Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells

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Human membrane cofactor protein (MCP, CD46) has been suggested, although no convincing evidence has been proposed, to be a fertilization-associated protein, in addition to its primary functions as a complement regulator and a measles virus receptor. We have cloned a cDNA encoding the murine homologue of MCP. This cDNA showed 45% identity in deduced protein sequence and 62% identity in nucleotide sequence with human MCP. Its ectodomains were four short consensus repeats and a serine/threonine-rich domain, and it appeared to be a type 1 membrane protein with a 23-amino acid transmembrane domain and a short cytoplasmic tail. The protein expressed on Chinese hamster ovary cell transfectants was 47 kDa on SDS/PAGE immunoblotting, ~ 6 kDa larger than the murine testis MCP. It served as a cofactor for factor I-mediated inactivation of the complement protein C3b in a homologous system and, to a lesser extent, in a human system. Strikingly, the major message of murine MCP was 1.5 kb and was expressed predominantly in the testis. It was not detected in mice defective in spermatogenesis or with immature germ cells (until 23 days old). Thus, murine MCP may be a sperm-dominant protein the message of which is expressed selectively in spermatids during germ-cell differentiation.

INTRODUCTION

Human membrane cofactor protein (MCP) is a complement regulatory protein which serves as a cofactor for factor I-mediated inactivation of complement proteins C3b and C4b [1], and thus plays a major role in protection of host cells from complement (reviewed in [2]). Recently, MCP was found to be a measles virus receptor (reviewed in [3]). The molecule consists of four short consensus repeats (SCR), a serine/threonine-rich domain (ST), 13 amino acids of unknown significance of homology, a transmembrane domain (TM) and a cytoplasmic tail (CYT), to yield a 45–70 kDa type 1 glycoprotein [2,4,5]. SCR1, 2 and 4 are N-glycosylated and the ST domain is O-glycosylated [2]. The functions of MCP are mapped on different sets of SCRs, based on experiments with deletion or substitution mutagenesis [6,7].

It has been reported that human sperm have abundant MCP, with no O-linked sugars, of 42 kDa [8,9]. This molecule is localized in the inner acrosomal membrane of spermatozoa and becomes expressed on the surface of spermatozoa through acrosomal reaction [10–12]. It has been proposed that the unusual structural and distribution features of sperm MCP may reflect a fertilization-associated protein [13–15]. This hypothesis was reinforced by the findings that monoclonal antibodies against human MCP can partially block fusion of human sperm to hamster eggs [16], that human oocytes may have a counter-receptor for MCP [17], and that in some idiopathic male infertility syndromes, MCP may be aberrant in a sperm-specific manner [18]. However, there is as yet no direct evidence that MCP is involved in sperm–egg interactions.

Recent studies in guinea pigs [19] have suggested that MCP may be expressed predominantly in the testis, whereas in pigs [20], monkeys [21] and humans [2,22] MCP is ubiquitously expressed. Here, we demonstrate that murine MCP is developmentally expressed in the testis, paralleling the formation of spermatids.

MATERIALS AND METHODS

Fetal and mutant mice

Mutant mouse strains, W/W^v [23], Sl/Sl^d [23] and jsd/jsd [24], and a mouse strain cryptorchidism [25], were raised in our laboratory as described in previous reports [26]. Infant and adult C57BL/6 and Balb/c mice were purchased from SLC, Shizuoka, Japan [24]. Testes were surgically excised, as described [26], and frozen at −140 °C, then kept at −80 °C.

cDNA cloning

Two methods were employed to clone cDNA of murine MCP. Firstly, human MCP cDNA was used as a probe to screen a cDNA library made from the testes of C57BL/6 mice. After ligation into the pAP3*neo* vector, colony hybridization (a total of 2×10^6 clones) was performed [27]. Although repeated attempts were made, no positive clones were obtained. Secondly, a conserved amino acid sequence was successfully identified through comparison of MCPs of different species (see Figure 2). Oligonucleotide reverse primers for PCR were synthesized according to the conserved amino acid sequences. PCR cloning was

Abbreviations used: CHO, Chinese hamster ovary; CYT, cytoplasmic tail; DACM, dimethylamino-(4-methylcoumarinyl) maleimide; DAF, decay-accelerating factor (CD55); MCP, membrane cofactor protein (CD46); RT-PCR, reverse transcription PCR; SCR, short consensus repeat; ST, serine/threonine-rich domain; TM, transmembrane domain.

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performed with the cDNA library, first using a forward primer outside the insert (vector sequence, 5′-actccgcccagttccgcccattctc-3′) and one of the reverse primers (5′-acatttgaccactttacaytc-3′). Thermocycle conditions were 20 cycles at 94 °C for 30 s, 50 °C for 60 s and 72 °C for 90 s for denaturing, annealing and extension respectively. Secondly, 1 μl of the product was added to a mixture of a vector primer in close proximity to the insert (5′-cctcagtggatgttgcctttact-3′) and the same reverse primer, and sequential PCR was carried out under the same thermocycle conditions for 35 cycles. This nested PCR cloning allowed us to pick up products of the expected size. The DNA sequence was determined by the dideoxy method on an Applied Biosystems DNA sequencer. Six independent cDNA clones were sequenced on both strands and were found to be identical. Finally, three clones containing a putative complete coding region were obtained by subsequent colony hybridization.

Message analysis

Total (20 μg) RNAs were isolated from the testes of normal and mutant mice using an RNAzol™ (TEL-TEST, Inc.). For Northern blotting, RNAs were denatured with glyoxal and DMSO at 50 °C for 1 h. The RNA was electrophoresed on 1 % agarose gels and transferred onto Hybond N^+ membranes (Amersham International, Amersham, Bucks., U.K.). Tissue blotting was performed with MTN™ (Clontech, Palo Alto, CA, U.S.A.). The blots were prehybridized for 30 min, hybridized for 1 h at 68 °C in Clontech hybridization buffer (ExpressHyb™, Clontech) with a ^{32}P-labelled 0.3 kb cDNA fragment corresponding to SCR2 and SCR3, and washed at high stringency [50 °C, 0.1 × SSC (SSC is 0.15 M sodium citrate/0.015 M sodium citrate)/0.1 % SDS]. The blots were rehybridized with a β-actin probe to ensure equivalent loading of RNA.

For reverse transcription (RT)-PCR, RNA (0.2 μg) isolated from Balb/c mice using an ISOGEN RNA isolation kit (Nippon GENE, Osaka, Japan) was reverse transcribed using random primers with RNAase H-free reverse transcriptase (Superscript, Gibco-BRL). The 254 bp murine MCP cDNA was amplified using primers 5′-cggccatttgaagctatggaac-3′ and 5′-tacgtaataaccttccatacaag-3′ with exTaq (Takara, Osaka, Japan). The thermocycle program was adjusted to semiquantitative conditions: 20–30 cycles of 94 °C for 30 s, 55 °C for 60 s and 72 °C for 120 s. The products were analysed by 1.5 % agarose gel electrophoresis.

Computer analysis

cDNA analysis was performed with Gene Works, GENETYX, Clustal W on a Macintosh 7200.

Preparation of anti-peptide antibodies recognizing murine MCP

The method for production of anti-peptide antibodies we employed has been described in previous reports [28,29]. Three peptides, ACELPRPFEAMELKGTPKLFYAC (the N-terminus of SCR1), MLQDPSFGKVYYIDGSFSWGAC (within SCR2), and IKVQCTMLQDPSFGKVYYIDGSC (the N-terminus of SCR2) were sequentially synthesized in order according to the combined information about the primary sequence of murine MCP (see Figure 1) and the predicted tertiary structure of human MCP [30]. Although the last two sequences partly overlap, only the last of the peptides successfully acted as an immunogen when rabbits were immunized with it [29]. Therefore the IKVQCT sequence could be implicated as the determinant antigen. The rabbit anti-peptide antibody raised against this peptide was used for subsequent experiments.

Isolation of stable transfectants expressing murine MCP

The cloned cDNA was ligated in a mammalian expression vector pCXN2, and Chinese hamster ovary (CHO) cells (5×10^7) were transfected with the vectors (10 μg) by lipofectamine [31]. The transfectants were maintained for 24 h in Ham's F12 medium containing 10 % (v/v) fetal calf serum and 0.06 % (v/v) kanamycin in an atmosphere of humidified 5 % CO_2/95 % air at 37 °C. Thereafter, the cells were transferred to the same medium containing 0.5 mg/ml G418 (Sigma, St. Louis, MO, U.S.A.) for selection [31]. Ten days later, the neomycin-resistant colonies were isolated with cloning cylinders and expanded in 24-well culture plates (Corning, Cambridge, MA, U.S.A.). Expression of murine MCP was tested by immunoblotting using the anti-peptide antibody. CHO cells transfected with vector containing anti-sense MCP cDNA, which were murine MCP protein-negative, were provided as control.

SDS/PAGE and immunoblotting

CHO cell transfectants or murine organs were washed with PBS and solubilized with 0.8 % (v/v) Nonidet P40/PBS (pH 7.4) containing 1 mM PMSF and 10 mM EDTA (solubilization buffer) [18,29]. After removal of the pellet by centrifugation [29], the supernatants were subjected to SDS/PAGE by the method of Laemmli as described previously [18]. Proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with 10 % of a blocking reagent (Morinaga Co., Japan) for 2 h, followed by the addition of 20 μl of anti-peptide antibody. One hour later, the membranes were extensively washed with PBS containing 0.02 % Nonidet P40, pH 7.4, and then incubated for 1 h with 2 μl of horseradish peroxidase-labelled goat anti-rabbit IgG antibody (Bio-Rad, Richmond, CA, U.S.A.). Again, after extensive washing, proteins were detected with an enhanced chemiluminescence (ECL) kit (Amersham) [18].

Factor I-cofactor activity

CHO cells with murine MCP ($\sim 10^8$ cells) were harvested and solubilized with the solubilization buffer [29,31]. Likewise, control CHO cells with no MCP protein were solubilized also. The pellets were removed by centrifugation (12000 ***g***, 15 min) and the supernatants of the cells were used as a source of MCP protein. Human factor I [7], factor H [7] and dimethylamino-(4-methylcoumarinyl) maleimide (DACM)-labelled human C3b [7], and murine factor I [32–34], factor H (referred to as H.1 in [34]) and DACM-labelled murine C3b [33,34] were prepared as described in the references. The DACM-labelled substrates (10 μg) were incubated in PBS/0.02 % Nonidet P40, pH 7.5, for 2 h at 37 °C with either murine (0.2 μg) or human (0.5 μg) factor I and the solubilized preparations (corresponding to 2×10^7 cells). In some experiments, human (0.3 μg) and murine factor H (0.5 μg) were used instead of the solubilized preparations as control. C3b to iC3b conversion was revealed by SDS/PAGE and illumination with a UV lamp (360 nm) [34].

RESULTS

Identification of a murine homologue of MCP

Based on a homology search for MCP of various species, degenerate oligonucleotide primers were designed to screen a cDNA library by the PCR method. The forward oligonucleotide

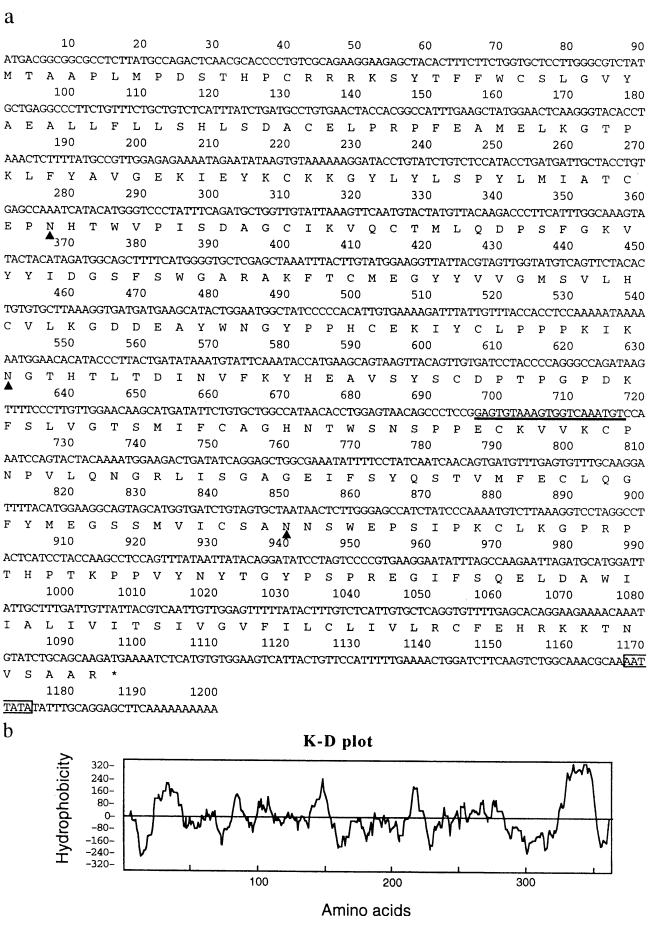

Figure 1 Complete nucleotide sequence and K–D hydrophobicity plot for murine cDNA

(**a**) The deduced amino acid sequence is shown below the nucleotide sequence using the single-letter code. The stop codon is shown by an asterisk. Closed arrowheads indicate possible N-glycosylation sites. An oligonucleotide primer for PCR cDNA cloning is indicated by an underscore. A putative polyadenylation signal, which is not typical, is boxed. The nucleotide sequence data is available from the EMBL Data Library/GenBank/DDBJ databases under the accession number AB001566. (**b**) K–D plot for hydrophobicity analysis.

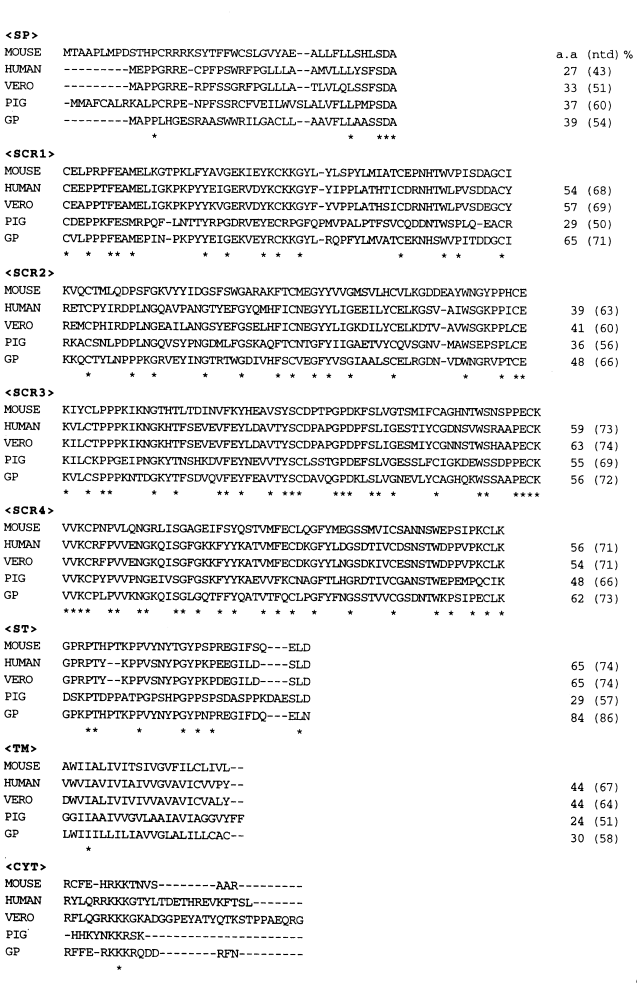

Figure 2 Alignment of the deduced amino acid sequences of mouse, human, Vero cell, pig and guinea pig MCPs

The values for amino acid (a.a.) and nucleotide (ntd) identities of each domain are shown in the right margin. Conserved amino acids among these species are indicated by asterisks below the amino acid sequence. In human, monkey and guinea pig cells, several isoforms with different ST and CYT domains have been reported. Here, we show the ST^c/CYT1 isoform of human [5] and monkey [21] MCPs and the GM1 isoform of guinea pig MCP (the signal peptide sequence is compensated for with the GM3 isoform) (see [19] for details).

was a vector sequence and the reverse was comprised of a mixture of degenerate primers. We tried several reverse primers. The first PCR products were used as a template for nested PCR. The second round of PCR allowed us to amplify a 717 bp cDNA. Sequence analysis revealed the consensus sequences for SCRs. Therefore we screened the cDNA library by colony hybridization using the 717 bp fragment as a probe.

Three clones contained $\sim$ 1.5 kbp inserts that had the same nucleotide sequence (Figure 1a). An ATG codon was detected downstream of a GC-rich sequence (CCCGGG) (not shown), suggesting that this was the initiating codon. If this is the case, the deduced amino acid sequence was 45.3% homologous to that of human MCP (Figure 2). The hydrophobicity plot suggested that this protein is a type 1 membrane protein with a signal peptide (Figure 1b), properties that are consistent with those of the reported human MCP [2]. The consensus amino acids in SCRs were fairly well conserved in the deduced protein sequence in comparison with human, monkey (Vero cell, an African green monkey kidney cell line), pig and guinea pig MCP (Figure 2). Thus, we named this protein murine MCP.

Alignment of the murine MCP amino acid sequence with those of MCPs of other species (Figure 2) revealed that: (1) there is a perfectly conserved sequence, PECKVVKC, at the junction of SCR3 and SCR4, across the species; (2) murine MCP exhibits the highest identity at both the amino acid and nucleotide levels with guinea pig MCP; and (3) SCR3, SCR4 and ST domains tend to be conserved among the species, except for those of pigs. The role and significance of the relatively long signal peptides observed in murine and pig MCPs are unknown. The appropriate selection of the PCR primer corresponding to the PECKVVKC sequence has led to the successful cloning of murine MCP. MCP of other species, as yet unidentified, will be cloned using primers generated from this amino acid sequence.

Protein expression and characterization of murine MCP

Murine MCP was permanently expressed in CHO cells by cDNA transfection. The protein expressed in CHO cells was solubilized and analysed by SDS/PAGE immunoblotting using anti-peptide antibody. Its molecular mass was 47 kDa, about 6 kDa larger

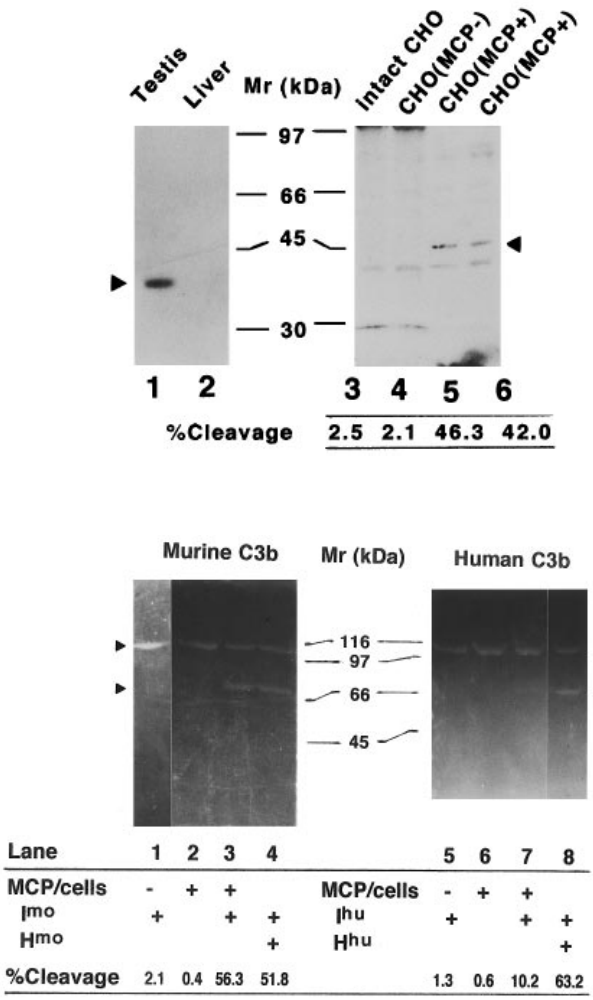

Figure 3 (a) Immunoblotting analysis of murine MCP and (b) factor I-cofactor activity in the solubilized CHO cell transfectants

(**a**) Solubilized preparations of the murine testis (2.0 mg) (lane 1) and liver (2.2 mg) (lane 2) and those (1×10^6) of intact CHO cells (lane 3), a clone with anti-sense MCP cDNA (lane 4) and two individual clones with murine MCP cDNA (lanes 5 and 6) were run on SDS/PAGE and transblotted onto nitrocellulose membrane. MCP proteins on the membrane (indicated by arrowheads) were detected by anti-peptide antibody, horseradish peroxidase-labelled secondary antibody and an ECL kit. Several bands common to the four lanes are non-specific. Protein markers No. 3 (Daiichi Pure Chemicals, Japan) are shown in kDa. Factor I-cofactor activity, evaluated with each clone as in panel (**b**), is shown beneath the lanes. (**b**) Solubilized preparations of CHO cells transfected with vector with anti-sense MCP cDNA (lanes 1 and 5) or with vector containing murine MCP cDNA (lanes 3 and 7) were incubated with murine factor I (I^{mo}) and fluorescence-labelled murine C3b (10 μg) (left panel) or human factor I (I^{hu}) and DACM-labelled human C3b (10 μg) (right panel). Without factor I, the solubilized preparation had virtually no C3b-cleaving activity under the conditions described in the Materials and methods section (lanes 2 and 6). Lane 4 with murine factor I and H.1 (H^{mo}) [34] and lane 8 with human factor I and H (H^{hu}) are positive controls. Arrowheads indicate the positions of α' chain (upper) and α'_1 fragment (lower) yielded by homologous factors I and H [30]. Protein markers No. 3 (Daiichi Pure Chemicals) in kDa are shown. The results are given as percentage cleavage based on the determination of fluorescence intensity using a spectrofluorometer [34]. A representative of five similar experiments is shown.

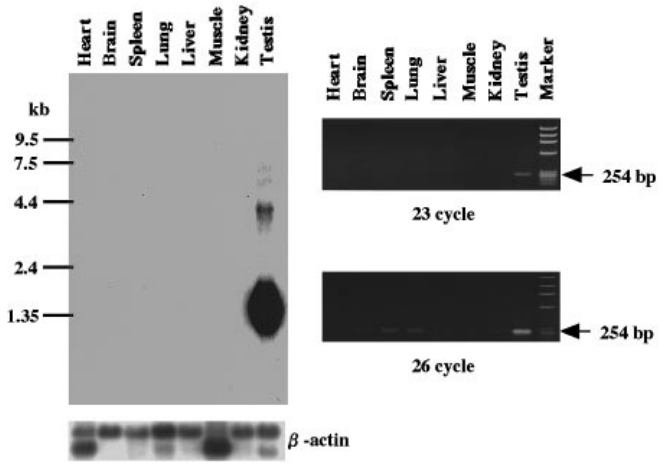

Figure 4 Northern blot and RT-PCR analysis

For Northern blot analysis (left panel) the membrane [Clontech MTN 7762-1, about 2 μg of poly(A)$^+$ RNA in each lane] was hybridized with a 0.3 kb fragment of murine MCP cDNA (upper panel). The same membrane was also rehybridized with human β-actin cDNA (lower panel). The hybridized membranes were exposed for 36 h (upper panel) and 2 h (lower panel). In the lower panel, a single 2.0 kb band was observed in all lanes, except for heart and muscle, which contained two forms of β-actin of 2.0 kb and 1.8 kb. Standard markers are indicated at the left side. For RT-PCR analysis (right panel) the 254 bp murine MCP cDNA (arrow) was amplified by PCR with the indicated cycles as described in the Materials and methods section. Marker No. 4 (Toyobo, Japan) is shown alongside.

than the murine testis MCP (Figure 3a). Factor I-cofactor activity in the solubilized preparation containing murine MCP was assessed using homologous substrate and protease according to an established method [32–34]. Murine C3b was effectively cleaved by murine factor I in the presence of the solubilized preparation (Figure 3b). Virtually no C3b cleavage was observed if the solubilized preparation was substituted with that containing no murine MCP. Cofactor activity of murine MCP was exerted for human factor I-mediated cleavage of human C3b, although its potency was lower in the human system than in the homologous system (Figure 3). Cofactor activity was assessed after 2 h incubation, since prolonged incubation allowed us to identify CHO cell cofactor activity, presumably due to the presence of CHO cell MCP [35] (results not shown). Thus, murine MCP serves as a factor I-cofactor as does murine factor H [34].

Message analysis of murine MCP

The murine MCP protein was clearly detected in the testis but barely in the liver (Figure 3a), spleen, heart, lung, kidney and muscle (results not shown). Northern blot analysis suggested that the murine MCP mRNA is 1.5 kb and is expressed predominantly in the testis (Figure 4a), consistent with the results of immunoblotting. Several faint bands with lower mobility ($\sim$ 3.8 kb) were observed, again only in the testis. No clear bands were detected in other organs tested (Figure 4a). Biased expression of murine MCP in the testis was further confirmed by RT-PCR (Figure 4b). The 254 bp band specific for murine MCP was detected only in the testis under the < 23 cycle PCR conditions. The message was detected in all organs tested under the > 26 cycle conditions, but the levels were far lower than in the testis.

The results of developmental blots indicated that the murine MCP message was barely expressed until day 29 and increased concomitantly with germ-cell development (Figure 5a). Therefore murine MCP mRNA was expressed in parallel with synthesis of spermatids in germ-cell development [23]. Supporting this finding (Figure 5b), MCP message expression was severely suppressed in mutant mice strains having no spermatids due to defects in germ-cell development [24].

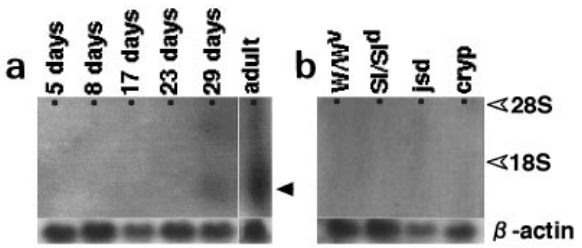

Figure 5 Northern blot analysis of murine MCP in (a) infant and (b) mutant mice

(**a**) Total RNA was extracted from the testes of infant Balb/c mice 5, 8, 17, 23 and 29 days after birth and also from adult Balb/c mice. The blot with 20 μg of total RNA in each lane was hybridized with the 0.3 kb murine MCP probe (upper panel). The same membrane was also rehybridized with the control actin probe (lower panel). The hybridized membranes were exposed for 7 days (upper panel) or 8 h (lower panel). The closed arrowhead indicates the $\sim$ 1.5kb specific message of murine MCP. (**b**) RNA was prepared from the testes of adult mutant mice as in (**a**). The blot with about 20 μg of total RNA in each lane was hybridized with the 0.3 kb MCP probe (upper panel). The same membrane was also rehybridized with the control actin probe (lower panel). The hybridized membranes were exposed for 7 days (upper panel) or 8 h (lower panel). Open arrows indicate the positions of 28S and 18S ribosomal RNA. cryp, mouse strain cryptorchidism.

DISCUSSION

We cloned a cDNA encoding murine MCP. The predicted amino acid sequence of murine MCP was 45.3 % identical with that of the human homologue [4], and the major domains, four SCRs, ST, TM and CYT, were conserved. The measles virus binding site [7] containing SCR2 was not very highly conserved compared with the other SCRs. The protein expressed on CHO cells was a singlet of 47 kDa, a little smaller than human MCP on CHO cells [35] and larger than murine testis MCP, like human conventional compared with testis MCP. Similar to murine factor H [33,34], murine MCP is compatible as a factor I-cofactor with murine factor I and C3b, but far less compatible with the human factors. Although compatibility of murine MCP with C3b of various species remains to be tested, murine MCP is likely to be an effective cofactor for homologous factor I.

Strikingly, its message was preferentially detectable in the testis. The structural properties and biased distribution profile of MCP observed may explain why mice are non-permissive to measles virus and why CRRY [31], a functional homologue of human MCP, has to be ubiquitously expressed on murine cells/cell lines to protect them from murine complement. In mice, MCP would be a spermatozoa-dominant protein involved in fertilization, as suggested for human MCP [12–14]. As murine MCP is expressed to a lesser extent in most organs compared with the human homologue, it should be less responsible for self-protection from autologous complement. Thus, CRRY and probably decay-accelerating factor (CD55) must be expressed ubiquitously for self-protection.

Many laboratories, including ours, have attempted to clone murine MCP for more than 6 years in vain, using human MCP cDNA as a probe. During the course of these studies, only CRRY, closely resembling complement receptor type 1 [32,36], was found on murine cells and tissues [32]. Most investigators in the fields of immunology, reproductive biology and virology believed that mice have no MCP. The present study has clarified that the failure to clone murine MCP by cross-hybridization and/or expression screening was due to its limited localization, low similarity and poor compatibility with human factor I, compared with human MCP.

Human MCP has YXXL [37], RRKKK [38] and FTSL sequences [39] in its CYT. Since YXXL is an endosome-targeting sequence [40], the common feature of proteins containing YXXL is internalization and endosomal uptake. MCP is also down-regulated by measles virus [3]. RRKKK serves as a moesin-binding site [38] and a targeting sequence for endoplasmic reticulum [40]. This sequence may contribute to the large intracellular pool of MCP. FTSL may be a retention signal in the cytoplasm [39]. Interestingly, none of these sequences are conserved in murine MCP. This may again reflect the divergence of the primary role of MCP in mice and humans.

We favour the interpretation that murine MCP is expressed in spermatids in germ-cell development. This was supported by our findings that it is not expressed in the testis until day 29 (spermatocytes and spermatides become predominant in 16–24-day-old and adult testis but not in 4-, 10- or 16-day-old testis [23]), and mutants lacking spermatogenesis have no MCP mRNA [23,24]. In addition, an unusual human MCP mRNA of 1.6 kb, which is $\sim$ 2 kb smaller than the conventional mRNA [4], is exclusively observed in the testis (A. Tsujimura and T. Seya, unpublished work), similarly to murine MCP mRNA. Although human spermatzoan MCP has been reported to be an egg-binding protein [12–14], no specific structural or genomic properties for sperm MCP have been discovered [41]. Based on the present findings of murine MCP and comparison studies with human MCP [42], our hypothesis is that the primary role of murine MCP is also associated with fertilization. Genomic and biological studies of murine MCP will be necessary to settle this issue. Furthermore, function-blocking antibodies against murine MCP and generation of MCP-deficient mice by gene targeting techniques will allow elucidation of the function of murine MCP. These studies are in progress in our group.

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