Germ cell-specific localization of immunoreactive riboflavin carrier protein in the male golden hamster: appearance during spermatogenesis and role in sperm function

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Abstract

Riboflavin carrier protein (RCP) is a phosphoglycoprotein (37 kDa) that is well studied in chicken. An immunologically cross-reacting protein was identified in mammals and active immunization of male rats and bonnet monkeys with chicken RCP lead to an ~80% reduction in fertility. However, the physiological mechanism responsible for inhibition of male fertility has not been investigated. Moreover, information on the cell type-specific localization and the origin of immunoreactive RCP during spermatogenesis is extremely limited. Hence, studies were carried out to determine the pattern of expression of immunoreactive RCP during spermatogenesis and its role in sperm function in the golden hamster. Immunoreactive RCP was germ cell-specific, found to be associated with the acrosome-organizing region of early spermatids and showed interesting patterns of immunolocalization during late stages of spermiogenesis. Mature spermatozoa exhibited acrosome-specific localization, mainly in the peri-acrosomal membrane. The immunoreactive protein was undetectable in (non)gonadal somatic cells tested. The protein had a molecular mass of 45–55 kDa and was biosynthesized by round spermatids. The acrosome-specific localization of immunoreactive RCP was unchanged during capacitation, but it was substantially lost during acrosome reaction. Functional studies indicated that treatment of spermatozoa with anti-RCP antibodies did not have any effect on either capacitation or acrosome reaction, but markedly reduced the rate of sperm penetration into zona-free hamster oocytes. These results show the existence of male germ cell-specific immunoreactive RCP, having a potential role in sperm–egg interaction in hamsters. Also the pattern of immunoreactive-RCP localization makes it an ideal marker to monitor development of acrosome in mammalian spermatozoa.

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Introduction

During mammalian spermatogenesis, the male germ cells undergo a complex and prolonged differentiation process to develop into spermatozoa. The post-meiotic phase of spermatogenesis, also known as spermiogenesis, is characterized by remarkable morphological and biochemical changes in germ cells with the formation of functionally important organelles, namely the acrosome and flagellum. A completely formed testicular sperm attains functional maturation upon capacitation and acrosome reaction, and thus acquires the ability to fertilize the oocyte (Yanagimachi 1994). The acrosome reaction, the hallmark of sperm functional maturation, involves the release of a number of proteases, which enable the sperm to penetrate the oocyte (Yanagimachi 1994). The acrosome, thus, is a storehouse for a large number of proteins known to play a role in the functional maturation of spermatozoa. A variety

of such proteins have been reported to be present/expressed specifically in the germ cells, particularly during sperm formation. Discovering the origin, distribution and functional role of such proteins is essential for a complete understanding of spermatogenesis. Several of these proteins could be potential markers of male fertility/infertility and/or cancer (Dean & Moul 1998, Jager *et al.* 2001). Furthermore, many of the germ cell proteins are immunogenic and serve as potential target molecules for immunocontraception in humans (Diekman & Herr 1997, Naz 2000). Hence, it is important to study in detail proteins associated with the male germ cells.

Among the proteins associated with the male reproductive system is a group belonging to the family of vitamin carriers. These include retinol-binding protein (Davis & Ong 1992) and riboflavin carrier protein (RCP; Adiga *et al.* 1997). These are believed to be involved in the transportation of micronutrients across the blood testis barrier

(Adiga *et al.* 1997). Immunoreactive RCP has been localized in the sperm head region in some mammals (Bhat *et al.* 1995) and is implicated to play an important role in mammalian reproduction. Earlier studies have indicated a marked reduction in male fertility (~80%), both in rodents and primates, following active immunization against chicken RCP (Adiga *et al.* 1997). However, the mechanism by which anti-RCP antibodies bring about this inhibition of fertility has not been well understood. Besides, the biochemical nature of the immunoreactive RCP, its origin and cellular localization during spermatogenesis as well as its role during sperm–egg interaction have not been defined clearly. In view of these shortcomings, we examined the occurrence and characterization of immunoreactive RCP in male reproductive tissues and attempted to characterize it and determine its functional role in the golden hamster. We chose the golden hamster for these studies since it is an ideal and important animal model for studies involving mammalian sperm structure and function.

Materials and Methods

Animals

Adult golden hamsters (*Mesocricetus auratus*) of 3 months old were used for the study. Animals were housed under a 14-h:10-h light/dark cycle with normal temperature and humidity. For some experiments, 21-day-old males were used. Animals were killed by cervical dislocation, and desired tissues were collected for various purposes as described below. All animal experiments were conducted according to the guidelines set by the Indian National Science Academy, New Delhi, India.

Preparation of enriched population of round spermatids

Enriched populations of round spermatids were obtained from hamster testis ($n = 4$) by centrifugal elutriation (Bucci *et al.* 1986). Briefly, testes were collected in Dulbecco's minimum essential medium (MEM), decapsulated, minced and digested with collagenase (0.04% in Dulbecco's MEM with 25 mM Hepes and 10 µg/ml DNase) for 30 min, at 32 °C in a water bath with gentle shaking. The cells were centrifuged at 1000 *g*, washed and resuspended in Ca^{2+} - and Mg^{2+} -free PBS containing 0.1% glucose and 0.2% BSA. These were then loaded into a JE6 elutriator rotor (Beckmann Coulter, Fullerton, CA, USA). Fractions (150 ml) containing enriched populations of specific cell types were obtained by varying the rotor speed and flow rates. The enriched cells in each fraction were recovered by centrifugation at 100 *g* for 7 min, washed once and resuspended in PBS. An aliquot of each cell population was fixed in 70% ethanol for DNA flow-cytometric analysis (FACS; Becton and Dickinson, Franklin Lakes, NJ, USA) and the percentages of individual germ cell types in

different fractions were assessed (Aravindan *et al.* 1990). About 70% enrichment of round spermatids was achieved and the preparation was used for the study.

[³⁵S]Methionine labeling of isolated round spermatids and immunoprecipitation

Enriched preparation of round spermatids, obtained by centrifugal elutriation, were incubated, in Dulbecco's MEM lacking L-methionine and L-cysteine, supplemented with 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA) and 100 µg/ml streptomycin for 8 h at 32 °C, in an atmosphere of 5% CO_2 in humidified air. [³⁵S]Methionine (specific activity, 1000 Ci/mmol; New England Nuclear, Boston, MA, USA) was added at a concentration of 50 µCi/ml (O'Brien 1987). Cells were cultured in 60 mm plastic tissue-culture dishes (Falcon Plastics, Oxnard, CA, USA) containing 6 ml medium, at a density of 5×10^6 cells/ml. At the end of the culture period, the cells were harvested and frozen at –70 °C.

The cells were later solubilized in an equal volume of RIPA buffer (0.5 M Tris/HCl buffer, pH 7.5, containing 150 mM NaCl, 0.1% SDS, 1% Triton X100 and 1% deoxycholate) with protease inhibitors (Complete Mini; Boehringer Mannheim, Mannheim, Germany) and 255 µmol each of L-methionine and L-cysteine (Sigma, St Louis, MO, USA). This was then subjected to four or five cycles of homogenization (30 s/cycle). The homogenate was clarified by centrifugation at 7000 *g* for 30 min at 4 °C. The supernatant was preadsorbed on a column containing rabbit non-immune IgG coupled to CNBr-Sepharose 6B (Sigma) for 4–12 h at 4 °C. The unbound fraction was divided into two halves and treated with either 100 µg/ml anti-RCP IgG (raised using published protocols against purified chicken RCP; White & Merrill 1988) or non-immune IgG and incubated for 12 h at 4 °C. The antigen–antibody complex was adsorbed on to 100 µl/ml Protein G–Sepharose (Sigma) for 4–6 h at 4 °C. The adsorbed beads were washed with chilled RIPA buffer followed by PBS, and subjected to electrophoresis by SDS/PAGE (10% gel; Laemmli 1970). The gel was processed for fluorography using Enhancer (New England Nuclear), dried and exposed to X-Omat K X-ray films (Eastman Kodak Company, Rochester, NY, USA).

Preparation of cauda epididymal (CE) spermatozoa

Epididymal spermatozoa were collected in Dulbecco's PBS (DPBS) by puncturing the distal tubules of the cauda epididymism, in the region devoid of capillaries, using a 26 gauge needle, as per standardized protocol (Bavister & Andrews 1988, Ain *et al.* 1999). For immunoblot analysis, the caudal content was filtered through a nylon mesh and the filtrate was spun at 1000 *g* for 10 min at 4 °C. The sperm pellet was washed thrice with PBS while the supernatant was further clarified at 12 000 *g* for 30 min at 4 °C and both were subjected to immunoblot analysis.

Immunoblot analysis

Total proteins (~100 µg) from either CE contents or CE sperm or CE contents devoid of sperm were separated by electrophoresis on SDS/10% polyacrylamide gels and were blotted on to nitrocellulose membrane (Towbin *et al.* 1979). After blocking the non-specific sites, the blots were treated with 1 µg/ml of either anti-RCP IgG or non-immune IgG or pre-adsorbed anti-RCP IgG (using a 100-fold excess of chicken RCP). After washing with PBS-T (PBS with 0.1% Tween-20), the blots were treated with 1:3000 dilution of anti-rabbit horseradish-peroxidase-conjugated antibodies raised in goat (Sigma). The immunoreactive bands were visualized using the enhanced chemiluminescence detection system (ECL Plus; Amersham Biosciences, Piscataway, NJ, USA).

Immunocytochemistry

Testis, epididymis and somatic tissues tested were all fixed in Bouin's fluid for 24 h. Tissues were washed, dehydrated, embedded in paraffin wax and 5 µm-thick sections were cut using a rotary microtome (1090A; Weswox Optik, MT, USA). Sections were placed on poly-L-lysine (0.01% in DPBS)-coated slides, deparaffinized in xylene and rehydrated in a graded series of ethanol. Similarly, round spermatids, obtained after centrifugal elutriation, and epididymal spermatozoa were resuspended in DPBS containing 1% BSA and spotted on to poly-L-lysine-coated slides. The slides containing tissue sections or cells were treated with methanol/hydrogen peroxide (49:1) to block endogenous peroxidase activity. Nonspecific sites were blocked using DPBS containing 1% BSA and 5% normal goat serum overnight at 4°C. After a brief rinse with PBS, slides were incubated with 100 µg/ml each of either anti-RCP IgG or non-immune IgG. Antibodies against peptide stretches 33–49 (CYA) or 64–83 (CED) of chicken RCP was also used at a dilution of 1:50 for some experiments. Similarly, antibodies to peptide stretch 130–147 (CGE) of avian RCP were used at 1:100 dilution (Monaco 1997, Subramanian *et al.* 2000), while those against SDS-denatured reduced carboxy-methylated RCP (SDS-RCM-RCP) were used at 1:1000 dilution (these antibodies were a kind gift from Professor PR Adiga). The slides were washed thrice with PBS-T and were further incubated with goat anti-rabbit horseradish peroxidase conjugate (1:1000) for 4 h at room temperature. After washing, immunoreactivity was detected using 0.05% diaminobenzidine and 0.015% hydrogen peroxide. The slides were counter stained with hematoxylin, dehydrated and mounted with DPX.

Immunogold electron microscopy

The CE spermatozoa were collected in PBS and fixed in 0.5% glutaraldehyde and 3% paraformaldehyde in PBS for 30 min. After blocking the aldehyde groups using 0.1 M glycine, they were dehydrated in graded ethanol series and cleared in propylene oxide. The sperm were

embedded in spur resin and gold interference sections were cut and placed on nickel grids. They were then incubated in DPBS containing 1% BSA and 5% normal goat serum overnight at 4°C. Anti-RCP IgG or non-immune IgG was used at 100 µg/ml in blocking solution and allowed to react with the sections at 4°C for 48 h. After extensive washing of the grids in drops of DPBS, sections were incubated for 12 h in a 1:50 dilution of goat anti-rabbit gold conjugate (5 nm gold particles; Sigma). Sections were washed in DPBS and fixed in 5% glutaraldehyde for 30 min. They were then stained with 1% uranyl acetate and lead citrate and observed at 80 KV accelerating voltage under a Joel 100CX electron microscope.

In vitro capacitation and induction of acrosome reaction of hamster sperm

Spermatozoa from the distal tubules of the CE were recovered into Tyrode's medium containing lactate, pyruvate and polyvinyl alcohol (TLP-PVA) supplemented with 3% BSA and cultured at 37°C in an atmosphere of 5% CO₂ in air. About 1×10^4 spermatozoa were added to either medium alone or medium containing 10 µg/ml of either non-immune IgG or anti-RCP IgG and cultured for 6 h. At every 2 h interval, percentage of sperm motility and quality of motility (score, 0–5) were assessed and sperm motility index (SMI) was calculated (Bavister & Andrews 1988, Ain *et al.* 1999). Acrosome reaction of viable spermatozoa (minimum of 700 sperm/treatment) was assessed after 4 h of culture by detecting the presence or absence of acrosomal cap under light microscope at 400× (Bavister *et al.* 1978). An aliquot of the spermatozoa undergoing capacitation or acrosome reaction was also washed and processed for immunocytochemistry.

Hamster egg-penetration assay (HEPA)

Adult female hamsters were superstimulated by i.p. injection of 30 IU pregnant mare's serum gonadotropin, on the day of post-estrous discharge, followed 56 h later by 30 IU human chorionic gonadotropin (hCG). Oocytes retrieved by puncturing the swollen ampullary region of excised oviducts at 16 h post-hCG, and were treated with 1 mg/ml hyaluronidase and 0.01 mg/ml soybean trypsin inhibitor to remove cumulus cells (Bavister 1989). The cumulus-free oocytes were denuded using acid Tyrode's solution for 1–2 min. These were washed and incubated with 1×10^4 spermatozoa exposed to either anti-RCP IgG or non-immune IgG or remained untreated for 2 h. After co-incubation, oocytes were washed, fixed with 3% glutaraldehyde and stained with lacmoid solution (0.25% in 45% acetic acid). They were then observed under an Olympus IMT2 inverted microscope.

Statistical analysis

The results of Fig. 8, Table 1 and acrosome-reaction data were from a minimum of three experiments. Values

Table 1 Assessment of fertilizing potential of anti-RCP-IgG-treated spermatozoa in the golden hamster Values shown are means $\pm$ S.E.M.

treatment		Number of oocytes co-cultured	Percentage of oocytes penetrated	Number of sperm per oocyte (range)	Number of sperm per penetrated oocyte
Oocyte	Sperm				
Untreated	Untreated	53	88.3 $\pm$ 4	7.2 $\pm$ 0.8 ^{a,b} (3–17)	8.1 $\pm$ 0.6 ^{a,b}
Untreated	Non-immune IgG	53	88.6 $\pm$ 4.3	7.3 $\pm$ 1.2 ^a (4–20)	8.3 $\pm$ 1.0 ^a
Untreated	Anti-RCP IgG	76	74.7 $\pm$ 5.2	1.9 $\pm$ 4 ^b (1–7)	2.5 $\pm$ 0.3 ^b

^aValues do not differ significantly ($P > 0.1$)^bValues differ significantly ($P < 0.001$)

shown were means $\pm$ S.E.M. and were analyzed by Student's *t* test. Compared values were considered significantly different if $P < 0.05$.

Results

Localization of immunoreactive RCP in testis, epididymis and spermatozoa

Testicular sections treated with anti-RCP antibodies showed intense staining in the germ cells located in the interior, towards the lumen of the seminiferous tubules (Fig. 1A and C). Immunostaining was undetectable in Sertoli cells, Leydig cells (Fig. 1A), sections of immature testis and accessory reproductive or non-reproductive organs (data not shown). No staining was seen in the testicular sections treated with either non-immune IgG (Fig. 1B) or antigen pre-adsorbed anti-RCP antibodies (Fig. 1D). Examination of immunostaining in germ cells revealed that the staining was specifically confined to spermatids (Fig. 2), with no staining associated with either spermatogonia or pachytene spermatocytes (Fig. 2B and C). The staining first appeared in pro-acrosomal granules/vesicles in the form of an arc in the peri-nuclear region of early spermatids (Fig. 2A and B). While the intensity of staining in the peri-nuclear region gradually increased during the later stages of spermatogenesis, diffused and mild staining was observed in the cytoplasm in the later stages of elongating spermatids (Fig. 2C).

Observations on immunostaining obtained with isolated spermatid population revealed the antigen to be localized during steps 1–17 of spermiogenesis (Fig. 3), as described elsewhere (Clermont 1954). Faint staining was first observed in pro-acrosomal granules/vesicles at the peri-nuclear region of the Golgi-phase spermatids (Fig. 3, steps 1–4). These migrate to the anterior part of the spermatid, i.e. the acrosome-organizing region (characterized by the presence of an eccentrically located nucleus). In the later stages of spermatid development, the staining was detected in the cytoplasm (Fig. 3, step 5). During steps 6–16 of spermiogenesis, intense and uniform staining was observed in both the cytoplasm and the developing acrosome. Interestingly, in the testicular sperm (steps 16 and 17) excess immunostaining, associated with the Golgi remnants/granules, was excluded from the tail region in the form of a residual body (Fig. 3, step 16). The immunoreactive protein finally localized specifically

to the acrosomal region of mature epididymal spermatozoa (Fig. 3, CE-sperm). A similar pattern of immunostaining was obtained in the mature epididymal sperm using antibodies to SDS-RCP or various peptide fragments (data not shown).

Following the establishment of organelle-specific localization of immunoreactive RCP during testicular development of spermatozoa, it was of interest to assess the pattern of immunostaining for RCP during epididymal and functional maturation of the sperm. In epididymal tissue sections, intense staining was observed on the luminal spermatozoa and was undetectable in the epithelial cells or the inter-tubular space (Fig. 4). Further characterization of this immunoreactive protein using immunoblot analysis confirmed its association with the total CE content and CE sperm (data not shown). Encouraged by cell-type and organelle-specific localization of the sperm-associated RCP, it was interesting to investigate the ultrastructural association of the protein in spermatozoa using immunogold electron microscopy. This indicated the distribution of immunoreactive RCP on the sperm head, specifically with the matrix and the peri-acrosomal membranes (Fig. 5A, outer acrosomal and plasma membranes). Also, a few gold particles were seen in the inner acrosomal membrane (Fig. 5A). No staining was observed in non-immune-IgG-treated sections (Fig. 5B).

Biochemical characterization of immunoreactive RCP in spermatids and spermatozoa

Immunoblot analysis of total proteins from spermatids showed the presence of three bands, of 29, 45 and 55 kDa (Fig. 6A). Moreover, [³⁵S]methionine radiolabeling of the enriched population of round spermatids in culture followed by immunoprecipitation using anti-RCP antibodies showed the ability of these germ cells to synthesize the immunoreactive protein during their development (Fig. 6B). Importantly, analysis of the immunoprecipitate showed a major protein of 45 kDa (Fig. 6B). Further analysis of sperm-associated RCP using urea-extracted lysates revealed an intense band in the 45 kDa region (Fig. 6C).

Role of immunoreactive RCP in sperm function

Following identification of the germ cell-specific immunoreactive RCP, it was interesting to investigate the possible role of this protein during sperm function. As an initial

Figure 1 Localization of immunoreactive RCP in testicular sections from hamster. Immunoreactive RCP was localized in elongating spermatids and later cell stages of spermiogenesis but not in pre-meiotic germ cells, Sertoli cells or Leydig cells (A, C). No staining was observed in non-immune-IgG-treated (B) or preadsorbed antibody-treated sections (D). Original magnifications: A and B, $\times 200$; C and D, $\times 500$. Scale bars: A and B, 69 μm ; C and D, 42 μm .

Figure 2 Localization of immunoreactive RCP in round and elongating spermatids. Immunoreactive RCP first appeared in the acrosome-organizing region in the early round spermatid (A; arrow). In the later stages of the round spermatid, the staining also appeared in the cytoplasm (B). In elongating spermatids, intense staining was observed both in the acrosome-organizing region and the cytoplasm (C; arrow and asterisk). No staining was observed in pachytene spermatocytes (B and C; star). Original magnification, $\times 500$. Scale bars: 17 μm .

Figure 3 Stage-specific localization of immunoreactive RCP in developing male germ cells in hamsters. Immunoreactive RCP was first detected in the form of a granule in step-1 spermatids. The staining was observed in the cytoplasm and developing acrosome, in step-4 spermatids. By step 7, cytoplasmic staining was intense. In step 16, most of the staining was found in the residual body and acrosomal region of the mature sperm. In epididymal sperm (CE-sperm), staining was solely present in germ cells in different stages of spermiogenesis. Spermatids in steps 1–3 constitute the Golgi phase, characterized by the assembly of Golgi components into the acrosome-organizing region. Steps 4–7 constitute the cap phase characterized by the appearance of a head cap. Steps 8–13 constitute the acrosomal phase characterized by formation of the acrosome. The maturation phase, constituted by steps 14–17, ends with the formation of a mature testicular spermatozoan. Original magnification: $\times 500$. The description of various steps of hamster spermiogenesis is based on Clermont (1954).

step, the pattern of localization of this protein was studied during capacitation and acrosome reaction (Fig. 7). Immunostaining of *in vitro*-capacitated sperm showed intense staining associated with the acrosomal region (Fig. 7A). Interestingly, however, most of the acrosome-associated immunoreactivity was lost during acrosome reaction (Fig. 7B). Thus acrosome-reacted sperm exhibited a very faint staining associated with the dorsal surface of the acrosome, presumably the inner acrosomal membrane (Fig. 7B). The staining was absent when non-immune IgG was used for detection (Fig. 7C).

In vitro sperm bioassay, carried out to study the role of RCP during capacitation, showed no change in the quality of motility of spermatozoa in the presence of anti-RCP

IgG (Fig. 8A). However, there was a significant ($P < 0.01$) reduction in the percentage motility (69.1 ± 1.1 versus $51.3 \pm 2.5\%$) at 15 min post-treatment, compared with untreated controls (Fig. 8B). By the sixth hour, the percentage of motility was significantly ($P < 0.01$) lower in antibody-treated samples ($43.8 \pm 3.1\%$) compared with controls ($59.7 \pm 2.7\%$; Fig. 8B). A decrease in motility upon antibody treatment was evident when the SMI values were compared (Fig. 8C); SMI at 6 h post-treatment was significantly ($P < 0.01$) lower in anti-RCP-IgG-treated sample compared with untreated sample (807.2 ± 103 versus 1211.3 ± 59.7 ; Fig. 8C).

The effect of antibody treatment on sperm acrosome reaction was also tested. Anti-RCP IgG treatment of sperm

Figure 4 Localization of immunoreactive RCP in epididymis. Immunoreactive RCP was localized to the luminal spermatozoa in the caput (A, B), corpus (C, D) and cauda (E, F) epididymis. No staining was observed in the epithelial cells and the intertubular connective tissues. Original magnifications: A, C, E, 200 $\times$; B, D, F, $\times$ 500. Scale bars: A, C, E, 69 μ m; B, D, F, 42 μ m.

did not significantly ($P > 0.1$) affect their ability to undergo the acrosome reaction. The percentage of spermatozoa undergoing acrosome reaction in the presence of anti-RCP IgG was $27.7 \pm 5.9\%$ compared with those in non-immune-IgG-treated ($29.7 \pm 1.3\%$) or -untreated ($29.9 \pm 1.8\%$) control samples. Because of the apparent inhibition of sperm motility in the anti-RCP-antibody-treated sperm sample, it was of interest to study the ability of such immunocompromised sperm to penetrate the zona-free oocyte, by carrying out HEPA (Table 1). In this assay, treatment of spermatozoa with anti-RCP IgG resulted in a marked reduction in the number of sperm penetrated per oocyte (by 74%), while the number of sperm per

penetrated oocyte decreased by 68%. Thus, the number of sperm penetrated per oocyte in the antibody-treated group was significantly ($P < 0.001$) low, i.e. 1.9 ± 0.4 , when compared with those in the untreated (7.2 ± 0.8) and non-immune-IgG-treated (7.3 ± 1.2) groups. Also, the number of sperm per penetrated oocyte in the antibody-treated group was significantly ($P < 0.001$) low (2.5 ± 0.3) when compared with those in the untreated (8.1 ± 0.6) and non-immune-IgG-treated (8.3 ± 1.0) groups (Table 1). However, the percentages of oocytes penetrated were not significantly different among the groups, in either the presence ($74.7 \pm 5.2\%$) or absence ($88.3 \pm 4\%$) of anti-RCP IgG (Table 1).

Figure 5 Immunogold electron-microscopic localization of immunoreactive RCP on hamster spermatozoa. Gold particles were found distributed in the matrix and peri-acrosomal membranes of the acrosomal region, in sections treated with anti-RCP antibody (A). A few gold particles were also found associated with the equatorial region of the inner acrosomal membrane (A). No gold particles were found in non-immune-IgG-treated sections (B). Original magnifications: $\times$ 20 000.

Figure 6 Biochemical analysis of immunoreactive RCP in round spermatid and spermatozoa. (A) Immunoblot analysis of urea-extracted proteins from round spermatids in one dimension showed three bands in the 55, 45 and 29 kDa regions (arrows). (B) Immunoprecipitation of [35 S]methionine-labeled proteins of round spermatids using anti-RCP antibodies showed an intense band at $\sim$ 45 kDa. (C) Immunoblot analysis of urea-extracted sperm proteins on one dimension gave an intense band at 45 kDa.

Discussion

In this study we demonstrate the existence of a germ cell-specific, strongly immunoreactive RCP of 45–55 kDa in the acrosomal region of hamster spermatozoa. The protein appears first in differentiating spermatids during spermiogenesis and in mature spermatozoa it is specifically localized in the acrosomal region. While the sperm RCP does not have any crucial role during capacitation or acrosome reaction, it appears to be involved in sperm–egg interaction.

One of the important findings is the conspicuous presence of immunoreactive RCP only in the post-meiotic germ cells, i.e. exclusively in round spermatids and spermatozoa. This is corroborated by a complete absence of immunoreactivity in immature testis (lacking spermatids or spermatozoa) and adult pre-meiotic germ cells, Sertoli cells and Leydig cells. Also, our results of [35 S]methionine radiolabeling of round spermatids followed by immunoprecipitation using antibodies to RCP clearly demonstrate the ability of post-meiotic germ cells to biosynthesize *de novo* the immunoreactive protein. Although RCP-translational activity in round spermatids is evident, the transcriptional regulation of its gene expression during earlier phases of spermatogenesis cannot be ruled out. Importantly, our data show that the immunoreactive RCP is not detectable in spermatocytes, though low amounts of the protein were detectable in spermatids of steps 1–4, with significant amounts being detected in the later steps of spermatid development, as well as in epididymal spermatozoa. The presence of strong immunoreactivity in the Golgi/acrosomal region, coinciding with the onset of acrosomal biogenesis, is consistent with the earlier report on

Figure 7 Changing pattern of localization of immunoreactive RCP during capacitation and acrosome reaction. In capacitated sperm, staining was found associated with the acrosomal region of the sperm (A; arrow). However, during the acrosome reaction most of the immunoreactivity was lost (B). A completely acrosome-reacted sperm showed faint staining on the dorsal surface (B; arrow). No staining was found in the non-immune-IgG-treated, acrosome-reacted sperm (C).

the association of this protein with the head region of a few mammalian spermatozoa (Bhat *et al.* 1995). Such a pattern of immunolocalization that we observed is shared by several other acrosomal-specific proteins, such as Tep22 (Neesen *et al.* 2002), proacrosin (Phi-Van *et al.* 1983), protein disulfide isomerase (Ohtani *et al.* 1993), SP-10 (Kurth *et al.* 1993), SIAA (Jimenez *et al.* 1994), rat 2B1 (Jones *et al.* 1996), ACIII (Gautier-Courteille *et al.* 1998), Kir3.2d (Inanobe *et al.* 1999), Acrin1 (MN7) (Oh-Oka *et al.* 2001) and CYP51 (Cotman *et al.* 2001). However, the site of biosynthesis of immunoreactive RCP in various mammalian species (Subramanian & Adiga 1996) remains to be investigated.

The cytoplasmic appearance of immunoreactive RCP in elongating spermatids and residual body may be functionally important from the point of view of sperm maturation in the hamster. One of the highlights of the present study is the exclusion of the bulk of the antigen, associated with Golgi remnants/granules, into the residual body during the terminal phase of spermiogenesis. This gains significance with the finding that most of the 29 kDa fragment observed on the immunoblot of round-spermatid proteins was lost in mature epididymal sperm. This could be indicative of partial or complete release of these immunoreactive components into the residual body of the developing spermatid at the end of testicular sperm development. It could, therefore, be possible to envision a role for these immunoreactive RCP fragments during spermiogenesis. Further studies would be required to assess their role, if any, during hamster sperm development.

The pattern of immunostaining in the acrosomal region of spermatozoa did not change during either epididymal transit or capacitation, indicating a role for the antigen in

Figure 8 Effect of RCP antibodies on capacitation of hamster spermatozoa in an *in vitro* sperm bioassay. Addition of anti-RCP IgG (10 µg/ml) to sperm in culture did not have any effect on the quality of motility (A) while significantly reducing the percentage of motile spermatozoa (B). This was reflected in the SMI value (C) that was significantly lower in anti-RCP-treated spermatozoa compared with controls. Significance levels, $P < 0.01$. TLP-PVA, Tyrode's medium containing lactate, pyruvate and polyvinyl alcohol.

post-capacitation events. Of interest here is the observation that most of the immunostaining is lost, following acrosome reaction, leaving only a marginal staining detectable in acrosome-reacted spermatozoa. This may be due to an incomplete loss of the acrosome and/or to the presence of a small amount of the antigen in the inner acrosomal membrane. This possibility is indeed consistent with our obser-

vation of the appearance of immunogold particles being associated with the inner acrosomal membrane and the matrix components. Loss of the immunoreactive RCP following acrosome reaction, coupled with its presence in the acrosomal matrix, may indicate a possible role for this antigen in regulating acrosomal function involving release of various hydrolytic enzymes required for sperm penetration into the oocyte (Dicarlantonio & Talbot 1988, Olson *et al.* 2003). Such a role has been assigned to the 22 kDa glycoprotein in the hamster (Longo *et al.* 1990).

In view of the above and also because we did not observe any discernible effect of the anti-RCP antibodies either on capacitation or acrosome reaction, we investigated the immunological interference of the protein on sperm-egg interaction, in order to understand its possible role in the downstream events of sperm function. Incubation of spermatozoa with antibodies to RCP resulted in a significant reduction in the number of spermatozoa per penetrated oocyte compared with the untreated or non-immune-IgG-treated control spermatozoa. This may be due to the effect of the antibody on the parameters governing sperm motility. Indirect evidence stems from our findings on the reduced percentage of motile spermatozoa following anti-RCP-IgG treatment and the sperm's interaction with oolemma being affected by the antibodies. However, it should be noted that a sperm's ability to interact with a zona-free oocyte is not the same as zona-intact oocyte. Pertinent to this is a report of anti-SP-10 antibody inhibiting hamster sperm binding to hamster oolemma but not to human zona pellucida (Hamatai *et al.* 2000). Despite these, we preferred HEPA in the current study to *in vitro* fertilization, since the former allows quantitative assessment on the sperm's fertilizing potential. It is known that antibodies to sperm antigens could affect sperm-oocyte interaction in many ways. For example, antibodies to MH61 inhibit sperm penetration into zona-free oocytes without affecting the numbers of sperm binding to the same oocyte (Kawamoto *et al.* 1999). Although, the mechanism of this inhibitory effect on sperm-egg interaction needs to be fully understood, based on our data it could be suggested that immunoreactive sperm-associated RCP could have an important role during sperm penetration into the oocyte.

It was quite interesting to note that there was an antibody-induced decrease in the percentage of motile spermatozoa in the *in vitro* sperm bioassay. This could be due to inactivation of a population of spermatozoa by anti-RCP antibodies by mechanism that is not clear at this time. One possibility could be the binding of antibodies to the Fc receptor on the sperm tail, disrupting their motility (Zouari *et al.* 1992). Antibody-induced agglutination has been reported for SMA-4 (Vernon *et al.* 1982) and SAGA-1 (Norton *et al.* 2001). Because we did not observe any sperm clumping in culture, we believe that antibody-induced agglutination is not the factor leading to a decrease in the percentage of motile spermatozoa.

Our data on the biochemical characteristics of the immunoreactive RCP in developing spermatids indicate

that the protein could be polymorphic with molecular masses ranging from 29 to 55 kDa, with the existence of these components being defined by the stage(s) of developing spermatids. Our preliminary studies attribute the observed variation in the molecular sizes of sperm RCP as being due to differentially denatured forms of the same protein (A Sreekumar & P B Seshagiri, unpublished observations). In this context, several sperm antigens with a role in fertilization have been reported to exhibit polymorphism in their molecular size (Diekman & Herr 1997, Naz 2000). This polymorphism has been attributed to proteolytic cleavage, as in sp42 (Berruti & Borgonovo 1996), differential glycosylation, as in SP-10 (Herr *et al.* 1990), or subunit structure, as in fertilin- β (Blobel *et al.* 1992).

Interestingly, the pattern of immunoreactivity observed for male germ cell-specific RCP using both immunocytochemical and immunoblot analysis was similar using antibodies against either native or denatured preparations of chicken RCP or against various peptide stretches of the protein. This is strongly indicative of the conservation of epitopes in the mammalian protein compared with its avian counterpart. No report, however, is available on the electrophoretic migratory profile and other biochemical characteristics of the immunoreactive mammalian sperm RCP except in the hamster, as described in the present study.

In summary, the present study reveals the existence in hamster spermatids and spermatozoa of an antigenic protein that has a molecular mass ranging from 29 to 55 kDa and is strongly immunoreactive to chicken RCP. The protein forms an integral part of the acrosome and does not appear to have any role during capacitation and acrosome reaction. It has, however, an important role in sperm-egg interaction. Its specific appearance at the Golgi/acrosome region of the developing spermatid makes sperm RCP a candidate marker to study the various steps of mammalian spermatogenesis. It could also provide an ideal tool for evaluating developmental defects in the acrosome during germ cell development, such as round cell syndrome (Jassim & Festenstein 1987).

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