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Molecular and Functional Characterization of the Rabbit Epididymal Secretory Protein 52, REP52[1]

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ABSTRACT

As part of a systematic study of rabbit epididymal proteins involved in sperm maturation, we have identified and characterized a novel glycoprotein (rabbit epididymal secretory protein 52 [REP52]) of 52 kDa. REP52 is synthesized and secreted in a tissue-specific manner by the mid (region 6) and distal (region 7) corpus epididymidis and associates weakly with the sperm surface overlying the principal piece of the tail. Sequencing of cloned *REP52* cDNA demonstrated that this protein represents a novel member of the highly conserved fibronectin type II (FN2) module protein family. The protein appears related but not homologous to ungulate seminal plasma proteins and is the first known example to be identified as a rabbit epididymal secretory protein. Consistent with other members of this protein family, REP52 possessed a high level of sequence identity within the FN2 module-encoding domains, but a highly variable N-terminal sequence that failed to show significant homology with published sequences. By analogy with evidence from studies of the ungulate seminal plasma proteins it is hypothesized that the tandemly arranged FN2 modules could facilitate the association of REP52 with sperm phosphatidylcholine residues on the outer leaflet of the sperm tail. It is also considered likely that these domains represent key elements for the function of this novel protein, a conclusion supported by the fact that antisera raised against the REP52 protein blocked in vitro fertilization in a concentration-dependent fashion.

epididymis, male reproductive tract, sperm maturation

INTRODUCTION

The mammalian epididymis represents the site where functionally incompetent spermatozoa originating from the testes undergo their final maturation, enabling them to complete the complex cascade of sperm-egg interactions that culminate in fertilization. Since sperm enter the epididymis as terminally differentiated cells with limited capacity for biosynthesis, maturational changes must be supported and possibly induced by sequential interactions with the luminal milieu; these changes are not an intrinsic property of spermatozoa themselves. In this context, one of the most remarkable features of the epididymis is the way in which the secretion of specific proteins is spatially restricted to precisely defined areas of this organ. As a consequence, the microenvironment in which spermatozoa undergo their maturation is constantly changing in a carefully orchestrated sequence.

Elucidating the molecular mechanisms by which constituents of the epididymal secretions interact with spermatozoa to promote their functional maturation has been an area of considerable interest for our laboratory. The approach we have taken to resolve these mechanisms is to focus on the male gamete, to explain the biological changes that are taking place in this cell during epididymal maturation and, from a knowledge of these changes, to deduce how the constituents of the epididymal secretions might be driving the maturation process to completion. Success in this context will have implications both for development of reversible male contraceptive agents and the etiology of male infertility, which frequently involves defects in aspects of sperm function, such as motility, zona binding, or zona-induced acrosomal exocytosis, all of which are acquired in the epididymis [1, 2].

In molecular terms, it is well established that the process of sperm maturation involves profound changes in the properties of the sperm plasma membrane. In recognition of this we have initiated a project to map and assign functional significance to these surface changes. In this paper we report the molecular and functional characterization of rabbit epididymal secretory protein 52 (REP52), a novel sperm-associated rabbit epididymal secretory protein that we have recently identified as part of this program [3].

MATERIALS AND METHODS

Chemicals

All chemicals were of reagent grade and, unless otherwise stated, were obtained from Sigma (Sigma Chemical Co., St. Louis, MO) or BDH (BDH Laboratory Supplies, Poole, England). Polyvinylidene fluoride (PVDF) membrane was purchased from DuPont (DuPont, Wilmington, DE). Fluorescein isothiocyanate (FITC)-labeled secondary antibodies were from Silenus (Silenus Laboratories, Melbourne, Australia), and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Bio-Rad (Bio-Rad Laboratories, Hercules, CA).

Animals

All experimental use of animals was approved by the Institutes Animal Ethics Committee in accordance with National Health and Medical Research Council guidelines. Male New Zealand white rabbits and female BALB/c mice (>8 wk) were obtained from breeding colonies housed at CSIRO, Sustainable Ecosystems, where they were maintained under a lighting regime of 16L:8D and supplied with food and water ad libitum. The rabbits used in the development study were 1, 2, 3, 4, 6, and 8 mo of age (two animals were used for each age group). Adult animals of at least 8 mo of age were used to study the androgen dependence of REP52 expression. Four animals were castrated, and two of these were administered an exogenous testosterone implant 14 days after castration as previously described [3]. Rabbits were killed by an intravenous overdose of Valabarb (Jurox, Sydney, Australia) 14 days postcastration (two animals) or 14 days postimplantation (two animals).

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Collection of Epididymal Fluid

To minimize blood contamination of tissue and epididymal fluid samples, the vascular system was cleared of blood by perfusion via the thoracic aorta with filtered saline (0.91% v/v) maintained at a constant flow rate of approximately 100 ml/min. When the desired organs were cleared of blood, they were either removed for collection of luminal fluids, or the saline perfusate was replaced by Bouin fixative, and the animals were perfuse-fixed for histology.

Luminal fluid was sampled from region 8 of the rabbit epididymis by retrograde perfusion as previously described [3] and from regions 2–5 by aspiration after piercing the duct with a sterile scalpel in isotonic sperm buffer (ISB; 103 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 400 mM EDTA, 30 mM Tris-HCl; pH 7.2). Immediately after collection, samples were gently centrifuged ($400 \times g$ for 5 min) to facilitate the separation of sperm and luminal fluid.

Isolation of REP52 and Preparation of Polyclonal Antibodies

REP52 was purified from the epididymal fluid of region 8 by preparative SDS-PAGE and electroelution according to the methods described by Nixon et al. [4]. Protein purity was assessed on silver-stained gels, and the crude protein preparations were then used to generate antibodies in female BALB/c mice as previously described [4]. Immune sera were collected from the orbital sinus of individual mice and purified over a Hi-Trap Protein G sepharose column (Pharmacia Biotech, Uppsala, Sweden), and the specificity of the purified IgG was analyzed by immunoblotting. The purified IgG was then used for characterization of the REP52 protein and isolation of the encoding cDNA as described below.

One- and Two-Dimensional SDS-PAGE and Immunoblotting

Proteins present in fluid from region 8 of the rabbit epididymis were solubilized in either reducing buffer (containing β-mercaptoethanol) or nonreducing buffer and separated by SDS-PAGE. Alternatively, proteins were separated by two-dimensional electrophoresis [3]. Following electrophoresis, proteins were electrotransferred onto Polyscreen PVDF transfer membranes using a Mini Trans-Blot Cell (Bio-Rad; 250 mA for 1 h).

The membranes were blocked overnight at 4°C in PBS containing 5% skim milk. Blots were then incubated in the presence of either preimmune or immune IgG diluted (1 μg per 50 ml) in PBS containing 1% skim milk for 1 h at room temperature. The blots were washed three times for 10 min in PBS with 0.05% Tween-20 and incubated with HRP-conjugated goat anti-mouse secondary antibody diluted 1:2000 in PBS containing 1% skim milk for 1 h at room temperature. The membranes were again washed three times, and cross-reactive proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions.

Selective Deglycosylation of Purified REP52

N-linked oligosaccharides, type-1 *O*-linked (Gal(β1,3)GalNAc(α1)) residues attached to serine or threonine, and α2–3 and α2–6 linked *N*-acetylneuraminic (sialic) acid residues were enzymatically cleaved from purified REP52 with peptide N-glucosidase F (PNGase-F; Boehringer-Mannheim, Germany), O-glycosidase DS (Bio-Rad), and NANase II (Bio-Rad), respectively, in accordance with the manufacturers' protocols. Cleavage reactions were terminated by the addition of SDS-PAGE loading buffer and heating of the samples for 5 min at 95°C. Proteins were then resolved by SDS-PAGE, stained with Coomassie Brilliant Blue, and analyzed for mobility shifts induced by deglycosylation.

Immunohistochemistry

Following perfusion fixation rabbit tissues were removed, carefully dissected free of connective tissue, and immersed in Bouin fluid for a further 24 h. The tissue was then dehydrated in 70% ethanol and processed for paraffin embedding and sectioning using standard procedures. Serial sections (5 μm) were mounted on poly-L-lysine-coated slides and, prior to use, were deparaffinized in successive changes of xylene and rehydrated through graded ethanol to PBS. Nonspecific antibody binding was blocked by overnight incubation in 3% BSA in PBS at 4°C. Sections were then incubated in a humidified chamber with the anti-REP52 IgG diluted (1 μg IgG per 200 μl) in PBS containing 1% BSA for 1 h at room temperature before being washed three times in PBS. Sections were then incubated in FITC-labeled sheep anti-mouse IgG (1:60 in PBS containing 1% BSA) for 1 h at room temperature. Slides were then mounted with antifade reagent (SlowFade; Molecular Probes, Eugene, Oregon) and viewed with a confocal microscope (Bio-Rad, MRC-1000). Sections incubated in either the presence of preimmune IgG or with secondary antibody only were included in all experiments as negative controls.

Following separation from epididymal fluid, sperm were diluted 1000-fold in ISB and washed three times by gentle centrifugation ($400 \times g$, 5 min). Washed spermatozoa ($\sim 5 \times 10^4$) were air dried onto poly-L-lysine-coated slides and fixed with methanol (10 min). Slides were then rinsed with ISB and immunostained with anti-REP52 IgG and FITC-labeled secondary antibody (diluted 1:200 and 1:60, respectively, in ISB containing 1% BSA) as outlined for tissue sections. The slides were then mounted with antifade and viewed under the confocal microscope.

Alternatively, washed suspensions of live spermatozoa were diluted to a final concentration of 1×10^6 ml^{-1} in ISB containing 3% (w/v) BSA and blocked for 1 h at 37°C. Blocking solution was removed by centrifugation ($400 \times g$, 4 min), and sperm were resuspended directly in ISB containing 1% BSA and immune IgG and incubated for 1 h at 37°C. The suspension was again washed by centrifugation ($400 \times g$, 4 min), and the pellet was resuspended in ISB containing 1% BSA and FITC-labeled secondary antibody and incubated in the dark for 1 h at 37°C. The suspension was washed as above, and the sperm pellet was resuspended for a final time in ISB. Sperm were then mounted onto poly-L-lysine-coated glass microscope slides and viewed immediately. The integrity of the sperm was established using the IT2A3 antibody, which reacts with an internal acrosomal antigen, using methods previously described [4].

Control samples of both live and methanol-fixed sperm in which the immune IgG was substituted for preimmune IgG, and in which the secondary antibody was omitted, were included in all experiments.

Preparation of Tissue Homogenates

Perfused tissues were removed into homogenizing buffer (0.25 M sucrose, 1.5 mM $MgCl_2$, 10 mM Tris-HCl; pH 7.4) and homogenized at 4°C for 2 min using a polytron. Homogenates were centrifuged at $100\,000 \times g$ for 30 min at 2°C in an ultracentrifuge (Beckman Optima TLX Ultracentrifuge, Beckman Instruments Inc., Fullerton, CA). The supernatant, interpreted as containing soluble tissue proteins, was carefully removed and dialyzed against three changes of distilled water over 24 h at 4°C. Protein concentrations were estimated using the Pierce Protein Assay kit (Pierce, Rockford, IL).

In Vitro Fertilization Assay

Rabbit oocytes were recovered from the oviduct of superovulated does as previously described [5]. Capacitated sperm were collected from the uterine horns of a mature doe mated with a buck of proven fertility. The capacitated sperm were diluted to a concentration of 5×10^5 in 1 ml Brackett Defined Medium (BDM) [5] and incubated (30 min at 37°C) with either preimmune IgG (400 μg/ml; control) or anti-REP52 IgG (40 μg or 400 μg/ml) before being washed with three changes of fresh BDM to remove unbound antibody. A 100-μl aliquot of this suspension ($\sim 5 \times 10^4$ sperm) was then added to cumulus intact oocytes (6–10 oocytes per treatment) and incubated under oil in 5% CO_2 (6 h at 37°C). Following incubation, oocytes were transferred to fresh BDM and incubated for a further 18 h at 37°C. Oocytes were then washed by gentle pipetting through a fine-bore pipette in three changes of BDM to remove loosely bound sperm. The number of sperm bound to the zona pellucida and present in the perivitelline space were recorded. Fertilization, defined as the presence of male and female pronuclei or cleavage of the embryo, was scored using Nomarski optics on a Zeiss compound microscope (Carl Zeiss, Thornwood, NY). This experiment was replicated three times, and values are presented as mean ± SEM calculated from the variance between replicates. Statistical significance was determined using an ANOVA.

Construction and Screening of a Rabbit Epididymal cDNA Expression Library

An amplified epididymal cDNA expression library was prepared with a commercial cDNA synthesis kit (GE Healthcare) as previously described [6]. Approximately 2×10^6 plaques were screened [7] with anti-REP52 IgG, and positive clones were isolated, eluted in phage diluent, and rescreened to purity [7]. Bacteriophage from purified, positive clones were propagated in liquid culture, and the cDNA inserts were characterized by restriction analysis. Complementary DNA inserts were subsequently purified with Nucleotrap (Macherey-Nagel, Duren, Germany) and subcloned into the Bluescript plasmid transcription vector pBSII SK- (Stratagene, La Jolla, CA).

DNA Sequencing

A series of nested deletions of the *REP52* cDNA inserts were generated in both 5′ and 3′ directions using the Discrete Delete (Epicentre Technologies,

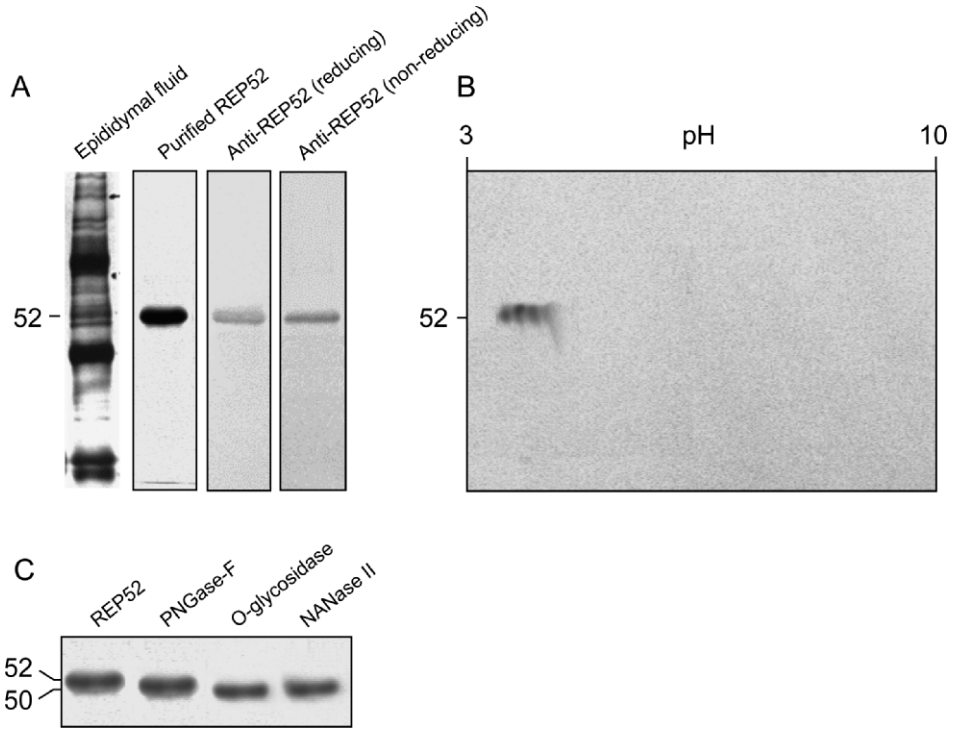

FIG. 1. Purification of REP52 from epididymal fluid and assessment of anti-REP52 IgG specificity. **A**) Lane 1: 12% SDS-PAGE gel of region 8 rabbit epididymal fluid (20 μg) showing the protein band corresponding to REP52; lane 2: 12% SDS-PAGE gel of REP52 (0.5 μg) purified by preparative electrophoresis and electroelution. This purified fraction was used for the subsequent generation of murine polyclonal antibodies; lanes 3 and 4: Western blots of region 8 epididymal fluid (10 μg) resolved under reducing (lane 3) and nonreducing (lane 4) conditions and immunostained with anti-REP52 IgG. **B**) Two-dimensional blot of region 8 epididymal fluid (100 μg) immunostained with anti-REP52 IgG. **C**) Purified REP52 (0.5 μg; lane 1) was selectively deglycosylated with PNGase-F (lane 2), O-glycosidase DS (lane 3), or NANase II (lane 4). Following treatment, the proteins were resolved on a 12% SDS-PAGE gel, stained with Coomassie Brilliant blue, and analyzed for mobility shifts. The numerical values on the left of each panel correspond to the molecular weight (kDa) of REP52 and the immunoreactive proteins.

Madison, WI) kit and the manufacturer's protocols. Plasmid DNA from overnight cultures was isolated using a FlexiPrep kit (GE Healthcare), and the double-stranded template was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer Applied Biosystems, Foster City, CA) and pUC/M13 reverse primers (Promega, Madison, WI). DNA sequences were analyzed using Geneworks software (IntelliGenetics Inc., Mountain View, CA) and a number of programs at the ExPASy Molecular Biology website (Swiss Institute of Bioinformatics; http://ca.expasy.org/). These included: ScanProsite (http://ca.expasy.org/tools/scanprosite), NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc), SignalP (http://www.cbs.dtu.dk/services/SignalP), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), and Compute pI/Mw (http://ca.expasy.org/tools/pi_tool.html). Searches of the Genbank and EMBL databases for related sequences were performed using the Basic Alignment Search Tool (BLAST; [8]) program at the NCBI.

RNA Extraction and Northern Blot Analysis

Tissue from male and female rabbits was frozen, and total RNA was extracted in the presence of Tri-reagent (Sigma) in accordance with the manufacturer's protocol. The quantity and quality of RNA was assessed spectrophotometrically at 260 and 280 nm and on 1% denaturing agarose gels run in 1× MOPS buffer (10 mM 3-[N-morpholino]propanesulfonic acid, 1.1 mM sodium acetate, and 0.25 mM EDTA) containing 1.85% (v/v) formaldehyde. Aliquots of 10 μg total RNA were resolved electrophoretically, stained by the addition of ethidium bromide directly to the loading/denaturing buffer (1× MOPS containing 6.5% [v/v] formaldehyde, 50% [v/v] formamide, and 0.05 μg/μl ethidium bromide) and visualized under an ultraviolet transilluminator. Ribosomal 28S (4.71 kb) and 18S (1.87 kb) bands were used as internal standards to estimate the relative size of cross-hybridizing RNA species. Following electrophoresis, the RNA was transferred by capillary blotting for 24 h to Hybond-N membranes (GE Healthcare). Filters were rinsed in 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate) and prehybridized in Rapid-hyb buffer (GE Healthcare) at 65°C for 1 h. A ^{32}P-labeled, full-length *REP52* cDNA probe was added directly to the hybridization solution, and the filters were hybridized at 65°C for 1–2 h. The filters were then rinsed in 2× SSC containing 0.1% SDS at room temperature and subsequently washed in 1× SSC containing 0.1% SDS at 65°C for 30 min, followed by several changes of 0.2× SSC containing 1% SDS at 65°C over 30 min. After washing, the wet membranes were wrapped in plastic and exposed to autoradiographic film (X-OMAT x-ray film, Eastman Kodak Co., Rochester, NY) with a single intensifying screen for at least 12 h at −70°C.

RESULTS

Purification of REP52 and Generation of Polyclonal Antibodies

REP52 was purified from region 8 epididymal fluid to apparent homogeneity (Fig. 1A) by three successive rounds of preparative SDS-PAGE. Typical yields of purified protein from 1 mg epididymal fluid were in the order of 20–40 μg, indicating that REP52 constitutes a minimum of 2%–4% of the total protein present in region 8 fluid. The anti-REP52 IgG generated against the purified protein cross-reacted with a single band of 52 kDa in rabbit epididymal fluid separated under either reducing or nonreducing conditions (Fig. 1A). A corresponding two-dimensional blot showed that this protein was composed of several acidic isoelectric charge variants with estimated pI values of 3.8–4.4 (Fig. 1B). Selectively, deglycosylation of the purified REP52 protein revealed that this microheterogeneity was most likely attributed to *N*- and/or *O*-linked sugars (Fig. 1C). Blots from which the primary antibody was omitted or substituted with preimmune IgG did not show any cross-reactivity (results not shown).

Identification and Sequencing of REP52 *cDNA*

Immunological screening of approximately 2×10^6 recombinant phages from a rabbit epididymal cDNA expression library identified a single clone, *REP52*-c1, expressing a fusion protein recognized by anti-REP52 IgG. Following isolation of this clone through secondary and tertiary rounds of screening, it was plaque purified. Verification that this clone expressed a fusion protein recognized specifically by the anti-REP52 IgG was obtained by rescreening plaque-purified *REP52*-c1 clones. All clones reacted positively. In control experiments where the primary antibody was either omitted or substituted with preimmune IgG, no positive signals were obtained.

Sequencing of *REP52*-c1 cDNA revealed a single, large open reading frame (orf) commencing at nucleotide 1 and

terminating at an in-frame stop codon (TGA) at nucleotide 220. This orf was in frame with the *Eco*RI site of the λgt11 vector, suggesting that it was responsible for expression of the recombinant polypeptide recognized by anti-REP52 IgG. It did not, however, contain a consensus signal for translation initiation, indicating that it was unlikely to contain the entire coding sequence of the gene. Based on these results, an oligonucleotide probe of 513 bp (*REP52-p*) was generated from the *REP52*-c1 insert by an *Eco*RI-*Hin*dIII double digest. Rescreening of the epididymal cDNA library by plaque hybridization with the α-^{32}P-labeled *REP52-p* probe led to the identification of an additional partial clone, *REP52*-c2, that overlapped the original *REP52*-c1A insert.

Sequencing of bidirectional overlapping subclones from *REP52*-c1 and *REP52*-c2 generated a contiguous nucleotide sequence comprising 2096 bp up to the start of the poly(A) tail (accession #NM_001082047). Analysis of the *REP52*-c1/c2 nucleotide sequence identified a consensus polyadenylation signal AATAAA [9] 209 bp upstream of the poly(A) tail and a single large orf. The first potential translation initiation codon capable of providing an uninterrupted coding sequence was preceded by a 5′ UTR of 371 bp. An in-frame stop codon (TGA) was located at nucleotide −24. The putative initiator methionine was flanked by the sequence CCGCCATGT, which closely resembles the consensus Kozak sequence, CCA(G)C-CATGG, for functional initiator codons by eukaryotic ribosomes [10]. Four additional ATG codons were detected in the same translational frame and therefore have the potential to function as additional translational initiation sites. However, only one of these sites, nucleotides 811–820, is homologous to that of the Kozak consensus sequence. The orf terminated at nucleotide 1408 (TGA) and was followed by a 3′ UTR of 318 nucleotides.

Southern blot analysis of rabbit genomic DNA digested with a number of restriction enzymes revealed simple hybridization patterns (Fig. 2) in which only one or two major hybridizing signals were detected. This pattern is consistent with DNA fragments produced by the restriction of a single gene, suggesting that *REP52* is represented by a single-copy gene in the rabbit genome.

Deduced Amino Acid Sequence of REP52-*c1/c2*

On the assumption that translation starts at the first initiation codon, the orf consists of 1407 bp and therefore encodes a hypothetical protein of 469 amino acids (Fig. 3A). The 5′ terminus of the orf encodes a typical signal peptide for secretory proteins with a hydrophobic core moiety of 15 amino acids [11]. The cleavage position for the signal peptide was predicted between amino acids H_{15} and D_{16} (Fig. 3A) [12]. Consistent with this prediction, the partial N-terminal amino acid sequence obtained from direct sequencing of the REP52 protein [3] commenced at amino acid D_{16} and was identical to that of the deduced amino acid sequence (Fig. 3A). This finding verifies that the cloned cDNAs (*REP52*-c1/c2) encode the REP52 protein. The mature protein of 455 amino acids resulting from signal peptidase cleavage has a theoretical isoelectric point of 9.09 and a deduced molecular mass of 49 613 Da. The differences between the predicted and observed pI (3.8–4.4) and molecular weight (52 kDa) are consistent with our evidence that the mature protein is glycoslyated.

Homology of REP52-*c1/c2 with Known Sequences*

Comparison of the entire coding region of *REP52*-c1/c2 with existing sequences in the GenBank and EMBL databases

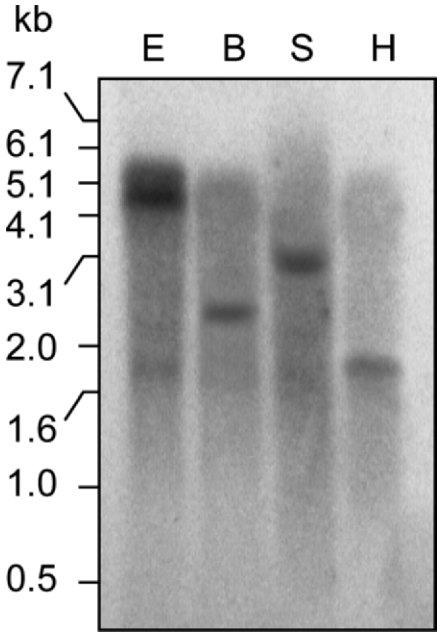

FIG. 2. Southern blot of rabbit genomic DNA. Rabbit genomic DNA (20 μg) was digested with the restriction endonucleases *Eco*RI (E), *Bam*HI (B), *Sac*I (S), and *Hin*dIII (H), electrophoretically separated on 0.8% agarose gels, and Southern blotted. Blots were hybridized with a truncated *REP52* oligonucleotide probe. The sizes (kb) of the molecular size markers are depicted on the left.

revealed that it shared significant sequence identity with a family of ungulate seminal plasma proteins, including bovine seminal vesicle secretion 8 (SVS8), bovine seminal vesicle secretory protein 109 (PDC-109), horse seminal plasma protein (HSP-1), porcine seminal plasma protein pB1 (PB1), and bovine seminal plasma 30K protein (LOC317699) in addition to a recently identified sperm-binding protein of epididymal origin (epididymal sperm binding protein 1 [ELSPBP1]; Fig. 3, B and C). At the protein level, homology was restricted to a 90-amino-acid overlap in the C-terminal portion of the deduced protein (D_{379}–C_{468}; Fig. 3B). This region corresponds to the two tandem fibronectin type II domains within REP52. In contrast, the N-terminus of REP52 (M_1–D_{379}) failed to show any significant homology with published sequence data.

Consistent with these results, *REP52* cDNA probes hybridized to the genomic DNA of all species examined (Fig. 4A). In the case of the closely related lagomorphs *S. audubonii*, *S. nuttallii*, and *L. californicus*, cross-hybridization signals identical to those demonstrated in Figure 2 were detected (Fig. 4B). However, dissimilar hybridization signals were observed in all nonrelated species examined, including the rat, mouse, guinea pig, bull, ram, boar, cat, dog, and tammar wallaby (Fig. 4A). Changing either the hybridization or washing stringency had no effect on the detection of positive signals (results not shown).

Similarly, anti-REP52 IgG demonstrated cross-reactivity with proteins of approximately 52 kDa in the cauda epididymal fluid and sperm extracts of all lagomorph species examined (Fig. 4D). However, among the limited number of nonrelated species examined, cross-reactive antigens were only detected in the rat and the mouse. A single immunoreactive protein band with an approximate molecular weight of 20 kDa was recognized in epididymal fluid and sperm plasma membrane preparations from both of these species (Fig. 4C). Immunofluorescent localization of the related molecules in rat and mouse spermatozoa demonstrated that the corresponding antigens

A

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MFLFSFLTLC LCLQHDLQQK SAEKGAFPAG SLLHQLHLLP GAPLQPVPRG QQLEAVLGGE   60
GHHDALDGLP VPLQREALLP GGHGEHVPWP EELRGVQPPQ PQRPGPGHGD HGHKGIERHI  120
FPGPARLGRR PRRKPLKPSG APRWAVTDPG HPPSLSSKAA RPRGCPFFHN NFLLLDKEST  180
LDPVTDTFNT HGTTISPADV FFGFRQSHQD FRSHSTNPTK SAWAHPTCRF GCLPNLLSIK  240
VNYSVTTSSG QSGFVGGCIV GKPPAVRQGK PAMAVCWGLC LMWVCAWAFL QMDCVNAAPG  300
LLSELLGFVD DSVVIATPGA ESYKAKTIKA SKITTTTPAA TTATHKPTTS QIQNKPPPGL  360
SSTPNAKNAL SPGPPGQSDK KCVFPFTFGN KKHFDCTVEG SIFHWCSLTD KYSGKWKYCT  420
DDDRARCVFP FIFEGHVYKD CITKGSLFRM AWCSLSPYYD HDKAWKYCY              469
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B

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REP52     379 DKKCVFPFTFGNKKHFDCTVEGSIFHWCSLTDKYSGKWKYCTDDDRARCVFPFIFEGHVYKDCITKGSLFRMAWCSLSPYYDHDKAWKYC 468

SVS8       26 DNKCVFPFIYGNKKYFDCTLHGSLFLWCSLDADYTGRWKYCTKNDYAKCVFPFIYEGKSYDTCIIIGSTFMNYWCSLSSNYDEDGVWKYC 115
PDC-109    46 DEECVFPFVYRNRKHFDCTVHGSLFPWCSLDADYVGRWKYCAQRDYAKCVFPFIYGGKKYETC-TKIGSMWMSWCSLSPNYDKDRAWKYC 134
HSP-1      31 ENKCVFPFNYRGYRYYDCTRTDSFYRWCSLTGTYSGSWKYCAATDYAKCAFPFVYRGQTYDRCTTDGSLFRISWCSVTPNYDHHGAWKYC 120
PB1        16 DDKCVFPFIYKGNLYFDCTLHDSTYYWCSVTTYYMKRWRYCRSTDYARCALPFIFRGKEYDSCIKEGSVFSKYWCPVTPNYDQDRAWRYC 105
LOC317699  72 GPACAFPFTYKGKKYYMCTRKNSVLLWCSLDTEYQGNWKFCTERDEPECVFPFIYRKKSYESCTRVHSFFWRRWCSLTSNYDRDKAWKYC 158
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C

Protein	Synonyms	Accession number	Species	Amino acid identity
SVS8	BSP-A3	P04557	Bovine	57/90 (63%)
PDC-109	BSP-A1, SVSP109	P02784	Bovine	56/90 (62%)
HSP-1	-	P81121	Equine	49/90 (54%)
PB1	-	P80964	Porcine	45/90 (50%)
LOC317699	BSP-30K	P81019	Bovine	44/90 (48%)
ELSPBP1	EP12	AAH15598	Human	39/90 (43%)

FIG. 3. Deduced amino acid sequence of REP52 and alignment with orthologous proteins. **A**) The deduced amino acid sequence of REP52. The underlined amino acid sequence corresponds to that obtained by Edmann sequencing of the REP52 protein [3]. **B**) Alignment of the *REP52* amino acid sequenced with the five proteins with which it shared greatest identity. This alignment is restricted to the C-terminal region comprising two tandem FN2 modules because the upstream N-terminus failed to display significant sequence homology with any published amino acid sequence data. **C**) Percentage identity of REP52 and orthologous proteins.

were expressed exclusively on the flagellum in a location consistent with that observed in the rabbit (see Fig. 8, G and H). However, the staining was punctate and of lower intensity than that recorded on rabbit spermatozoa.

Functional Motifs of the Putative REP52 Protein

A PROSITE motif [13] search performed on the deduced amino acid sequence of *REP52*-c1/2 identified a number of potential posttranslational modifications including: three myristylation sites, one N-glycosylation site, one amidation site, nine protein kinase C phosphorylation sites, five casein kinase II phosphorylation sites, and two regions in the C-terminus that displayed consensus fibronectin type II (FN2) domains. With the exception of the latter, FN2 domains, the majority of these motifs have a high probability of occurrence, and their functional significance must therefore be interpreted with caution [13]. Prediction of potential O-glycosylation (O-GalNAc; mucin type) sites based on sequence context and surface probability [14] identified 15 potential O-glycosylation sites.

Tissue Specificity of REP52 Synthesis

The tissue specificity of REP52 expression was initially examined by immunoblotting of crude tissue extracts prepared from the epididymis, testis, ovary, brain, kidney, liver, spleen, and muscle with anti-REP52 IgG (Fig. 5A). This approach demonstrated that, among the tissues examined, REP52 expression was restricted to the epididymis. In contrast, blots from which the primary antibody was omitted or substituted with preimmune IgG did not show any cross-reactivity for REP52 (results not shown). The tissue-specific nature of REP52 expression was confirmed by Northern blotting of total RNA isolated from a similar panel of tissues. Under conditions of high stringency, the *REP52* probe hybridized to a single abundant epididymal transcript of approximately 2.1 kb (Fig. 5B). Northern analyses also demonstrated that *REP52* mRNA was expressed in a highly regionalized manner within region 6 (proximal corpus) of the epididymis (Fig. 5B). No hybridization signals were detected in any other tissue examined. Reduction of hybridization and washing stringency and increasing the length of autoradiographic exposure had no effect on the detection of positive signals (results not shown).

To confirm the restricted pattern of REP52 synthesis and explore its secretion into the epididymal lumen, we conducted immunocytochemical staining of paraffin sections from sequential sites along the epididymis. As shown in Figure 6, the appearance of detectable antigen was first noted in the proximal portion of epididymal region 6. Within this region, immunostaining was restricted to isolated principal cells, while other cells in the same tubule were only faintly stained or were unstained (Fig. 6A). Distal to this zone the proportion of positive cells increased rapidly, and the epididymal epithelium became uniformly stained by the midportion of region 6 (Fig.

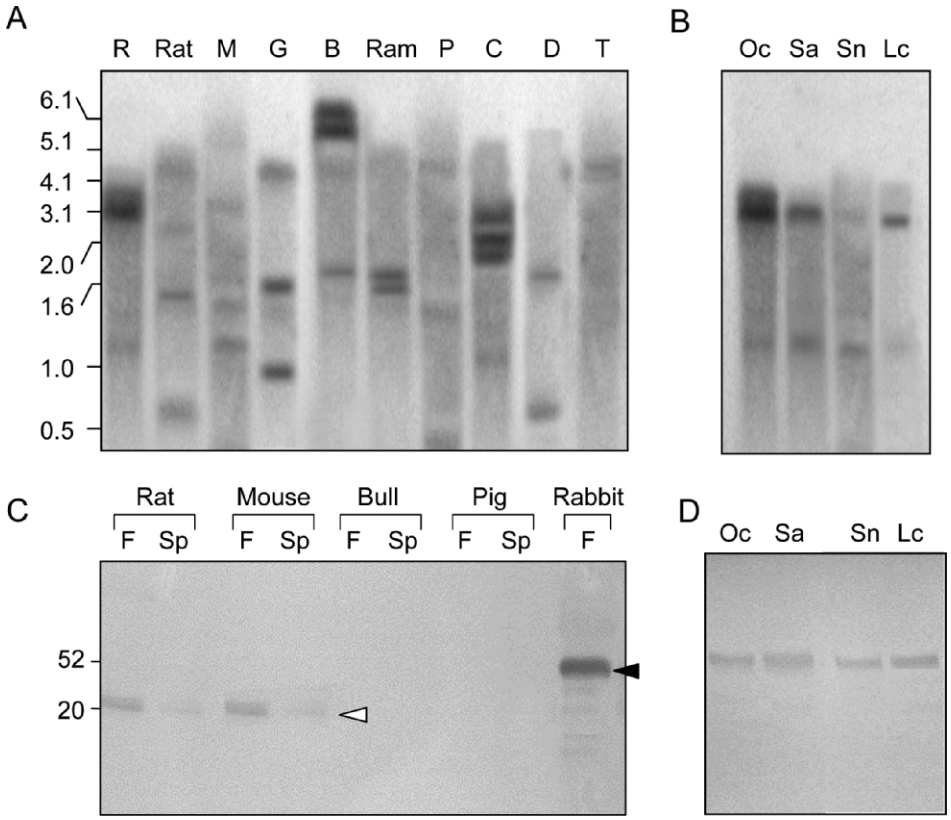

FIG. 4. Detection of REP52 orthologous proteins in nonrelated species. **A**) Genomic DNA (20 μg) from rabbit (R), rat (Rat), mouse (M), guinea pig (G), bull (B), ram (Ram), pig (P), cat (C), dog (D), tammar wallaby (T), **B**) *O. cuniculus* (Oc), *S. audubonii* (Sa), *S. nuttallii* (Sn), and *L. californicus* (Lc) were digested with the restriction endonuclease *Eco*RI and electrophoretically separated on 0.8% agarose gels and Southern blotted. Blots were hybridized with a truncated *REP52* oligonucleotide probe. **C**) Proteins present in sperm plasma membrane extracts (Sp) and cauda epididymal fluid (F) from rat, mouse, bull, and pig were resolved on 12% SDS-PAGE gels and transferred to PVDF. Caudal fluid (F) from domestic rabbits was run for comparison. **D**) Similarly, proteins present in sperm plasma membrane extracts from *O. cuniculus* (Oc), *S. audubonii* (Sa), *S. nuttallii* (Sn), and *L. californicus* (Lc) were also prepared for Western blotting. Blots were immunostained with anti-REP52 IgG. Numerical values at left correspond to the molecular weight (kDa) of the immunoreactive bands in rabbit fluid (shaded arrow) and rat and mouse fluid (unshaded arrow).

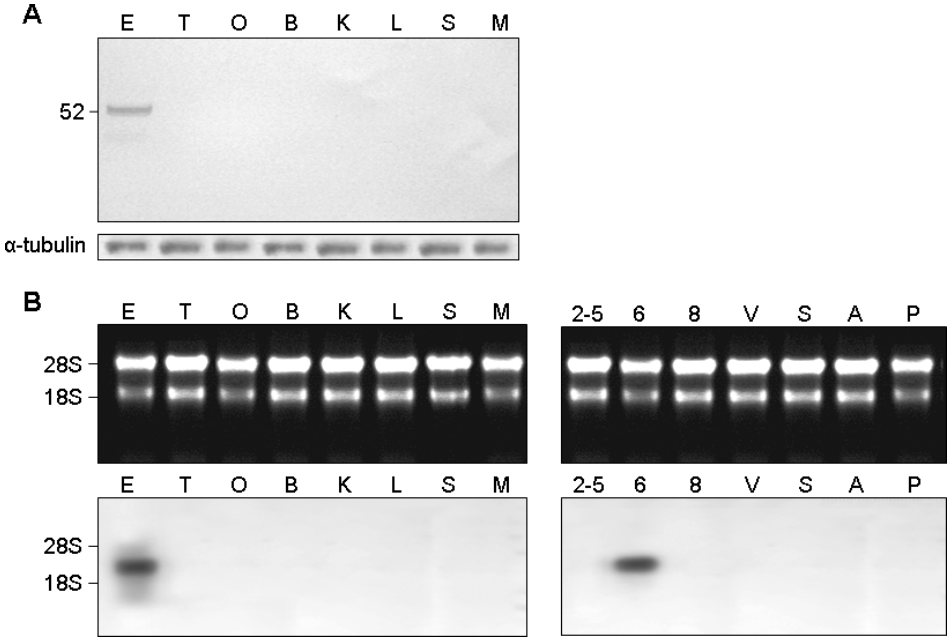

FIG. 5. Tissue specificity of REP52 synthesis. **A**) Proteins (20 μg) extracted from the epididymis (E), testis (T), ovary (O), brain (B), kidney (K), liver (L), spleen (S), and muscle (M) were resolved on 12% SDS-PAGE gels under reducing conditions. Proteins were then transferred to PVDF, and blots were immunostained with anti-REP52 IgG. Following detection of REP52, membranes were stripped and reprobed with anti-α-tubulin to ensure equal protein loading. The numerical value on the left of the panel corresponds to the molecular weight (kDa) of the REP52 protein. **B**) Denaturing agarose gels were loaded with 10 μg of total RNA extracted from: rabbit testis (T), epididymis (E), ovary (O), brain (B), spleen (S), kidney (K), liver (L), muscle (M), epididymal region 2–5 (2–5), epididymal region 6 (6), epididymal region 8 (8), vas deferens (V), seminal vesicles (S), ampulla (A), and prostate (P). Resolved RNA was visualized using an ultraviolet transilluminator to ensure equal loading and RNA integrity and subsequently transferred to nylon membranes by capillary blotting. Northern blots were hybridized with a truncated oligonucleotide probe constructed from the 5′ region of *REP52*-c1 and exposed to autoradiographic film. The positions of the internal molecular size markers, 28S (4.71 kb) and 18S (1.87 kb) ribosomal RNA, are depicted on the left of the relevant panels.

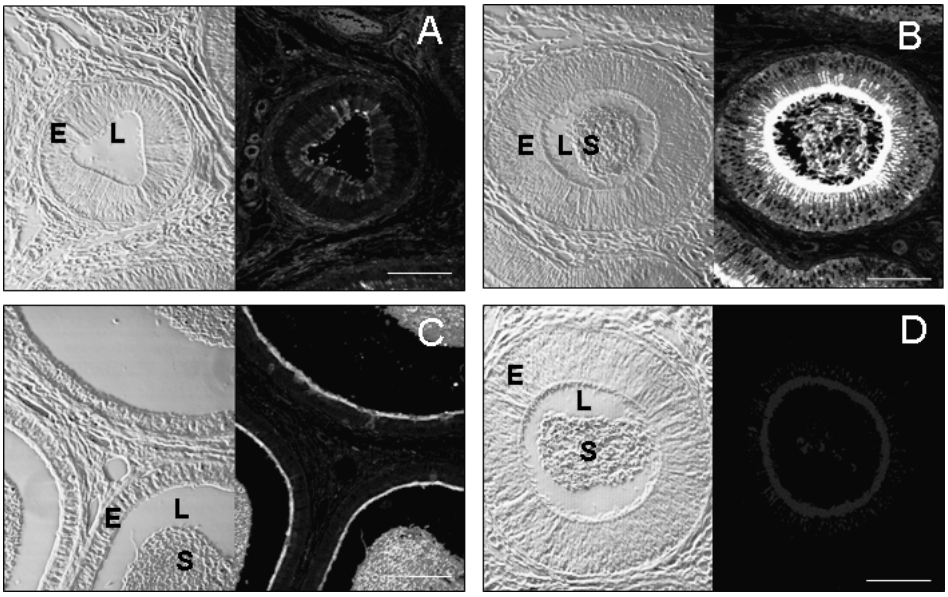

FIG. 6. Immunofluorescent localization of REP52 within the epididymis. Composite phase contrast (left-hand side of each panel) and immunofluorescence (right-hand side of each panel) photomicrographs demonstrating the localization of anti-REP52 IgG in the proximal (**A**) and middle (**B**) segments of region 6 and region 8 (**C**) of the rabbit epididymis. Confocal settings were adjusted so that the weak fluorescence displayed by control sections incubated with preimmune IgG (**D**) was below the recorded level, and all images were taken under these settings. E, Epithelium; L, lumen; S, sperm. Bar = 50 μm.

6B). Staining in these regions was concentrated on the apical surface and within the supranuclear cytoplasm of principal cells. Beyond the middle segment of region 6, intracellular staining of principal cells gradually decreased and was not detected distal to epididymal region 7. However, intense immunofluorescence persisted in the lumen and at the apical margin of the epithelium in region 8 (Fig. 6C). No staining was detected in clear cells, basal cells, or peritubular or vascular tissue.

Testicular Regulation of REP52 *Expression*

The ontogeny of *REP52* gene expression during neonatal development of the rabbit epididymis was examined by Northern analysis. As demonstrated in Figure 7A, the induction of *REP52* gene expression appeared between 2 and 3 mo of age. Northern blot analysis demonstrated a minor increase in the steady-state mRNA levels between 3 and 4 mo of age, after which time the relative signal intensity remained constant until at least 8 mo of age. No discernible differences in the size of the transcript were observed throughout ontogenesis.

Hormonal deprivation induced by bilateral orchidectomy for a period of 14 days led to a marked reduction in *REP52* transcription (Fig. 7A) and protein synthesis (Fig. 7B) in epididymal tissue. The administration of testosterone to orchidectomized rabbits resulted in only a minimal recovery of *REP52* mRNA and protein to levels far below precastrate values, implying that testosterone alone is insufficient to induce optimal expression of *REP52*.

Interaction of REP52 with Spermatozoa

The temporal appearance of anti-REP52 immunoreactivity in the epididymal epithelium coincided with the detection of

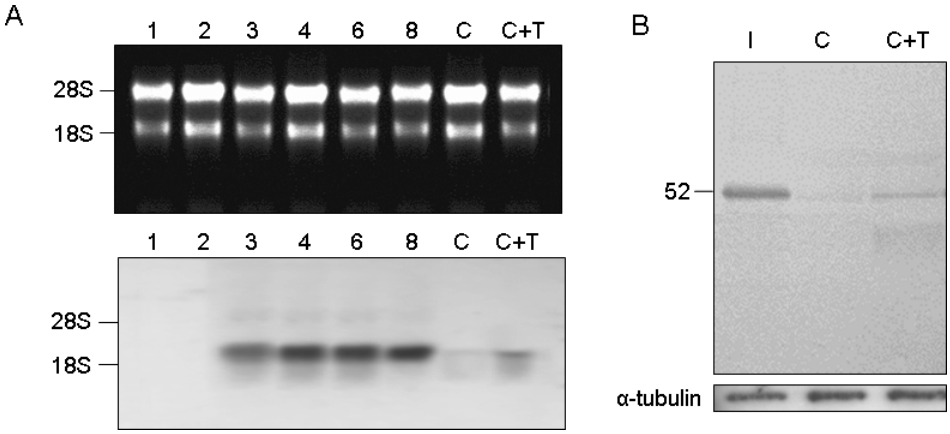

FIG. 7. Developmental and androgen regulation of REP52 synthesis. **A**) Denaturing agarose gels were loaded with total RNA (10 μg) extracted from region 6 of the epididymis of rabbits aged 1–8 mo and mature rabbits that were castrated (C) or castrated and administered exogenous testosterone therapy (C+T). Resolved RNA was visualized, and Northern blots were hybridized with a truncated probe constructed from the 5′ region of *REP52*-c1A. The positions of the internal molecular size markers, 28S (4.71 kb) and 18S (1.87 kb) ribosomal RNA, are depicted on the left of each panel. **B**) Proteins (20 μg) present in epididymal tissue homogenates prepared from intact animals (I), castrate animals (C), and castrate animals receiving testosterone therapy (C+T) were resolved on 12% SDS-PAGE gels and transferred to PVDF. Blots were immunostained with anti-REP52 IgG. Following detection of REP52, membranes were stripped and reprobed with anti-α-tubulin to ensure equal protein loading. The numerical value on the left of the panel corresponds to the molecular weight (kDa) of the REP52 protein.

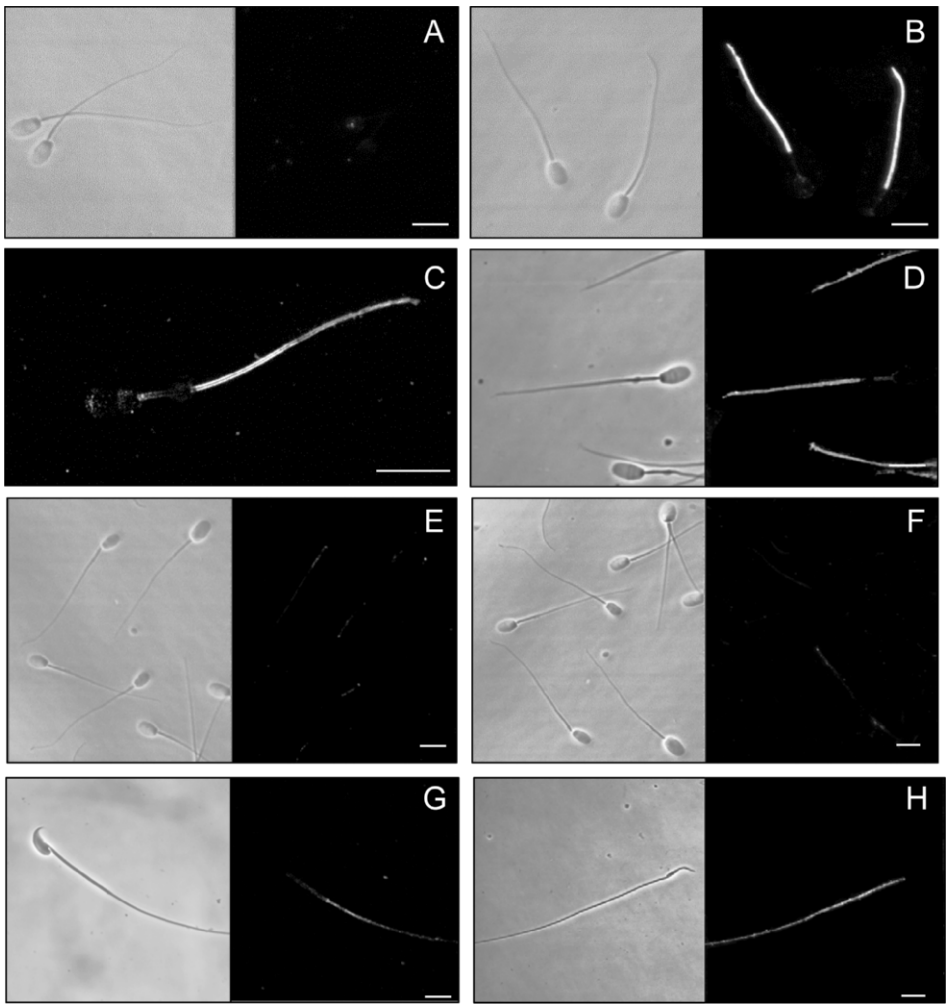

FIG. 8. Immunofluorescent localization of REP52 on spermatozoa. Photomicrographs demonstrating the localization patterns of REP52 on rabbit (**A**–**F**), mouse (**G**), and rat (**H**) spermatozoa. Live and methanol fixed sperm (**A**) from epididymal regions 2–5 did not display cross-reactivity with anti-REP52 IgG. In contrast, live (**B**) and methanol-fixed (**C**) spermatozoa from region 8 displayed immunofluorescent labeling predominantly over the principal piece of the sperm flagellum. Capacitated sperm (**D**) recovered from uterine flushings of mated female rabbits (12 h postcoitum) displayed a similar pattern of fluorescence to that of region 8 spermatozoa. Confocal settings for all treatments were adjusted so that the weak fluorescence displayed by control sperm incubated with either preimmune IgG (**E**) or in the absence of primary antibody (**F**) was below that recorded, and subsequent images were taken under identical settings. The localization of anti-REP52 IgG on live mouse (**G**) and rat (**H**) spermatozoa was also assessed. Bars =10 μm.

immunoreactivity on the principal piece of the tail of live spermatozoa (Fig. 8B). Weak immunoreactivity was first apparent on approximately 10% of the spermatozoa recovered from the proximal portion of epididymal region 6. However, commensurate with the increase in cellular staining, the labeling intensity of spermatozoa also increased distally, reaching a maximum on sperm from region 8, where greater than 90% of live spermatozoa displayed strong fluorescence. REP52 labeling persisted on ejaculated sperm recovered from the uterine flushings of mated female rabbits (12 h postcoitum; Fig. 8D). However, the protein was able to be dissociated from the surface of live sperm relatively easily by increasing the ionic strength of the sperm incubation buffer (Fig. 9).

In Vitro Fertilization

The role of REP52 in sperm-egg interaction was evaluated in an in vitro fertilization assay. The effects of anti-REP52 IgG on the number of sperm bound tightly to the zona pellucida, the number present in the perivitelline space, and the number of eggs fertilized were quantified (Table 1). Preincubation of sperm with anti-REP52 IgG induced a significant concentration-dependent inhibition of sperm binding to zona-intact oocytes and subsequent fertilization in vitro. At a concentration of 400 μg IgG per ml, the immune sera completely blocked fertilization (100% inhibition). At the lower concentration of 40 μg IgG/ per ml, the reduction in the mean percentage of fertilized oocytes appeared directly proportional to the reduction in the number of sperm bound to the zona pellucida; here both the number of sperm binding to oocytes and the fertilization rate were reduced to around 40% of the in vitro control levels (60% inhibition).

To determine if this inhibitory effect was caused by agglutination or immobilization of spermatozoa, a portion of the sperm recovered from uterine flushings of mated female

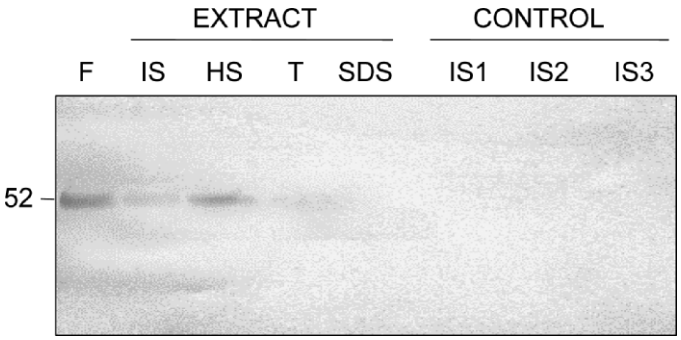

FIG. 9. Sequential extraction of proteins from region 8 spermatozoa. Sperm proteins were extracted as previously described [4], and an equivalent amount of protein (20 μg per lane) was resolved on 12% SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed with anti-REP52 IgG. Extract: region 8 epididymal fluid (F), proteins extracted by treatment with isotonic buffer (IS), high-ionic-strength buffer (HS), 0.1% Triton X-100 (T), and 2% SDS (SDS). Control: proteins extracted in three sequential washes in isotonic buffer (IS1–IS3) after the initial treatment with isotonic buffer.

TABLE 1. Effect of anti-REP52 IgG on fertilization rates in vitro.

Variable	Antibody treatment		
	REP52 (40 μg/ml)	REP52 (400 μg/ml)	Control
Experiments	3	3	3
Total eggs	27	17	24
Zona-bound sperm (mean ± SEM)	1.8 ± 1.5*	0.5 ± 0.4*	5.2 ± 2.9
Sperm in perivitelline (mean ± SEM)	0.3 ± 0.2*	0*	0.7 ± 0.5
Percentage of eggs fertilized	29*	0*	71

* Values are significantly different compared to in vitro controls; one-way ANOVA ($P < 0.05$).

rabbits (12 h postcoitum) was incubated with anti-REP52 IgG (under identical conditions to those used in in vitro fertilization assays). In the presence of 400 μg IgG per ml, virtually all spermatozoa were agglutinated compared with approximately 80% at the lower concentration of 40 μg IgG per ml. Agglutinated complexes were predominantly formed by tail-to-tail cross-linking of spermatozoa. Spermatozoa exhibited strong flagellar beating even when agglutinated, indicating that anti-REP52 IgG did not exert a cytotoxic effect. Agglutination did, however, virtually eliminate forward motility, especially at high concentrations of antibody. Preimmune IgG did not elicit tail-to-tail sperm-agglutinating activity.

DISCUSSION

Polyclonal antibodies raised against REP52 were used to detect fusion proteins produced by a rabbit epididymal cDNA expression library. This approach successfully identified a contiguous sequence of 2096 bp, which encoded the REP52 protein. Northern blot analysis of epididymal RNA revealed the presence of a single 2.1-kb mRNA transcript that hybridized to the *REP52* oligonucleotide probe. This implies that the isolated cDNA for *REP52* represents the near-full-length coding sequence of the single-copy *REP52* gene. Differences between the observed and predicted pI of REP52 (3.8–4.4 and 9.09, respectively) infer that the protein is synthesized as a precursor that is subject to extensive posttranslational modification. Structural analysis of the deduced REP52 protein sequence indicated that such modifications are likely to include the addition of negatively charged carbohydrate side chains. Consistent with this notion, selective deglycosylation experiments demonstrated that REP52 contains both *N*- and *O*-linked sugars and that at least some of the attached carbohydrate side chains were shown to possess terminal, negatively charged *N*-acetylneuraminic acid residues. Such modifications could also account for the differences observed between the predicted and experimental molecular weights of the mature protein (49.613 kDa and 52 kDa, respectively).

Northern analyses and immunolocalization studies demonstrated that the REP52 protein is expressed in a tissue-specific and highly regionalized manner within the rabbit epididymis. Following its secretion in region 6 of the epididymis, REP52 forms a weak ionic association with the plasma membrane overlying the sperm flagellum, inviting speculation that it may contribute to the development of the potential for sperm motility during epididymal transit. Consistent with this hypothesis, it has been suggested that the inability of testicular spermatozoa to move is due, at least in part, to the immaturity of the plasma membrane, since these sperm can move almost as actively as mature caudal sperm if they are demembranated and exposed to ATP, cAMP, and magnesium ions (Mg^{2+}) [15–18]. However, whether structural modifications such as the acquisition of proteins during epididymal transit contribute to the development of the capacity for sperm motility remains a contentious issue [19, 20]. Furthermore, the fact that REP52 associates with rabbit spermatozoa distal to the region in which they acquire the capacity for forward progressive motility [21, 22] precludes the possibility that it is directly involved in the initiation of motility.

Prediction of the molecular structure of the deduced REP52 protein identified an N-terminal hydrophobic core sequence of 15 amino acids. These amino acids are apparently cleaved from the mature form of the protein isolated from epididymal fluid [3] and are therefore likely to represent a signal peptide [11] capable of directing the protein toward the secretory pathway [23]. Further structural analysis identified a putative single pass transmembrane domain of 18 amino acids, raising the possibility that REP52 is capable of plasma membrane insertion, thereby offering a putative model for interaction of the protein with epididymal spermatozoa. However, if this were the case, then one would expect the protein to behave as an integral membrane protein and require solubilization of the sperm plasmalemma for extraction. This is not consistent with the demonstration that REP52 readily dissociates from the sperm surface after treatment with high-ionic-strength media.

An alternative mode of sperm association is suggested by the presence of two consensus FN2 domains in a region of the deduced amino acid sequence with a high probability of surface exposure. FN2 domains are approximately 40 residues long and contain four conserved cysteines that are involved in disulphide bond formation [24]. They constitute part of the collagen-binding region of fibronectin and have been implicated in a variety of extracellular binding events [24, 25]. Of particular interest in the context of the present study is the demonstration that FN2 domains possess phosphatidylcholine-binding activity [26]. This property has been shown to facilitate the binding of a family of seminal plasma proteins (SVS8, PDC-109, HSP-1, PB1, LOC317699) to sperm surface lipids containing the phosphatidylcholine group [27–30].

Nonetheless, the potential importance of REP52 in sperm maturation is highlighted by the demonstration that its developmental expression is temporally correlated with the onset of puberty and entry of spermatozoa into the epididymis. Although this finding suggests that *REP52* gene expression is induced in response to rising concentrations of circulating plasma androgens during pubertal development, it was shown that androgens alone were not sufficient to restore the steady-state levels of *REP52* mRNA in castrated animals. It is considered unlikely that this result was a reflection of inadequate levels of circulating androgen since these values were measured directly. Furthermore, the steady-state levels of another epididymal protein, REP38, have previously been shown to be restored to precastrate levels in the same animals [6]. Similarly, we also consider it unlikely that this result was influenced by the duration of testosterone therapy. In this context, Jones et al. [31] established that testosterone treatment for a period of 14 days is adequate to restore protein synthesis to normal values in regions 6–8 of the rabbit epididymis following castration. Taken together, these results raise the possibility that steady-state levels of *REP52* mRNA are regulated by the synergistic action of androgens and additional lumicrine factors [32] originating upstream in intact animals.

Interestingly, lumicrine factors have been implicated in the gene regulation of a number of additional epididymal proteins, including proenkaphalin [33], retinoic acid binding protein [34], CRES gene (cystatin-related epididymal specific) [35], 5

alpha-reductase [36], gamma-glutamyl transpeptidase (GGT) mRNA IV [37], A-raf [38], glutathione peroxidase (GPX5) [39], polyomavirus enhancer activator 3 (PEA3) [40], a disintegrin and metalloprotease 7 (ADAM7) [41], and sperm adhesion molecule 1 (SPAM1) [42]. These factors may, therefore, prove to be as important to the regulation of epididymal gene expression as the more widely studied endocrine factors. The identification of these lumicrine factors and the mechanisms by which they affect rate-limiting steps in transcription initiation remain largely unknown. Evidence from Hinton et al. [32] indicates that, at least for the GGT gene, this mode of regulation may be mediated by growth factors acting via a complex signal-transduction pathway involving the interplay of second messengers and transcription factors. Initiation of mRNA in response to these extra- and intracellular cues has also been proposed as the primary control point in the regulation of differential gene expression along the epididymis [39, 40, 43]. It remains to be established whether the steady-state levels of *REP52* mRNA are under similar complex control.

The presence of antigenically related proteins in rat and mouse epididymal fluid indicates that REP52 is not unique to the rabbit epididymis. Consistent with the localization of rabbit REP52, the rat and mouse homologues are synthesized and secreted by the epididymal epithelium and associated with luminal spermatozoa. The functional analogy between these proteins remains to be explored, although it is noteworthy that anti-REP52 IgG localizes specifically to the rat and mouse sperm flagellum. Nevertheless, structural differences between the proteins are inferred by different relative mobilities in SDS-PAGE gels. Future work will focus on the functional characterization of REP52 and the orthologous proteins present in mouse spermatozoa.

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