

Molecular Characterization of Mammalian Cylicin, a Basic Protein of the Sperm Head Cytoskeleton

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Abstract. The cytoskeletal calyx structure surrounding part of the nucleus of the mammalian sperm head contains two major kinds of basic proteins, i.e., the ~60-kD calicin and a group of very basic (IEP > pH 10) polypeptides ranging in size from ~58 to ~100 kD ("multiple band proteins," MBPs). We have produced MBP-specific mAbs and have isolated a bovine and a human cDNA clone encoding one of these proteins, termed "cylicin" (from the Greek word *ἡ κύλιξ* for cup or beaker). Bovine cylicin I of a calculated molecular weight of 74,788 contains a high proportion (29%) of positively charged amino acids, resulting in an IEP of 10.55, numerous KKD tripeptides, and is

characterized by an organization of the central part of the molecule in nine repeating units of maximally 41 amino acids each of which according to prediction analysis should tend to form an α helix. The identity of the polypeptide has been proven by direct amino acid sequencing of > 14 different fragments and by experiments using antibodies raised against a partial cDNA-derived protein segment produced in *E. coli*. By Northern blot analysis we have identified the 2.4-kb cylicin I mRNA only in testis. The unusual cytoskeletal protein cylicin is compared with other proteins and its possible architectural role during spermiogenesis is discussed.

DURING differentiation the cell type-specific architecture is established and maintained by structural protein assemblies, collectively referred to as the cytoskeleton. Over the past two decades major cytoskeletal elements of a number of somatic cells have been characterized, including "insoluble" filaments, such as the intermediate-sized filaments (IFs),¹ and various kinds of membrane-associated plaques such as the plasma membrane-attached desmosomal plaques anchoring the IFs (for references see Troyanovsky et al., 1993).

The molecular composition of the cytoskeleton of mammalian spermatozoa is much less clear. This is particularly true for the sperm head and its largest cytoskeletal component, i.e., "perinuclear theca" with the large funnel-shaped calyx (see Fig. 1 a) surrounding the posterior part of the nucleus ("postnuclear cap"; see Fawcett, 1975; Bellvé and O'Brien, 1983; Longo et al., 1987). By EM several substructures have been resolved within the calyx such as the plasma membrane-associated "paracrystalline sheet," the "postacrosomal sheath," and the "postacrosomal layer" which is closely applied to the nuclear envelope.

Calyx structures of bovine sperm heads have been shown to be resistant to treatment with high salt buffers and non-denaturing detergents (e.g., Bellvé and O'Brien, 1983; Longo et al., 1987). SDS-PAGE of such calyx fractions has

revealed two kinds of prominent basic polypeptides: calicin of ~60 kD and a group of "multiple band proteins" (MBPs) in the range of 58–74 kD with very similar isoelectrical charges around pH 10 (Longo et al., 1987). Using calicin-specific antibodies, Paranko et al. (1988) have then shown the widespread occurrence of this cytoskeletal protein in calices of a wide range of mammalian species, from mice to men. Using the antibodies, Escalier (1990) and Courtot (1991) have further described an altered distribution of calicin in defective ("round-headed") spermatozoa of infertile men. Similarly, MBP antisera were used to localize this protein in the postacrosomal sheath of spermatozoa of several species (Longo et al., 1987; Longo and Cook, 1991).

Because of the unusual biochemical properties of these proteins and their possible morphogenetic and architectural role during spermiogenesis we have studied them in greater detail, using antibodies of high specificity and cDNA clones. Here we report the identification and molecular biological characterization of a bovine and a human MBP, designated "cylicin I," which represents a novel kind of protein.

Materials and Methods

Calyx Isolation

The isolation of calices from bovine sperm was done as described (Longo et al., 1987) with minor modifications. All solutions except the sperm collecting buffer (PBS, 25 mM EDTA) contained 5 mM DTT, 1 μ g/ml leupeptin and 1 μ g/ml pepstatin.

1. *Abbreviations used in this paper:* IF, intermediate-sized filament; MBP, multiple band protein.

Gel Electrophoresis and Immunoblotting

Proteins of calyx fractions were separated by two-dimensional gel electrophoresis with NEPHGE in the first, and SDS-PAGE in the second dimension (for details see Longo et al., 1987). The separated polypeptides were transferred to nitrocellulose sheets and visualized by Ponceau S staining. The nitrocellulose filters were incubated for 2–4 h with PBS containing 0.05% Tween at room temperature, to reduce the background of nonspecific binding. Antibodies bound were visualized by alkaline phosphatase-coupled second antibodies (Promega via Serva, Heidelberg, FRG).

Protein Sequence Analysis

Calices were isolated and their polypeptides separated by two-dimensional polyacrylamide gel electrophoresis (see Longo et al., 1987). Polypeptide spots were excised from the gels after staining with Coomassie blue. 8–10 individual samples of a specific spot were then combined and digested in the gel matrix (Eckerskorn and Lottspeich, 1989) using different proteases (sequencing grade) obtained from Boehringer Mannheim (Mannheim, FRG) or Promega (via Serva). The subsequent analytical procedure included several modifications (the contribution of A. Bosserhoff and R. Frank, Center for Molecular Biology, University of Heidelberg, FRG, is gratefully acknowledged). For example, reaction tubes and pipet-tips were coated with polypropylenglycol (PPG 4000), gel pieces were allowed to shrink with pure acetonitrile, and residual SDS was extracted with *n*-heptane:3-methyl-1-butanol (4:1).

The peptides obtained were separated by HPLC, using either a Brownlee C18 or C4 column (220 × 2.1 mm) and a 130A HPLC separation system (Applied Biosystems, Weiterstadt, FRG). Material of selected peaks was rechromatographed using a Brownlee C8 column (100 × 2.1 mm) in 20 mM sodium acetate-water as solvent A and 15 mM sodium acetate in 80% acetonitrile as solvent B. The HPLC-separated fragments were either frozen and stored at –20°C or directly sequenced on Polybrene treated filters ("BioBrene," Applied Biosystems) using a 477A protein sequencer apparatus from Applied Biosystems. Alternatively, samples were electro-transferred to Immobilon (Millipore, Molsheim, France) or to ProBlot (Applied Biosystems) membranes and amino acid sequences of native amino termini or of fragments produced during staining with Ponceau S in 1% acetic acid were determined.

Antibodies

For the preparation of murine mAbs against bovine calyx proteins a 6-wk-old female Balb/c mouse was immunized by a subcutaneous injection with 50 µg of bovine calyx material isolated as described (see above). The protein material was resuspended in 250 µl 8 M urea, 10 mM Tris-HCl, pH 7.4, mixed with the same volume complete Freund's adjuvant (Sigma, Munich, FRG) and emulsified. Booster injections were given subcutaneously on day 28 in incomplete Freund's adjuvant and intraperitoneally on day 56 in PBS, without adjuvant. Spleen cells harvested on day 59 were fused with cells of the mouse myeloma line X63-Ag8.653 at a ratio of 3:1 in the presence of 40% polyethylenglycol 4000 (Roth, Karlsruhe, FRG). Hybridoma cell clones were selected as described (Moll et al., 1992) and medium was tested by immunoblots and by immunofluorescence microscopy using methanol-acetone fixed bovine spermatozoa collected from epididymides. Positive clones were subcloned twice and immunoglobulin subclasses determined as described (Schmelz et al., 1986). In the present study the mAbs Cal-22 (IgG1) and Cal-144 (IgM) were used in most experiments.

Specific guinea pig antisera (for immunization and isolation see

Benavente and Krohne, 1986) against cylicin were prepared using "partial proteins," synthesized in *E. coli* transfected with subclones from the partial cDNA-clone H23 (1.9 kb, see below), using the pOE-bacterial expression vector system of Qiagen (Diagen, Düsseldorf, FRG).

Construction of an Expression Library, Screening, and Sequencing

A guinea pig antiserum raised against a mixture of calicin and some MBPs obtained by dissection of a fraction of SDS-PAGE-separated calyx-proteins was used for screening a λgt 11 human testis cDNA expression library (Clontech via ITC Biotechnology, Heidelberg, FRG). A partial cDNA-clone (1.4 kb; the contribution of Dr. Paranko is gratefully acknowledged) was used for re-screening the same library, and two longer cDNA-clones H6 and H23 (both 1.9 kb), were obtained. These were subcloned in the M13mpl8 and M13mpl9 vectors (Yanisch-Perron et al., 1985), and one of them (H23) was sequenced in both directions using the "T7 sequencing kit" of Pharmacia (Freiburg, FRG).

The human cDNA-clone H23 was then used to screen a bull testis library prepared as follows. Internal parts of bull testis, rich in tubuli seminiferi, were frozen in liquid nitrogen, and RNA was isolated as described (Kreis et al., 1982; Magin et al., 1983). Poly(A)⁺ RNA was prepared ("mRNA purification kit"; Pharmacia), and cDNA was made from 5 µg poly(A)⁺ RNA using oligo-dT as primer and the "time saver" cDNA-kit from Pharmacia. The cDNA was inserted into the EcoRI site of the λ ZAP II vector (Stratagene GmbH, Heidelberg, FRG). For in vitro packaging the "gigapack II gold" packaging extract from Stratagene was used. 3 × 10⁶ phages were used for amplifying and 1 × 10⁶ phages of the amplified library were plated together with *E. coli* XL1-blue cells (Stratagene) onto LB-agar plates (50,000 plaque forming U/plate). For screening of plaques, the plates were blotted on nitrocellulose filter paper, and for hybridization the radiolabeled ("random prime") partial cDNA-clone H23 was used. Plaques were purified as described (Koch et al., 1990), and cDNA-inserts isolated by the "in vivo excision method" (Stratagene). 11 clones were isolated and all clones were partially sequenced. Clone B11 (2.1 kb) was used for generating deletion clones with the "nested deletion kit" of Pharmacia and sequenced in both directions as described above. In addition, the "T7 deaza-sequencing kit" of Pharmacia was used for sequencing the 5'-region of clone B11.

Sequence analyses were done with the HUSAR (Heidelberg Unix Sequence Analysis Resources) software program package.

RNA-Isolation and Northern Blot Hybridization

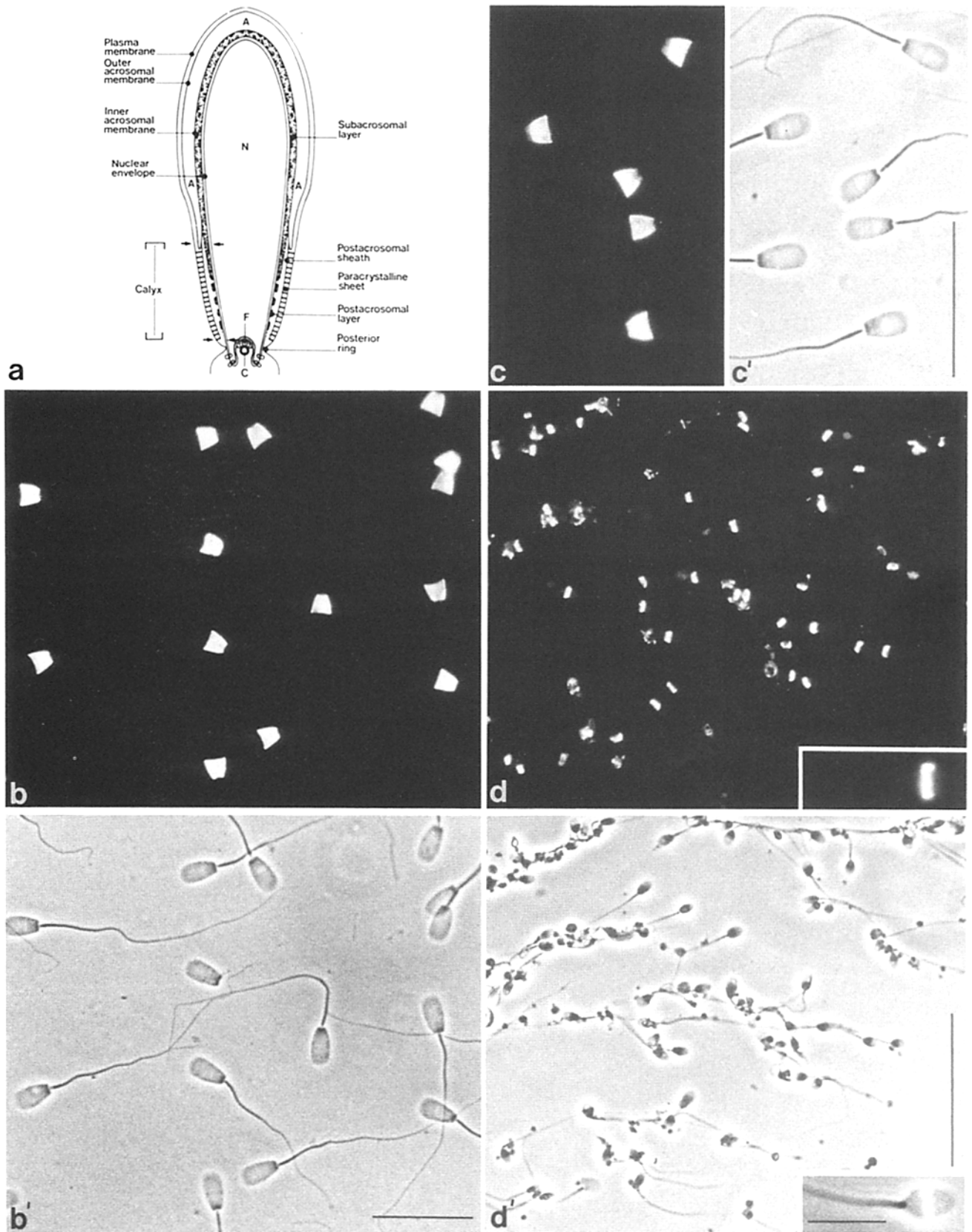
Total and poly(A)⁺ RNA was isolated as described above. RNA was denatured with glyoxal, separated on 1% agarose gels, transferred to Biotyde A filters (Pall, Dreieich, FRG) and hybridized (see Bader et al., 1988), using radiolabeled "antisense" RNA obtained by in vitro transcription (Bluescript manual; Stratagene) of cDNA-clone B11.

Light and Electron Microscopic Immunolocalization

Immunofluorescence microscopy with cryosections of bovine and human testes and methanol-acetone fixed bovine sperm from epididymides or ejaculated human spermatozoa, kindly provided by free healthy donors, was done as described (Longo et al., 1987).

Pieces of bovine testicular tissue were obtained from freshly killed young bulls (see Longo et al., 1987). For electron microscopic localization ~5-µm-thick cryosections of bovine epididymides were fixed for 20 min in PBS containing 2% formaldehyde freshly prepared from paraformaldehyde and

Figure 1. Schematic drawing of a mammalian sperm head (taken from Longo et al., 1987) and calyx visualization by immunofluorescence microscopy using mAbs Cal-22 and Cal-144. (a) The "perinuclear theca," a large and complex cytoskeletal component of the sperm head is subdivided into the anterior "subacrosomal layer" and the posterior calyx with the "postacrosomal layer" closely applied to the nuclear envelope and the "postacrosomal sheath," which is connected to the plasma membrane by the ridges of the "paracrystalline sheet." The posterior ring at the bottom of the sperm head and the various membranes of the sperm head are also indicated. *N*, nucleus; *A*, acrosomal interior; *F*, implantation fossa; *C*, centriolar complex. (b–d') The cylicin-containing structures of spread bovine (b–c') and human (d and d') spermatozoa are decorated by mAb Cal-144 (b and d; b' and d' show the corresponding phase-contrast pictures) or mAb Cal-22 (c, epifluorescence; c', phase contrast optics). The "equatorial belt" arrangement of the calyx structure is shown at higher magnification in the specific inset (d and d'). The bovine sperm-specific second mAb Cal-22 reacts also with the calyx of spread bovine sperm from the epididymides (c, immunofluorescence; c', phase-contrast). For reasons unexplained some individual spermatozoa show no reaction with Cal-22. An example is seen in c and c' at the lower left margin. Bars: (b' and c') 25 µm; (d) 50 µm; (inset) 10 µm.



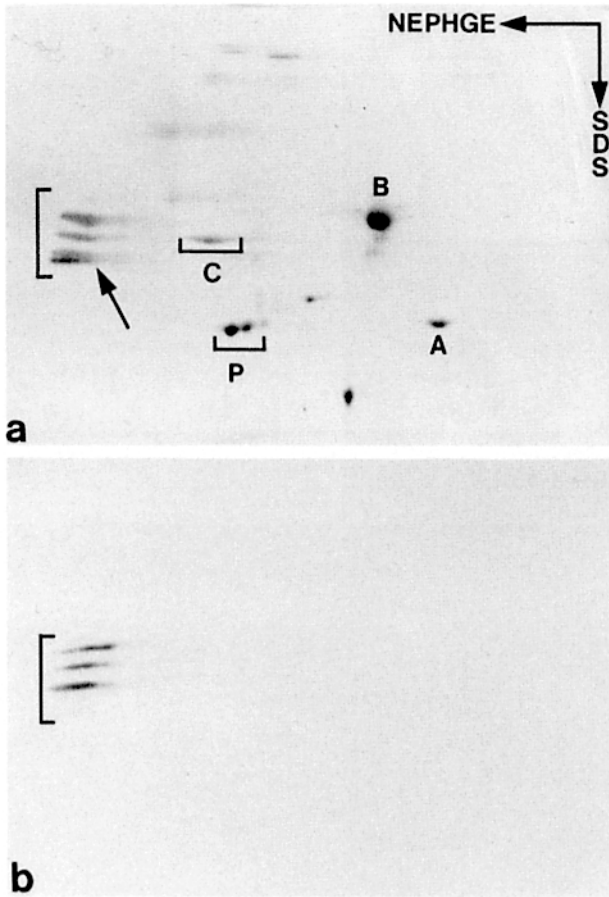


Figure 2. Immunoblot of the calyx fraction proteins of bovine spermatozoa, separated by NEPHGE (horizontal arrow) in the first and SDS-PAGE (downward arrow) in the second dimension and stained with Coomassie blue (a) or transferred to nitrocellulose sheets (b) and reacted with the mAb Cal-22 and visualized by secondary reaction with goat antibodies to murine immunoglobulins coupled to alkaline phosphatase. The bracket denotes the series of major multiple band proteins at the basic end of the gel of estimated M_r values of ~58, ~63, ~69 and ~74 kD. Coelectrophorized reference proteins are BSA (B), actin (A), phosphoglycerokinase (P), and bovine sperm calicin (C; see Longo et al., 1987). Note the reaction with most of the MBP components.

subsequently permeabilized by incubation in PBS containing 0.1% saponin for 10 min. After fixation the cryosections were incubated for 2–4 h with the guinea pig antibodies, washed in PBS, incubated with the second gold-conjugated antibody, fixed again, and treated with the “silver enhancement” method as described (Franke et al., 1984; Blessing et al., 1993).

Results

Monoclonal Antibodies

In immunofluorescence microscopy mAbs Cyl-22 and Cyl-144, which specifically recognize several polypeptides of the MBP group (for example see below), decorated exclusively and intensely the calyx of bovine spermatozoa (Fig. 1, *b–c'*), except for some rare individual cells (Fig. 1, *c* and *c'*). While mAb Cyl-22 was found to be specific for bovine sperm cells (Fig. 1, *c* and *c'*), Cyl-144 cross reacted with human spermatozoa (Fig. 1, *d* and *d'*), in which it immunostained a calyx-structure in the form of a near-equatorial belt (see in-

sert in Fig. 1, *d* and *d'*), as this has also been reported for calicin (Paranko et al., 1988).

On immunoblots of calyx-associated polypeptides separated by two-dimensional gel electrophoresis, mAb Cyl-22 reacted only with three of the MBPs (Fig. 2, *a* and *b*), whereas Cyl-144 reacted with all discernible MBPs of 58, 63, 69, and 74 kD (data not shown).

cDNA Cloning and Sequencing

By screening bovine and human cDNA-libraries we isolated several bovine cDNA-clones and two partial human cDNA-clones of various lengths.

Fig. 3 shows the nucleotide sequence of the bovine cDNA-clone B11 and the amino acid sequence deduced therefrom. The clone consists of 2160 nucleotides and contains a putative polyadenylation signal as well as the beginning of a poly-A tail, indicating that it represents most of the corresponding mRNA of 2.4 kb detected in Northern blots (see below). Taking the first possible start codon of the open reading frame as translational start site, the polypeptide consists of 667 amino acids with a calculated molecular weight of 74,788 and contains an exceptionally high (45%) proportion of charged amino acids of which two thirds (29%) are basic (estimated isoelectric point pH 10.55) and a high number of putative phosphorylation sites. The difference of this calculated molecular weight and the 58 kD estimated from SDS-PAGE might be due to the high charge of this molecule although we cannot formally rule out the existence of a precursor protein.

The identity of the polypeptide encoded was established by amino acid sequencing of peptides proteolytically derived from all the major and minor basic polypeptide spots visual-

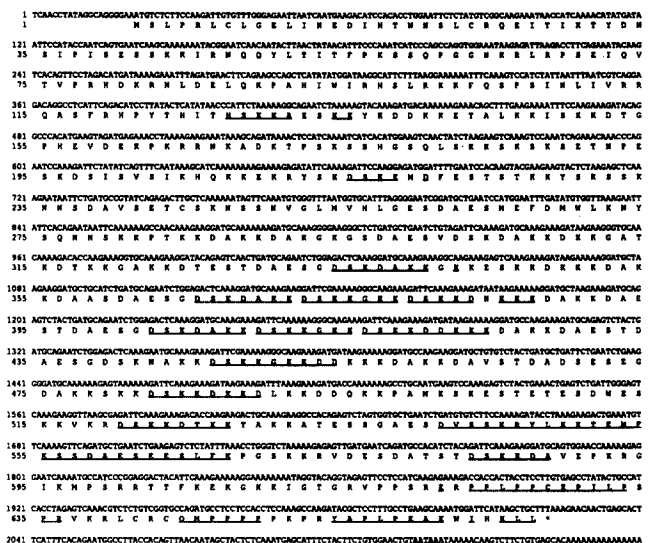


Figure 3. Nucleotide sequence (upper lines and numbers) and deduced amino acid sequence (one letter code, lower lines and numbers) of cDNA-clone B11 encoding cylicin I. The cDNA clone B11 of 2,160 nucleotides contains a putative polyadenylation signal (underlined) and the beginning of the poly(A) tail. Taking the first possible start codon of the open reading frame the encoded polypeptide consists of 667 amino acids (asterisk indicates stop codon). Amino acid sequences of a number of proteolytically derived peptides of cylicin I are underlined. The sequence data are available from EMBL under accession number Z22779.

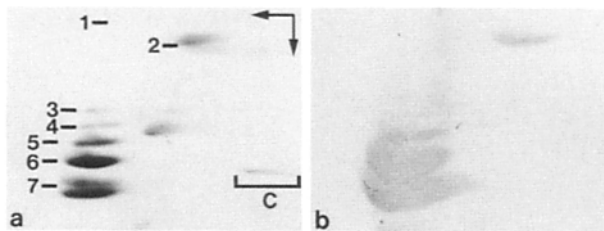


Figure 4. Gel electrophoresis separation of bull sperm calyx proteins and immunoblot reaction of a guinea pig antiserum raised against recombinant human cyclin. A number of Coomassie blue-stained basic polypeptides are visible after two-dimensional gel electrophoresis of bovine calyx proteins (*a*; NEPHGE, *horizontal arrow*; SDS-PAGE, *downward arrow*). The numbers in *a* designate the specific MBPs (MBP1 was not observed in every preparation), most of which cross react with guinea pig antibodies raised against bacterially expressed human cyclin (*b*; alkaline phosphatase reaction).

ized on two-dimensional gel electrophoresis (Figs. 2 and 4). Amino acid sequences of the major polypeptides (for numbering see Fig. 4 *a*) are presented in Table I. The only polypeptide presenting sequences occurring in the translation product shown in Fig. 3 is the component "MBP7" of which a total of nine distinct proteolytic peptide fragments and more than four fragments obtained during acid hydrolysis (see Bodanszky, 1988; Stewart and Young, 1984) were identified in the cDNA-deduced sequence. This polypeptide, termed "cyclin I," was clearly different from calicin, which presented an unblocked amino terminus allowing the determination of the first 30 amino acids, and from all other MBPs, including those showing immunological cross-reaction with "cyclin I" (Fig. 4 *b* and Table I). Polypeptide MBP2 displayed one peptide sequence, that was similar to but not identical with "cyclin I," i.e., peptide 1 (Table I). While this proves the identity of the cDNA-derived sequence shown in Fig. 3 with MBP7, i.e., "cyclin I," and its difference

Table I. Amino Acid Sequences of Peptides Obtained from Major Polypeptides of the Complex of MBP

Polypeptide	Sequence	Hydrolysis
MBP2	1) XLPPXP I LPXSYX I	Try
	2) SLGXGLV I SPXLASS	"
	3) EP I LG (G/M) GXPL	"
MBP5	1) XAYDNYVPVS	Chym
	2) (I /S) SGTP (Y/S) VYLAA	Try
	3) DSEAES I V	AspN
	4) DLPAVYLAA	"
	5) DLPAVY I VQ/Y	"
	6) DNYVPVS	"
	7) DVNA	"
MBP6	1) YLDX I NLVY	Chym
	2) XKPXLLQ	Try
	3) SKPSLLQENK	"
	4) I S (G/E) XPSVYLAA	"
	5) DSEASX I VS	AspN
	6) DAKQAAPSSPPV	"
MBP7 Mixture	1 5 6 7 8 9	Acid
	DSKK (K/G/D/A) (K/A/D/T) (K/T) (K/D) (K/A/D)	Acid
	DSKD "	
	DSKE "	
	HSKK "	
Distinct peptides	1) XAPLPXAKX I XKLL	ArgX
	2) YAPLPEAK	Try
	3) KTEMFK	"
	4) YLKKTEMFK	"
	5) XQMPPPP	"
	6) EXPPLPPCEP I LPXPR	"
	7) SSDAESEEXLF	"
	8) DAESEESLFPKGSKKRV	AspN
	9) DVSSKRYLKKTEMFK	"

Major basic polypeptides of bovine sperm head calices were excised from polyacrylamide gels after two-dimensional gel electrophoresis, digested with various proteases (listed under "Hydrolysis"), and the peptides obtained sequenced. Only the amino acid sequences, which were obtained from MBP7 occur within the translate of cyclin I cDNA (see Fig. 3). In addition, we observed mixtures of peptides produced during treatment with Ponceau S in 1% acetic acid on Immobilon membranes (*Acid*), which reflect the acid labilities at aspartyl residues (see Bodanszky, 1988; Stewart and Young, 1984). These mixtures of peptides are due to the occurrence of nine repeating units in the central part of the molecule (see Figs. 3 and 5). The number above the sequence correspond to the position of the specific amino acids within the peptide (alternatives in brackets). X, unidentified amino acid; Try, trypsin; Chym, chymotrypsin; AspN, endoprotease AspN; ArgX, endoprotease ArgX.

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1  MSLPRCLGELINEDIHTWNSLCRQEITIKTYDNSIPISSESKIRNQOY
.....
51  LITIFPKSSQPGGNKRLRPEIQVTVPRHRDKRNLDLOKPAHIWRHSLR
1  .....GQITVTRHRDKRLEEGQKPAHKWIRHSFR
.....
101  KKFQSPSINLIVRRQASFRHPYTHITHSKKAESKYYKDDKKETALKKISK
30  KILQWPPITYAAREQTPFRHLYTSKTHLKAEEKSKDEKGGTPLKKDSK
.....
151  KDTG----PHE---VDEKPKRRNKADKTPSKSSHGSQLSKSKSKSETN
80  KKGGSYATNPESQIIVEEKKRQNEADKTPKLSHENEQSKSKSSSETN
.....
193  PESKDSISVSIKHO-KKEKRYSKDSKEMD--FESTST--KKYSKSSKNNS
130  PESQNSKTVS-KNCSQKDKDSKSKKNTTEFLHTKNNPKKDLKRSKTSN
.....
238  DAVSETCSKNSSNVG-LMVHLGESDAESMEFDMWLKNYSQNNKPKTKKD
177  DPISIEICSENSLNVDLML-VGQSDDESINFDAWLRNYSQNNKSNYS-
.....
283  AKKD-----AKGKG--SDAESVD-SKD  RU 1
225  LKY-TKY-TKGD--TKKN----AKK--SSDAESD-SKD
.....
306  AKKD-KKGATKD--TKKG-----AKDTESTDAESGD-SKD  RU 2
253  AKKDSK-KVKN--VKKDDKKKDKKTESTDAESGD-SKD
.....
338  AKKG-KKESKKD--KKKDAKKDAA----SDAESGD-SKD  RU 3
290  ERKDTKKD-KKK--LKKDDKKKDTKKYESTDTESGD-AKD
.....
369  AKKDSKKG-KKD--SKKDNKKKDAKKDAESTDAESGD-SKD  RU 4
327  ARNDSRNL-KKA--SKNDDKKKDAKKITFSTDESESELESKE
.....
406  AKKDSKKG-KKD--SKKDDKKKDAKKDAESTDAESGD-KGD  RU 5
365  ---SKKDEKKDKKDSK-TDNKKSVKNDEESTDADSE-PKGD
.....
443  AKKDSKKG-----KKDDKKKDAKKDAVSTADSE-SEG  RU 6
.....
476  AKK-SKKDSKKDKKDLKKDDOKKPKAMKSKESTETESDWESEK  RU 7
401  SKKG-KKDEKKGKDKKDKKDKKDAKKNAESTEMESDLE-
.....
517  VKRDSKDKTKT-----AKKATESGAEEDVSSKRY  RU 8
439  LKKD-KKHSK-EKKGSKDKK-DARKDTESTDAEFDESSKTKG
.....
548  LKK-TEMFK-----SDAESSEELFKPG  RU 9
479  FK--TST-KIKGS-----DTE-SE-ES--LYKPG
.....
570  SKKRVDES DATSTDSKDDAVEPKRGIKMP SRRTTFKEGKKIGTRVPPS
501  AKKKIDESDGT SANSKMEGLESKRGRMS SKKTFNEKGEKASTGRVPPS
.....
620  RERPLPPCEPI LPSRVKRLCRQCMPPPPPKPRYAPLPEAKWIHKLL
551  REKPLPACEPSLSPKYRRLCWCKMPPPPPKPRYAPLPEAPWIHKLL

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Figure 5. Comparison of the amino acid sequence (see Fig. 3) of the bovine cDNA clone B11 (upper numbers) and the partial human cDNA clone H23 (lower numbers). Note high degree of identical (asterisks) or conservative exchange (points) amino acids and the existence of nine repeat units (RU 1-9, right margin). Identical or conservative exchange amino acids at the same position within each repeat are indicated by bold face print; in some cases there are non-homologous amino acids in bold face print, because they can be found in the repeat with almost the same frequency at this position. Horizontal bars indicate omissions introduced to optimize the alignment. The nucleotide sequence of the human cDNA clone H23 is available from EMBL under accession number Z22780.

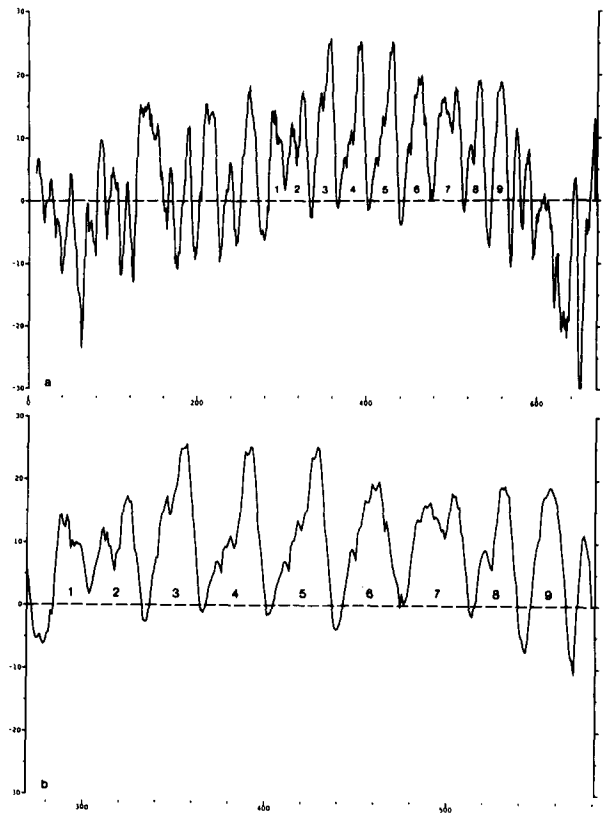


Figure 6. Prediction of the secondary structure of the deduced amino acid sequence of the cDNA clone B11. The prediction was made using the HUSAR software program package (see Materials and Methods) according to Garnier et al. (1978). (a) The most part of the molecule, except the amino- and carboxy-terminal ends, is predicted to consist of α helices, which are spaced by short "linkers." Areas above the middle line represent putative α helices. (b) A section of the middle part of the molecule shows the α helices (indicated by numbers) which represent the repeat units (see Fig. 5). The lower scale numbers in a and b represent the amino acid positions.

from all other MBPs, it does not exclude limited sequence homologies between cyclin I and the other MBPs.

Comparison of the amino acid sequences of bovine cyclin I and the sequence deduced from the 1.9-kb human cDNA-clone H23 revealed a high interspecies sequence homology between these two cyclins, showing in the comparable region a total of 61% identical and 68% homologous (including conservative exchanges) amino acids in corresponding positions (Fig. 5).

Molecular Character and Domain Organization of Cyclin

Closer inspection of the amino acid sequences of bovine and human cyclin and computer-assisted comparisons with the known sequences stored in the EMBL data library showed that the proteins of this family are not related to any other protein so far sequenced and display some very unusual sequence motifs.

The greatest contribution to the high electrical charge and the overall basic character comes from the numerous tripeptides of the type KXX, i.e., lysine dipeptide with a third variable amino acid, which in most cases is aspartic acid, result-

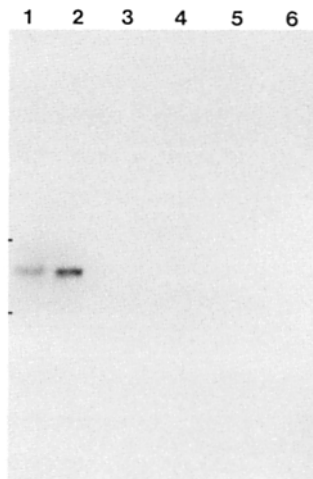


Figure 7. Autoradiogram showing the result of Northern blot hybridization of mRNA encoding bovine cylicin I in RNAs from different bovine tissues. Using an antisense riboprobe from cDNA-clone B11 the cylicin I mRNA is recognized as a 2.4-kb band in testicular RNA (20 μ g total RNA in lane 1 and 5 μ g of poly(A)⁺-RNA in lane 2) but not in RNAs from any of the other tissues (20 μ g per lane): snout (lane 3), thymus (lane 4), lymph nodes (lane 5), and spleen (lane 6). All RNAs have been routinely tested in parallel experiments, for the

presence of other protein mRNAs, notably plakoglobin (3.5 kb; see Franke et al. 1989), type 1 desmocollin (6.0 kb; see Koch et al., 1991), type 2 desmocollins (4.0 and 4.2 kb; see Koch et al., 1992), and desmoglein (7.6 kb; see Koch et al., 1990). The positions of the size markers (*E. coli* 16S [1.6 kb] and 23S [3.0 kb] rRNA) are indicated on the left margin.

ing in KKD. This particular tripeptide KKD occurs 25 times in bovine cylicin I and 21 times in the human polypeptide (Figs. 3 and 5). These KXX tripeptides are spread over most of the molecule, except for the carboxyterminal ("tail") portion of ~55 amino acids. Instead, the tail region impresses by proline-rich segments: about one third (17) of the last 50

amino acid residues are prolines with a pronounced cluster tendency (e.g., PPS, PPL, PPC, PPKPPK).

The most striking feature of cylicin, however, is the arrangement of the central part of the molecule in sequence elements forming a repeating unit (RU, Fig. 5). These repeating units with a maximal number of 41 residues vary considerably in size, the shortest being partly deleted units of only 19 amino acids. Bovine cylicin I comprises nine such units, whereas the human cylicin contains only eight. The KKD motifs extend throughout these repeating units (Figs. 3 and 5).

The conformational importance of the repeating units became also apparent from secondary structure predictions according to Chou and Fasman (1978) or Garnier et al. (1978): While the amino-terminal head and the tail domains of cylicin are clearly not arranged in α helices, also due to the frequency of proline residues in these regions, the repeating units stand out by a predicted tendency to form individual short α helices interrupted by short linker segments (Fig. 6). We are currently examining the conformation of cylicin and calicin and fragments thereof by proteolysis protection experiments and optical methods.

Testis Specific Synthesis of Cylicin as Detected by Northern Blot Hybridization and Antibody Reactions

Using the bovine cDNA-clone B11 in Northern blot hybridization experiments with various RNAs, we detected a 2.4-kb mRNA in poly(A)⁺ RNA from bovine testis but not in various other tissues (Fig. 7).

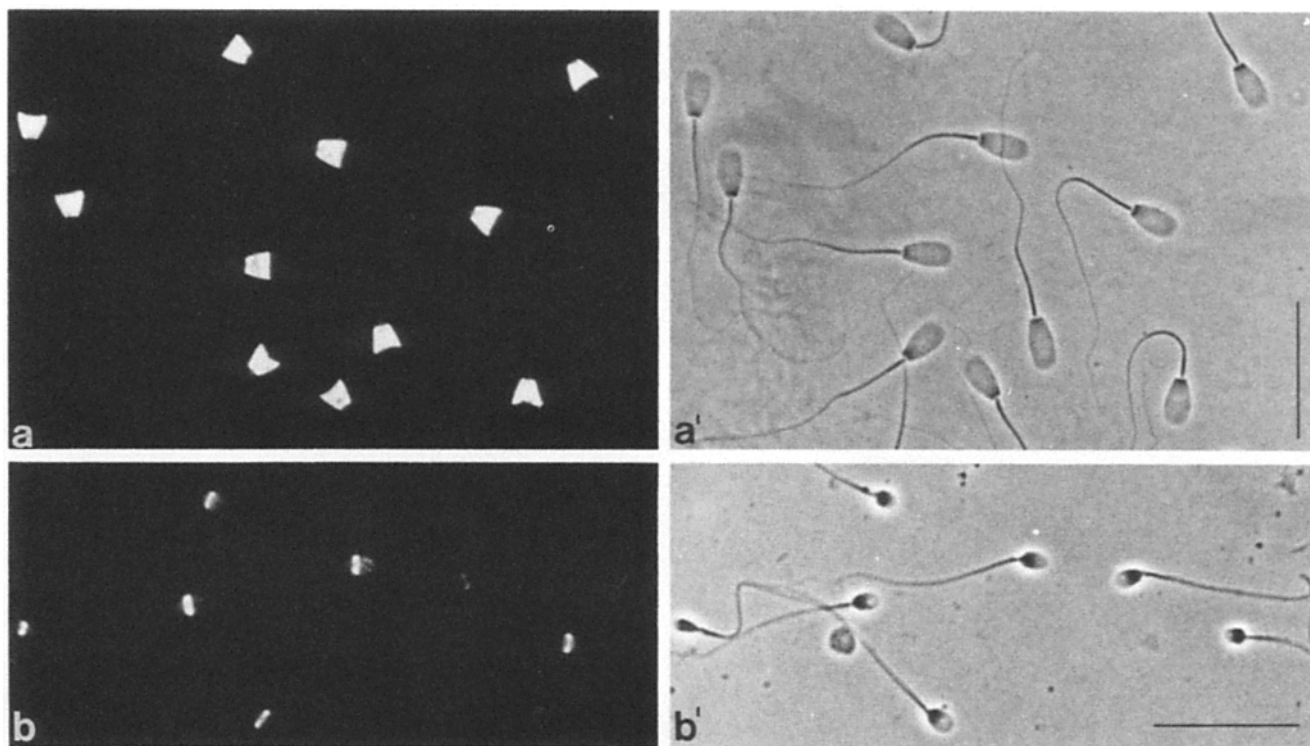


Figure 8. Calyx structure visualization of bovine and human spermatozoa with a specific guinea pig antisera by immunofluorescence microscopy. (a) The guinea pig antisera cross react to the calyx of spread bovine spermatozoa (a, epifluorescence; a', phase-contrast; compare with Fig. 1, b and b'). (b) On spread human spermatozoa the reaction results in the more belt-like appearance of the calyx structure as known from Fig. 1, d and d' (b, epifluorescence; b', phase-contrast). Bars, 50 μ m.

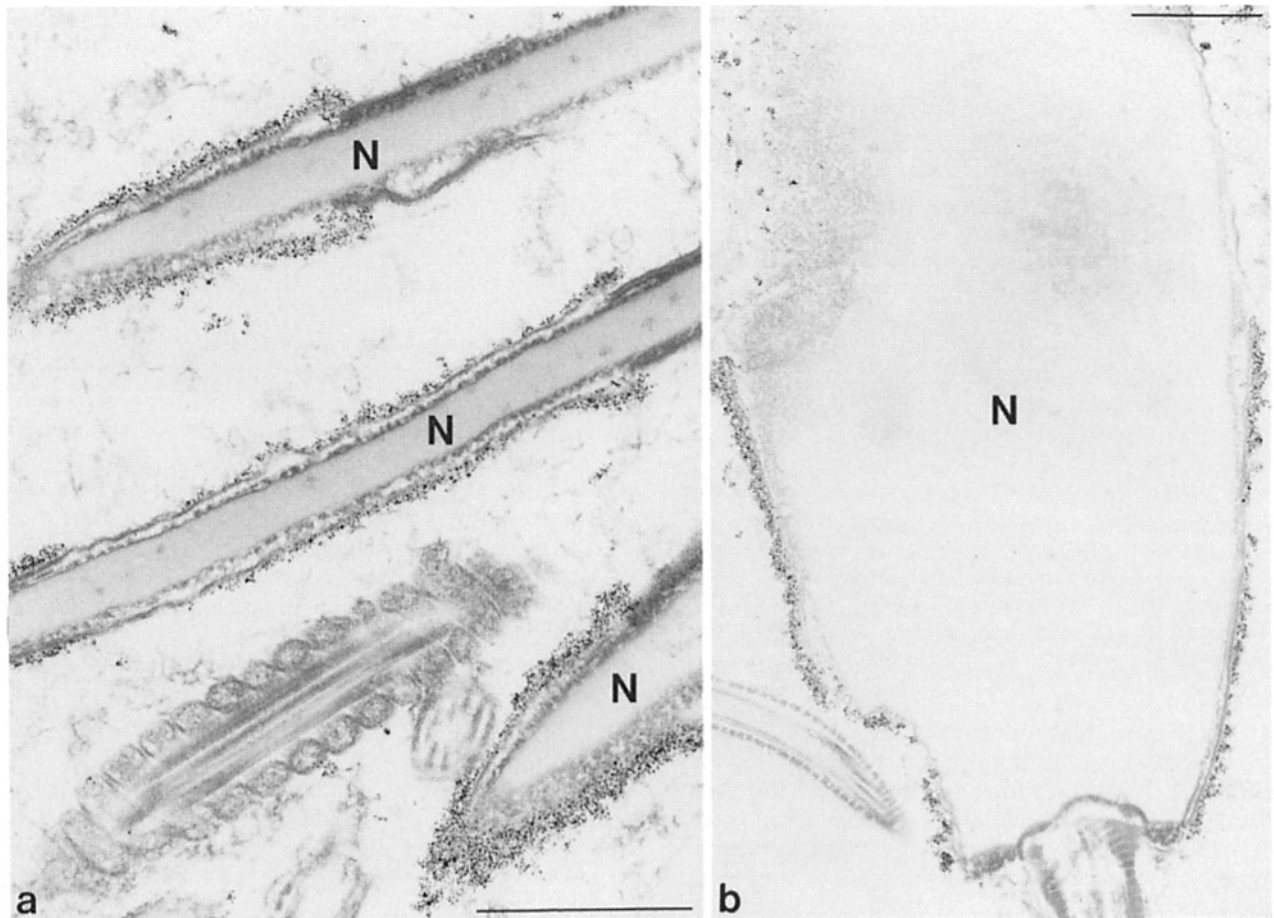


Figure 9. Electron microscopic immunolocalization in bovine spermatozoa with a specific guinea pig antisera. Cryosections ($\sim 5 \mu\text{m}$) of bovine sperm from the epididymides were used for the immunogold localization with a cylicin specific antiserum. Longitudinal (*a*) or frontal (*b*) sections of bovine sperm heads show a strong immunogold-labeling of the postacrosomal sheath in the electron microscope, indicating an association of cylicin with the calyx. *N*, nucleus. Bars, $1 \mu\text{m}$.

Specific guinea pig antibodies raised against partial polypeptides of human cylicin as encoded by clone H23 reacted with two polypeptides of ~ 60 and ~ 80 kD (data not shown) and showed cross-reactions in immunoblots with bovine cylicin I from total spermatozoa (data not shown).

The specific association of cylicin with the calyx was demonstrated by immunofluorescence microscopy using these antibodies which decorated the calyx of spread bovine and human spermatozoa (Fig. 8) and of spermatids and maturing spermatozoa in frozen sections of testicular tissue (data not shown). While in bovine sperm the cylicin-positive structure appeared in the typical funnel shape (Fig. 8 *a*), human sperm cells again revealed an immunostained near-equatorial belt (Fig. 8 *b*).

Using these antibodies for immunolocalization in the electron microscope, we observed a strong and specific immunogold labeling of the dense calyx structures in the posterior head domain of bovine spermatozoa (Fig. 9). In human sperm, the more belt-like appearance of the calyx seen in the immunofluorescence microscope was confirmed by the results of the immunogold labeling (data not shown). On bovine sperm cells, results obtained with this antiserum were identical to those obtained with mAb Cal-144 (data not shown).

Discussion

This study provides the first sequence information on a sperm-head calyx-associated protein and thereby has led to the identification of a new kind of cytoskeletal proteins, with cylicin I as the first known representative. Cylicin I and other MBP molecules have so far been detected only in spermatids and spermatozoa. With a calculated isoelectric point of pH ~ 10.5 they are basic cytoplasmic proteins, comparable in charge with the chromatin-bound core histones (for review see Johns, 1982; van Holde, 1989). The most conspicuous sequence feature of cylicin I, the abundant KXX tripeptides, notably those in which X is a negatively charged residue ($\text{KK}^{\text{D/E}}$), is also seen in certain histones of the H1 family (Wu et al., 1986; van Holde, 1989), and the proline-rich carboxy-terminal portion is another property in common with H1 histones. Besides these general similarities, however, cylicin do not reveal any sequence homology to any histone.

In this context it should be noted, that $\text{KK}^{\text{D/E}}$ clusters are also frequent in certain other cytoplasmic proteins such as in the microtubule-binding region of protein MAP 1B (Noble et al., 1989) and in a subterminal segment of the neurofilament protein NF-H (Lees et al., 1988). This sequence ele-

ment is also conspicuously frequent in certain structurally important nuclear proteins such as topoisomerase I (for example, see D'Arpa et al., 1988), the pore complex-associated protein NSP1 (Hurt, 1988) and the "arginine-rich" nucleoplasmic protein described by Chaudhary et al. (1991). It has also not escaped our attention that the repeating units, predicted to form α helices, contain AK(K)-rich elements known to occur in some DNA-binding proteins, including histone H1 (for review see Churchill and Travers, 1991).

The arrangement of a large central portion of cylicin in relatively short repeating units of ~ 40 amino acids, which presumably represent individual structural and possibly also functional elements, has also been noted in other cytoskeletal-associated proteins, the most prominent example being the family of junctional plaque proteins of the plakoglobin/ β -catenin/armadillo gene product family (Franke et al., 1989; Peifer and Wieschaus, 1990; McCrea et al., 1991).

The relationships of cylicin I to the other MBPs is not yet clear. On the one hand, certain antibodies show cross-reactions between cylicin I and other MBPs. On the other hand, we have not identified identical amino acid sequences in cylicin I and other MBPs, except of limited similarities in MBP2. We are currently using our cylicin cDNA probes to examine the possible existence of a broader multigene family to which cylicin I may belong.

From the primary sequence of cylicin, particularly its high charge density, it is not obvious what makes this protein so insoluble that it is, at least to a considerable proportion, associated with the cytoskeletal calyx structure as isolated by sequential extractions, including treatment with high salt buffers (for partial extractions of cylicin in these buffers see, however, also Longo et al., 1987). As calicin is also very basic, the entire calyx may represent an overall positively charged cytoskeletal structure, an unusual situation in view of the negative charges of most other cytoplasmic molecules. At present, however, we cannot exclude that calicin, cylicin and other MBPs are complexed with negatively charged elements of the calyx which have not yet been identified. Clearly, cylicin reconstitution experiments using protein either from purified calyx fractions, from recombinant DNA expressed in *E. coli*, or by expression of cDNA, transfected into heterotypic cells, will have to be performed to assess the assembly behavior and structure-forming potential of this protein.

The specific synthesis of cylicin I and calicin in late spermiogenesis, and the specific enrichment in the perinuclear calyx (Longo et al., 1987; Longo and Cook, 1991) suggest that these proteins are involved in spermatid differentiation, probably in sperm head morphogenesis. This is also in line with reports of Escalier (1990) and Courtot (1991) that the distribution of one of these proteins, i.e., calicin, is drastically changed in morphologically altered "round-headed" spermatozoa (for the distribution of other cytoskeletal proteins in round-headed spermatozoa see the review of Baccetti et al., 1988). However, the obvious interspecies differences in sperm head shape and calyx arrangement indicate that neither cylicin I nor the other common MBPs are sufficient to establish the species-specific morphogenesis in spermatozoa. With the amino acid sequence and cDNA clones in hand, it is now experimentally possible to ask the question of function with the adequate methods. i.e., in molecular and genetic terms.

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