Basic Proteins of the Perinuclear Theca of Mammalian Spermatozoa and Spermatids: A Novel Class of Cytoskeletal Elements

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Abstract. The nuclei of bovine spermatids and spermatozoa are surrounded by dense cytoplasmic webs sandwiched between the nuclear envelope and the acrosome and plasma membrane, respectively, filling most of the cytoplasmic space of the sperm head. This web contains a complex structure, the perinuclear theca, which is characterized by resistance to extractions in nondenaturing detergents and high salt buffers, and can be divided into two major subcomponents, the subacrosomal layer and the postacrosomal calyx.

Using calyces isolated from bull and rat spermatozoa we have identified two kinds of basic proteins as major constituents of the thecal structure and have localized them by specific antibodies at the light and electron microscopic level. These are an $M_r$ 60,000 protein, termed calcin, localized almost exclusively to the calyx, and a group of multiple-band polypeptides (MBP; $M_r$ 56,000-74,000), which occur in both the calyx and the subacrosomal layer. The polypeptides of the MBP group are immunologically related to each other, but unrelated, by antibody reactions and peptide maps, to calcin. We show that these basic cytoskeletal proteins are first detectable in the round spermatid stage. As we have not detected any intermediate filament proteins and proteins related to nuclear lamins of somatic cells in sperm heads, we conclude that the perinuclear theca and its constituents, calcin and MBP proteins, are the predominant cytoskeletal elements of the sperm head. Immunologically cross-reacting polypeptides with similar properties have been identified in the heads of rat and human spermatozoa. We speculate that these insoluble basic proteins contribute, during spermiogenesis, to the formation of the perinuclear theca as an architectural element involved in the shape changes and the intimate association of the nucleus with the acrosome and the plasma membrane.

During the past decade it has become increasingly clear that the overall shape of a cell and its internal architecture is dependent not only on membranous elements but also on a group of structures that resist cell lysis and extraction by nondenaturing detergents and buffers of various pH and ionic strength. Such proteinaceous structures are now usually referred to under the collective terms cytoskeleton, as to the cytoplasm, and karyoskeleton, for nuclear architectural elements. Among the cytoskeletal components some of the most prominent structures are the actin-containing microfilaments and the tubulin polymers, i.e., microtubules. The term cytoskeleton also includes certain filamentous webs such as membranous coats in the cell periphery, many of which contain proteins of the spectrin family, the regular clathrin coats of coated pits and vesicles, and the plaques of adhering junctions. Remarkably, some residual cyto- and karyoskeletal components are preserved even after extraction in high salt concentrations, and maintain typical architectural features. Structures of this category include the intermediate-sized filaments (IFs) of the cytoplasm and the biochemically related structure in the cortex of the nucleus, the nuclear lamina. It also includes various kinds of plasma membrane-associated plaques, notably those associated with the desmosome, and, in the nucleus, the subunits of the pore complex.

Several routes of cell differentiation are characterized by spectacular shape changes of both the nucleus and the cytoplasm, one of the most prominent being spermiogenesis in the seminiferous tubules of the mammalian testis (reviewed in references 8 and 23). Contributions of microfilaments and microtubules to shape changes taking place during the development of spermatids and spermatozoa have been discussed, although controversially. In the sperm tail, microtubules are undoubtedly important elements of spermatozoan functions. In addition, various stabilizing elements that are resistant to high salt treatment have also been identified in the sperm tail, including the outer dense fibers and the fibrous sheath (e.g., 3, 8, and 23).

The role of cytoskeletal elements in the formation and maintenance of the nucleus-containing sperm head is even less clear (reviewed in reference 8). In this respect observations that the sperm head also contains structural elements resistant to extractions with detergents and high salt buffers...
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Figure 1. Schematic drawing showing the major components of an idealized mammalian sperm head. The scheme is a modified version of the classic diagram of elements of the primate sperm head (23). N, nucleus; A, acrosomal content. The membrane types are listed in the upper left; the posterior ring, the centriolar complex (C) and the dense layer of the implantation fossa (F) are also indicated. The major cytoskeletal elements, as perceived by electron microscopy (indicated on the right hand side), form the perinuclear theca, which can be distinguished, and under certain conditions fragments, into an anterior portion, the subacrosomal layer, filling the subacrosomal space, and a posterior complex, designated here as calyx (arrows). The latter is located in the postacrosomal segment of the sperm head (13) and consists of (a) the postacrosomal layer intimately applied to the nuclear envelope, (b) the postacrosomal sheath, (c) the paracrystalline sheath of filamentous ridges (stripations; reference 35), and (d) some less distinct material in between (dotted area). The degree and extent of regularity of the material in the paracrystalline sheath varies between different species and in relation to fixation and isolation conditions. The term postacrosomal sheath (a synonym is postacrosomal dense lamina) is used here in the more restricted definition (23, 52), whereas others have also used it to describe the thin sheath plus the paracrystalline and other material (e.g., 40 and 44). The material of the subacrosomal and postacrosomal layers, plus the interspersed material (dotted area), has also been referred to as perinuclear substance in the literature (17). Depending on the type of fixation used the material of the perinuclear theca often appears with a finely filamentous texture (20, 40).

appear to be relevant. Bellvé and O'Brien (8) have attracted attention to the existence of a large, extraction-resistant, extranuclear structural element, the perinuclear theca (Fig. 1). This structure represents a dense layer of variable thickness sandwiched in between the outer nuclear membrane on the one side and the inner acrosomal membrane, and caudally to the posterior ring, the postacrosomal segment of the plasma membrane, on the other side (reviewed in references 17, 23, 40, and 50). The postacrosomal segment of the peri-

nuclear theca is very complex and forms a continuous, rigid, funnel-shaped entity. Since this structural complex can be isolated as a whole we shall specifically refer to it as the calyx. On the basis of ultrastructural and cytochemical observations the calyx complex has been further subdivided into a thin postacrosomal layer that is tightly applied to the nuclear envelope continuous with the subacrosomal layer (perinuclear substance) sense (8, 17), and a thin (~5 nm) layer parallel to the postacrosomal plasma membrane (postacrosomal sheath) that is connected to the inner aspect of the plasma membrane by a paracrystalline sheet of ridges or filaments of 10–14 nm in diameter (Fig. 1). In addition, some indistinct, loosely webbed material is located in between these elements.

Although various procedures for the isolation of detergent- and high ionic strength–resistant structures from mammalian sperm heads have been described, no protein has so far been positively localized to any of these structures. On the other hand, there have been a number of controversial reports on the existence of IF proteins and nuclear lamins in mammalian sperm heads (for details see Discussion). In the course of a study aiming at the clarification of whether mammalian spermatids and spermatozoa possess IFs or nuclear lamins we have found that practically the only cytoskeletal element of the sperm head that is resistant to extractions with detergent and high salt buffers is a scaffold of the perinuclear theca, and we have identified, by the use of antibodies as specific probes, two types of basic proteins as major components of this dense-web structure.

Materials and Methods

Collection and Fractionation of Spermatozoa

Vasa and epididymides were obtained from adult Wistar rats and from bulls slaughtered at a local meat processing firm. Ejaculates containing human spermatozoa were from outdated reference samples obtained from the anology unit of the Department of Dermatology, Mannheim Medical School, University of Heidelberg, FRG. All procedures were carried out at 4°C unless otherwise indicated and all solutions contained 1 mM phenylmethylsulfonyl fluoride (PMSF). Vasa and epididymides were minced with scissors in PBS and filtered through fine-mesh gauze. Sperm were recovered by centrifugation at 1,000 g for 5 min and washed twice in PBS containing 25 mM EDTA (PBS–EDTA) and either stored frozen as a sediment or directly fractionated.

Sperm resuspended in PBS–EDTA (2–3 ml packed sperm in ~30 ml) were fractionated into heads and tails by sonicication with a sonifier (output setting of 7, three bursts, each 15 s in duration; Branson Sonic Power Co., Danbury, CT). The suspension of heads and tails was made 50% in sucrose and layered over 75% sucrose (80 mM Tris-HCl, 1 mM EDTA, 2 mM dithioreitol [DTT], 150 mM NaCl, pH 7.5) and centrifuged at 26,000 g for 45 min in a rotor (SW 27; Beckman Instruments, Inc., Fullerton, CA). Tails concentrated as a sharp band at the sucrose interface and heads formed a pellet at the bottom of the tube. Purity of head and tail fractions was assessed with a Zeiss microscope equipped with phase-contrast optics.

Sperm heads were collected and washed once in PBS–EDTA. In this and the following incubations sperm heads were pelleted by centrifugation at 1,000 g for 10 min and resuspended with a motor-driven homogenizer. Sperm heads were demembraned by incubation for 30 min with 10% Triton X-100 in PBS–EDTA and then treated with 2 M NaCl, 5 mM DTT, 10 mM Tris-HCl buffer (pH 7.4) for a total extraction period of 40 min. After the second extraction, sperm heads were treated with DNase I (100 μg/ml Cooper Biomedical, Wiesbaden, Federal Republic of Germany) in 10 mM Tris-HCl buffer (pH 8.5) containing 2 mM MgCl₂ for 30 min at 20°C. The resulting residual structures were extracted a third time for 15 min with 2 M NaCl (same buffer as above), then with DNase (80 μg/ml; 30 min at 20°C), and again with 2 M NaCl (10 min). The final residue was washed two times in 10 mM Tris buffer (pH 7.4) and pelleted. In some ex-
perirnents 1 instead of 2 M NaCl was used. Tail fractions were also treated in parallel using essentially the same extraction protocol. To determine the effects of the various treatments used in this protocol on the morphology of spermatozoa and sperm heads, samples were taken after each incubation, washed in PBS, and fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 30 min. Samples were washed for 1 h in 50 mM cacodylate buffer, postfixed in 1% OsO4, for 30 min, dehydrated in ascending concentrations of ethanol, and embedded in Epon (cf. 26). Thin sections, stained with uranyl acetate and lead citrate, were examined with a Siemens 101 electron microscope.

**Gel Electrophoresis, Immunoblotting, and Peptide Mapping**

Total spermatozoa or pellets of cell fractions were boiled for 5 min in SDS sample buffer (5% SDS, 10 mM sodium phosphate buffer, pH 7.2, 10% 2-mercaptoethanol; cf. 39). For subsequent two-dimensional gel electrophoresis the solubilized proteins were precipitated by addition of 90 µl cold acetone, dried, and either used directly or after further removal of DNA. For DNase treatment the dried pellets were resuspended in 200 µl 1 ml DNase buffer containing 50 µg DNase I and incubated for 10-15 min at 20°C. The polypeptides were then either collected directly by centrifugation (10,000 g, 5 min) or precipitated with 6 vol acetone. The pellets were washed with 90 and 100% acetone and then solubilized in sample buffer (47).

In some experiments, nonextracted sperm heads (sediment of 100-µl vol) were boiled directly in 1 ml SDS sample buffer (see above) and precipitated with 6 vol acetone. The pellet was washed with 90 and 100% acetone, dried, and resuspended in 2 ml DNase buffer containing 50 µg DNase I and incubated for 30 min at 20°C. The polypeptides were then collected and processed as described above.

SDS-PAGE was carried out essentially according to Laemmli (39) using variable loads (up to 50 µg/lane); two-dimensional gel electrophoresis using nonequilibrium pH gradient gel electrophoresis (NEPHGE) was as described (47) with minor modifications (2). For immunoblotting, gels were processed as described (2). Proteins transferred to nitrocellulose paper were visualized by staining with Ponceau S in distilled water (Sigma, Munich, FRG). The stained nitrocellulose paper was washed in distilled water and photographed to identify the exact position of proteins. Periodic acid Schiff reaction for glycoprotein was performed as described (21).

Tryptic peptide maps from polypeptide spots after two-dimensional gel electrophoresis were done as described (19).

**Antibodies**

The following antibodies were used. (a) Guinea pig antibodies obtained after immunization with protein eluted from an SDS-PAGE band containing the M, 66,000 polypeptide, calcin, obtained from the calyx fraction of bull sperm heads (for methods of elution and immunization see reference 11). Antibodies were affinity-purified as described (37) when necessary. (b) Guinea pig antibodies prepared similarly but directed against the multipolypeptide bands (MBP) (M, 66,000-34,000) were also affinity-purified and used as described above. (c) Guinea pig antibodies specific for lamin B that recognizes the two lower bands of cell types and species (reference 11). (d) Guinea pig antibodies against cytokeratin and vimentin were as described (reference 27-29). (e) Monoclonal antibody IFA which reacts with a highly conserved epitope common to all classes of IF proteins (54). (f) Monoclonal antibody K8.13 reacting with basic (type II) cytokeratin and cytokeratin No. 18 (reference 30; commercially available from Bio-Makor, Rehovot, Israel). (g) Monoclonal antibody K8.13 reacting with several cytokeratins (reference 32; commercially available from Laboratories Oy, Helsinki, Finland). (i) Monoclonal antibody VIM9 reacting with vimentin (Biochron, Berlin, FRG). (j) Monoclonal antibody PK88 reacting with lamin A, B, and C (38). Fluorescein- or Texas red-labeled secondary antibodies were from goat (Dianova, Hamburg, FRG).

**Light and Electron Microscopic Immunolocalization**

Immunofluorescence microscopy on 5-10 µm cryostat sections of shock-frozen tissue samples from testes and other tissues (liver, intestine, and heart) of bulls and rats was performed as described (27, 38). For immunolocalization experiments on whole mount preparations, suspensions of washed spermatozoa were placed as droplets on coverslips and gently spread. Cells adhering to the coverslips were then either rinsed briefly in PBS or were first permeabilized by brief incubation in PBS containing 0.5% Triton X-100 and then washed. Thereafter, cells were either directly incubated with antibodies for 30 min or were first immersed in -20°C methanol for 5 min and in -20°C acetone for 2 min before antibody application. All subsequent steps were essentially as described for tissue sections. In double label immunolocalization experiments both primary antibodies were applied simultaneously (cf. II).

For electron microscopy of tissue samples, small pieces were fixed for 30 min in 2.5% glutaraldehyde (50 mM sodium cacodylate, 50 mM KCl, 2.5 mM MgCl2, pH 7.2) in the cold (0-4°C), followed by three washes in 50 mM cacodylate buffer and secondary fixation in 2% OsO4 for 60 min.

Further processing was as described (26). For electron microscopic immunolocalization in tissue specimens, cryostat sections were processed as described (71), using colloidal gold-labeled goat antibodies to guinea pig or mouse Ig (5-5 nm particle diameter; Janssen, Beerse, Belgium). Alternatively, immunogold labeling was performed on ultrathin sections of Lowicryl K4M-embedded samples as described (58). Samples used for Lowicryl embedding were prefixed for 20 min with 2% formaldehyde (0.1 M sodium phosphate, pH 7.3, 50 mM KCl, 2.5 mM MgCl2). For immunoelectron microscopy of whole spermatozoa one of the two following protocols was used. (a) Spermatozoa were resuspended in PBS containing 0.5% Triton and washed in the same buffer. Antibodies were then added (final concentrations ranging from 5-50 µg/ml) and the suspension incubated for 2 h at room temperature. After three washes in PBS to remove unbound Ig, gold-labeled secondary antibodies (see above) were added and incubated overnight. After another three washes in PBS the pellet material was sequentially fixed with glutaraldehyde and OsO4 and processed for electron microscopy as described. (b) Spermatozoa attached to coverslips were briefly treated with PBS, with or without 0.5% Triton X-100, and then with -20°C methanol and acetone as described for light microscopic preparations. The coverslips were then incubated for 30 min with the primary antibodies (same range of concentrations), washed three times in PBS, incubated with secondary antibodies for 4 h, washed in PBS, fixed, and flat-embedded as described above for immunoelectron microscopy processing of cryostat sections (cf. II and 71).

Controls were done in parallel using other primary antibodies unreactive on spermatids and spermatozoa but positive on various other cell types (including Sertoli cells), such as antibodies to vimentin, nuclear lamins (see above and reference II), and plakoglobin (18), or secondary antibodies alone.

**Results**

**Isolation and Fractionation of the Perinuclear Theca**

Nuclei isolated from bull or rat sperm heads with buffers containing 1% Triton X-100 maintained their specific shape and remained delimited by the perinuclear theca, with variable portions of the nuclear envelope persisting (Fig. 2, a, b, and c). Depending on the specific preparative conditions, the perinuclear theca often tended to split, to variable extents, into two sublayers separated by a space, which probably represents an artifact (Fig. 2, d and e), in a similar pattern as described after cavitation (50) and treatment with low concentrations of Triton X-100 (72). One layer, which was of rather uniform width, undercoated the plasma membrane and included, in the calyx region, the postacrosomal sheath and the material of the paracrystalline layer. The other layer was usually thinner, irregularly patchy in its distribution and conformation, and remained closely associated with the outer margin of the nuclear envelope.

Incubation of such nuclear preparations in high salt buffer containing 2 M NaCl and 5 mM DTT resulted in a pronounced increase in transparency and swelling of the anterior region of the sperm nucleus (Fig. 2, b and c). This swelling usually disrupted the thin subacrosomal layer in several places (Fig. 2 c). Throughout the swelling and extraction of chromatin, however, the cuplike formation surrounding the posterior aspect of the sperm nucleus, i.e., the calyx, maintained its overall size and shape (Fig. 2, b and c).
Figure 2. Morphology of extracted and nonextracted bull sperm heads. When isolated bull sperm heads (a) were treated with Triton X-100, then incubated in buffer containing 2 M NaCl and 5 mM DTT for 15 (b) and 45 (c) min, and viewed with phase-contrast optics, large amounts of material were extracted concomitant with chromatin swelling and disruption of the anterior aspect of the sperm head (subacrosomal layer), whereas the posterior part (calyx) by and large retained its original size (the triangle in b indicates an intact sperm head of a suspension that was mixed with extracted specimens just before examination). Electron micrographs (d-f) of ultrathin sections showing the calyx (fork brackets in d and e, bracket in f) in nonextracted sperm heads (d), after incubation with Triton X-100 (e), and
Absence of Intermediate Filament Proteins and Nuclear Lamins in Karyo- and Cytoskeletons of Spermatozoa and Spermatids

The presence of known IF proteins and nuclear lamins was examined in immunoblots of proteins from total rat and bull spermatozoa, isolated whole sperm heads, and high salt buffer sperm head residues obtained after extraction with Triton X-100 (isolated calyx preparations), using a panel of highly specific and well characterized antibodies. Cytoskeletal and nuclear lamina preparations were used as positive controls. In all cases from both species the sperm material tested was negative for the presence of vimentin (Fig. 3, a and a'), a broad range of cytokeratins (one example is shown in Fig. 3 b and b'), and the highly conserved epitope recognized by antibody IFA (54), which is common to all classes of IF proteins (Fig. 3, c and c'). These samples were also negative when tested with the cytokeratin antibodies AE1 and AE3 (data not shown). Antibodies recognizing all three the final residue of sperm heads after extraction with Triton, 2 M NaCl, and DNase (f). During the isolation procedure the calyx is often split into two leaflets (small brackets in d). In f, the area formerly occupied by the sperm nucleus is indicated by the star. N, sperm nucleus. Bars: (a-e) 10 μm; (d-f) 0.1 μm.
Figure 4. Absence of IF proteins and nuclear lamins in bovine spermatids and spermatozoa as determined by immunofluorescence microscopy. Frozen sections of bull testis reacted with guinea pig antibodies to vimentin (a); murine monoclonal antibody IFA to all classes of IF proteins (b); guinea pig antibodies to cytokeratins (c); and nuclear lamins A, B, and C (d). (a', c', and d') Corresponding phase-contrast micrographs demonstrating sperm within the seminiferous tubule lumen (L). Antibodies to vimentin (a) and antibody IFA (b) react strongly with Sertoli and peritubular cells (IS, interstitium) but not with sperm cells. Antibodies to cytokeratins (c) show no reaction to sperm or spermatogenic cells but (only in the bull) react with cells located along the periphery of the seminiferous tubule. Antibodies to nuclear lamins (d) react exclusively with the nuclear periphery of somatic and early spermatogenic cells. Bars, 25 μm.
mammalian lamins (A, B, and C) also failed to react with any polypeptide of sperm heads and subfractions made therefrom in bull (e.g., see Fig. 3, d and d') and rat (not shown). These results were confirmed by immunofluorescence microscopy with frozen sections from testicular tissue of both bull (Fig. 4) and rat (data not shown). With antibodies to vimentin, Sertoli cells as well as cells of the interstitium and the wall of the seminiferous tubule were positive, whereas spermatids and spermatogenic cells were negative (Fig. 4 a). Identical results were obtained with the monoclonal antibody IFA to the highly conserved epitope of IF proteins (Fig. 4 b). A broad range of antibodies to cytokeratins were negative for all cell types examined in rat testis, i.e., both somatic and spermatogenic cells (data not shown). In bull testis sperm, spermatogenic cells, Sertoli cells, and components of the interstitium were negative (Fig. 4, c and c'). In this species, however, cells that, remarkably, were positive for both cytokeratins and desmin (probably myoid cells) were present within the wall of the seminiferous tubules (Fig. 4 c).

Frozen sections of testes incubated with antibodies to nuclear lamins demonstrated an intense staining along the periphery of all somatic cell nuclei (Fig. 4, d and d'). Weak staining was apparent in nuclei of early spermatogenic cells (data not shown), whereas spermatids and spermatocytes were completely negative.

Identification of an Mr 60,000 Polypeptide as a Major Component Specific to the Calyx

Gel electrophoretic analyses of proteins from bull and rat whole sperm and isolated sperm heads showed an enrichment of several polypeptide bands in the Mr range from 56,000 to 74,000. Isolated calyces revealed a prominent Mr 60,000 polypeptide (Fig. 5, a and c) that showed a pink-reddish metachromatic staining with Coomassie Blue (Fig. 5 a, lanes 2 and 6). In addition, between Mr 56,000 and proteins separated by SDS-PAGE and (a') corresponding autoradiograph of a parallel immunoblot reacted with affinity-purified antibodies against the Mr 60,000 polypeptide, calicin. Lanes 1-4 contain proteins from bull sperm; lanes 5 and 6 contain proteins from rat sperm. Lanes 1 and 3, sperm tails; lanes 2 and 6, fractions of calyx-enriched perinuclear theca material obtained after extraction of sperm heads in buffers containing Triton X-100, 2 M NaCl, and DNase I; lane 3, whole sperm heads; lane 4, whole sperm. Bars on the left margin of a indicate the position of reference proteins: β-galactosidase (Mr 116,000), phosphorylase a (Mr 94,000), BSA (Mr 68,000), and skeletal muscle α-actin (Mr 42,000). The arrowhead designates calicin found in both bull and rat sperm heads to be enriched during extractions. (b and c) Coomassie Blue-stained two-dimensional polyacrylamide gel electrophoresis of polypeptides from whole bull sperm heads (b) and fractions of perinuclear thecal material (c) obtained after extraction as in a, lane 2. First dimension, NEPHGE; second dimension, SDS-PAGE. The left arrow points to the Mr 60,000 protein (calicin) that has not entered the first dimension gel and the right arrow denotes the calicin migrated into the gel. The bracket indicates a triplet of MBP (Mr 74,000-56,000) that are abundant in isolated sperm heads and can be partially extracted between 1 and 2 M NaCl (c). Reference proteins (acidic proteins are to the left) used for coelectrophoresis are: BSA (B), actin (A), and yeast phosphoglycerokinase (P). (b') Autoradiograph of an immunoblot corresponding to the gel shown in b and reacted with the same affinity-purified calicin antibodies used in a. The antibodies react exclusively with calicin (a' and b').
The specificity of the antibodies used, the presence of the affinity-purified antibodies to the Mr 60,000 protein nor the MBP (Fig. 5 a, lanes 1 and 5). In both ca-
lyx preparations and isolated sperm heads, the MBP showed, when examined in immunoblot assays (Fig. 5 b and b'), this also indicated that the Mr 60,000 does not form an extended series of polypeptide modifications. Tryptic peptide map analysis revealed that the Mr 60,000 polypeptides of bull (Fig. 6 a) and rat (Fig. 6 b) are closely related to each other and that the MBP (Fig. 6 c and d) represent a group of polypeptides that is clearly different from the Mr 60,000 polypeptide. Details of the interspecies relationships of the corresponding proteins in bull, rat, and human sperm will be presented elsewhere.

When the localization of the Mr 60,000 protein was examined by immunofluorescence microscopy with the affinity-purified Mr 60,000 antibodies on cryostat sections of various frozen tissues, positive reaction was noted only in the seminiferous tubules of all species examined (Fig. 9 shows bull testis; data on the other two species examined will be presented elsewhere). The immunostaining was exclusive to the posterior, i.e., calyx, region of the sperm heads. The earliest stages found positive were round to slightly elongated spermatids (Fig. 8 a), in which the staining was, by and large, restricted to a subacrosomal ring (Fig. 8 b) near to, but not identical with, the equatorial segment (for definition see references 8 and 23).

Because of the specific location of the Mr 60,000 polypeptide in the calyx and the finding that it constitutes the major protein of detergent- and high salt buffer-resistant sperm heads, we shall use the name calicin for this protein.

Identification of MBP as Components of the Entire Perinuclear Theca

In most analyses the very basic MBP appeared in the form of four polypeptide components (Fig. 5, a and b and Fig. 10). Mr values of 56,000, 58,000, 63,000, and 74,000 could be resolved (Fig. 5 b and Fig. 10). The relative amounts of the individual bands within the MBP, however, varied from one experiment to another. Characteristically, these polypeptides appear at the same position on two-dimensional gel electrophoresis using the NEPHGE technique, indicating that they are very similar in electrical charge and/or migrated in a common complex. They migrated either close to, faster (Fig. 5 b), or slower (Fig. 9 a) than calicin.

When affinity-purified antibodies against the MBP were examined by immunoblotting, all bands of this group reacted (Fig. 9 a' presents an example of a NEPHGE separation).

Figure 6. Comparison of [3H]-labeled tryptic peptides of basic cytoskeletal proteins of the nuclear theca from bull and rat spermatzoa. (a) Mr 60,000 polypeptide (calicin) of bull obtained from a preparation as shown in Fig. 5 c. (b) Same protein (calicin) from rat (see Fig. 5 a, lane 5). (c) Mr 74,000 polypeptide of the MBP group from bull (for preparation see Fig. 5, b and c, bracket). (d) Mr 56,000 polypeptide of the MBP group from bull. E, first dimension by electrophoresis; C, second dimension by chromatography.

74,000 a group of MBP were present as major components in isolated sperm heads and in sperm heads extracted with 1 M NaCl (data not shown), but were less prominent in calyx preparations obtained with 2 M NaCl (Fig. 5 a). In both calyx preparations and isolated sperm heads, the MBP showed, upon staining with Coomassie Blue, a strong scattering effect to obliquely incident light, as is characteristic of several very basic proteins (cf. 34). Both the Mr 60,000 polypeptide and the MBP were not stained with the periodic acid Schiff reaction, indicating that they are not considerably glycosylated. Fractions of rat and bull sperm tails did not reveal the Mr 60,000 protein nor the MBP (Fig. 5 a, lanes 1 and 5).

Because the Mr 60,000 band represented the predominant polypeptide of isolated calyces it was cut out from preparative gels (fraction is shown in Fig. 5 a, lane 2) and the protein was eluted and used to generate antibodies. The reaction of affinity-purified antibodies to the Mr 60,000 polypeptide, when examined in immunoblot assays (Fig. 5 a'), indicated the specificity of the antibodies used, the presence of the same antigen in both rat and bull calyces, the enrichment of the antigen in isolated sperm heads and in extracted calyx fractions, and its absence in sperm tails.

Two-dimensional gel electrophoresis of calyx preparation and nonextracted sperm head proteins showed that both the Mr 60,000 and the MBP are very basic proteins. During NEPHGE the Mr 60,000 polypeptide migrated exceptionally fast (Fig. 5, b and c), indicative of an isoelectric point >pH 8 (given the limitations of current isoelectric focusing techniques the exact isoelectric point could not be deter-
Figure 7. Immunofluorescence localization of $M_r$ 60,000 cytoskeletal polypeptide (calicin) in whole spermatozoa of bull. Spread preparation of epididymal bull sperm incubated with affinity-purified antibodies to calicin (a, epifluorescence; a', phase-contrast optics). The antibodies localize exclusively to the posterior aspect of the sperm head and, in the three-dimensional perspective afforded by this preparation, demonstrate that the protein is located in a funnel-shaped calyx surrounding the posterior margin of the nucleus. Bar, 20 μm.

Figure 8. Immunofluorescence microscopy of calicin ($M_r$ 60,000) in bull testis. (a and b) Frozen sections through bull seminiferous tubules incubated with affinity-purified antibodies to calicin showing (a and b, epifluorescence optics) areas with mature sperm (a) and spermatids (b). (a' and b') Corresponding phase-contrast micrographs showing luminal spermatozoa (L) and spermatogenic cells (SC). Luminated spermatozoa (a) are stained in the entire posterior aspect corresponding to the calyx. Antibody staining is first detectable in round to slightly elongated spermatids (b) in a ringlike fashion. Bars, 20 μm.
Figure 9. Immunoblot with antibodies to the MBP group. (a) Coomassie Blue staining of residual proteins of Triton X-100- and 1 M NaCl-extracted fractions of perinuclear thecal material from bull sperm heads, separated by two-dimensional gel electrophoresis (as in Fig. 5, b and c), and corresponding autoradiography of immunoblot (a') reacted with affinity-purified antibodies against the upper polypeptide of the MBP (Mr 74,000). The arrow indicates the position of calicin. The brackets denote the position of the triplet polypeptide of the MBP that react with the purified antibody. Internal reference proteins as in Fig. 5, b and c.

Affinity-purified antibodies prepared by elution from one of the bands using the nitrocellulose strip purification method (37) reacted with the other three bands, thus indicating immunological cross-reactivity. Analysis of the individual polypeptide spots by tryptic peptide mapping (Fig. 6, c and d) resulted in peptide patterns different from each other and from the pattern obtained from calicin. Based on these results, as well as the different metachromatic appearances after Coomassie Blue staining, we conclude that the MBP are different from calicin and are a group of polypeptides that are immunologically related to each other but exhibit significant peptide map differences that make it unlikely that they form a series of proteolytic degradation products.

When frozen sections of testis and whole mount preparations of spermatozoa were examined by immunofluorescence microscopy during affinity-purified antibodies specific for the basic MBP, an exclusive reaction on spermatozoa (Fig. 10, a–c) and spermatids (Fig. 10 d) was found. Unlike the calicin reaction, however, the MBP antigens were detected within the entire perinuclear theca, i.e., in the postacrosomal calyx as well as in the subacrosomal layer (Fig. 11, b and c). In spermatids the MBP immunoreactivity appeared to cover an area smaller than in spermatozoa.

Electron Microscopic Localization of Calcin and the MBP

The electron microscopic appearance of the major components of the perinuclear theca is shown in Fig. 11, a and b. The subacrosomal layer was mostly composed of a web of fibrillar granular material without distinct substructures. The postacrosomal calyx was more complex (see also Fig. 1), revealing a similar fibrillar granular web immediately covering the nuclear envelope (postacrosomal layer) and the ~5- to 7-nm thick, electron-dense postacrosomal sheath connected, in some regions, to the plasma membrane by the paracrystalline sheet formed by parallel filamentous ridges of 10–14 nm in diameter and a center to center spacing of ~16 nm.

Immunoelectron microscopy, using colloidal gold–coupled secondary antibodies on frozen tissue sections of testicular tissue as well as permeabilized (suspended or spread) spermatozoa, confirmed our light microscopic observations of intense calicin labeling in the calyx (Fig. 11, e and f). MBP were also concentrated in the calyx, notably in spermatids fixed in situ (Fig. 11 c), but were also detected in the subacrosomal layer (Fig. 11 d). Within the calyx most of the immunogold label seen after incubation of specimens with either antibodies to calicin (Fig. 11 f) or MBP (Fig. 11 d) was associated with the material of the postacrosomal layer on the outer nuclear membrane, where it appeared to be rather homogeneously distributed. At present we are unable to decide with which of the major substructures of the postacrosomal layer of the perinuclear theca the calicin and the MBP label is associated. A more detailed morphological and immunoelectron microscopic study of the components of the perinuclear theca in spermatozoa and during spermiogenesis will be presented elsewhere (Paranko, J., F. Longo, G. Krohne, and W. W. Franke, manuscript in preparation).

Discussion

The most important conclusions from our study are (a) that the major cytoskeletal element of the heads of bovine spermatozoa is the perinuclear theca, a densely woven web connecting the nuclear envelope with the acrosome and postacrosomal plasma membrane, respectively, and (b) that this cytoskeletal structure contains a class of basic proteins un-
Figure 11. Morphology of perinuclear theca in bull sperm heads and immunolocalization of the M, 60,000 protein (calcin) and MBP at the electron microscopic level. (a, b) Electron micrographs of ultrathin sections through in situ fixed testicular sperm (a and b) showing details of perinuclear structures at the transitional region from the anterior to the posterior part (a) and of the posterior region (calyx) of the sperm head (b). S, Sertoli cell cortex with microfilament bundles; A, acrosome; SL, subacrosomal layer; N, nucleus; NE, nuclear envelope. The arrow in a denotes the junction between the subacrosomal layer and the calyx region. The arrow in b denotes the plasma...
related to all cyto- and karyoskeletal proteins described so far. We have also presented evidence to show that the insoluble perinuclear theca is a compositionally complex structure comprising common proteins such as the MBP, as well as at least one protein specific for the calyx. Cytoskeletal proteins similar to calcin and the group of MBP also occur in sperm of rat and man, suggesting that they may be widespread, if not ubiquitous, in mammalian sperm, in agreement with electron microscopic descriptions of perinuclear theca structures in a broad range of species (e.g., 13, 17, 23, 24, 40, 44, 50, 52).

Previously, several authors have noted that on extraction of sperm heads with detergents and high salt buffers under reducing conditions a residual nuclear structure is obtained (8, 9, 53, 57, 65); most authors have ascribed this stability of a residual nuclear cortex to the preservation of intranuclear matrix and/or a nuclear lamina as described for a variety of somatic cells. However, our present finding that the perinuclear theca is the only major structural complex of the spermatozoon resistant to treatments with non-denaturing detergents and a broad range of salt concentrations supports the notion of Bellvé and colleagues (8, 9, 45). These authors have reported that the perinuclear layer that withstands extractions in buffers containing 1% SDS and high ionic strength as well as the removal of nuclear DNA and protamins (190 mM divalent cations) is derived from a structure external to the nuclear envelope and does not contain the nuclear lamins characteristic of somatic cell nuclei (8, 33). The extraction protocols used by these authors were less rigorous than our extractions in 2 M NaCl-containing buffer and, like our extractions in 1 M NaCl, left the perinuclear theca as a more or less continuous layer around the entire nucleus, thus not allowing the separation of calyces.

The discovery of a novel class of basic proteins as the major components of the perinuclear theca as a whole and the calyx specifically provides biochemical support for the morphologically derived concept of the perinuclear theca as a distinct structural entity (8, 17, 40). Clearly, both kinds of thecal proteins, calcin and MBP, are different from all known cytoskeletal proteins, notably IF proteins and nuclear lamins. We cannot explain why these abundant proteins have not been detected earlier in fractions of the similarly extracted preparations of Pruslin and Rodman (53) and in fractions of basic proteins recovered in nuclear residual structures from mouse sperm heads (45, 56). Perhaps interspecies differences also contribute to the differences of composition reported in the literature. On the other hand, the basic character of the major perinuclear theca proteins provides an explanation for previous conclusions, based on cytochemical staining reactions with phosphotungstic acid, that the dense perinuclear material is composed mainly of non-glycosylated, lysine-rich proteins (17, 31, 44), and probably also explains its known argyrophilia (36).

The localization of the abundant MBP in the entire perinuclear theca suggests that they represent a major component of the perinuclear substance, a finely filamentous web common to both the subacrosomal and postacrosomal layers (Fig. 1). Actin has also been localized by some authors to the filamentous web of the subacrosomal layer, including the apicalmost subacrosomal pit, the perforatorium of some rodents and birds (4, 14–16, 25, 26, 59, 64, 68–70). However, as actin is extracted in high salt buffers, we consider it unlikely that this protein makes a quantitatively important contribution to the cytoskeletal theca. Moreover, a cystein-rich polypeptide of Mr 13,000 has been described in fractions of perforatorium material from rat spermatozoa (49), but it does not occur in the subacrosomal material of other species lacking a perforatorium (15). A basic Mr 15,000 murine sperm protein has also been identified in the perinuclear theca but this protein is also extractable in high salt concentrations (57). Acid phosphatase activity has also been reported to occur in the subacrosomal layer (13).

In contrast to the anterior portion of the perinuclear theca, the calyx is characterized by a greater stability and complexity, both in morphological terms and in its protein complement. We have shown that calcin is a major protein of this structural complex, in addition to the MBP and probably other proteins. The topological specificity of the distribution of calcin is remarkable. Its immunofluorescence microscopic distribution correlates with that of a surface-exposed membrane polypeptide of a very similar Mr value, the posterior head protein described in guinea pig spermatozoa (43). It will be important to determine the molecular basis for such domain restrictions within the plasma membrane, restrictions which are also indicated by the higher frequency of membrane-intercalated particles as seen by freeze-cleavage in this area (reviewed in reference 23), and to identify possible cytoskeletal components involved in the establishment and maintenance of such constraints. While our immunoelectron microscopic findings have identified the presence of calcin in patches of material of the postacrosomal layer covering the nucleus, at present we cannot exclude that this protein also contributes to the postacrosomal sheet and the filamentous ridges of the paracrystalline sheet, as accessibility of these tightly packed structures to the gold-coupled antibodies used might have been insufficient.

We have taken great care to examine the presence of structures and proteins similar to those known from somatic cells, notably IF proteins and lamins. The results are negative, and, because of the perplexingly controversial literature, shall be discussed in some detail.

(a) IFs have never been reported to occur in spermatids

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and spermatozoa in any of the numerous electron microscopic studies with these cells. The 10–14-nm filaments or ridges of the paracrystalline sheet of the calyx and the 14–15-nm filaments described in the subacrosomal space of certain spermatid stages (e.g., 59) differ in their structural details from IFs and are not decorated with antibodies to IF proteins (see also reference 50). The 11–13-nm filaments located between the acrosome and the plasma membrane in certain regions of hamster sperm heads (48) also look different from IFs. Our finding of a lack of reactivity of antibody IFA (54), which reacts with all classes of IF proteins, including their homologues in several invertebrates (6), also speaks against the presence of IFs and IF proteins. Our present study and most reports in the literature agree that in mature testis vimentin IFs are expressed in myoid and Sertoli cells but are absent from spermatids and spermatozoa (27, 51, 55, 67). The significance of reports that certain vimentin antibodies react with the equatorial segment of ejaculated human spermatozoa (46, 68) or in the postacrosomal sheath (5) remains unclear, and the minuscule amounts of antigen reported might be due to the notorious contaminations of ejaculates by nonspermiogenic material. Negative results were also obtained when testicular tissue was examined by the same group (69). In summary, from the weight of previously published experimental evidence and that presented here we conclude that IF proteins are either not expressed during spermiogenesis or are present in amounts too low to be detected.

Our findings by immunoblotting, immunocytochemistry, and peptide mapping that proteins immunologically and biochemically similar to nuclear lamins of somatic cells are not expressed, or are present only in undetectable amounts, in spermiogenic cells of bull and rat are in agreement with several previous reports of an absence, in avian and mammalian spermatids and spermatozoa, of a nuclear lamina structure and of karyoskeletal proteins identical with, or closely related to, lamins of somatic cells (8, 22, 33, 38, 63). Only sparse and spotty immunofluorescence with lamin antibodies on murine sperm has been reported by Schatten et al. (60), who concluded that lamins are vastly reduced during spermiogenesis. In contrast, Pruslin and Rodman (53) and Maul et al. (41) have reported the presence of lamin-related proteins in mouse sperm heads, as judged from immunofluorescence staining and gel electrophoretic positions, but without positive identification of the individual polypeptides localized. At present this discrepancy cannot be satisfactorily resolved. We do not exclude the possibility that low amounts of cell type-specific lamin(s), which have epitopes in common with somatic lamins recognized by the antibodies used by some authors (41, 53) but not by the antibodies used by us and others, may be expressed during spermiogenesis. In-deed, a spermatogenesis-specific nuclear lamin has been described in *Xenopus laevis* by Benavente and Krohne (10).

Therefore, we conclude that the largest contribution to the densely webbed residual structures of sperm heads is made by the perinuclear theca and, hence, the basic cytoskeletal proteins described in this paper. It is tempting to speculate that both kinds of proteins, calycin and MBP, are involved in spermiogenesis in that they contribute to the reduction of the cytoplasm in the forming sperm head and to the compression of the residual cytoplasm into the densely woven web, which practically represents the only vestigial cytoplasm left above the posterior ring. They may also contribute to the nuclear–acrosome association and provide some sort of intermembranous cement (cf. 8 and 17). In discussing possible functions of these proteins it should also be remembered that it is the posterior aspect of the sperm head, including the calyx region, which makes contact and fuses with the egg plasma membrane (e.g., 7, 62, and 73), so that possible contributions of thecal proteins to the fertilization process should also be taken into consideration.

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