–PAGE (8–16% linear gradient polyacrylamide gel) of pellets (P) and supernatants (S) of proteins incubated without (– actin) or with (+ actin) preformed actin filaments; (b) corresponding immunoblot with antibodies specific for human cylicin II. Left side: molecular weight markers; (c) immunoblot of two-dimensional gel (NEPHGE in the first dimension, SDS–PAGE in the second dimension) of proteins of the F-actin pellet probed with anti-human cylicin II antibodies. Apparent isoelectric points, as deduced from the position of marker proteins (on a gel run in parallel), are indicated at the top. Left side: molecular weight markers. (B) Cosedimentation of boar cylicin II with F-actin. Purified protein was incubated with 1 μM F-actin (a) or without F-actin (b). Pellets (P) and supernatants (S) were resolved by SDS–PAGE (8–16% linear gradient polyacrylamide gel) and visualized by silver staining procedure. Purified protein was at final concentration: 0.018 μM (1); 0.0371 μM (2); 0.0725 μM (3); and 0.15 μM (4). Left side: molecular weight markers.

antibodies specific for human cylicin II (Fig. 4A, part b, +actin). Proteins of the F-actin pellet were resolved by two-dimensional gel electrophoresis; a spot of $M_r$ 66 kDa and *p*I of about 10 was detected by anti-human cylicin II antibodies (Fig. 4A, part c). Cosedimentation studies with the purified 66 kDa protein homologue of human cylicin II confirmed its actin-binding ability (Fig. 4B); the binding was found saturable; the dissociation constant was estimated to be about 7 nM.

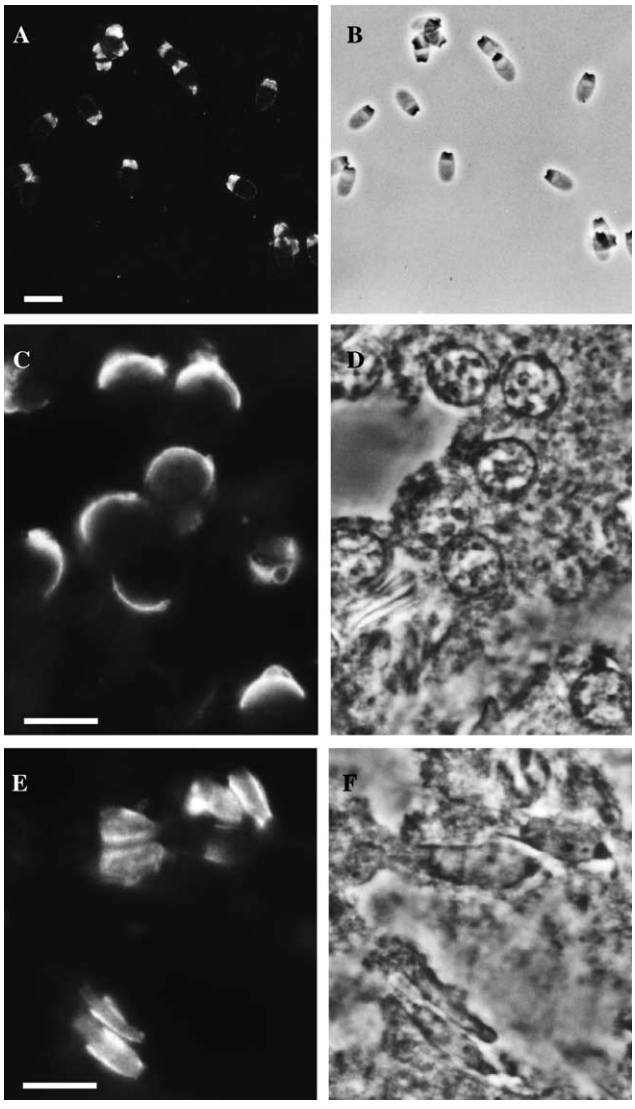


Fig. 5. Immunohistochemical characterization of cylicin II. (A,B) Immunofluorescence localization in spread preparations of epididymal boar sperm heads. (A) immunofluorescence detection with anti-cylicin II antibodies (antibodies to peptide P2 of porcine cylicin II); (B) corresponding phase-contrast micrographs. Scale bar: 10 μm. (C–F) Immunofluorescence localization in boar testis. The testicular tissue was fixed with Bouin and paraffin-embedded. Sections (4 μm) were labelled by indirect immunofluorescence with anti-cylicin II antibodies (antibodies to peptide P2 of porcine cylicin II). (C,E) immunofluorescence detection of cylicin; (D,F) phase-contrast micrographs of the same fields. Note, in C, the acrosomal staining of round spermatids, and, in E, the acrosomal and postacrosomal staining of elongating or elongated spermatids. Scale bar: 10 μm.

*Immunolocalization of cylicin II in boar sperm and testis*

Localization of the porcine homologue of bovine and human cylicin II was studied in boar sperm and testis. Antibodies to human cylicin II and antibodies to peptide P2 of porcine cylicin II were used; the same results were obtained with both antibodies. Immunofluorescence microscopy on boar sperm heads (Figs. 5A and B) showed the labeling of the calyx structure; for some sperm heads, a punctated staining in the acrosomal region was also found. Immunofluorescent staining of boar testis sections showed the presence of the porcine homologue of cylicin II in a region corresponding to the acrosome of round spermatids (Figs. 5C and D). In elongating or elongated spermatids (Figs. 5E and F), immunostaining was localized either both in the acrosomal and in the postacrosomal regions or restricted to the postacrosomal region.

## Discussion

One of the main purposes of this study was to identify the actin-binding properties of proteins that compose the sperm perinuclear cytoskeleton. Extraction of sperm thecal proteins by the procedure of Oko and Maravei [20] which uses 0.1 M NaOH did not allow one to perform functional studies because of a protein denaturation by such extreme alcaline conditions. So, our extraction protocol included a prior removal of proteins with 1 M sodium carbonate, pH 11, a reagent already used to recover intracellular membrane proteins [35]. A 66 kDa protein, with a basic p*I* (of about 10), was characterized in proteins removed with sodium carbonate from boar sperm heads. Internal sequence studies of the protein suggested that it is the homologue of bovine and human cylicins II, already described in sperm perinuclear theca [8]. This was confirmed by showing the reaction of the 66 kDa protein with antibodies to human cylicin II, which recognized the protein in boar sperm extracts, more especially in the proteins extracted with sodium carbonate, and the purified 66 kDa protein. F-actin binding of the porcine homologue of human cylicin II was first detected by probing with anti-human cylicin II antibodies, F-actin pellets, and supernatants of proteins extracted from sperm heads with sodium carbonate. This F-actin binding property was further confirmed with the purified 66 kDa protein.

Cylicin II is a member of the multiple band polypeptides (MBP), a group of very basic proteins first characterized by Longo et al. [2]. The sequences of two MBP: cylicin I [7] and cylicin II [8], are known both in bovine and in human. Extraction of porcine cylicin II with 1 M sodium carbonate, pH 11, as well as the small amount present in 1 M NaCl extract are reminiscent of

the work performed by Longo et al. [2]; these authors reported that MBP are partially removed from bovine sperm heads with 2 M NaCl, pH 7.4.

The binding of cylicin II to F-actin is probably in part related to the polycationic nature of this perinuclear theca protein. The important role of polycationic structures for binding to acidic sites on F-actin and for bundling of actin filaments has been highlighted by Tang and Janmey [36] and Tang et al. [37]. Thus, the F-actin bundling properties of calponin or MARCKS are clearly dependent on electrostatic interactions [36,37]. The actin-binding site of MARCKS contains a large number of lysine residues; some peptide sequences of bovine and human cylicin II (for example, sequence 20–27) share a weak homology with the binding site of MARCKS. Tang and Janmey [36] also reported that polymers of lysine or arginine are able to bundle actin filaments, without the presence of an identified actin-binding site, and with a $K_d$ in the micromolar range. Results from our study show that porcine cylicin II has a high affinity for F-actin ($K_d$ about 7 nM) and thereby suggest the presence of a specific actin-binding site rather than non-specific electrostatic interactions.

Inspection of the amino acid sequences of bovine and human cylicin II and computer-assisted comparison with protein sequences in all databases did not show any obvious relationship with known actin-binding proteins, except with caldesmon. Nevertheless, no clear homology with the two actin-binding sites of caldesmon was found [38,39]. Bovine and human cylicins II [8] are basic proteins composed of an amino-terminal part (about 100 amino acids in bovine cylicin II), a central part arranged in nine repeating units in bovine cylicin II and largely deleted in human cylicin II, and a carboxy-terminal part containing sequences resembling nuclear localization signals [40]. We are now investigating which part of cylicin II binds to F-actin, the exact localization of the actin-binding sequences, and whether cylicin I, the other member of MBP family, is an actin-binding protein.

Cylicin II is the third protein of the perinuclear cytoskeleton with actin-binding properties. The two others are: calicin [28] and testis-specific capping protein, an heterodimer formed by the association of testis-specific isoform subunits: CPα3 [11,12] and CPβ3 [13] (although the direct binding of this putative F-actin capping protein has not been demonstrated by in vitro experiments). Another recently described candidate is testis fascin FSCN3 [41]; however, its localization in the perinuclear theca has not been demonstrated, either by stepwise extractions from sperm heads or by immunoelectron microscopy. The respective role of actin-binding proteins of the perinuclear theca is not clearly defined during spermiogenesis. Using anti-peptide antibodies specific for cylicin II, we have observed an immunocytochemical localization in testis similar to the one previously reported for MBP [9]. More especially, an acrosomal localization in round spermatids was found, a localization also reported for actin [22–24,28], for calicin [28], and for CPα3 [12]. We have suggested that actin-binding properties of calicin allow its targeting at the subacrosomal space of round spermatids [28], a region filled with F-actin. Such a hypothesis is also relevant for cylicin II. Nevertheless, this protein may have additional functions, for example, binding to the nuclear membrane. Indeed, cylicin II has two potential bipartite nuclear localization signals (positions 431–448 and 461–478 of bovine cylicin II).

Another role for the actin-binding ability of cylicin II may be its interaction with actin present in mature sperm. The presence of actin and its exact localization in spermatozoa are somewhat controversial and seem to be variable according to mammalian species [23]. We have shown in our study that actin is removed from boar sperm heads together with proteins extracted with 1 M NaCl (i.e. membrane proteins [20]) while cylicin II is further extracted with an alkaline buffer of high molarity: 1 M $Na_2CO_3$, pH 11. Moreover, immunoelectron microscopic studies of boar sperm [42] have shown that actin is present, at the equatorial segment, between plasma membrane and outer acrosomal membrane, while studies on cylicins [2,9] have localized these proteins in the postacrosomal segment (or calyx) of spermatozoa from various mammalian species. Thereby, a direct association of actin and cylicin II in mature boar sperm seems unlikely.

At last, it is important to note that sperm perinuclear cytoskeleton needs to be removed from sperm heads at the early steps of sperm entry in the oocyte. This removal seems to be a crucial prerequisite for sperm nuclear decondensation and chromatin remodeling [43,44] and depends on the presence of cortical F-actin in oocyte microvilli [43]. This process is delayed in the case of intra-cytoplasmic injection of a sperm cell into the oocyte [44]. Whether actin-binding properties of some sperm cytoskeletal proteins such as cylicin II (or calicin) may be involved in the fast removal of perinuclear theca in the early events of fertilization deserves further investigations.

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