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Multidrug resistance-associated protein 9 (ABCC12) is present in mouse and boar sperm

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The human and murine genes for MRP9 (multidrug resistance-associated protein 9; ABCC12) yield many alternatively spliced RNAs. Using a panel of monoclonal antibodies, we detected full-length Mrp9 only in testicular germ cells and mouse sperm; we obtained no evidence for the existence of the truncated 100 kDa MRP9 protein reported previously. In contrast with other MRPs, neither murine Mrp9 nor the human MRP9 produced in MRP9-transfected HEK-293 cells (human embryonic kidney cells) appears to contain N-linked carbohydrates. In mouse and boar sperm, Mrp9 localizes to the midpiece, a structure containing all sperm mitochondria. However, immunolocalization microscopy and cell fractionation studies with transfected HEK-293 cells and mouse testis show that MRP9/Mrp9 does not localize to mitochondria. In HEK-293 cells, it is predominantly localized in the endoplasmic reticulum. We have been unable to demonstrate transport by MRP9 of substrates transported by other MRPs, such as drug conjugates and other organic anions.

Key words: ATP-binding-cassette protein (ABC protein), drug transport, mammary tumour cell, multidrug resistance-associated protein 9 (MRP9), sperm, testis.

INTRODUCTION

The largest family of mammalian primary active drug transporters is the MRP (multidrug resistance-associated protein) family, which belongs to the ABCC subgroup of ABC (ATP-binding-cassette) proteins. MRP1 (ABCC1) was discovered by Cole et al. [1] in 1992, and eight other MRPs have since followed one by one [2–4], as reviewed by us [5–12] and others [13–19]. The eight human MRPs that have been characterized are all able to transport organic anions, such as drugs conjugated to glutathione, sulfate or glucuronate. In addition, selected MRPs may transport a variety of endogenous compounds, such as leukotriene C4 (MRP1), bilirubin glucuronides (MRP2 and MRP3), prostaglandins E_1 and E_2 (MRP4), cGMP (MRP4, MRP5 and MRP8) and several glucuronosyl or sulfatidyl steroids. The absence of two of these transporters is associated with human disease. Defective MRP2 results in Dubin–Johnson syndrome; defective MRP6 causes pseudoxanthoma elasticum. A defect in MRP8 was recently associated with the inability to produce wet, brownish, sticky earwax [20].

The most recent addition to the MRP family is MRP9 (ABCC12), independently described in 2001 by Yabuuchi et al. [21], Tammur et al. [22] and Bera et al. [23]. MRP9 is most closely related to MRP5 and MRP8. In humans, MRP9 is located next to MRP8 on chromosome 16q12.1 [21], presumably as a result of a recent duplication [22]. Whereas no mouse homologue of MRP8 (ABCC11) has been found [24], the cDNA of murine Mrp9 (Abcc12) was cloned and characterized by Shimizu et al. [24]. Both the human MRP9 and the mouse Mrp9 genes yield a plethora of alternatively spliced RNAs, and this has led to uncertainty about the true nature of the protein encoded by MRP9. The gene structure looks unambiguous and is predicted to contain 29 exons in both human and mouse, encoding a classical 'short' MRP, lacking the N-terminal domain present in MRP1, 2, 3, 6 and 7 but absent from MRP4, 5 and 8. The predicted amino acid sequences of murine Mrp9 and human MRP9 are 84.5 % identical and their hydropathy profiles are nearly identical [24]. Human MRP5 and human MRP9 share 42 % identity and all conserved elements in MRPs are also present in MRP9 [22].

A transcript encoding full-length human MRP9 has not yet been successfully cloned, however. Tammur et al. [22] identified transcripts of approx. 5000 bp on blots of human testis, ovary and prostate RNA, but were unable to clone a full-length cDNA containing all MRP9 exons. Yabuuchi et al. [21] showed that the predominant MRP9 splice variants present in human tissues do not encode a canonical MRP, but they did detect a small amount of RNA containing all putative exons in testis RNA [21]. The relevance of this RNA is supported by the results obtained with RNA from mouse testis [24]. The predominant transcript in this tissue is a 5.4 kb RNA, which encodes the complete Mrp9 protein

Abbreviations used: ABC, ATP-binding-cassette; AEC, 3-amine-9-ethylcarbazole; BTS, Beltsville thawing solution; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; EST, expressed sequence tag; FCS, foetal calf serum; HEK-293 cells, human embryonic kidney cells; HRP, horseradish peroxidase; KO, knockout; LAMP-1, lysosome-associated membrane protein-1; mAb, monoclonal antibody; MRP, multidrug resistance-associated protein; MTC, multiple tissue cDNA; PDI, protein disulfide-isomerase; QPCR, quantitative PCR.

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sequence. Two minor variants were cloned as well, both encoding truncated proteins [24].

In contrast, Bera et al. [23] concentrated on the main splice products of the human *MRP9* gene [25]. They cloned two major RNAs: a 4.5 kb RNA lacking exons 5, 16 and 26, which was found only in testis and encoded a truncated protein of 930 amino acids; and a 1.3 kb RNA present at high levels in brain and encoded a putative protein of 233 amino acids. Interestingly, substantial amounts of the 4.5 kb RNA were also found in some breast cancer cell lines. Moreover, tumour cells in some samples of breast cancer showed strong RNA *in situ* hybridization with the *MRP9* probe. A band of approx. 100 kDa, presumably the 930-amino-acid translation product of the 4.5 kb RNA, was detected in testis extracts and in a breast cancer cell line extract by an IgG fraction purified from rabbit antisera raised against MRP9 synthetic peptides [25].

We have focused on the full-length canonical versions of murine Mrp9, as well as human MRP9. We have tried to determine whether these proteins are actually synthesized *in vivo* and what their transport function could be. In the present paper, we report that murine Mrp9 is present in murine sperm and sperm cell precursors.

MATERIALS AND METHODS

Chemicals and reagents

DMM (1-deoxymannojirimycin) was made as described by Broxterman et al. [26]. A polyclonal antibody against the human mitochondrial outer membrane protein Sam50 [27] was generated. Sam50 fused to glutathione S-transferase was produced in *Escherichia coli* BL21 cells, purified over glutathione beads and was eluted using thrombin. The thrombin was removed with a heparin column (Amersham) and the purified Sam50 was injected into rabbits according to standard protocols. The rabbit antisera recognized a mitochondrial protein as demonstrated using confocal microscopy and stained a 50 kDa band on Western blots of human, mouse and pig tissues.

Commercial antibodies came from the following sources: polyclonal rabbit anti-(human calreticulin) (Upstate Biotechnology); polyclonal rabbit anti-[human EEA1 (early endosome antigen 1)] (Upstate Biotechnology); polyclonal rabbit anti-(bovine catalase) (Abcam); monoclonal rat anti-(mouse CD107a) [LAMP-1 (lysosome-associated membrane protein-1)] (BD Pharmingen); monoclonal mouse anti-(pigeon cytochrome *c*) (BD Pharmingen); polyclonal rabbit anti-(human calnexin) (Santa Cruz Biotechnology); polyclonal goat anti-[human GRASP65 (Golgi reassembly stacking protein 65)]; (Santa Cruz Biotechnology); rabbit anti-[PDI (protein disulfide-isomerase)] rec 442 (a gift from Dr Hidde Ploegh, The Whitehead Institute, Massachussetts Institute of Technology, Cambridge, MA, U.S.A.); HRP (horseradish peroxidase)-conjugated goat anti-rabbit (Santa Cruz Biotechnology); HRP-conjugated goat anti-rat (Santa Cruz Biotechnology); HRP-conjugated rabbit anti-mouse (Dako); HRP-conjugated rabbit anti-goat (Dako); rabbit anti-rat (Vector Labs); Alexa Fluor® 488-conjugated goat anti-rat (Molecular Probes); Texas Red-conjugated goat anti-rabbit (Molecular Probes).

Protein immunoblot (Western blot) analysis

Western-blot analysis was performed essentially as described previously [28,29]. Protein concentration was quantified using the Bio-Rad protein assay (Bio-Rad), fractionated by SDS/PAGE (8 % polyacrylamide slab gel) and transferred to a nitrocellulose membrane by electroblotting. After blocking for 1 h in PBS containing 1 % (w/v) non-fat milk powder, 1 % (w/v) BSA and 0.05 % (v/v) Tween 20, the blots were incubated for 2 h with the MRP9 rat mAbs (monoclonal antibodies), with subsequent use of an HRP-labelled goat anti-rat antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence for detection.

Transfected cell lines

HEK-293 cells (human embryonic kidney cells) were grown in DMEM (Dulbecco's modified Eagle's medium; Gibco BRL) containing 10 % (v/v) FCS (foetal calf serum), 100 units of pencillin and 100 μg/ml streptomycin. The cloning of the full-length human MRP9 cDNA has been described previously [21]. To make MRP9-expressing cell lines, the MRP9 cDNA was inserted into the EcoRI and HindIII sites of the pcDNA3.1(–) vector (Invitrogen). By using calcium phosphate precipitation, HEK-293 cells were transfected with the pcDNA3.1(–)-MRP9 vector. After selection with G418 (1 mg/ml), positive colonies were picked and separately expanded in DMEM containing 10 % FCS and penicillin/streptomycin for further studies (HEK-293/MRP9-PC-4).

Although the details are not described in the present paper, several other transfected cell lines were also generated to characterize human MRP9. Briefly, to generate pEGFP-N2-*MRP9*, the 5′ part of an EcoRI and PstI *MRP9* cDNA fragment of pcDNA3.1 containing full-length *MRP9* cDNA was inserted into the corresponding restriction sites of the pEGFP-N2 vector. To replace the stop codon, PCR was used to amplify a 1327 bp section of DNA from an *MRP9* cDNA template. The PCR fragment was digested with PstI and SmaI and inserted into pEGFP-N2 containing the 5′ part of MRP9. The orientation and fidelity of the fragment were verified by sequence analysis. By analogous procedures, an MscV-MRP9-IRES-EGFP construct (where MscV is murine stem cell virus, IRES is internal ribosome entry site, and EGFP is enhanced green fluorescent protein) was generated and expressed in HEK-293 cells. Finally, *MRP9* was expressed in insect Sf9 cells using a baculovirus construct: the EcoRI *MRP9* cDNA fragment from plasmid pcDNA3.1(–) was inserted into the corresponding restriction sites of the pFastBac-1 vector. After the orientation of the MRP9 cDNA insert in the vector was verified, DH10Bac cells were transformed with the pFastBac-1-MRP9 construct to generate the recombinant bacmid DNA. The sequence of the resulting recombinant bacmid DNA was verified by PCR analysis. Sf9 cells were transfected with the bacmid DNA to produce recombinant baculovirus, and the MRP9-producing Sf9 cells were used to prepare inside-out membrane vesicles for transport studies. The presence of MRP9 in these vesicles was confirmed using our new anti-MRP9 mAb M_9I-27, with 1 μl of vesicle protein producing a clear 150 kDa band on a Western blot.

Cloning of rat Mrp9 (Abcc12) cDNA

The rat *Mrp9* gene, which is homologous with mouse *Mrp9* or human *MRP9* genes, was identified in the NCBI (National Center for Biotechnology Information) mouse database, as well as the EMBL/UCSC database. By using the GENSCAN program [30] (http://genes.mit.edu/GENSCAN.html), we have predicted plausible exons in the rat *Mrp9* gene. Based on the predicted exons, rat EST (expressed sequence tag) clones were extracted from the EST database. We have screened MTC (multiple tissue cDNA) panels (MTC™; Clontech, Palo Alto, CA, U.S.A.) by means of PCR using the following primers: forward primer, 5′-GACTATCGGATGAGATACAGAGACAACAC-3′,

and reverse primer, 5′-CAAAGCAGCTGGCGTTCTCCTACTGAGAAG-3′. Among the tissues screened, the highest expression of rat Mrp9 was detected in the testis.

To clone the rat Mrp9 cDNA, we designed the following four sets of PCR primers: c12-1 (forward primer, 5′-GTCCACAGAGGAGGAAGCTAGAGTGAAC-3′ and reverse primer 5′-GACTCACCTGCCCTAGAAGAGCAGAAATG-3′), c12-2 (forward primer 5′-GATACGTCCAAAGTGGGAACTCAGCCCTG-3′ and reverse primer 5′-CTGCATGGAGGAGGTGATGTGAGAGAAC-3′), c12-3 (forward primer 5′-CACATGTACCAGTTGGTTTACATAGCAAG-3′ and reverse primer 5′-CAAAGCAGCTGGCGTTCTCCTACTGAGAAG-3′), and c12-4 (forward primer 5′-GACTATCGGATGAGATACAGAGACAACAC-3′ and reverse primer 5′-GCGAGGAGCGCTGTTACCTCCAGCCTTG-3′). The PCR reaction was performed with rat testis cDNA (Clontech) and Ex Taq™ polymerase (TaKaRa, Osaka, Japan). The PCR conditions consisted of 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s. After agarose-gel electrophoresis, the PCR products were extracted from the gels and subsequently inserted into TA cloning vectors (Invitrogen) according to the manufacturer's protocol. The sequences of the resulting inserts were analysed with an automated DNA sequencer (Toyobo Gene Analysis). Finally, the whole cDNA sequence of rat Mrp9 was obtained by assembling those partial sequences.

QPCR (quantitative PCR) analysis of human *MRP9*, mouse *Mrp9* and rat *Mrp9*

The expression of human *MRP9*, mouse *Mrp9* and rat *Mrp9* in different organs was examined by QPCR with human, mouse and rat MTC panels respectively. The PCR was performed with TaKaRa Ex Taq™ R-PCR version in a TaKaRa Smart Cycler™ System (TaKaRa), where amplification of DNA was monitored with SYBR Green I. The reaction mixture contained 2 μl of cDNA (MTC panel), 2.5 μl of 10× PCR buffer, 0.75 μl of 10 mM dNTP mixture, 0.75 μl of 10 μM PCR primers, 0.3 μl of 250 mM Mg^{2+} solution, 0.25 μl of 1:300-diluted SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, U.S.A.), and 0.25 μl of Ex Taq™ polymerase in a total volume of 25 μl. For this analysis, the following specific primers were used; for human (forward primer 5′-GTAAGGTACAACTTGGATCCCT-3′ and reverse primer 5′-TGCTGCTAGTAACATCGCAA-3′), for mouse (forward primer 5′-TATGGCCCGGGCACTTCTCCGTAA-3′ and reverse primer 5′-GACCTTTACAGTCCAACCTCTGCAGCTAGT-3′) and for rat (forward primer 5′-GTAGGAGAACGCCAGCTGCTTTGTATGG-3′ and reverse primer 5′-GACCTTTACAGTCCAACTTCTGCAGCTAGT-3′). The PCR cycle consisted of four steps, i.e. denaturation (95 °C, 15 s), annealing (60 °C, 15 s), amplification (72 °C, 15 s) and quantification (87 °C, 10 s).

Rat mAbs

For the generation of antibodies against MRP9, segments of MRP9 cDNA (Figure 1) were subcloned into the pMal-c vector to produce of a fusion protein of *E. coli* maltose-binding protein with MRP9 segments. The fusion protein was produced in *E. coli* strain JM101 and purified by amylose affinity chromatography. The use of the fusion protein for the production of rat mAbs was as described in [31]. Briefly, the mAbs were selected by an ELISA test, and positive clones were cross-checked on cytospins of HEK-293s/MRP9 and HEK-293 cells. The antibodies obtained were finally screened on Western blots of cellular lysates prepared from HEK-293 cells transfected with MRP9 cDNA. Three mAbs of the M_9I series against the N-terminal segment, and four of the

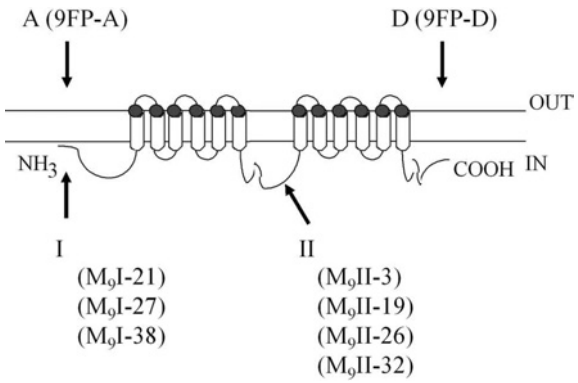

Figure 1 Segments of MRP9 used for raising antibodies

The Figure shows the putative topology of MRP9 in the membrane [23]. Arrows above the membrane indicate the protein segments used to raise rabbit polyclonal antibodies against a fusion protein containing amino acids 1–33 (9FP-A) or amino acids 1317–1349 (9FP-D). Arrows below the membrane indicate the MRP9 segments used to generate rat mAbs. Series I was raised against a mixture of fusion proteins containing amino acids 1–33 and 1–42; series II against amino acids 690–734. All series I antibodies react with the fusion protein containing amino acids 1–33. See the Materials and methods section for details.

M_9II series against the linker segment were used for the present study.

Immunohistochemistry of cells and tissues

For immunolocalization of the MRP9 protein in cells, HEK-293 cells were grown for 2 days on glass coverslips. After fixation with acetone, we used the EnVision Plus HRP system (K4008; Dako), which uses AEC (3-amine-9-ethylcarbazole) as a substrate, with minor modifications for staining. Several rat monoclonal anti-MRP9 antibodies were used for MRP9 immunostaining. A rabbit anti-(rat IgG) (Vector Laboratories AI-4001, pre-absorbed with mouse IgG, 1:200 dilution) was used as the secondary antibody. Before mounting, the coverslips were counterstained with haematoxylin.

Adult FVB mice were used for the isolation of testis and epididymis. Immunohistochemistry of testis cytosections fixed with acetone was performed as described for HEK-293 cells.

Isolation and immunostaining of sperm

Mouse sperm were washed out of the caudal epididymis and centrifuged at 400 ***g*** for 10 min at 20 °C. The pellet was washed in PBS and a drop was placed on a glass coverslip, spread out like a blood smear, and air-dried. After fixation in acetone for 10 min at room temperature (20 °C), the coverslip was rinsed with PBS/0.05 % Tween 20 for 5 min and the smear was then stained using the EnVision Plus HRP system (Dako). Preblocking was carried out with a mixture of PBS, 0.05 % Tween 20 and 5 % (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories). Rat anti-MRP9 mAbs were used in undiluted form (culture supernatant) for 2 h at room temperature; after rinsing with PBS/0.05 % Tween 20, the coverslip was pre-incubated with rabbit anti-rat IgG (Vector lab, pre-absorbed with mouse IgG in 1:200 dilution), followed by the polymer–HRP incubation with the AEC substrate, as specified by Dako. The coverslips were counterstained for 5 s with haematoxylin solution.

Boar semen was collected in the Cooperative Centre for Artificial Insemination in Pigs 'Utrecht en de Hollanden' (Bunnik, The Netherlands). Semen was filtered through gauze to remove gelatinous material and diluted to 150 million cells/ml in BTS (Beltsville thawing solution: 0.2 M glucose, 20 mM sodium

citrate, 15 mM $NaHCO_3$, 3.36 mM sodium EDTA, 10 mM KCl and 20 mM Hepes, pH 7.4) and kept at 17 °C for transport or storage in airtight 80 ml insemination tubes. Prior to further experimentation, the sperm samples were first washed through BTS by centrifugation for 5 min at 400 ***g*** and the resuspended sperm (1 billion sperm/ml) was then spun through a discontinuous (35 %/70 %) Percoll® (Amersham) density gradient to remove epithelial cell contamination and immature sperm [32]. After washing the pellet with PBS, sperm smears were stained as for the mouse sperm, but preblocking before adding anti-MRP mAbs was carried out with 4 % BSA in PBS without the donkey serum.

Confocal microscopy of immunofluorescent cells

Confocal fluorescence images were obtained on a Leica TCS SP2-AOBS confocal system (Leica Microsystems, Heidelberg, Germany), using the 488 nm line from an argon laser and the 594 nm line from an HeNe laser as the excitation source. Images were taken using an HCX PL APO CS ×63 NA (numerical aperture) 1.4 oil objective. Possible cross-talk between the different fluorochromes, which could give rise to false-positive co-localization of the signals, was avoided by careful selection of the imaging conditions. For the analysis of the intracellular location of MRP9 in transfected cells, the MRP9-HEK-293 cells were grown overnight on poly(L-lysine)-coated coverslips. After rinsing with PBS, the cells were fixed with either methanol (5 min in ice-cold methanol, rehydration in PBS for 5 min) or formalin (20 min in formalin at room temperature, three PBS washes, 30 min PBS incubation, permeabilization for 5 min in a mixture of 1 % BSA and 0.2 % Triton X-100 in PBS). For antibody incubations, the cells were preblocked with 1 % BSA in PBS for 30 min, incubated with primary antibody for 1 h, washed with 1 % BSA/PBS, incubated with the secondary antibody for 1 h, washed with 1 % BSA/PBS and PBS, and mounted in a Vectashield.

The rat anti-MRP9 antibodies M_9I-38 and M_9II-19 were used undiluted on formalin-fixed cells and 1:2 diluted on methanol-fixed cells. The secondary antibodies used were Alexa Fluor® 488-conjugated goat anti-rat or Texas Red-conjugated anti-rabbit.

Analysis of protein-linked oligosaccharides

Total cellular lysates were incubated with an N-glycosidase cocktail (2.67 m-units/μl), a mixture of endoglycosidase F and peptide N-glycosidase F (Boehringer Mannheim, Mannheim, Germany), at 30 °C overnight to remove N-linked oligosaccharides. High-mannose oligosaccharides were removed by incubation with endoglycosidase H (2 units/ml; Boehringer Mannheim) in 50 mM sodium citrate (pH 5.5) and 0.2 % SDS, at 37 °C overnight.

Cell fractionation studies

Mouse testis was dissected into small pieces in ice-cold mito-buffer (0.2 mM sodium-EDTA, 0.25 M sucrose and 10 mM Tris/HCl, pH 7.8) and homogenized by 15 strokes with a Wheaton Dounce tissue grinder with tight pestle clearance (0.05 mm). The homogenate was centrifuged at 1000 ***g*** for 10 min at 4 °C. The supernatant (S1) was centrifuged at 8000 ***g*** for 15 min at 4 °C (Sorvall SS34 rotor), the pellet (P2) was washed twice with mitobuffer containing protease inhibitors (Roche Complete™ tablets) and stored frozen. P2 was thawed, centrifuged at 8000 ***g*** for 15 min at 4 °C and resuspended in a minimal volume of 0.8 M sucrose, containing 0.2 mM Tris/EDTA (pH 7.8), and layered on to a 5 ml 0.9–2.0 M sucrose gradient (in 0.3 mM sodium-EDTA and 10 mM Tris, pH 7.8). After centrifugation to equilibrium (2 h, 4 °C and 25 500 rev./min in a Beckman SW40 rotor), approx. 15–20 fractions were collected through a hole punched in the bottom of the tube. The membranous material in each fraction was collected by centrifugation for 10 min at 200 000 ***g***. The pellets were resuspended in mitobuffer and analyzed by Western blotting.

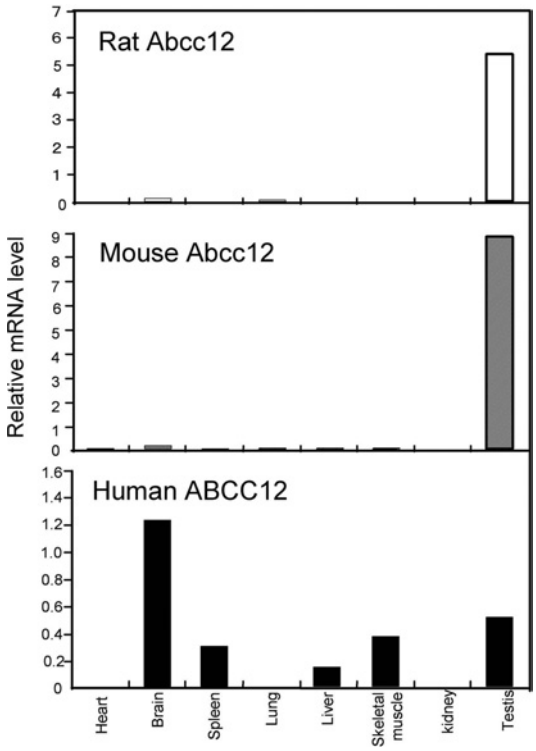

Figure 2 MRP RNA levels in rat, mouse and human tissues, determined by semi-quantitative RT (reverse transcriptase)–PCR

Note differences in scale for human and rodent tissues. See the Materials and methods section for details.

RESULTS

Generation of antibodies against MRP9

Whereas transcripts of *MRP9* have been detected in various human, rat and mouse tissues including brain, the expression levels are relatively low, with the exception of rat and mouse testes (Figure 2). Most of the RNA in human testis consists of alternative splice products, but our previous analysis has also shown the presence of a minor fraction of an RNA able to encode full-length MRP9 [21]. To test whether this protein is actually synthesized in cells, we generated a series of mAbs and two polyclonal rabbit antibodies against human MRP9 segments, with little sequence identity with other MRPs (Figure 1). The MRP segments were fused to the *E. coli* maltose-binding protein, and purified fusion proteins were used as immunogen (see the Materials and methods section). The mAbs were raised in rats in the hope of generating antibodies that would react not only with human MRP9, but also with murine Mrp9.

To characterize the antibodies, we used HEK-293 cells transfected with a MRP9 cDNA construct. Figure 3(A) shows the results with four of the seven different mAbs. Each of the mAbs recognizes a single 150 kDa band in the transfected cells, but not in the parental cells, and the same result was obtained with the three mAbs not shown in Figure 3(A). Figure 3(B) presents immunohistochemical results with three of the mAbs. In the HEK-293 cells, MRP9 does not localize to the plasma membrane, as

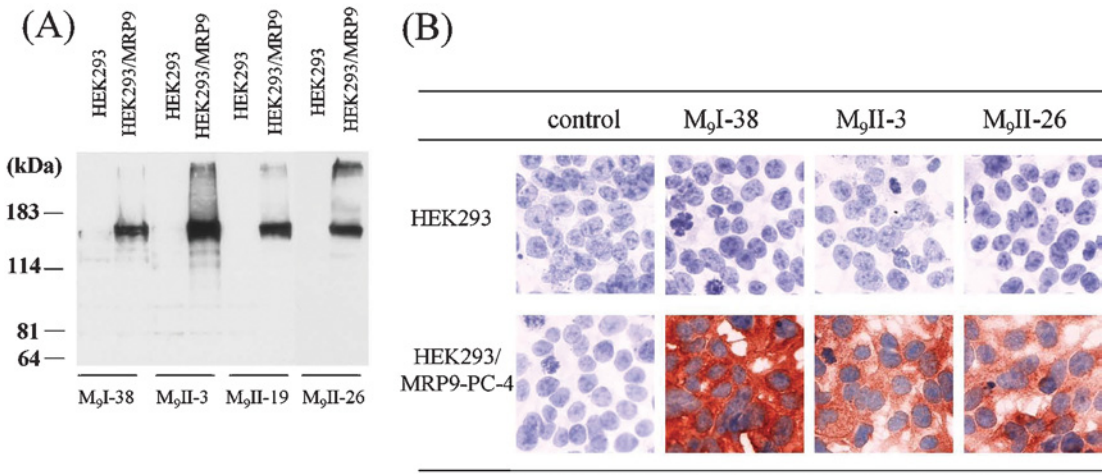

Figure 3 Detection of MRP9 in HEK-293 cells transfected with an MRP9 cDNA construct

(**A**) Western blot of cell lysates from transfected and parental HEK-293 cells stained with four different rat anti-MRP9 mAbs. (**B**) Immunocytochemical staining of transfected cells (HEK-293/MRP9-PC-4) and parental HEK-293 cells stained with three different rat mAbs. See the Materials and methods section for details.

other MRPs do, but remains intracellular. The two rabbit antisera raised against MRP9 segments also recognized a 150 kDa band, but as these sera gave more background than the mAbs, they were not used in most of the further experiments.

Detection of full-length Mrp9 in mouse tissues

With the antibodies generated against MRP9 we were unable to detect any form of MRP9 in human tissues or cell lines, or on Western blots of extracts from tissues or cell lines. These included testis and mammary tumour cell lines, in which a short 100 kDa form of MRP9 has been detected by others [25]. We therefore turned to murine tissues. Figure 4(A) shows a Western blot of mouse tissue extracts, incubated with mAb M_9II-19. Only testis extracts contained a 150 kDa band that co-migrated with the 150 kDa band in HEK-293/MRP9 cell extracts. More rapidly migrating bands were observed in mouse liver extracts, but these were non-specific as they were also present in the control incubated with second antibody only (results not shown). The 150 kDa band in mouse testis reacted with six of our seven anti-MRP9 mAbs; only M_9II-26 did not react (Figure 4B), although it reacted as intensely with human MRP9 as the other mAbs (Figure 3A). mAb$_9$II-26 therefore provided a convenient control for further mouse studies. Whereas the mAbs directed against the N-terminus of MRP9 (M_9I series) only stained a single 150 kDa band, the mAbs directed against the linker region (M_9II series; see Figure 1) also stained a band of approx. 105 kDa (Figures 4A and 4B, indicated by arrowheads). This is unlikely to be a protein corresponding to one of the known alternatively spliced RNAs of mouse Mrp9 [24], as both RNAs encode the N-terminal sequence of Mrp9 and thus the corresponding proteins should also react with the mAbs of the M_9I series (Figure 1). It is therefore either a degradation product of Mrp9 or the result of an internal translation start. The variable amount of this band and its absence in tissues other than testis makes the degradation product alternative the more likely theory.

In a (failed) attempt to generate a mouse Mrp9 KO (knockout) by oligonucleotide targeting [33], we obtained mice producing MRP9 without the N-terminal segment used to generate the M_9I series of antibodies. Western blots of testis extracts from these mice contained a 150 kDa band that reacted with the M_9II series of mAbs, but not the M_9I series, confirming the specificity of the M_9I antibody series for MRP9 (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/406/bj4060031add.htm). We estimate that the missing segment in truncated Mrp9 is only 31 amino acids long (see Supplementary material at http://www.BiochemJ.org/bj/406/bj4060031add.htm), explaining why the truncated Mrp9 co-migrates with wild-type Mrp9.

We studied the distribution of Mrp9 in mouse testis by immunocytochemistry using three mAbs. Figure 4(C) shows strong staining of the testicular tubules with the Mrp9-specific mAbs M_9I-38 and M_9II-3, but not with M_9II-26, which does not react with murine Mrp9 (Figure 4B). No significant staining was observed in Leydig cells or other interstitial elements. Within the seminiferous tubules we found no staining of the Sertoli cells, and Mrp9 staining appeared to be specific for testicular germ cells.

During mouse testis development, Mrp9 was first detected in mice at 3 weeks of age (Figure 5A). Only a few cells were positive in 3-week testis (Figure 5B), the pachytene spermatocytes. As the mice aged, more Mrp9-positive cells appeared (Figure 5B). In the adult seminiferous tubules, specific association of subsequent generations of spermatogonia, spermatocytes and spermatids at particular development steps occurs in a cyclic manner referred to as stages of the cycle of the seminiferous epithelium [34]. To study which types of cells during spermatogenesis were positive for Mrp9, we investigated sections of seminiferous tubules in the 12 different epithelial stages in the mouse. During germ cell differentiation in the adult mouse testis, pachytene spermatocytes in stage VI of the epithelial cycle were the first germ cells to show Mrp9 expression (Figure 6B). Subsequently, Mrp9 was detected during further development of the spermatocytes, round spermatids and elongating spermatids (Figure 6A). During maturation of the spermatids, Mrp9 accumulated in the tail of elongating spermatids (Figure 6B). There was no significant staining of spermatogonia or pachytene spermatocytes in stage I–IV (Figure 6A), of type B spermatogonia in stage VI (Figure 6B), in leptotene spermatocytes in stage VIII–IX (Figure 6C) or in leptotene/zygotene spermatocytes in stage X (Figure 6D).

Analysis of Mrp9 in mouse sperm

To study whether mature mouse sperm contain Mrp9, we analysed sperm from the epididymis. Figure 7(A) shows a Western blot

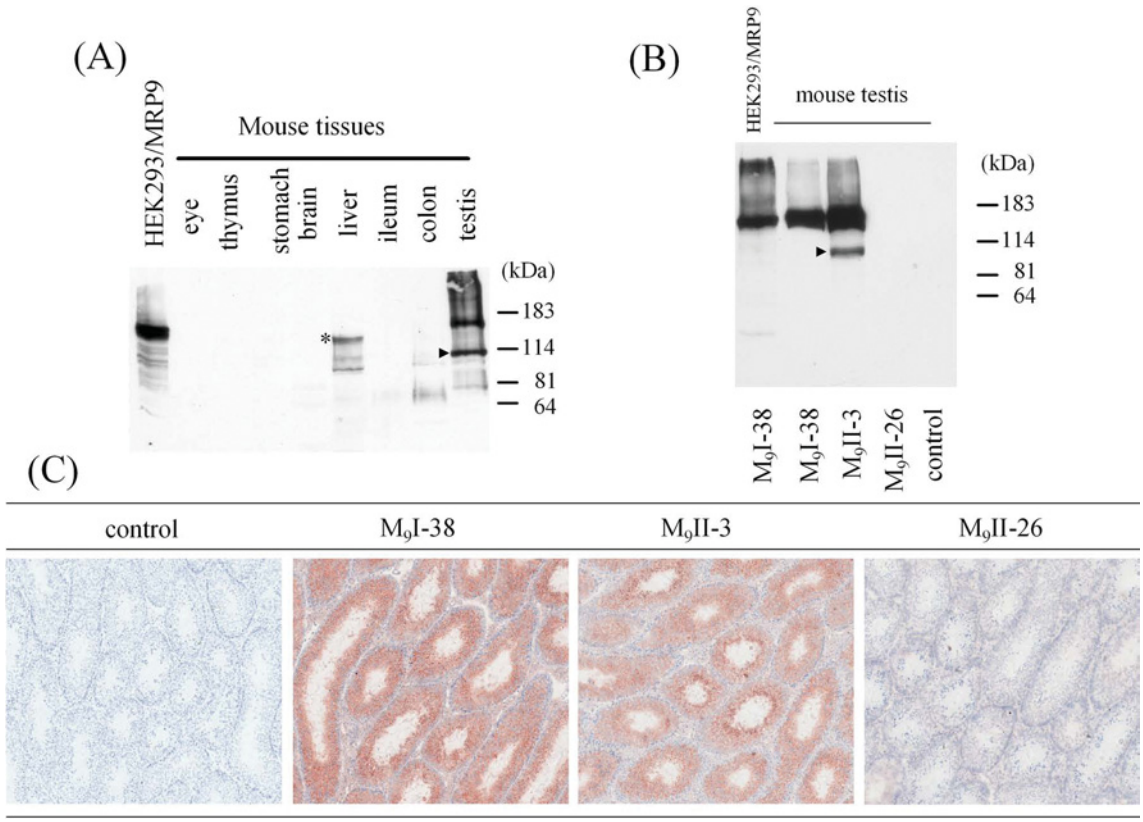

Figure 4 Tissue distribution of MRP9 in mice

(**A**) Western-blot analysis of MRP9 in protein extracts from various mouse tissues, using rat mAb M_9II-19. The asterisk indicates a non-specific band reacting with the second antibody. The arrowhead indicates the 105 kDa band. (**B**) Western-blot analysis of MRP9 in mouse testis extracts, using three different rat anti-MRP9 mAbs. The arrowhead indicates the 105 kDa band. (**C**) Sections of adult mouse testis, stained with two rat mAbs that detect MRP9 and a control mAb M_9II-26 that does not react with mouse MRP9 [as shown in (**B**)]. See the Materials and methods section for details.

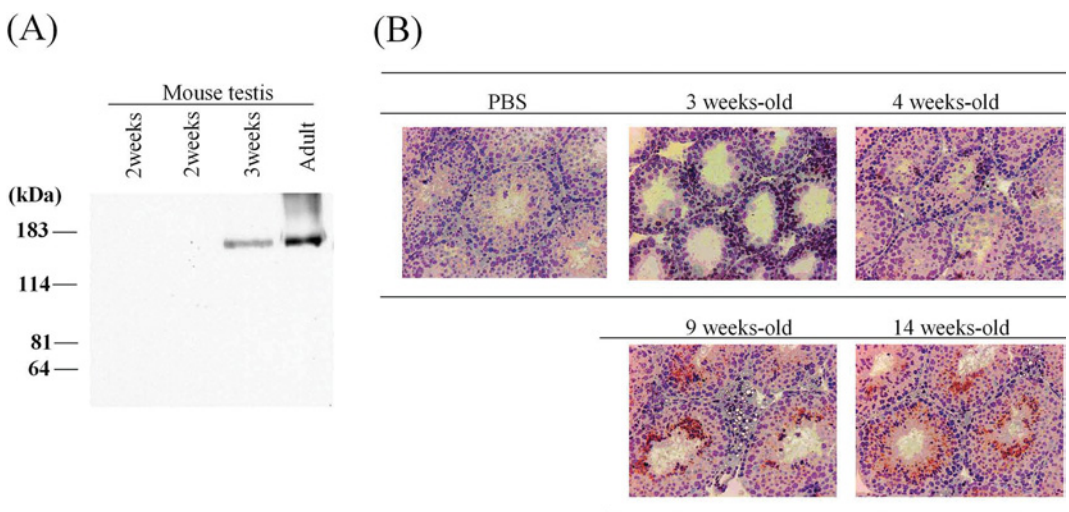

Figure 5 Appearance of Mrp9 during mouse development

(**A**) Western blots of testis extracts from mice 2 weeks (two different mice) or 3 weeks after birth, with adult testis extract for comparison. (**B**) Immunocytochemistry of testis sections of mice of different ages. AEC was used instead of DAB (diaminobenzidine) to visualize the antibody signal. mAb M_9I-38 was used in both (**A**) and (**B**).

of mature sperm lysates analysed with six different antibodies. All three antibodies of the M_9I series detect the characteristic 150 kDa Mrp9 band, M_9II-3 and M_9II-19 detect this band and the additional 105 kDa band, whereas the control mAb M_9II-26 does not detect any proteins. The background with control mAb M_9II-26 is somewhat higher in epididymis (Figure 7B) than in testis (Figure 4C). The upper part of Figure 7(B) shows specific staining in the lumen of the epididymal ducts and this originates

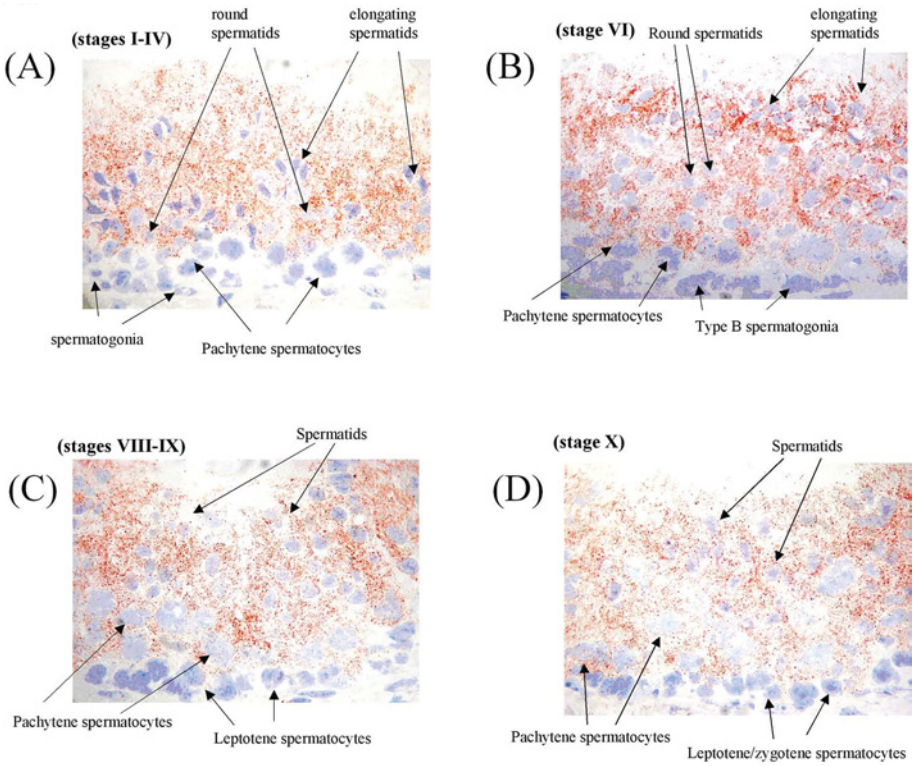

Figure 6 Immunohistochemical staining of Mrp9 in different stages of the seminiferous tubule cycle, using anti-MRP mAb M_9II-3

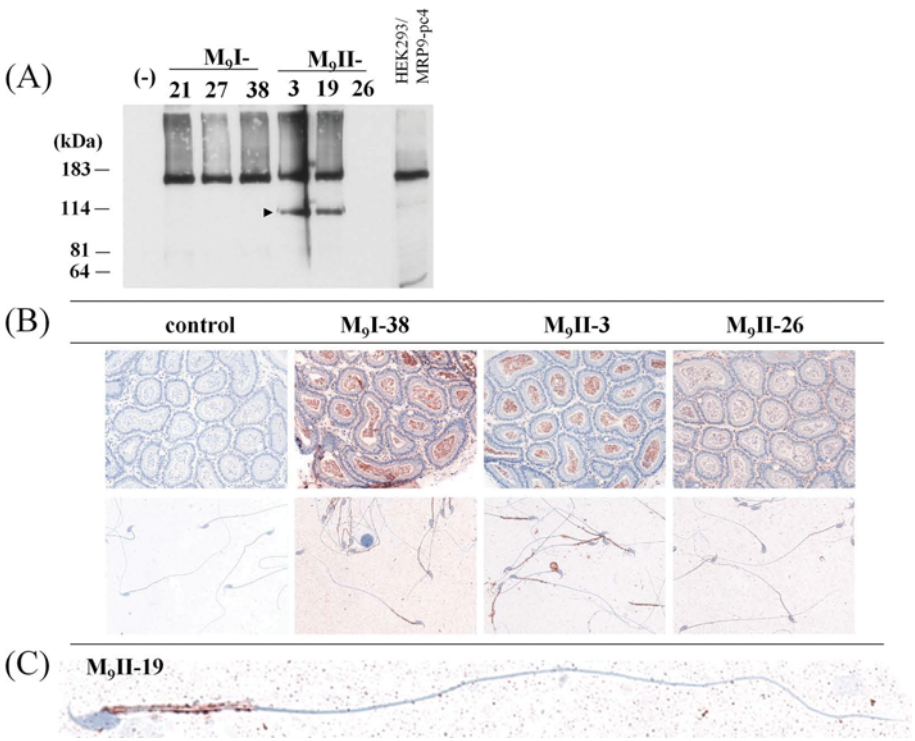

Figure 7 MRP9 in murine epididymis and in aspirated cauda epididymal sperm

(**A**) Western blot of murine sperm extracts probed with six different rat anti-MRP mAbs. An extract of HEK-293/MRP9 cells probed with mAb M_9I-38 is shown for comparison. The arrowhead indicates the 105 kDa band. (**B**) The upper panel shows sections of mouse epididymis stained with the rat mAbs indicated. The control was treated only with the second antibody. The lower panel shows mouse sperm stained with the same set of antibodies. See the Materials and methods section for details. (**C**) A single mouse sperm stained with mAb M_9II-19 at higher magnification.

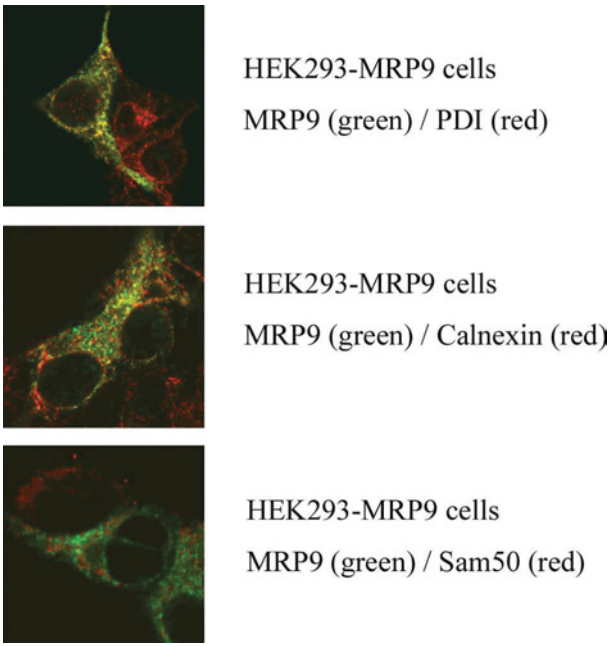

Figure 8 Immunolocalization of MRP9 in HEK-293/MRP9 cells

In the top and middle panels, cells were fixed for 20 min with formalin at room temperature. After washing and permeabilization they were incubated with mAb M_9II-19 (undiluted), rabbit anti-PDI (1:200) (top panel) or rabbit anti-(human calnexin) (middle panel) for 2 h at room temperature. After washing, the anti-MRP9 mAb was detected with goat anti-rat coupled with Alexa Fluor® 488 (green); the anti-PDI antibody was detected with goat anti-rabbit coupled with Texas Red (upper panel; 1:200); and the anti-calnexin antibody was detected with goat anti-rabbit coupled with Texas Red (middle panel; 1:400) by incubation for 1 h at room temperature. In the lower panel, cells were fixed for 5 min with ice-cold methanol. MRP9 was detected with mAb M_9I-38 (1:2 diluted); Sam50 was detected with a rabbit polyclonal antibody raised against human Sam50 (1:200). The secondary antibodies were the same as in the top panels. See the Materials and methods section for further details.

from the sperm that are migrating towards the ductus deferens, but not in the epithelium. That the sperm cells are responsible for the luminal staining is confirmed in the lower half of Figure 7(B) and in Figure 7(C). The Mrp9-specific mAbs stain the midpiece of sperm; the control mAb M_9II-26 gives no staining at all.

Post-translational modification and intracellular location of MRP9 in transfected HEK-293 cells

Human MRP9 and murine Mrp9 run at a higher rate than MRP5 on SDS/PAGE, although these proteins are of a predicted similar size on the basis of their amino acid sequences. The MRP9 band is also much sharper on Western blots than the other MRPs, which are all heavily glycosylated. To test whether any MRP9 glycosylation was detectable, we incubated HEK-293/MRP9 and mouse testis cell lysates with endoglycosidases, prior to Western-blot analysis. As shown in Supplementary Figure 2 (http://www.BiochemJ.org/bj/406/bj4060031add.htm), there was no indication of glycosylation of MRP9/Mrp9.

The lack of detectable N-linked glycosylation of MRP9 suggests that it is not routed through the Golgi cisternae to the plasma membrane or to a post-Golgi endosomal compartment. Indeed, Figure 3(B) does not show MRP9 in the plasma membrane or in lysosomes, the usual final destinations of MRPs in transfected cells [35]. To analyse the distribution of MRP9 in the transfected HEK-293 cells in more detail, we performed co-localization studies with markers for intracellular membranous compartments. As shown in Figure 8, MRP9 is not located in the mitochondria (represented by the mitochondrial outer membrane marker

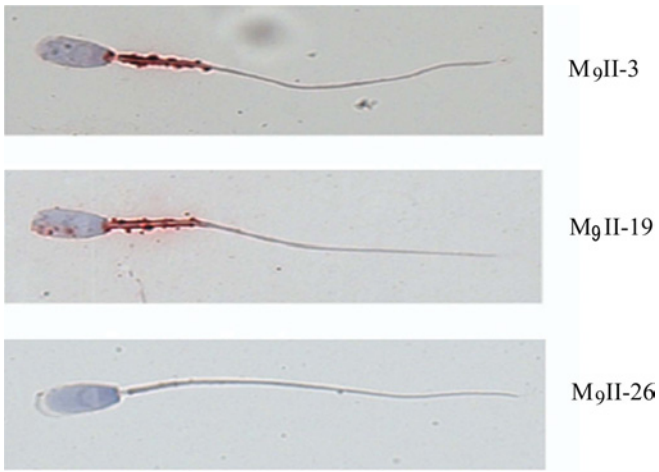

Figure 9 Immunolocalization of MRP9 in boar sperm, using rat mAbs directed against human MRP9

The panel shows from top to bottom: boar sperm stained with mAb M_9II-3, M_9II-19, or M_9II-26 (which does not react with pig MRP9). The presence of the anti-MRP9 mAb was detected by peroxidase staining as described in the Materials and methods section, resulting in coarse granular precipitates of the peroxidase reaction product.

Sam50) and is also not in lysosomes (analysed with the lysosomal marker LAMP-1; results not shown). Co-localization was most extensive with the ER (endoplasmic reticulum) markers PDI and calnexin, as also shown by the pixel analysis in Supplementary Figure 3 (http://www.BiochemJ.org/bj/406/bj4060031add.htm). Lack of co-localization of MRP9 with mitochondrial markers was also observed in HEK-293 cells transfected with other MRP9 constructs and with an EGFP–MRP9 fusion construct (see the Materials and methods section).

Localization of Mrp9 in murine and boar sperm

Although the staining for Mrp9 in the sperm midpiece shown in Figure 7(B) is clear, we were unable to resolve the intracellular localization. The staining was too weak for reliable immunofluorescence staining; none of our six mAbs was suitable for (cryo) electron microscopy and neither were the two anti-MRP9 sera raised. We therefore decided to use cell fractionation. Sucrose gradients of subcellular fractions of mouse testis showed that Mrp9 clearly separated from the mitochondrial markers Sam50 and cytochrome *c*, confirming that Mrp9 is not in the mitochondria (Supplementary Figure 4 at http://www.BiochemJ.org/bj/406/bj4060031add.htm). In contrast, MRP9 did not separate unambiguously from the lysosomal marker LAMP-1 or the ER markers calnexin (Supplementary Figure 4) or calreticulin (results not shown).

Attempts to fractionate mouse sperm cells failed owing to lack of material and we therefore decided to use boar sperm. Of the seven MRP9-specific mAbs, two reacted well with the boar sperm midpiece, as shown in Figure 9, and these antibodies also detected a band of approx. 150 kDa on Western blots (results not shown). Unfortunately, our attempts to separate a boar sperm homogenate into subcellular fractions failed, as the membranous fractions aggregated so strongly that we were unable to separate them in a sucrose gradient.

DISCUSSION

MRP9 was the last member of the MRP family to be cloned and no more will follow. The genome sequence is complete and the

only other candidate MRP left, MRP10 (ABCC13), is clearly a pseudogene [36,37]. MRP9 is an unusual MRP in many respects; it contains no detectable N-linked glycosylation; the full-length protein is only made in detectable amounts in testicular germ cells and sperm; it appears to be localized mainly in the ER in transfected HEK-293 cells, and in a still poorly defined (non-mitochondrial) membrane fraction in sperm. We have tried to find a substrate for MRP9 by testing the ability of MRP9 to induce cellular resistance to cytotoxic compounds and to transport known MRP substrates in vesicular transport, without any positive result so far (see Supplementary material at http://www.BiochemJ.org/bj/406/bj4060031add.htm).

We have been unable to detect any form of MRP9 in any human tissue or cell line. This is puzzling, given the fact that MRP9 RNA was readily detected in many tissues and in cell lines from various tumours. Each of the seven mAbs that were raised against human MRP9 (Figure 1A) should have sensitively detected both full-length MRP9 and all long splice variants described. This includes the 100 kDa band detected by Bera et al. [25] in testis and in a breast cancer cell line. It is possible (but unlikely) that the single polyclonal antibody used by Bera et al. [25] was a more sensitive tool than the seven mAbs that we generated. It is also possible that the antiserum produced by Bera et al. [25] reacted with a 100 kDa cross-reacting band and that there is very little translation of the MRP9 RNA detected with PCR. It may be significant that there are hardly any published RNA blots containing MRP9 transcripts; most MRP9 transcripts were so far detected by PCR, indicating low expression of MRP9.

MRPs evolve rapidly and substantial differences have been observed between human MRPs and their murine counterparts. The most striking so far is the absence of MRP8 in mice [20]. However, it seems unlikely that MRP9 is a pseudogene in humans, as the basic structure of MRPs is fully conserved in MRP9 and as it is 84.5 % identical in amino acid sequence with murine Mrp9, with a high level of identity conserved over all the 29 putative exons of MRP9.

Our results suggest a role for MRP9 during the latter part of the male meiotic prophase, the development of spermatids and/or possibly in sperm function. MRP9 is clearly and exclusively localized in the sperm midpiece, the anterior part of the flagellum characteristically containing all of the sperm's mitochondria. Nevertheless we are confident that MRP9 is not in mitochondria. Conventional wisdom states that sperm does not contain ER, the subcellular fraction in which we find most of the MRP9 made in transfected HEK-293 cells. However, Olson and Winfrey [38] have described a midpiece-specific submitochondrial reticulum in mammalian spermatozoa, and this structure remains a possible location for MRP9. The generation of an Mrp9 KO mouse may shed more light on MRP9 function.

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