Immunology 1999 **98** 137–143

Molecular cloning, expression and characterization of the rat analogue of human membrane cofactor protein (MCP/CD46)

R. MEAD, S. J. HINCHLIFFE & B. P. MORGAN *Department of Medical Biochemistry, UWCM, Cardiff, UK*

SUMMARY

In humans, host cells are protected from homologous complement by membrane proteins encoded in the regulators of complement activation (RCA) gene cluster. These include complement receptor 1 (CR1), decay-accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46). In mouse and rat a single membrane inhibitor, Crry, appeared to perform the functions of both DAF and MCP and was proposed to be the functional analogue of both. Recently, however, murine homologues of DAF and MCP have been identified, prompting a search for the rat counterparts. We have described the identification of rat DAF and here describe the cloning of rat MCP from cDNA and genomic libraries, using a probe based on the mouse MCP cDNA sequence. The domain structure for rat MCP was identical to that of mouse MCP with four short consensus repeats (SCRs) followed by a STP domain, transmembrane segment and cytoplasmic tail. Overall identity of rat and mouse MCP was 77% at the amino acid level and 88% at the nucleotide level. Northern blot analysis from a range of tissues indicated that high-level expression was limited to the testis, although expression in other tissues was detected using reverse transcription–polymerase chain reaction. Rat MCP mRNA localized to Sertoli cells and spermatogonia in seminiferous tubules by *in situ* hybridization, but was absent in mature sperm. In cofactor assays utilizing human factor I, a recombinant soluble form of rat MCP catalysed cleavage of human C3ma.

INTRODUCTION

Human membrane cofactor protein (MCP, CD46) was first described in 1986. It is a broadly distributed membrane protein which acts as a cofactor for the cleavage of C3b and C4b by factor I.[1–3] In man, MCP plays an important role in the regulation of C3 and C5 convertases, protecting host cells from homologous complement activation. In mice and rats the membrane regulator Crry is broadly distributed and combines the functions of decay-accelerating factor (DAF) and MCP, provoking the suggestion that Crry replaced MCP and DAF in rodents.[4–6] However, both DAF and MCP have recently been identified in mice,[7,8] prompting us to search for their counterparts in rats. Rat DAF has recently been cloned in our laboratory.[9] Here we describe the cloning of rat MCP.

Received December 1998; revised 7 May 1999; accepted 14 May 1999.

This work was presented at the XVIIth International Complement Workshop (Rhodes, Greece, October 1998) and published in abstract form (*Mol. Immunol.* 1998; **35**, 377).

Correspondence: Professor B. P. Morgan, Department of Medical Biochemistry, UWCM, Cardiff CF4 4XN, UK.

MATERIALS AND METHODS

General and molecular biology

All general reagents were from Sigma Chemical Co. (Poole, UK) unless otherwise stated. Rat cDNA and genomic libraries were from Stratagene (La Jolla, CA). Ultraspec RNA isolation medium was from Biotecx (Houston, TX). Superscript reverse transcriptase was from GibcoBRL (Paisley, UK). Nick™ columns for radioactive probe purification, Taq polymerase, and buffers were from Pharmacia (Milton Keynes, UK), dNTPs were from Bioline (London, UK). RNase inhibitor rRnasin® was from Promega (Southampton, UK). Geneclean II DNA purification kit was from Anachem (Luton, UK) and plasmid purification kits were from Qiagen (Dorking, UK). Hybond-N nylon membranes, Rapid-Hyb buffer, *redi*prime DNA labelling system and ^{32}P-labelled α-dCTP were from Amersham International (Little Chalfont, UK). Oligonucleotide primers were synthesized in house on an ABI Model 394 synthesizer (Applied Biosystems, Warrington, UK).

Screening a rat testis cDNA library

A 1·1 kilobase (kb) fragment of mouse MCP cDNA was polymerase chain reaction (PCR) amplified from mouse testis cDNA, using primers derived from the published sequence.[8] The probe was radiolabelled with [^{32}P]α-dCTP using the Rediprime kit (Amersham) according to the manufacturer's

protocol. The labelled probe was purified from residual nucleotide using a Nick column (Pharmacia) and used to screen 5×10^5 clones from both a rat testis cDNA library (Stratagene, λZAPII) and a rat genomic library (Stratagene, λFIXII) essentially as described previously.[9]

Agar plugs containing positive plaques were picked, eluted in 1 ml SM buffer and replated. The screening protocol was then repeated, individual positive plaques picked and eluted in SM buffer and the cDNA recovered from the phage using the Exassist™/SOLR™ system (Stratagene). Individual bacterial colonies containing recombinant phagemid were grown up in LB broth containing 50 μg/ml kanamycin and the phagemid DNA was purified using a QIAprep spin plasmid mini-prep kit (Qiagen). Automated sequencing was carried out in house using an ABI model 377 DNA sequencer (Applied Biosystems, Warrington, UK).

Northern blotting and reverse transcription–polymerase chain reaction (RT-PCR)

Northern blots were performed as described previously.[9] RNA was isolated from fresh rat tissues using the Ultra-spec total RNA isolation reagent (Biotecx Labs) according to the manufacturer's instructions. Denatured total RNA (10 μg) was electrophoresed on 1·5% agarose, 2% formaldehyde gels and blotted by capillary action onto Hybond-N membranes (Amersham). Membranes were probed with a 462-base pair (bp) rat MCP PCR product amplified with the primers GCTTAATGGCAACGGTGATG (nucleotides 450–469 sense strand) and GGAGGCTTGGTAGAATGAGG (nucleotides 911–886 anti-sense strand). The probe was labelled with [^{32}P]α-dCTP using the Rediprime kit (Amersham). The blots were rehybridized with a labelled GAPDH PCR product to check for equivalence of sample loading.[9]

For RT-PCR analysis, RNA was isolated as above and reverse transcribed with SuperScript reverse transcriptase (GibcoBRL) using random hexamer DNA primers. Rat MCP cDNA was amplified with the specific primers noted above. As a control, rat GAPDH was amplified from the same cDNA using specific primers. Negative control contained no cDNA template, positive control was 100 ng vector containing the full-length rat MCP cDNA.

In situ *hybridization*

A 420-bp region of rat MCP cDNA encoding the signal peptide, SCR1 and half of SCR2 was cloned and used as template for synthesis of DIG-labelled sense and anti-sense RNA probes using T7 and SP6 RNA polymerases and the DIG RNA labelling kit (Boehringer Mannheim, Lewes, East Sussex). Rat testis sections were prepared on a cryostat from frozen tissue blocks, digested with proteinase K (5 μg/ml in 100 mM Tris/50 mM ethylenediaminetetraacetic acid EDTA; 200 μl per section) for 15 min at 37° and immediately immersed in 4% paraformaldehyde for 10 min at room temperature. Sections were washed twice for 5 min with diethyl pyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS), once in DEPC-treated water and allowed to air dry. Sections were then prehybridized in Rapid-Hyb buffer (Amersham) for 1 hr at 55°. RNA probes were denatured at 95° for 3 min before diluting 1 : 200 in fresh Rapid-Hyb buffer. Sections were hybridized overnight at 55°, washed once for 10 min in 1 × SSC at room temperature, twice for 10 min in 1 × SSC at 65° and equilibrated in buffer C (100 mM Tris pH 7·5, 150 mM NaCl, 0·2% Tween-20) for 15 min at room temperature. Sections were blocked in buffer C containing 1·5% bovine serum albumin (BSA) for 1 hr, before incubation with sheep anti-DIG antibody diluted 1 : 2000 in buffer C containing 1·5% BSA. After washing (twice for 10 min in buffer C and once for 10 min in detection buffer; 0·1 M Tris–HCl, 0·1 M NaCl, 50 mM $MgC1_2$, pH 9·5), specific signal was detected by staining for 16 hr with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; 175 μg/ml) and Nitro-blue tetrazolium (NBT; 337·5 μg/ml) in detection buffer 2 [0·1 M Tris–HCl, 0·1 M NaCl, 5% (w/v) polyvinyl alcohol, pH 9·5]. Sections were washed in PBS for 10 min and counterstained with Eosin.

Cofactor assay

For these assays, human C3 and factor I were purified by classical methods and purity was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE); C3ma was generated by incubating purified human C3 with 0·1 M methylamine for 1 hr at 37°, inactivated C3 was separated from active C3 by anion exchange on a Mono-Q column (Amersham Pharmacia Biotech). A recombinant soluble form of rat MCP (signal peptide and SCRs 1–4) was engineered into a transfer vector and used to create a replication-deficient adenovirus expressing rat MCP (rAd MCP) (McGrath *et al.* in preparation). The adenovirus is E1 deleted and E3 partially deleted serotype Ad 5 with the gene of interest under the control of the cytomegalovirus (CMV) IE promotor with a simian virus 40 (SV40) polyadenylation signal.[10] Human fetal foreskin fibroblasts (HFFF) grown in serum-free medium were infected with rAd MCP and the supernatant was collected on day 3 postinfection. Control supernatant was obtained from HFFF infected with control adenovirus containing no insert. The concentration of soluble MCP in the supernatant was estimated by SDS–PAGE analysis and protein assay. Cofactor assays were performed by incubating 45 μl of supernatant (approximately 0·45 μg sMCP) with 0·5 μg of purified methylamine-inactivated human C3 (C3ma) and 0·75 μg human factor I in a total volume of 50 μl. In control experiments, C3ma was incubated with factor I alone (negative control) or with factor I and 5 μg soluble human CR1 (sCR1, T Cell Sciences, Needham, MA). Following incubation at 37° for 16 hr the mixture was diluted in an equal volume of SDS–PAGE sample buffer and subjected to SDS–PAGE on 10% acrylamide gels under reducing conditions (BioRad minigel system, BioRad Laboratories, Hertfordshire, UK). The gel was blotted onto nitrocellulose and probed with polyclonal rabbit antihuman C3c (generated in house) followed by goat anti-rabbit horseradish peroxidase-conjugated antibody (BioRad) and the blot developed using the ECL system (Pierce, Rockford, IL).

RESULTS

Molecular cloning of rat MCP

From screening of the rat testis cDNA library, several positive clones were isolated, sequenced and shown to contain sequence compatible with an MCP analogue. The longest clone identified contained the entire coding sequence of rat MCP except for the initiation codon and a portion of the signal peptide. From screening of the rat genomic library, a single clone was isolated

and sequencing of this genomic clone (pBSRG-1) with a reverse primer designed from the cDNA sequence provided the sequence of the missing portion of the signal peptide through to the start codon. The entire nucleotide and predicted amino acid sequence for rat MCP is shown in Fig. 1. Comparison of the derived amino acid sequence of rat MCP with the sequences of MCP analogues in human and other species revealed strong homology among the rodent MCPs but weaker homology with human and pig MCP (Fig. 2). The cDNA was designated rat MCP based on its high degree of homology with mouse MCP, 77% at the nucleotide level and 88% at the amino acid level. The domain structure was identical to mouse MCP, with a signal peptide followed by four short consensus repeats (SCRs), a Ser, Thr, Pro (STP) -rich domain, a transmembrane domain and a short cytoplasmic tail. The cDNA contained a short STP domain, corresponding to the STP-C isoform of human MCP.[2]

Tissue expression by Northern blotting and RT-PCR

In Northern blots, a strong hybridization signal was obtained for testis RNA, revealing a single product of $\approx 1{\cdot}6$ kb (Fig. 3a). No signal was detected in any of the other tissues tested, although GAPDH signal was detected in all tissues (not shown). In order to detect low level expression of rat MCP mRNA, RT-PCR was performed (Fig. 3b). In addition to testis, a clear signal for MCP mRNA was obtained in lung, small intestine and kidney, with weak expression in brain, spleen, skeletal muscle, liver and heart. No message was detected in the ovary.

Localization in testis by *in situ* hybridization

In order to identify the cells expressing MCP in the rat testis, *in situ* hybridization was performed (Fig. 3c). The anti-sense probe hybridized to areas containing Sertoli cells and sperma-

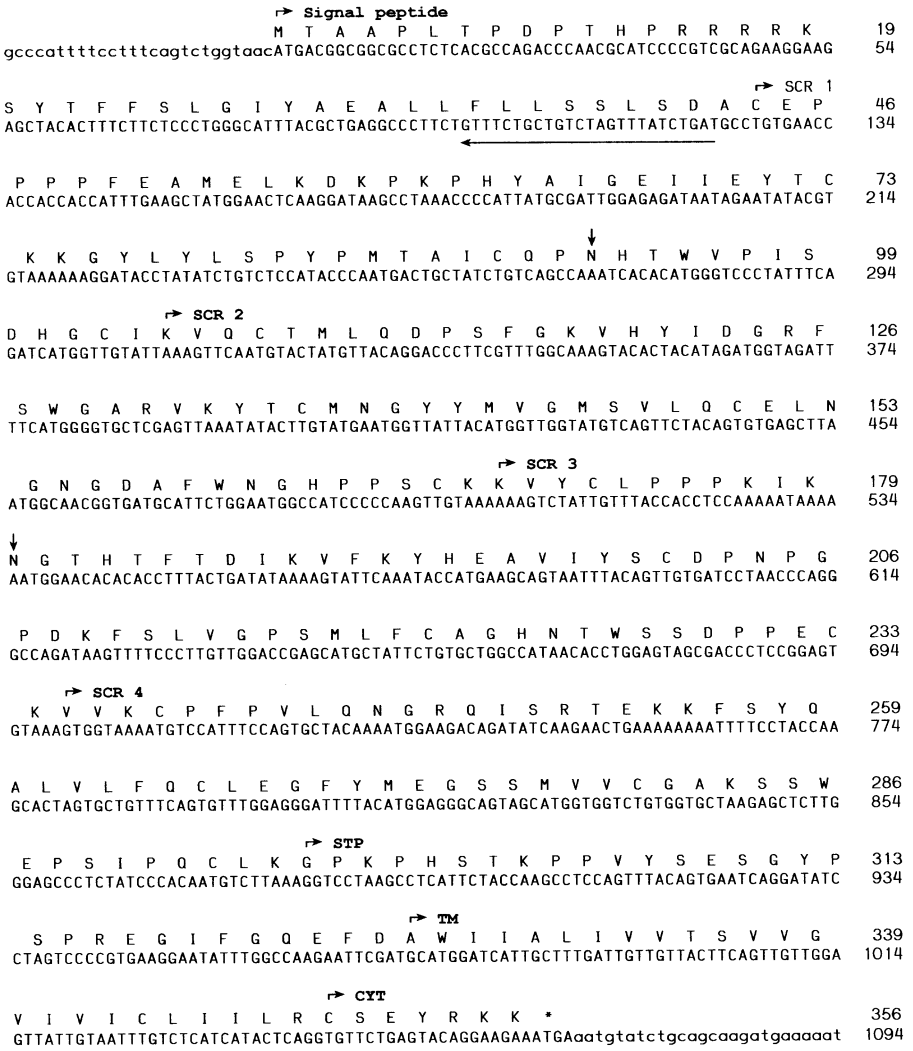

Figure 1. Complete nucleotide and deduced primary amino acid sequence of rat MCP. Location of the primer used to sequence the genomic clone is shown underlined with an arrow. The cDNA clone began at nucleotide 40 of the coding sequence. Potential glycosylation sites are indicated by arrows (↓). Boundaries between domains are also indicated: SCR, short consensus repeat; STP, serine/threonine/proline-rich domain; TM, transmembrane domain; CYT, cytoplasmic tail. The first residue of the mature protein is C44. Sequence submitted to Genbank.

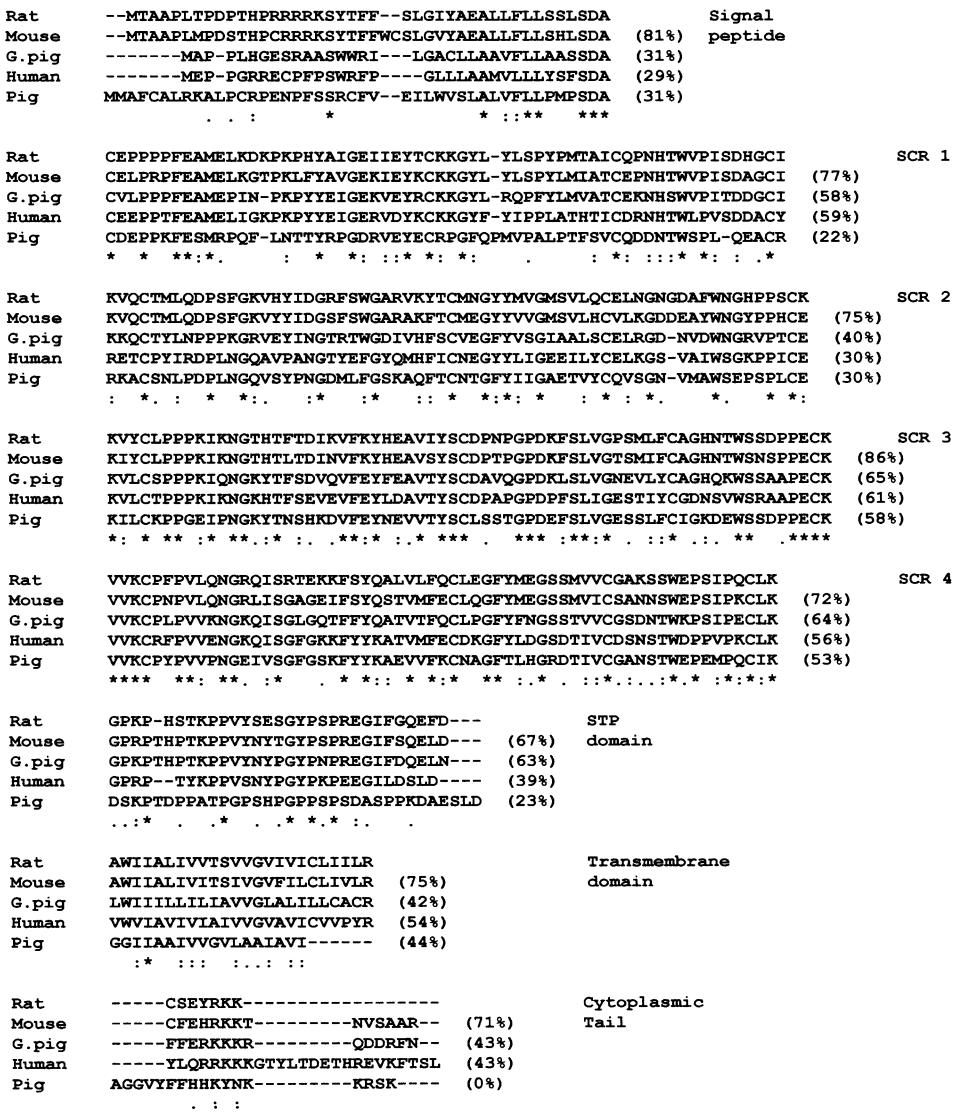

Figure 2. Alignment of MCP/CD46 from rat, mouse, guinea pig, human and pig. The domain structure is taken from ref. 8. Percentage homologies for each domain compared to rat MCP are shown on the right. Alignment performed using Clustal W version 1.7 (ref. 11). The STPc/CYT 1 isoform of human MCP[2] and the GM1 isoform of guinea pig MCP[12] were used for the alignment. The (*) indicates positions with residues fully conserved across all species, the symbol (:) indicates positions with conservation in most species (.) indicates positions with weaker conservation.

togonia of seminiferous tubules, whereas the Leydig cells, connective tissue supporting the tubules, and mature sperm in the lumen of the tubules were negative. Minimal staining was observed when the sense probe was used as a control.

Soluble rat MCP has cofactor activity for cleavage of C3

Soluble rat MCP was expressed from an adenoviral construct in HFFF. SDS–PAGE and silver staining identified in rAd MCP supernatants a single major protein species of apparent MW 25 000, compatible with the predicted Mr of soluble MCP, absent in control supernatant. Total protein in the supernatant was 20 μg/ml by Coomassie assay and the MCP band accounted for approximately 50% of total protein. The soluble rat MCP was tested for cofactor activity in a system using purified human factor I and C3ma. Soluble rat MCP exhibited cofactor activity for human C3ma in the presence of human factor I, as demonstrated by the appearance of C3 cleavage products of MW 43 000 and 46 000 (Fig. 4). In contrast, supernatant from HFFF infected with control virus demonstrated only a very low level of background cofactor activity. Purified human sCR1, used as positive control, demonstrated excellent cofactor activity. Controls in which factor I was omitted revealed no cleavage.

DISCUSSION

We here describe the molecular cloning and partial characterization of rat MCP. The full-length rat MCP sequence was obtained from a screening approach involving a combination of cDNA and genomic libraries. Rat MCP was highly homologous to mouse MCP (88% at the amino acid level).[8] As in

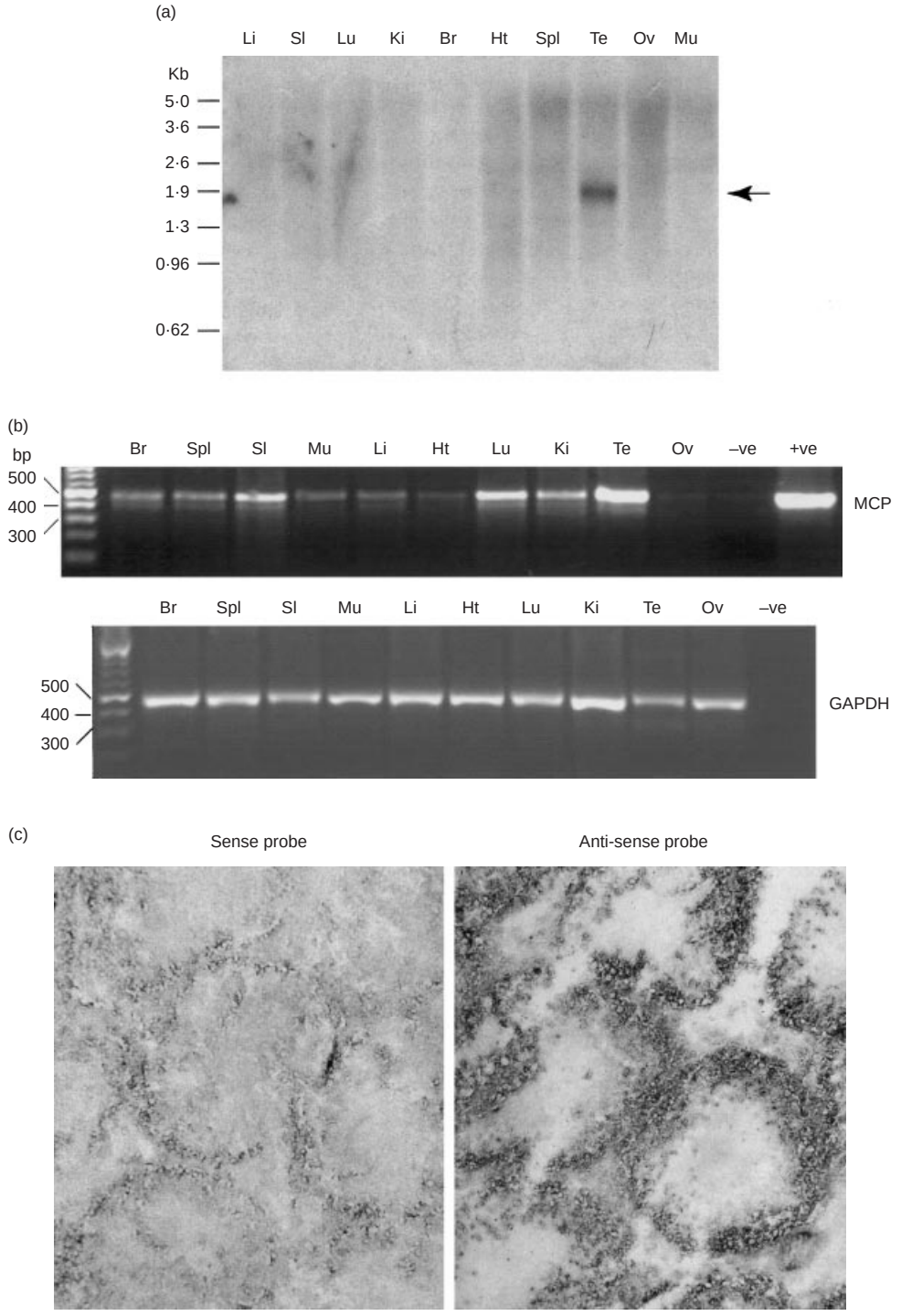

Figure 3. Analysis of expression of rat MCP by Northern blotting, RT-PCR and *in situ* hybridization. (a) Northern blot analysis. RNA was isolated from fresh tissue and subjected to Northern analysis as described in the Materials and Methods. Positions of RNA markers are shown on the left in kilobases (kb), a single band of $\approx$1·6 kb is detected in testis (arrow). (b) RT-PCR analysis. RNA was isolated, reverse transcribed and amplified as described in the Materials and Methods. Size in base pairs (bp) of 100 bp DNA ladder are shown on the left. Li, liver; SI, small intestine; Lu, lung; Ki, kidney; Br, brain; Ht, heart; Spl, spleen; Te, testis; Ov, ovary; Mu, skeletal muscle; $-$ve, negative control (no template); $+$ve, positive control (vector containing full-length rat MCP cDNA). (c) *In situ* hybridization. Probes were made and sections probed as described in Materials and Methods. Results are shown for sense (control) and anti-sense (positive) probes. Magnification $\times$100.

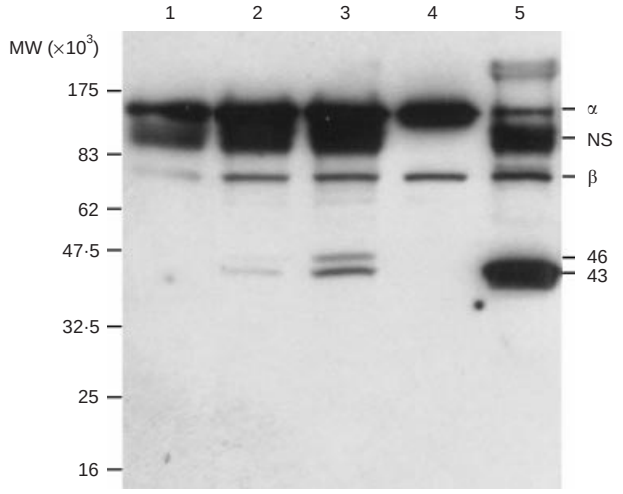

Figure 4. Cofactor assay for rat MCP. Cofactor assays were performed by incubating rat MCP (0·45 μg in serum-free medium) with 0·5 μg of purified methylamine inactivated C3 (C3ma) and 0·75 μg factor I in a total volume of 50 μl (lane 3). In control incubations, C3ma was incubated with factor I alone (lane 1; negative control), factor I plus supernatant from HFFF infected with empty adenovirus (lane 2), 5 μg soluble human CR1 (sCR1, T Cell Sciences, Needham, MA; lane 4) or factor I and 5 μg soluble human CR1 (lane 5). Positions of molecular weight markers are indicated on the left. Cleavage products of the α-chain of C3 (46 000 and 43 000 MW) are indicated on the right, together with the α- and β-chains of C3ma and a non-specific band (ns) present in all lanes containing factor I.

the mouse, tissue expression as assessed by Northern analysis was restricted to testis. However, by RT-PCR it was apparent that message was present, albeit at low levels, in numerous other tissues.

The expression of MCP at high level only in testis in both rat and mouse supports the suggestion that MCP might have specific roles in reproduction independent of its role in regulation of complement. Such a scenario has previously been suggested in humans.[13,14] It was therefore of some interest to examine which cell in testis expressed MCP, particularly as such information was not available for the mouse. By *in situ* hybridization, rat MCP message was localized to areas of the seminiferous tubules containing Sertoli cells and spermatogonia.

The restricted pattern of expression of MCP in rodents raises the possibility that these molecules have evolved functions distinct from complement regulation and, indeed, may have no complement inhibiting activity. We thus set out to examine whether rat MCP displayed factor I cofactor activity for cleavage of C3b. Rat MCP in membrane extracts of transfected CHO cells (data not shown) or expressed as a soluble recombinant protein (Fig. 4) exhibited Factor I cofactor activity for cleavage of methylamine-treated C3, demonstrating that the protein is a functional complement regulator. Nevertheless, the restricted expression pattern makes it unlikely that MCP is a major factor regulating complement in rodents. Rodents have an additional C3 convertase regulator, Crry, not present in man, which possesses both decay accelerating and cofactor functions, is broadly expressed and is essential for complement homeostasis *in vivo*.[5,6] We have also shown that rat DAF is a functional regulator,[9] which, like Crry, is widely distributed.[15] Taking into account the activities and distributions of these three regulators, we suggest that Crry is the major regulator of alternative pathway activation on most rodent cell types *in vivo*. Classical pathway regulation in the rat is likely to involve both DAF and Crry, although a definitive analysis of their relative importance *in vivo* is lacking. Although MCP may provide some additional alternative pathway regulation in testis, its unique expression at this site strongly implies some other role in spermatogenesis or sperm survival. Current work is focused on the generation of antibodies against rat MCP in order to further assess tissue distribution of MCP and to facilitate studies of the relative roles of Crry, DAF and MCP in regulating complement activation *in vivo* in the rat.

ACKNOWLEDGMENTS

This work was funded by The Wellcome Trust. B.P.M. is a Wellcome Senior Fellow, S.H. is a Wellcome Prize Fellow. R.M. is supported by a Departmental Studentship. We thank Yvonne McGrath for generating the soluble recombinant rat MCP, and Drs Carmen van den Berg, Claire Harris and Philippe Gasque for reagents and useful discussion.

REFERENCES

1. Seya T., Turner J.R. & Atkinson J.P. (1986) Purification and characterization of a membrane protein (gp45) that is a cofactor for the cleavage of C3b and C4b. *J Exp Med* **163,** 837.
2. Liszewski M.K., Post T.W. & Atkinson J.P. (1991) Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. *Annu Rev Immunol* **9,** 431.
3. Johnstone R.W., Loveland B.E. & Mckenzie I.F. (1993) Identification and quantification of complement regulator CD46 on normal human tissues. *Immunology* **79,** 341.
4. Holers V.M., Kinoshita T. & Molina H. (1992) The evolution of mouse and human complement C3-binding proteins: divergence of form but conservation of function. *Immunol Today* **13,** 231.
5. Matsuo S., Ichida S., Takizawa H. *et al.* (1994) *In vivo* effects of monoclonal antibodies that functionally inhibit complement regulatory proteins in rats. *J Exp Med* **180,** 1619.
6. Funabashi K., Okada N., Matsuo S., Yamamoto T., Morgan B.P. & Okada H. (1994) Tissue distribution of complement regulatory membrane proteins in rats. *Immunology* **81,** 444.
7. Spicer A.P., Seldin M.F. & Gendler S.J. (1995) Molecular cloning and chromosomal localization of the mouse decay-accelerating factor genes. Duplicated genes encode glycophosphatidyl-anchored and transmembrane forms. *J Immunol* **155,** 3079.
8. Tsujimura A., Shida K., Kitamura M. *et al.* (1998) Molecular cloning of a murine homologue of membrane cofactor protein (CD46): preferential expression in testicular germ cells. *Biochem J* **330,** 163.
9. Hinchliffe S.J., Spiller O.B., Rushmere N.K. & Morgan B.P. (1998) Molecular cloning and functional characterisation of the rat analogue of human decay accelerating factor (CD55). *J Immunol* **161,** 5695.
10. Graham F.L. & Prevec L. (1995) Methods for construction of adenovirus vectors. *Molecular Biotechnology* **3,** 207.
11. Thompson J.D., Higgins D.G. & Gibson T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673.
12. Hosokawa M., Nonaka M., Okada N., Nonaka M. & Okada H. (1996) Molecular cloning of guinea pig membrane cofactor protein. *J Immunol* **157,** 4946.

13. Anderson D.J., Abbott A.F. & Jack R.M. (1993) The role of complement component C3b and its receptors in sperm–oocyte interaction. *Proc Natl Acad Sci USA* **90,** 10051.
14. Taylor C.T., Biljan M.M., Kingsland C.R. & Johnson P.M. (1994) Inhibition of human spermatozoon–oocyte interaction *in vitro* by monoclonal antibodies to CD46 (membrane cofactor protein). *Human Reproduction* **9,** 907.
15. Spiller O.B., Hanna S.M. & Morgan B.P. (1999) Tissue distribution of the rat analogue of decay-accelerating factor. *Immunology* **97,** 374.